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Petunia

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With 73 Figures



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Preface

The solanaceous family is one of the most interesting and diverse plant groups among the dicotyledons. Plants of various genera are grown for their edible parts, for drugs, or for ornamental purposes. The six genera grown primarily as ornamental plants are: *Browallia, Brunsfelsia, Datura, Nicotiana, Petunia,* and *Salpiglossis.* Of these, *Petunia* is the most economically important in terms of worldwide use as an ornamental plant.

In addition to its horticultural value and prominence, the genus *Petunia* plays a key role as experimental plant material in a broad range of horticultural and biological research endeavors. Because of the broad range of commercial and research interest in *Petunia,* the collaborating authors believed it was appropiate to prepare a monograph dealing in a comprehensive manner with all economic and biological facets of the genus *Petunia.* The authors sincerely hope that this monograph will be of resource value and stimulating to students, researchers, and workers in industry interested in having a compendium of the knowledge available on the genus *Petunia.*

KENNETH C. SINK

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1 Introduction

K. C. Sink [1]

This chapter is devoted to a broad overview and appreciation of the many and diverse biological and economic aspects of the genus *Petunia*. The use of *Petunia* as a model plant species for biological investigations stems in part from the fact that plants can be readily grown from seed to flower in approximately 70–80 days, that the greenhouse or growth chamber cultural requirements are simple, and that abundant seed harvest occurs about 4 weeks after pollination. *Petunia* may also be studied as a result of a culture problem or because it presents a unique biological system.

1.1 Survey of *Petunia* as a Research Species

Breeding lines and commercial hybrid varieties of *Petunia hybrida* Vilm., a composite horticultural species, are the types among the *Petunia* germplasm used most frequently in biological studies. The experimental use of *Petunia* species is limited to research projects concerned primarily with understanding taxonomic, anatomical, and morphological relationships within the genus. Since the genus was established in 1803 by Jusseau, the early studies were mainly concerned with taxonomic, cytological, and inheritance studies. Starting in the 1930's, papers were published on chromosomally induced variegation, mosaic color patterns, chromosomal behavior, self-incompatibility, and the biochemistry of flower color pigmentation. In the 1940's and 50's, further genetic reports were issued on incompatibility, anatomical and reproductive studies, cytoplasmic inheritance, and the inheritance of flower color. Research reports in the 1960's were concerned with biochemical studies of the pigments responsible for flower color, in vitro fertilization, cytoplasmic sterility and restoration genes, karyotype analysis, inheritance of the grandiflora-multiflora character, air pollutant studies, refined biochemical analysis of incompatibility reactions, pollen grain composition, guidelines for seed germination, and early seedling growth. From the early 1970's to the present time, studies were reported on the enzymatic and other molecular changes in styles of self-compatible and -incompatible lines, ultrastructure of the female reproductive apparatus, and additional information on flower color pigmentation, genetics of air pollutant resistance, cytoplasmic sterility, and cytogenetics. Plant nutrient levels were also established, the biochemical and metabol-

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ic response to pathogens and parasites, inheritance studies, and tissue, anther, protoplast culture, and related genetic transformation studies were reported.

1.2 Survey of *Petunia* as an Economic Plant Species

Floricultural glasshouse crops are generally divided into four groups: pot plants, cut flowers, foliage, and bedding plants. *Petunia* is a bedding plant because it is generally grown as an annual from seed each year for outdoor decorative purposes in prepared ground beds and window boxes. Some cultivars can be grown in various types of containers, their pendulous growth-flowering habit adapting them as decorative plants often termed hanging baskets. *Petunia* was probably the first plant species to be grown and sold extensively as a flowering hanging basket plant.

Petunia currently ranks first among bedding plants, comprising approximately 30% of the quantity of seeds sold (Anonymous 1977). With respect to plant type, single-flowered grandifloras comprise about 70%, single-flowered multifloras 15%, and double-flowered types 15% based on number of seeds sold. With respect to popularity, pink, rose and salmon combined, and red each account for about 20%, white for 18%, bicolors and blue for 15% each, and mixtures for 10%. In the U.S., petunias comprise two-thirds of the dollar value of all bedding plants.

2 Taxonomy

K. C. SINK [1]

2.1 Geographical Distribution

To date, approximately 30 *Petunia* species have been described. Their geographic origin is South America, where the various species have been documented from collections made in Argentina, Brazil, Bolivia, Paraguay, and Uruguay. The majority of the species have been found in three Brazilian provinces: Parana, Santa Catarina, and Rio Grande do Sul, and in areas of the other countries, particularly in interior areas where they share a common border. *Petunia* species, described in the literature of the 1800's, were commonly found along river banks in isolated areas, for example, at the mouth of the Rio de la Plata, the Rio Negro, and the Paraná. *Petunia parodii*, described by Steere in 1930, was only reported to occur in the Formosa province of Argentina. *Petunia parviflora* has been found in the above countries and also in Cuba, Mexico, and in the United States in New Jersey, California, Texas, Arizona, and Florida. Whether the plants of *P. parviflora* found in geographic locations outside of South America are indigenous ecotypes or have become established through seed and plant dispersal remains to be established. Contemporary ecological, adaptive, and evolutionary research studies of *Petunia* in the native habitat is a complete void.

2.2 Status of *Petunia* Within the Solanaceae

The information presented in this section dealing with the relationship of the genus *Petunia* to other genera in the Solanaceae is based primarily on the observations and inferences made by Goodspeed (1954). The genera *Cestrum* and *Petunia* are believed to have affinities to *Nicotiana*. This general assumption is based upon several lines of taxonomic evidence. Their natural geographic ranges overlap to a considerable extent, primarily in Brazil and Argentina (Goodspeed 1954). In particular, members of the *Nicotiana* subgenus *Petuniodes*, section *Alatae* are commonly found in Uruguay and Brazil at stream beds, river banks, and upland sites, as are some species of *Petunia*. Goodspeed (1954) postulated that common progenitors of pre-*Nicotiana*, pre-*Cestrum*, and pre-*Petunia* were lowland inhabitants east of the geosyncline in South America, with pre-*Nicotiana* in the La Paz region of Bolivia between the eastern territory occupied by pre-

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Petunia and the north-western territory occupied by pre-*Cestrum*. Later, progenitors of the section *Noctitlorae* and *Alatea* spread southward and eastward with *Alatea* migrating into the territory also occupied by pre-*Petunia*. The subsequent intermingling of *Alatea* and *Petunia* is evident in certain *Nicotiana* species which have a high degree of similarity to *Petunia*, particularly in the shape of the corolla limb, flaccid daytime relaxation, estivation, stamen insertion, fragrance, inflorescence, and capsule.

With respect to chromosome number, Goodspeed (1954) suggested that 6 may have been the basic number at the early stage of evolution. *Petunia* generally possesses an $n=7$ base number which may have arisen as an increase over 6, but in higher plants changes in chromosome number are due more often to decreases. Median type chromosome morphology exists both in *Petunia* and the *Petuniodes* subgenera of *Nicotiana* reflecting similarity of primitive types. Within the section *Alatae*, those species most commonly having *Petunia* characters also have the closest chromosome number of $n=9$.

2.3 Early Taxonomic Studies

The genus *Petunia* was founded by Jussieu in 1803 based on his examination of herbarium specimens of Commerson that were collected near the mouth of the La Plata River, and included the two species *P. nyctaginiflora* and *P. parviflora* (Stout 1952). The name *Petunia* is derived from Petun, the name given tobacco in Brazil. Actually, petunias had been described prior to Jussieu's founding of the genus and much of the early literature is quite confusing since the taxonomists of that era did not have the opportunity to observe each others, herbarium specimens or plants in the native habitat.

The species *P. axillaris* was described by Lamarck in 1793 as *Nicotiana axillaris*. According to Stout (1952), Jussieu identified *Nicotiana axillaris* as *Petunia nyctaginiflora*, a species with a corolla about 1–3/4 in. in diameter and a corolla tube about 1–1/2 in. long. Until Fries (1911) used the name *Petunia axillaris*, it was designated *Nicotiana nyctaginiflora* and *N. axillaris* (Lehmann 1818, Kuntze 1898) or *Petunia nyctaginiflora*. Since the publication of the *Petunia* taxonomic monograph by Fries (1911), *P. axillaris* has been widely used as the accepted designation.

The true plants of *P. axillaris* according to Stout (1952) are self-incompatible. He also observed "intra-genotypic cross-incompatibilities", and one mutant plant that was self-fertile. Stout (1952) further claimed that the plants utilized and depicted by Mather (1943) and Ferguson and Ottley (1932) as *P. axillaris* was in fact *P. parodii*.

Confusion also existed with respect to the early designation of *P. integrifolia*. It was described by Hooker (1831) as *Salpiglossa integrifolia*, a species with small violet flowers. Don (1833) named it *Nierembergia phoenicea* and Lindley (1833) described it as *P. violacea* as did Fries in 1911. Fries (1911) created a subgenus *Eupetunia* for the many forms of violet-colored *Petunia*. He described the new species *P. inflata* and *P. occidentalis* in the new subgenus. In 1904 Chodat and Hassler

described five forms of *P. violaceae*. Finally, Smith and Downs (1966) designated *P. violaceae* and *P. inflata* as *P. integrifolia* var. *integrifolia* based on their study of *Petunia* species in Santa Catarina, Brazil.

2.4 Species Described by Fries, Steere, Smith, and Downes

Contemporary *Petunia* taxonomy began with Fries' (1911) monograph. His key is presented on p. 5 and 6. In 1930, Steere described *P. parodii*, a white-flowered species, previously unreported in the *Petunia* taxonomic literature. Steere (1930) stated that *P. parodii* can be delineated from its allies by the larger, more erect stems, with longer internodes, unusually long corolla tube, small limb, and pointed lobes with deep purple veining. The stamens are distinctive because of the very minute differences in length among the four long stamens, as opposed to *P. axillaris* which is didynamous.

The most recent *Petunia* taxonomic study was conducted by Smith and Downs (1966) on plants growing in the field in the Santa Catarina province of Brazil. Seventeen species were classified by Smith and Downs, including *P. axillaris, P. integrifolia*, and seven others which had previously been documented. *Petunia parviflora* was also mentioned as occurring widely throughout the province. The taxonomic key for *Petunia* developed by Smith and Downs (1966) is given on p. 6 and 7.

Petunia Juss. (Fries 1911)

Subgenus *Pseudonicotiana*: Corolla alba, tubo subcylindrico; filamenta tubo medio affixa.

1. Planta elata: folia elliptica vel spathulata; corolla 3.5 cm longa vel ultra; pedicelli fructiferi erecti. *axillaris.*
2. Planta minor: folia linearia; corolla 1.5 cm vix attingens; pedicelli fructiferi deflexi. *pygmaea.*

Subgenus *Eupetunia*: Corolla violacea, tubo sursum sensim ampliato; filamenta infra medium affixa.

I. Calyx fere ad basin divisus, incisuris obtusis, laciniis sursum plus minus (interdum leviter) dilatatis.

A. Folia ovata, rhomboidea vel spathulata, corolla magna (18 mm longa vel ultra).

1. Pedunculi fructiferi deflexi. *violacea.*
2. Pedunculi fructiferi erecti.

a) Corolla ventricoso-infundibuliformis, tubo basi breviter tubuloso-cylindrico; capsula 5–7 mm longa. *inflata.*
b) Corolla aguste infundibuliformis, tubo basi longe tubuloso-cylindrico; capsula 9–11 mm longa. *occidentalis.*

B. Folia lineri-spathulata; corolla minima, 1 cm haud attingens. *parviflora.*

II. Calyx minus alte incisus, incisuris acutis vel obtusis, lobis apicem versus angustatis.

A. Stigma truncatum, clavatum vel plus minus discoideo-dilatatum.

1. Ramuli, folia calyxque pilis longis crispulis vestita.

a) Folia lineari-oblongs, margine revoluta. *ledifolia.*

b) Folia obovata-spathulata, plan.
Tubus corollae basi longe cylindricus, dein ampliatus; stamina ca. 10 mm corollae adnata. *Sendtneriana.*
Tubus corollae basi breviter cylindricus; stamina ca. 3 mm corollae adnata. *Regnellii.*

2. Ramuli, folia calyxque pilis brevibus, vulgo rigidis vestitata vel subglabra.

a) Folia (vetustiora saltem) margine revoluta. Semina reniformia.
Ramuli foliaque pilis rigidis dense vestita. Folia brevia (nectimentrum vix excedentia), sessilia.

α) Folia ovata-oblonga; calyx profunde (ad 3/4) divisus. *micrantha.*
β) Folia lineari-oblonga; calyx vix ad medium incisus. *ericaefolia.*

**)Ramuli foliaque glabriuscula, glandulis subsessilibus instructa. Folia longiora (centimentrum conspicue superantia), basin versus sensim angustata.

α) Folia 1–3 mm lata; corolla magna, 1.8–2.2 mm longa. *paranensis.*
β) Folia 0.5–1 mm lata; corolla minor, an 1.7 mm longa. *rupestris.*

b) Folia margine plana. Semina subglobosa vel globosa-reniformia.

*) Folia latiora (spathulata, ovata vel lanceolata).

α) Folia spathulata, basin versus in petiolum alatum conspicuum angustata.

c) Ramuli foliaque brevissime glanduloso-puberula.
Corollae tubus ventricoso-infundibuliformis, longitudine limbum vix aequante vel conspicue brevior. Pedicelli fructiferi foliis sustinentibus sub duplo brevior. *excellens.*

3. Folia minuta, centimentrum haud vel vix attingentia. Tota planta longe glanduloso-pilosa. *Dusenii.*

Petunia Juss. (Smith and Downs 1966)

1. Calyx-lobes linear or more or less enlarged toward apex, usually much longer (except *P. integrifolia* var. *depauperata*) than the short or very short tube; plants herbaceous.
2. Corolla-tube wholly subcylindric; corolla white; filaments inserted in the middle of the tube. 1. *P. axillaris*
2. Corolla-tube infundibuliform at least toward apex; corolla violet or purple; filaments inserted below the middle of the tube.
3. Corolla-tube ample.
4. Stems densely viscid-pilose. 2. *P. integrifolia*
4. Stems nearly glabrous.

5. Leaves elliptic, to 20 mm wide. 6. *P. schiedeana*
5. Leaves narrowly lanceolate, not more than 7 mm wide. 4. *P. littoralis*
3. Corolla-tube slender, about three times as long as wide.
6. Leaves narrowly lanceolate, 7 mm wide; stems viscid-pilose. 3. *P. reitzii*
6. Leaves elliptic, up to 17 mm wide; stems nearly glabrous. 5. *P. saxicola*
1. Calyx-lobes distinctly attenuate, usually about equaling or slightly longer than the distinct tube; plants often shrubby.
7. Leaves with inrolled margins, linear or linear-spatulate, thick. 7. *P. ericifolia*
8. Leaves entire, obtuse, 3–6 mm long.
8. Leaves serrulate, acuminate, 12–20 mm long. 8. *P. serrulata*
7. Leaves with flat margins, wider, usually rather thin.
9. The slenderly cylindric base of the corolla long and about equaling the calyx. 9. *P. sendtneriana*
9. The slenderly cylindric base of the corolla short to nearly lacking, much shorter than the calyx.
10. Glands of the leaves minute, sessile or subsessile, making the leaves appear glabrous; leaves 2–6 mm wide. 10. *P. caesia*
10. Glands or hairs of the leaves long, evident.
11. Stems prostrate; leaves 0.5–5 mm wide.
12. Leaves with the central nerve obscure above, flat, straight, fleshy; larger leaves few and lax, not more than 2 mm wide, obtuse. 11. *P. heterophylla*
12. Leaves with the central nerve evident above at least as a depression; sometimes conduplicate when old.
13. Leaves obtuse or subacute, sublinear, 0.5–3 mm wide, drying pale, usually curved. 12. *P. selloviana*
13. Leaves broadly rounded, spatulate, 4–5 mm wide, drying dark, straight. 13. *P. spathulata*
11. Stems erect or suberect.
14. Leaves dense, dimorphic, narrowly oblanceolate, not more than 7 mm wide.
15. Calyx much enlarged in fruit, up to 12 mm long. 14. *P. alpicola*
15. Calyx little enlarged in fruit, not more than 8 mm long. 15. *P. kleinii*
14. Leaves lax, subuniform, oblanceolate to elliptic, 3–15 mm wide.
16. Calyx-lobes 2–3 times the length of the short tube; calyx enlarged in fruit; stems covered with short, rigid, glandular hairs. 16. *P. macrodactylon*
16. Calyx-lobes about as long as the tube. 17. *P. linoides*

2.5 Origin of the Cultivated *P. Hybrida* Vilm.

Loudon (1840) stated that seeds of the white petunia were sent to Europe in 1823. In 1830, Tweedie, a botanical collector, discovered the plant *(P. integrifolia)* in Buenos Aires and sent seeds to the Botanic Garden at Glasgow. The species first flowered there in July, 1831. Loudon (1840) further stated that "there are many hybrids and varieties of this species, as it is found to hybridize freely with the white petunia." In the 1918 edition of the Curtis Botanical Magazine, an anon-

ymous author stated that a hybrid (between *P. nyctaginiflora* = *P. axillaris* and *Salpiglossa integrifolia* = *P. integrifolia*) was first obtained in 1834 by a nurseryman Atkins of Northampton, England. The hybrid was described and a figure presented in Sweet's Brit. Fl. Gard. Ser. 2, Vol. 3. The same writer remarked that modern day petunias are the prime result of the skillful efforts of E. Benary of Erfurt, Germany, who apparently was the first individual to produce seed varieties that bred true. He also selected against vigor to obtain "the best petunias."

Ferguson and Ottley (1932) stated the general belief in the 1950's that *P. axillaris* and *P. violacea* were the species involved. As far as these authors were concerned, there was no doubt as to the white-flowered species' parent being *P. axillaris*. However, with respect to the purple-flowered ancestor, it was not, in their opinion, first described and named based on seeds collected in South America, but rather on plants grown in England from seeds of unknown origin. Ferguson and Ottley (1932) thought that it was possible the parental seeds came from South America, but they also thought there was a strong possibility the plants described as the parental species were, in fact, natural hybrids between *P. axillaris* and the South American pigmented ancestor of the garden petunia. This assessment was based on their comparative evaluation of *P. violacea* as described by J. W. Hooker, D. Don, and Lindley with their own material.

Stout (1952) reported that pure strains of *P. integrifolia* and *P. axillaris* were apparently not maintained in Europe. He believed this because the hybrids between the two species were far superior in their horticultural attributes. Furthermore, *P. "violacea"* probably has been used for a long time as an incorrect designation for the garden types.

Since the introduction of *P. hybrida* Hort. (Vilm.) a limited number of research reports have focused on describing the breeding, taxonomy, and chemotaxonomic relationships between the putative parental species and including the cultivated types in the analyses. Lamprecht (1953) made the intercrosses between *P. axillaris*, *P. inflata*, and *P. violacea*, all of which were fertile in the various combinations, and he examined the hybrid and subsequent segregating populations for various taxonomic characters. The characters examined were those used by Fries (1911) for composing his taxonomic key and included: shape of the corolla tube, length of adnation of the filament to the corolla tube, peduncle shape, symmetry of the corolla, and degree of curvature on the corolla tube. Lamprecht (1953) observed that all the traits examined were found to segregate in a discontinuous fashion; thus, several genes were responsible for their expression. He concluded that the three species cannot be considered as separate and suggested that *P. axillaris* had priority and that the other two were synonyms.

Mather and Edwardes (1943) reported the inheritance of flower color differences between *P. axillaris* and *P. violaceae* in a study of species divergence. They maintained that the major differences between species would be expressed in a polygenic manner. Their *P. violacea* was self-incompatible and the species hybrid could only be produced when *P. axillaris* was used as the female parent. The flower color differences were determined by two major genes *W* and *M* and a third *D* for dilution. The expression of the two major genes was modified by polygenes. Speciation with respect to flower color was believed to be due to natural selection operating on the polygenes that interacted with the two major alleles.

Chlebowski (1967) reported that the gene controlling small flower size (multiflora) in the species *P. axillaris* and his line of *P. hybrida grandiflora* were identical; thus, indicating that the 1 monogenic locus was common to *P. axillaris* and its derived horticultural type. Steere (1932) produced triploid ($2n = 3x = 21$) offspring for cytological study by crossing a tetraploid *P. hybrida* race with *P. axillaris*. He found the regularity of meiotic processes in the triploids to exceed that of certain diploid *P. hybrida* types. Sink (1975) utilized three marker lines: *apt*, apetalous flower; *D*, flower doubleness; *G*, grandiflora in *P. hybrida* Hort. and hybridized them with *P. axillaris, P. inflata, P. parodii*, and *P. violacea*. Monogenic inheritance was found except for certain crosses involving the *D* line with *P. inflata* and *P. violacea*. These observations lend further support to the involvement of the species studied in the ancestory of the cultivated *P. hybrida* Hort., and to a general conclusion that there is a high degree of genetic homology among these *Petunia* species.

Natarella and Sink (1974) conducted a chemotaxonomic study using phenolic compounds from *P. axillaris, P. inflata, P. parodii,* and *P. violacea*, five interspecific hybrids, and several cultivars of *P. hybrida* Hort. Only the *P. axillaris* × *P. parodii* and *P. axillaris* × *P. violacea* chromatographic patterns were significantly correlated with the cultivars. The latter hybrid was calculated as being closest to the cultivars and likewise it had the highest matching coefficient based on presence-absence of homologous bands. Natarella and Sink (1975) reported an electrophoretic analysis of proteins and peroxidases extracted from leaf tissue of the same *Petunia* spp., interspecific hybrids and cultivars as mentioned above. For peroxidase banding patterns, the average correlation of each *Petunia* species with all of the cultivars was: *P. axillaris*, 0.74; *P. inflata*, 0.73; *P. violacea*, 0.52; *P. parodii*, 0.68. Analysis of the protein bands revealed that only *P. inflata* was significantly correlated, on average, with all of the cultivars. The overall results indicated that *P. axillaris* and *P. inflata* may be considered putative parents of *P. hybrida*. Thus, while chemotaxonomic studies have provided further insight and verification of the origin of *P. hybrida*, conflicting experimental results were obtained from the two techniques employed.

Brunaud et al. (1977) in a study of 12 lines of *P. hybrida* plus *P. axillaris, P. violacea*, and *P. parodii* found that the presumed genetic differences in these lines were also reflected in growth habit variation and that the expression of some growth traits was modified by environmental factors.

3 Anatomy and Morphology

K. C. SINK [1]

Extensive anatomical and morphological studies have not been reported for *Petunia*. However, pollen biology and the reproductive apparatus and early embryo development appear to be thoroughly documented, including some recent (1970's) fine structural analyses. The purpose of this segment of the monograph is to present the available basic botanical information on the genus *Petunia*.

3.1 Plant Structure and Development

3.1.1 Seed

Gunn and Gaffney (1974) described *P. hybrida* seed as "0.6–0.7 mm in length, 0.5–0.6 mm in diameter, spheroidal-angular, light to dark brown coloration, reticulate, reticulum well defined at 10 × with apparently straight, thin walls, darker color than interstices." The hilum is conspicuous, sub-basal, same color of seedcoat, nipplelike, and minute. The embryo is linear (Fig. 3.1) and bent. The inner margin of the seed coat is appressed to the endosperm.

Stout (1952) observed both curved and straight embryos in the seeds of *P. axillaris, P. integrifolia,* and *P. parodii*. He also observed that the seed surface of these species is reticulate and the ridges on *P. integrifolia* are even in height with smooth sides; *P. parodii* and *P. axillaris* have serrated ridges. Normal seeds of all three species have considerable endosperm with a well-formed embryo.

3.1.2 Fruit

Gunn and Gaffney (1974) found that the fruit of *Petunia* is a capsule containing 130–1 065 seeds each depending on the species. Sink and Power (1978) counted the number of seeds per capsule for all currently available *Petunia* species. The average seed count per capsule varied from 30 for *P. violacea* to 1 224 for *P. parodii*. Seed capsule size varies from 10–13 mm in length, 7–8 mm in diameter, is conic, widest at the base and tapering to the apex, brown, glabrous, and smooth. The mature fruit is surrounded by an enlarged, gladular-hairy calyx composed of five strap-shaped lobes which are equal to or longer than the capsule depending on the species (Gunn and Gaffney 1974). There are approximately 250 000 seeds per ounce of *P. hybrida* Hort. cultivars.

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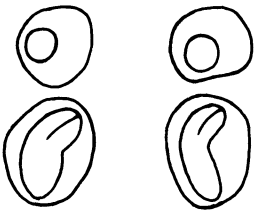


Fig. 3.1. Schematic cross and longitudinal sections of *Petunia hybrida* Vilm. seed, approximately × 7. (Gunn and Caffney 1974)

3.1.3 Vegetative Growth

Petunia seeds imbibe water, exhibit obvious swelling, and the radicle is observed protruding from the cracked seed coat 48–60 h after sowing. The seeds commonly exhibit an after-ripening requirement which is more pronounced in white-flowered cultivars and species, and also in some grandiflora genotypes. Although not examined in detail, seed storage at room temperature for 3–4 months will satisfy the after-ripening requirement. Freshly harvested seed can be readily germinated by pre-soaking in 100 ppm gibberellic acid for 24 h prior to sowing (Ascher 1978, pers. comm.). Jassey et al. (1977) studied the seed germination requirements of 25 *Petunia* lines at harvest and following GA_3 or red-light exposure. Three lines, TLh-2, Tbl-3, and *P. parodii* were chosen for detailed analyses. Seeds of Tbl-3 and *P. parodii* would not germinate at harvest or at 25° or 30 °C; their dormancy was broken by GA_3 or red-light treatment. The seeds of Tbl-3 germinated after harvest at 15 °C and progressively lost dormancy during dry storage. Jassey et al. (1977) did not obtain germination of isolated embryos, indicating a complex interaction between embryo, endosperm, and seed coat in the control of seed germination. Optimum germination was obtained with intact seeds given the required treatment of a moist condition in the dark or, GA_3 or red light. According to Bianchi and Walet-Foederer (1974) eight-day old seedlings have a well-defined shoot apex composed of two independent tunica layers and 1 corpus (Fig. 3.2).

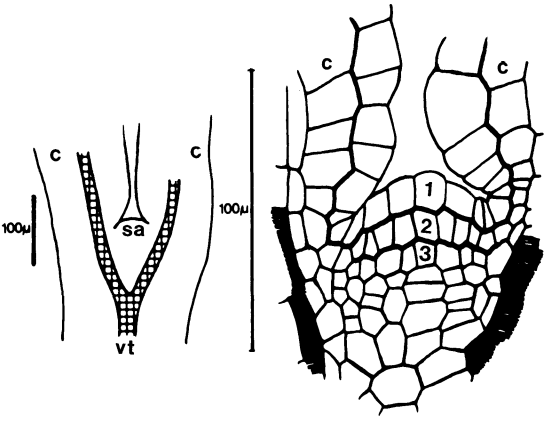


Fig. 3.2. Longitudinal section of a shoot apex of an 8-day-old *Petunia* seedling indicating the two independent tunica layers, *1* and *2*; corpus, *3*; *vt* vascular tissue; *sa* shoot apex; *c* cotyledons. (Bianchi and Walet-Foederer 1974)

Table 3.1. Summary of *Petunia* apex dimensions in microns. (Data from Prior 1957)

Species	Apex height			Apex width			Width/height ratio	No. of apices
	Avg	Min	Max	Avg	Min	Max		
Petunia hybrida								
Vegetative	22	6	28	135	65	155	6:1	28
Inflorescence	60	32	102	180	118	217	3:1	35

Phyllotaxy of seedling growth is opposite, four-ranked, and when flowering commences, it changes to alternate usually with a solitary flower arising from each leaf node. Flowering in *Petunia* is photoperiodically controlled with long days or a night interruption with incandescent light promoting early flowering (Piringer and Cathey 1960).

Prior (1957) described the dynamic changes that occur in the vegetative apex of *Petunia hybrida* at flowering. The vegetative stem apex is broad and low-domed with average width of 135 μm, height of 22 μm, and a width to height ratio of 6: 1. A triseriate tunica is retained throughout the plastochron and cells of this region average 9 μm wide and 10 μm high. The range in apex dimensions is shown in Table 3.1.

Leaf primordia are initiated by cell divisions in the inner tunica layer followed by meristematic activity to a depth of four or five cells. The second tunica layer also becomes involved in leaf production by dividing periclinally. Axillary buds are observed only after two, mostly three, leaf primordia are produced in the vegetative phase. These axillary buds do not elongate until flowering starts.

Petunia species bear an indeterminate number of solitary axillary flowers, and the flowers do not always arise at every node. Prior (1957) observed that at flower initiation approximately one-third of the apex undergoes increased cell division, forming the new terminal meristem. The remaining two-thirds of the original apex rapidly divide to form a flower. This sequence is depicted in Fig. 3.3, camera lucida drawings from Prior's (1957) study.

At flowering, the apex increases in size to an average width of 180 microns and a height of 60 microns. The tunica becomes reduced from three to two layers at transition and is composed of cells averaging 8 μm in width and height. Cornu and Bugnon (1971) also observed that the shoot apex in *P. hybrida* Hort. is composed of three separate cell layers. The second tunica layer of cells begin dividing periclinally at the initiation of each flower bud. Increased meristematic activity of the corpus, extending to a depth of 8–10 cells, ultimately produces the new terminal meristem. Every flower on the stem arises directly from the apex and the terminal meristem is renewed by cell division on the flank of the enlarging primordium. Prior (1957) stated that the most striking feature of transition to flowering in *Petunia* is the manner in which the stem apex is renewed. The apex does not simply cut off floral primordia on its flanks; it is transformed from a region of indeterminate to one of determinate growth, i.e., the flower primordium. Concomitant with this transformation, the new shoot apex is initiated by cell di-



Fig. 3.3. Camera lucida reconstruction of the *Petunia* shoot apex at transition to flowering. *A* apex; *F1–F2*, successive flowers. (Prior 1957)

visions on one side, which increase in scope and frequency during the subsequent plastochron.

The scorpioid, sympodal flowering sequence in *Petunia* was further examined by Cornu and Bugnon (1971). Their observations paralleled those of Prior (1957) that the flower does not arise from a stem meristem but from a nonstructural mass into a flower with an overlapping contiguous bud.

3.2 The Flower

3.2.1 Carpel and Placental Divergences

Murray (1945) observed that each of the five sepal divergences of *Petunia hybrida* trifurcates to form one median and two lateral sepal bundles. The sepal divergences are not accompanied by parenchymatous gaps, but small ones may be associated with the petal divergences. The cylinder remaining after the stamen divergences is divided into four segments – two large ones opposite each other and two small ones on alternate radii. An abaxial bundle and two bundles for the capillary wall are derived from each small segment. In the mature ovary, the adaxial bundles are amphicribral. In *Petunia*, as in *Datura* and *Nicotiana*, fusion of the sepal tissue with the style occurs before ovule development.

3.2.2 The Stamen, Style, and Stigma

Konar and Linskens (1966a) studied the morphology and anatomy of the stigma of *P. hybrida*. They observed that the pentamerous flower has five epipetalous stamens which terminate growth at various levels below the stigma. The stamens are of three types with respect to filament length. Two long ones (17 mm) dehisced first, followed by two others with shorter filaments (14 mm); and the shortest (10 mm) dehisced last.

The style was observed to be bent in the flower bud and to become straight at anthesis. The total style and stigma length ranged from 25–30 mm at anthesis. The stigma was described as a flat, bilobed structure with the middle portion of each lobe raised slightly upward. Muszynski et al. (1976) observed the stigmatic surface of allotetraploid petunias by scanning electron microscopy and confirmed the bilobed structure with a distinct central depression. However, the papillate hairs are much more dense in allotetraploids than in diploids. The central area is depressed and slit-like, gradually narrowing to become the style. A mature stigma has four to six large, shiny exudate drops. Numerous smaller drops, varying in size, also occur. The stigmatic surface has a large number of papillate hairs distributed all over. The style is solid and the stigmatic tissue is single-stranded and multilayered. The pollen tubes move through this tissue by intrusive growth. The mature stigma has a single epidermal layer subtended by subepidermal cells. A thin cuticle overlays the epidermis; the subepidermal cells are densely cytoplasmic and meristematic. The center of the developing style, the stigmatoid tissue, is composed of small cells with prominent nuclei and cytoplasm which is in continuity with the subepidermal cells of the stigma. Two provascular strands traverse the ground tissue and terminate at the base of the stigma. The bi-celled stigmatic papillae arise from transverse division of the epidermal cells. Early in development, the stigmatic cells become filled with starch and oil globules as do the epidermis and papillae. Stigmatic exudation occurs in two stages: first, the epidermal and papillate cells release the oily exudate that accumulates between the cuticle and the cellulose wall. As the amount of exudate increases, the cuticle extends and finally ruptures at various places.

Initially, the exudate passes through the discontinuities, but later the cuticle is thrown off in the form of flakes and the entire stigmatic surface is covered with a thin layer of oily exudate. Secondly, after anthesis, the exudate located in the secretory zone is released quickly between the epidermal cells. The latter are loose at maturity. When the exudation begins, it carries the remnants of the cuticle with it so that nothing of it remains at maturity.

3.2.3 Embryo Sac

Ferguson (1927) found that the structure of the mature embryo sac of *Petunia* is very similar to the type ordinarily described for angiosperms (Fig. 3.4). Usually, the polar nuclei fuse before the pollen tube enters the sac, but may be delayed until it has penetrated the wall of the embryo sac and extended to or beyond the egg nucleus. The pollen tube generally penetrates the embryo sac 72–92 h after pol-

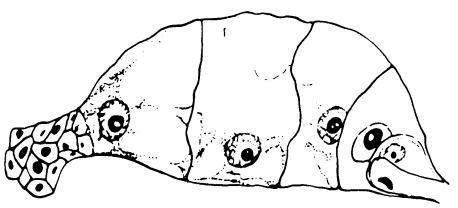


Fig. 3.4. *Petunia* embryo sac with three endosperm cells derived from the primary, basal, endosperm cell. The primary micropylar endosperm has not divided, but its nucleus and the lower sperm nucleus have united to form a large fusion nucleus. The second sperm nucleus is still within the pollen tube. (× 600, Ferguson 1927)

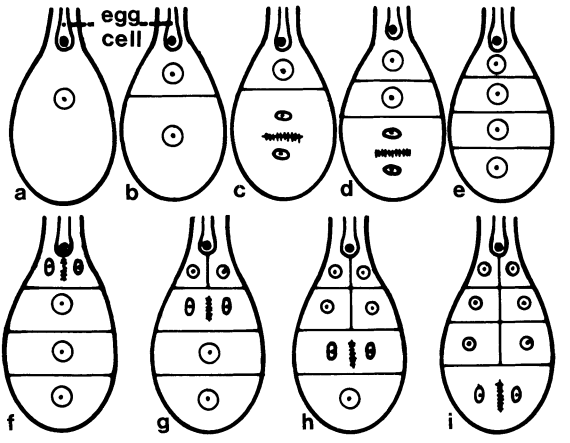


Fig. 3.5. Schematic diagrams of the steps in early endosperm formation in *Petunia*. (Ferguson 1927)

lination. The endosperm nucleus divides about the time the pollen tube growing tip has reached the egg nucleus, but prior to the discharge of the sperm nuclei from it. After the first mitosis of the endosperm nuclei, a delicate wall is laid down dividing the sac into a large basal cell and a smaller micropylar cell (Fig. 3.5). *Petunia* is similar to other members of the Solanaceae in that free nuclear divisions do not occur in the early stages of endosperm development.

Ferguson (1927) further observed that primary divisions of the *Petunia* endosperm are completed before the sperm nuclei leave the pollen tube. After the first division of the endosperm, its subsequent developmental pattern is not always the same. Before further mitosis of the endosperm, the lower sperm nucleus may be released from the pollen tube and associate with the nucleus of the primary micropylar endosperm cell, or, before the sperm is released, the primary basal endosperm cell divides to form a large basal cell and a much smaller upper cell. Lastly, before either sperm nucleus is discharged, the larger lower cell may divide to yield two daughter cells, equal in size, with their wall parallel to previously formed cells. Whichever pattern is followed, when the micropylar "fusion nucleus" forms as in early prophase, the endosperm always consists of a tier of four cells.

The lower sperm nucleus leaves the tube, comes in contact with the upper endosperm nucleus, and increases somewhat in size. Ferguson (1927) found no evidence that the sperm nucleus enters the endosperm nucleus, but rather the two press closely together and the latter increases in size, losing its dense appearance and becoming more reticulate, enclosing a large nucleolus. Later the two nuclei are so closely associated that the resulting nucleus gives no structural suggestion of its dual nature, i.e., the "fusion nucleus."

After the first mitosis of the upper endosperm – "fusion nucleus" – the wall is laid down at right angles to those cells previously formed. Successive divisions proceed regularly with the first division at the micropylar end and each succeeding division in the adjacent basal cell (Fig. 3.5, Ferguson 1927). When the anticlinal walls are laid down, they lie at right angles to both the horizontal walls and the longitudinal walls. Thus, each of the four layers of endosperm cells is divided into four cells or quadrants. Hence, 12 of the cells descend from the primary basal

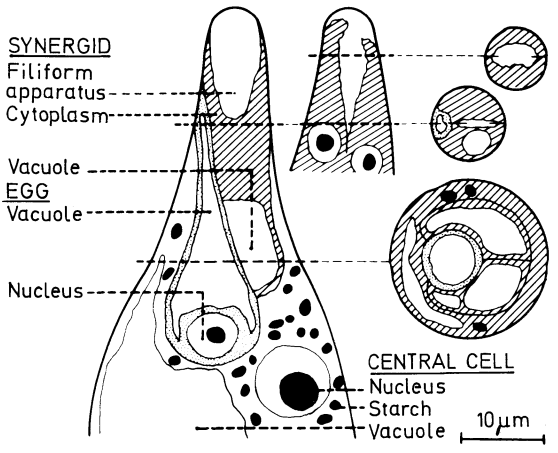


Fig. 3.6. Schematic reconstruction of longitudinal and cross sections of the micropylar region of the mature embryo sac of *Petunia*. (Van Went 1970 a)

endosperm cell and 4 derive from the primary micropylar endosperm cell after its union with a sperm nucleus.

Ferguson (1927) confirmed the developmental sequence of the embryo sac by chromosome counts of the upper endosperm cell after fusion with the sperm nucleus and subsequent mitosis. She observed 21 chromosomes in the upper endosperm-fusion cell and 14 in the primary basal endosperm cell. Thus, triple fusion does not occur in *Petunia* and the endosperm is composed of a mixed group of 21 and 14 chromosome cells.

Van Went (1970 a) observed that the synergids of *Petunia* prior to anthesis are approximately 14 µm long by 11 µm wide and are surrounded by a wall with an average diameter of 0.1 µm. The nucleus is centrally located, a few organelles are present, randomly distributed, and a few small vacuoles are present. After anthesis, the synergid structure is completely different (Fig. 3.6). The synergids and the egg cell are long, pear-shaped cells, approximately 35 µm long and 15 µm wide. The latter two surround the egg cell to form the egg apparatus which in turn is surrounded, two-thirds, by the central cell. A filiform apparatus (FA) lies between the tips of the synergids. The FA is cap-shaped where it borders the micropyle, and one of its two halves, covering the synergids, is always thinner than the other half. Most of the synergid cytoplasm is located at the micropylar end, their chalazal parts are filled with a number of vacuoles and the nucleus is located above the vacuoles.

The synergid nucleus is spherical, approximately 6.5 µm in diameter and surrounded by a porous envelope. The outer nuclear membrane is covered with ribosomes and the nucleoplasm is homogeneous. Numerous small concentrations of chromatin are regularly spread over the whole nucleus. A single nucleolus is located in the nucleus, which has a network of fine granular material in which is embedded, especially at the periphery, large numbers of ribosome-like particles.

The synergids also contain high numbers of mitochondria, uniformly distributed in the cytoplasm and all similar in appearance. They contain short cristae that appear partly tubular. The number of plastids in the synergids is low, about one to every ten mitochondria and they are evenly distributed in the cytoplasm.

Their shape varies from dumbell to spherical with an average diameter of 1.5 μm. They have a few thylakoids, usually a long one lying parallel to the outer membranes, and a few short ones extending into the interior of the plastid. The plastids do not contain starch, as do all other cell types in the ovule. Numerous dictyosomes, randomly disbursed in the cytoplasm, are found in the synergids. They consist of three to five flat cisternae with an average length of 0.7 μm, the middle ones being the longest. An extensive endoplasmic reticulum (ER) system is present in the synergids, most lying parallel to the long axis of the cell. Around the nucleus they are parallel to the nuclear envelope. The ER sheets are concentrated in groups of two to six and the group membranes are always parallel. The ER membranes are covered with ribosomes and the synergids also have free ribosomes.

Van Went (1970a) also speculated on the possible role and function of the synergids. He earlier observed (Van Went and Linskens 1967) that the *Petunia* pollen tube grows through the micropyle and enters the embryo sac growing through the FA and into one of the synergids. Since the micropyle is very narrow, smaller than the pollen tube diameter, he felt that a stimulus would be needed to induce the pollen tube to grow into the FA. Van Went (1970a) thought that the stimulus may be provided by secretion of chemotropic substances in the synergids. This assumption was based on the observed accumulation of mitochondria and extensive, rough ER in the micropylar area of the synergid after anthesis; both indicate increased metabolic activity. The activity might be related to the production of the chemotropic substances secreted through the large plasma membrane area into the FA and subsequently diffusing into the micropyle.

Van Went (1970b) also observed the ultrastructure of the egg and the central cell of *Petunia*. The immature egg is acentrally located below the two synergids. It is pyramid-like in shape, average diameter 11 μm, with a centrally located nucleus and a few small vacuoles. The immature central cell has a single large vacuole with the two polar nuclei, most of the cytoplasm is located at the micropylar end of the cell. In many respects, these two immature cells are quite similar. The number of oval to spherical mitochondria is moderate. Within them are a number of short, randomly distributed cristae. Few plastids are present and they are very irregular in shape with an average diameter of 1.5 μm. They contain a small number of thylakoids, and sometimes, a small starch grain. A few dictysomes are present and the ER is poorly developed.

After anthesis, the mature pear-shaped egg cell is longer, 35 μm long and 15 μm wide at the base. The top of the egg is situated 10 μm below the top of the embryo sac. It is in contact with the embryo sac wall at the top only; otherwise, it is freely pendulous. The mature egg has a single large vacuole with the nucleus and most of the cytoplasm located at the chalazal pole of the cell, it has few mitochondria, plastids, and dictysomes and a poorly developed ER. The organelles have the same ultrastructure as the immature egg.

Fusion of the polar nuclei occurs at different times. Their diameter is 10 μm and they lie close to each other, separated by a thin layer of cytoplasm in which organelles occur. Each nucleus has a large nucleolus and no chromatin clumps. The ultrastructure of the cytoplasm of the central cell changes significantly during anthesis. The mitochondria do not change in number or structure. The plastids

become large, 3–5 μm in diameter, and completely filled with starch grains. The ER is poorly developed, and located primarily near the plasma membrane and nuclear envelope.

3.2.4 Pollen

Ferguson and Coolidge (1932) found that pollen grains of *Petunia* are bilateral in origin. When observed in dry air, they are ellipsoidal in outline when lying horizontal. There are normally three germ pores each located on a furrow extending the length of the grain. The furrows are equidistant, thus giving the grains a triangular shape when viewed from the end. Pollen grains from polyploid plants are generally very irregular in shape and size.

Hesemann (1971), using cytophotometric analysis, determined the DNA content of the generative and vegetative nuclei in the pollen grain and tube of *Petunia hybrida* mutants. The results indicated that DNA replication continues in the mature pollen grain and terminates when the nuclei enter the pollen tube. Vegetative nuclei of the mutants exhibit differential onset of DNA degredation which Hesemann (1971) interpreted as playing an important role in pollen tube development.

3.3 Fertilization and Embryogenesis

Cooper (1946) observed that the pollen tube penetrates the embryo sac by growing between the apices of the synergids and egg cells. It enlarges to form two cone-like projections at its apex; one cone extends into the central cell and the tip of the other becomes closely appressed to the egg. The male nuclei are assumed to enter the embryo sac via the projections. Van Went (1970c) observed that the pollen tube enters the embryo sac by growing through the filiform apparatus (Fig. 3.7). The tube ceases growth, and an opening forms at its apex shortly after it penetrates the cytoplasm of one of the synergids – the penetrated synergid. The penetrated cytoplasm increases greatly in volume and in cytoplasmic content. The large chalazal vacuole disappears with only a few small ones remaining; the cell bursts at the chalazal pole with part of the cytoplasm lodged between the central cell (primary endosperm cell) and the chalazal parts of the egg and the other synergid. Thus, the sperm cells are "transported" to the egg and central cell, and fertilization occurs through a fusion type process (Fig. 3.7). Van Went (1970c) observed incomplete fusion of the male and female nuclei more frequently in the primary endosperm cell than in the zygote.

Vallade et al. (1978) found, using cytophotometric methods, that soon after fertilization the DNA content of the zygote nucleus of *P. hybrida* increases to about 3 c. All of this DNA is subsequently replicated during the S stage. During the first two cellular generations, there is a progressive decrease in DNA from 5.92 c in the G_2 zygotic nuclei to 4.94 c in the G_2 four-celled proembryo nuclei.

Embryo development in *Petunia* has not been comprehensively studied aside from root embryogenesis by Vallade (1972). Initial cells are laid down early and

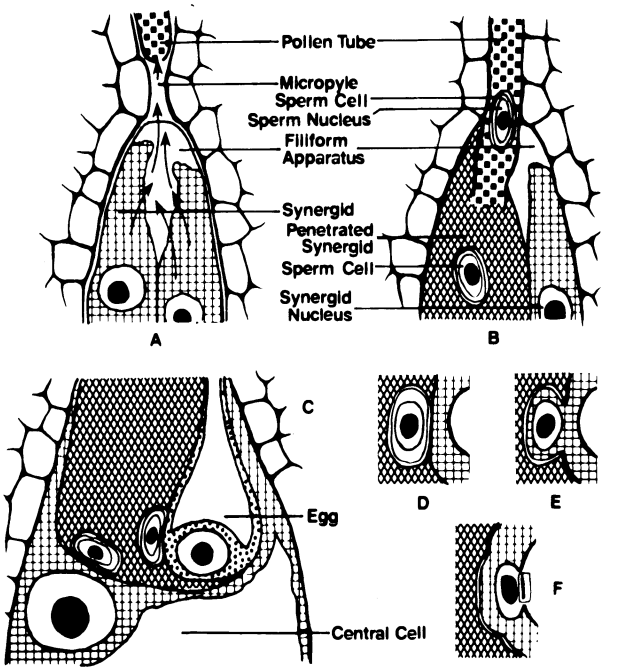


Fig. 3.7 A–F. Diagrammatic steps in the proposed manner of fertilization in *Petunia*: **A** mature synergids produce and secrete substances which direct the growth of the pollen tube. **B** pollen tube grows through the filiform apparatus and discharges its content into one of the synergids. **C** the penetrated synergid bursts at its chalazal pole and the sperm cells are transported to the egg and central cell. **D, E, F** the two sperm cells fuse with the egg and central cell, respectively and their nuclei become located near the female nuclei, and fusion occurs thereafter. (Van Went 1970c)

in a regular manner at the base of the globular embryo (Fig. 3.8). These cells are the origin of the primary portion of the future root. Following this segmentation, new types of growth occur, particularly as related to cell division of the subapical initials. These divisions are generally amplified after germination, when the root becomes functional. The quiescent center, previously four cells, grows by division of its elements and by attachment to neighboring cells.

3.4 Chimeras

Muszynski (1964) irradiated seed of *P. axillaris* and *P. violacea* (tetraploid) with gamma rays from a Co^{60} source. One plant had a sectorial chimera containing green and variegated tissue. The lower epidermis had the same number of chlorophyll granules in stomata cells from the white portion of the variegation as did the normal tissue. Cross sections of the variegated leaves showed numerous chlorophyll granules in the mesophyll cells of green sections and relatively few in

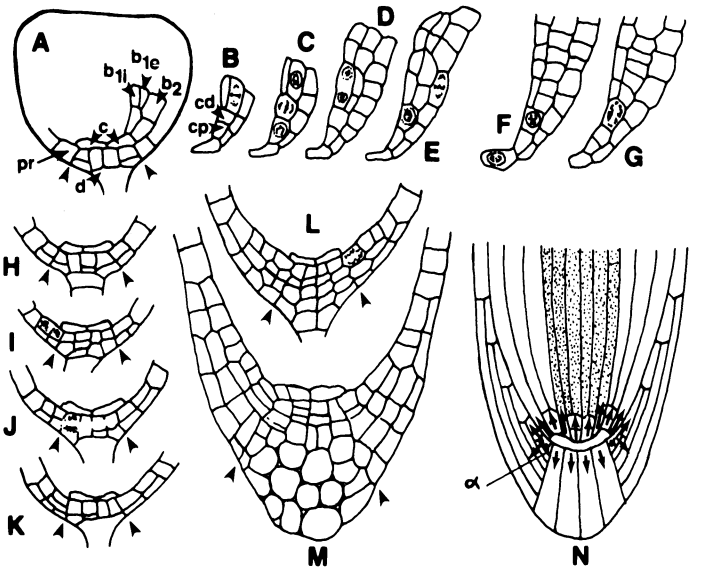


Fig. 3.8 A–N. Root embryogenesis in *Petunia*. **A** heart-shaped embryo stage with cortical files (of cells), (b_{1i}, b_{1e}, and b_2), original quiescent center (*c*), protoderm (*pr*), and stage *d* which constitute the principal elements in the (root) cap. **B–E** Method of elongation of the cortical files during embryogenesis; *cd* distal cell; *cp* proximal cell. **F–G** Cortical files at the level of the young roots (postgermination phase). **H–M** Method of formulation of the cap; *arrows* limit of the axial zone and the lateral portions. **N** Diagrammatic scheme of the method of growth of a young root; *arrows* presumed role played by the terminal cells in the files in providing cellular material; only cells α, in contact with both cortex and the *axial* zone of the cap, assure a bidirectional growth. (Vallade et al. 1978)

the mesophyll cells of white parts. The light-green color was due, therefore, to an absence of chlorophyll in the palisade cells.

Bianchi et al. (1974) found that a spontaneously occurring dwarf mutant of *Petunia* was conditioned by a somatic mutation – histogenic layer reversion mechanism. Genetic analysis indicated that the dwarf phenotype is inherited in a monogenic recessive pattern. However, frequent back mutations (to normal) are presumed to occur randomly in the three histogenic shoot layers. Thus, depending upon which layer carried the dwarf cell type, two dwarf types of plants are identified. These mutants are present in the outer and subepidermal layers respectively and thus had considerable influence on the leaf and flower morphology since these tissues are derived primarily from LI and LII cell types.

4 Cytology

D. MAIZONNIER [1]

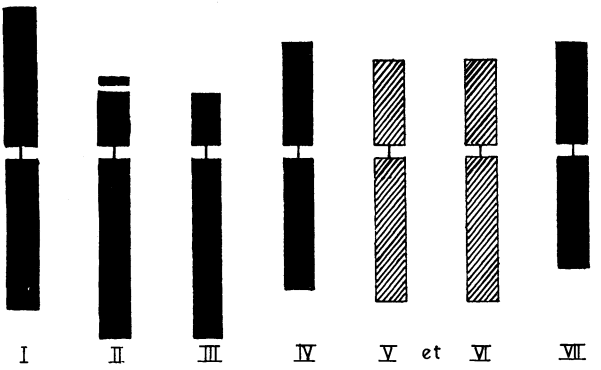
4.1 Chromosome Numbers Within the Genus and Karyogram of *Petunia Hybrida*

The genus *Petunia* is a compound of the diploid species, 5 with 14 chromosomes [*P. axillaris* (Lam) B.S.P., *P. violacea* Lindl., *P. inflata* R. Fries, *P. parodii* W.C.S., and *P. hybrida*] and one with 18 chromosomes (*P. parviflora*). Several authors have studied *P. axillaris* and *P. violacea* under the respective names of *P. nyctaginiflora* and *P. integrifolia* but more details concerning these species are given in Chap. 2. All species with 14 chromosomes were crossed and gave progenies except when *P. inflata* was pollinated by *P. parodii*. *P. parviflora* does not cross with species having 14 chromosomes. Recently however, somatic hybrids were obtained by Cocking (1980); see also Chaps. 10 and 11.

Maizonnier (unpublished data) analyzed karyotypes of the five species with 14 chromosomes but did not detect any differences.

The karyogram of *Petunia hybrida* was first studied by Marthaler (1936) who described 7 pairs of chromosomes and concluded that they were easily distinguishable from one another. More recently, Takehisa (1963), using more suitable techniques (pretreatment and squash), gave a karyotypic formula of *Petunia* chromosomes similar to that reported by Marthaler (1936). Thereafter, Maizonnier (1971), using classical parameters applied to the karyotypes of 4-haploid plants, established a karyogram in which five chromosomes were rather easily distinguished, but chromosomes V and VI remained indistinguishable. Finally chromosomes V and VI were discerned by Smith and Oud (1972) and Smith et al. (1973) through fluorescence and scanning reflectodensitometer analyses which gave intensity curves for each of the seven chromosomes. Both karyograms given by Maizonnier (1971) and Smith et al. (1973) are presented in Fig. 4.1. According to Smith et al. (1973), the seven chromosomes were classified into three groups on the basis of their centromere position: group A with a median or submedian centromere (chromosomes I, IV, and VII), group B with a subterminal centromere (chromosomes II and III), and group C with a centromere in an intermediate position (chromosome V and VI). Chromosome II carries the nucleolus organizer as well as satellites of varying sizes; sometimes chromosome III also bears small satellites (Smith et al. 1973 and Maizonnier 1976 b). Another character that could be used to discriminate the seven chromosomes is the location of constitutive heterochromatin; unfortunately, a recent study by Dietrich et al. (1981)

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THE INBRED LINE V10. SURVEY OF DATA RELATING TO CHROMOSOME MORPHOLOGY AND FLUORESCENCE PATTERN

Chromosome group	Chromosome no.	Centromere index $l/l+s$	Relative length % T.C.L.	Chromosome type	Fluorescence intens. curve
A	I	0.50–0.54	9.1–8.3		
	IV	0.50–0.55	7.1–6.5		
	VII	0.50–0.55	6.5–5.9		
B	II	⩾ 0.63	7.9–7.2		
	III	⩾ 0.66	7.6–6.4		
C	V	0.57–0.60	7.6–6.2		
	VI	0.57–0.60	7.6–6.2		

Fig. 4.1. The karyogram of *Petunia hybrida* Hort. *Upper part,* the karyogram published by Maizonnier (1971); *lower part,* data from Smith et al. (1973)

showed that heterochromatin is mainly localized near the centromeres of all the chromosomes and at the secondary constriction of satellite chromosomes and, is therefore, not applicable.

4.2 Structural and Numerical Changes in *Petunia* Chromosomes

4.2.1 Structural Changes

In most cases structural changes are induced by physical mutagenic treatments such as X-rays or γ-rays (Rick 1943; Müller 1958; Maizonnier and Cornu 1971; Maizonnier 1976 b). A section in Chap. 5 is devoted to this subject. This section is a summary of structural changes found in *Petunia* and the details of a particular translocation that produces breakage-fusion-bridge cycles.

The simplest and most current chromosomal change obtained after irradiation is the deletion which is due to a single break (terminal deletion) or a double break (intercalary deletion). Genetic markers and cytology allowed the detection of terminal deletions on chromosome I, V, and VI (Maizonnier 1976 b; Cornu and Maizonnier 1979, and Maizonnier et al., in preparation). The lack of a pachytene map and the difficulty in obtaining this meiotic stage with completely separated bivalents, without stickiness and overlapping (Dietrich et al. 1981 and Maizonnier, unpubl. data) did not allow, until now, the observation of the classical loops corresponding to intercalary deletions.

A cytologically confirmed case of duplication evidenced by genetic markers was found in the progeny of a plant with an induced ring chromosome (Maizonnier 1976 b, Maizonnier and Cornu 1979).

Ring chromosomes, as well as some translocations, may be classified into a group of chromosome rearrangements giving rise to breakage–fusion–bridge cycles. The behavior of a ring chromosome was analyzed by Maizonnier and Cornu (1979), Cornu and Maizonnier (1983); a summary is presented in Chap. 5 and illustrated in Fig. 4.2. Only a brief synopsis of the behavior of a telocentric translocation described by Maizonnier and Cornu (1971) is presented in this section. After γ-ray treatment (Chap. 5) was performed on heterozygous zygotes, striped plants were observed in the progeny. The striped phenotype is produced by the instability of gene *Hf1*, yielding dark sectors with petunidin (presence of *Hf1*) and light sectors with paeonidin (absence of *Hf1*). This phenotype is associated with a telocentric translocation in which one arm of chromosome I is transferred to the end of the long arm of chromosome II. During mitotic division, the chromatids of the translocated arm fuse at their end, originating anaphase chromatid bridges. Unequal breakages of these bridges produce different situations in which *Hf1* may be present (in single or double doses) or absent in the daughter cells, explaining the presence of petunidin in dark sectors and its absence in light ones. Although it is responsible for a gametic semisterility, this translocation is transmitted by selfing and crossing but it does not tolerate

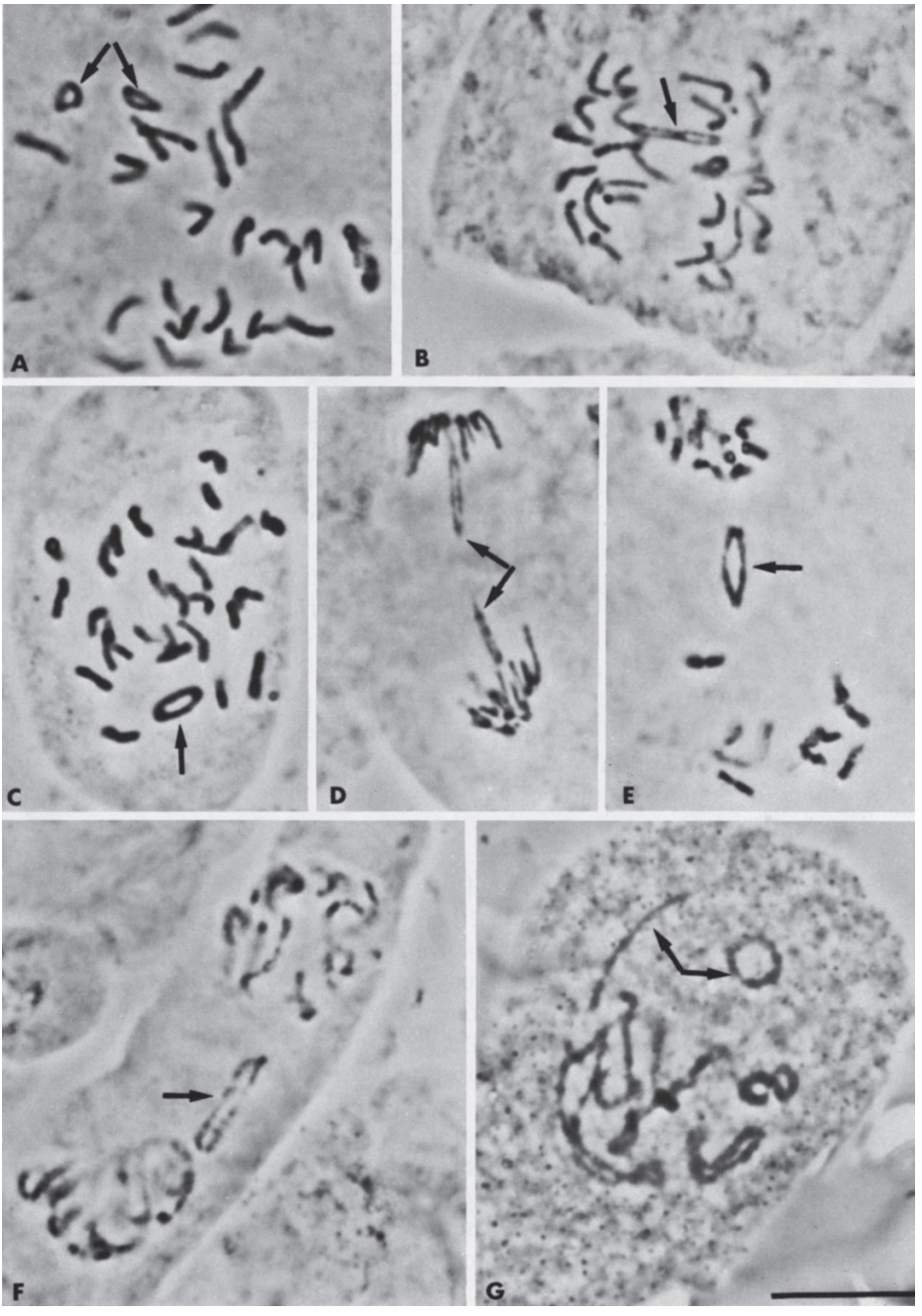


Fig. 4.2 A–G. Mitotic and meiotic behavior of a ring chromosome (Maizonnier 1976 b). **A** Mitotic anaphase showing ring splitting without bridge formation. **B** Mitotic anaphase showing a bridge due to both sister-chromatid exchange and interlocking of the daughter-rings. **C** Dicentric chromatid ring due to sister-chromatid exchange. **D** Late mitotic anaphase showing two chromatidic rings resulting from breakage of a bridge from interlocked rings. **E** Late mitotic anaphase showing a bridge from a dicentric chromatid ring. **F** Mitotic telophase with a lagging ring-shaped chromatid bridge. **G** Meiosis at diplotene stage in which the ring chromosome does not pair with its homolog. (Bar represents 5 μm)

homozygosity. This chromosomal change permitted the first accurate gene localization performed in *Petunia* by Maizonnier and Cornu (1971), *Hf1* on chromosome I.

More classical type translocations have also been observed. For instance, Maizonnier (1976b) describes an interchange between chromosome V and VI that is revealed only by genetic markers (*Po* and *An2*) which exhibit a change in their natural linkage relationship. This interchange breeds normally but is not detectable by means of classical cytological techniques. Another type was found (Maizonnier 1976b) in which the rearranged chromosomes and, consequently, the new phenotype are not transmitted sexually. The initial deletions, necessary to yield the interchange, are probably incompatible with a normal development and functioning of the haplophase.

4.2.2 Numerical Changes

Numerical changes in *Petunia* may be classified into three groups: haploidy, polyploidy, and aneuploidy. A complete Chapter (13) is devoted to haploidy; thus in the present Chapter only the following two sections dealing with polyploidy and aneuploidy are presented.

4.2.2.1 Polyploidy

a) Tetraploidy

The first known tetraploid *Petunia* appeared in California at the end of the nineteenth century. Other spontaneous tetraploid plants have been described by Vilmorin and Simonet (1927), Kostoff (1930), Dermen (1931), Steere (1932), and Matsuda (1934). More recently, Maizonnier (1976a) has described a genetic system yielding natural tetraploids based on a recessive gene which causes meiotic disturbances. Such a system probably explains the occurrence of the tetraploids observed by the previous authors. With respect to the induction of tetraploids, Levan (1938) reported experiments carried out by no less than six research workers in the first years after the discovery of colchicine.

As in most other species, tetraploidy in *Petunia* may lead to an increase in plant and flower size (Levan 1938); however, in certain cases, chromosome counts were absolutely necessary to distinguish them from diploids in a mixed population of the multiflora type (Maizonnier, unpublished data). Furthermore, some genes for flower size and shape, for instance *Un*, yield an increase in flower size and, with such a genetic background, polyploidy effects are difficult to distinguish from genetic ones.

Induced and spontaneous tetraploids behave similarly at meiosis. They both form typical meiotic configurations and result in classical meiotic disturbances, aneuploidy, and poor fertility (Matsuda 1934; Levan 1938).

Due to seed production problems and also to slow growth habits, tetraploids remain rather scarce among cultivars, and it seems that attempts to improve them by Muszynski (1975) have been unsuccessful.

b) Triploidy

Spontaneous triploids found among diploid progenies may be attributed to the functioning of unreduced gametes which is a frequent phenomenon in the species (Maizonnier, unpublished data). In the past, triploids were obtained by crossing tetraploids with diploids and the reciprocal cross (Dermen 1931; Steere 1932; Matsuda 1935; Levan 1937). Among these results, it is worth mentioning the occurrence of a great number of diploid progeny with combined parental characters, using a diploid as the female parent (Steere 1932); this observation was not confirmed (Levan 1937).

Meiosis of triploids has been reported as being surprisingly regular, with trivalent configurations only (Steere 1932) or with abnormal configurations involving a mixture of trivalents, bivalents, and univalents (Dermen 1931). According to Matsuda (1935), the discrepancy may be due to genetic differences in the material. Dermen (1931), Matsuda (1935), and Levan (1937) analyzed the progeny of self- and cross-pollinated progenies involving triploids and their results constitute the basis of most of the chromosome number series reported by Levan (1937) and others.

4.2.2.2 Aneuploidy

According to Rick (1971), *Petunia hybrida* is "an example of extreme tolerance of aneuploidy". It is true that the list of aneuploid progenies given by the author and also by Levan (1937) is quite varied, ranging from 15 to 20 chromosomes, if only hyperdiploids are considered. Our own experience (unpublished data) is in complete agreement with the observations of the previous authors.

a) Monosomics

Among 2168 plants cytologically analyzed by several authors, Levan (1937) did not mention any monosomics. This kind of aneuploid does not occur spontaneously due to the lack of viability of gametes with only six chromosomes. However, Rick (1943) found 3 monosomics among 33 mutants possessing gross chromosomal deviations and following X-ray treatment. Apparently, Rick did not study progenies of his monosomics.

Among the numerous mutants cytologically observed at Dijon since 1970, Maizonnier (unpublished data) observed only two confirmed monosomics. The first plant was a sectorial cytochimera: one part of the plant with 14 chromosomes, the other with 13. The monosomy concerned chromosome V as revealed by the disappearance of dominant markers carried by this chromosome. The monosomic part of the chimaera was isolated and propagated by cuttings but self- and cross-progenies did not yield monosomics. The second monosomic appeared after a γ-ray treatment applied on parental pollen. Genetic markers and cytology indicated monosomy for chromosome VI. In spite of numerous self- and cross-pollination attempts, this monosomic did not yield any progeny. Thus, in *Petunia* monosomy is not a spontaneous numerical change; it is induced by mutagenic treatment but until now, it is not transmissible.

b) Trisomics

According to *Cytogenetics of aneuploids* (Kush 1973) "all the trisomics are very distinct" for *Petunia* and have been described by Levan (1937). But although it is true that Levan found numerous plants with 15 chromosomes, he did not give any description of the trisomics; he only envisaged such a work: "Certain of the trisomic classes can be morphologically identified, and in a future work I shall give a description of them" (Levan 1937). To our knowledge, this future work never appeared. Thereafter, Heseman (1964) tried to distinguish six types of trisomics by studying stomata and pollen characters, but did not try to correlate these trisomic types with any supplementary chromosome of the karyogram. Finally, Smith et al. (1975) obtained some trisomics from $3x \times 2x$ crosses and formulated the project to analyze their karyotypes by scanning reflectodensitometry in order to correlate the presence of supplementary chromosomes with modifications of morphological traits. They observed a trisomic V karyotype but the corresponding phenotype remained difficult to recognize among a mixed ($2x$ and $2x+1$) population without counting the chromosomes. However, this work allowed them to ascribe markers to both chromosome V and VI and, in this way, to distinguish genetically trisomics V and VI and to separate genetically chromosomes V and VI which were previously indistinguishable without using reflectodensitometry. The aim of the present section is to give a morphological description of each trisomic of the complete series found at Dijon by Maizonnier (1976 b) and correlated with the observed karyotypes (Fig. 4.3) in order to determine the main characters which would permit their recognition and utilization in genetic experiments. In addition, the results of a quantitative analysis of 14 general and floral characters carried out by Maizonnier (1976 b) and which provide the basis of a flower scheme for each trisomic compared to the $2n$ controls of the same progenies are presented.

α) Morphological Description of Trisomics

Trisomic I. Trisomic I can be recognized among a mixed population as early as the first two leaf stages: the plantlet is weak, deformed, with poor rooting; the young leaves are more or less lanciform. The rosette (Fig. 4.4) is flat and the leaves remain narrow and petiolate. The first flower opens at the end of a significantly shorter stem than normal plants. Flower characters analysis indicates a narrower flower than in the control (Fig. 4.5).

Trisomic II. The first stage at which the trisomic plants can be distinguished from the disomic controls is the rosette with three or four leaves (Fig. 4.4). These leaves are short, rounded, and pubescent. The chlorophyll pigmentation of trisomic II results in a yellow-green hue, but this character is only applicable during winter when plants etiolate in the greenhouse. Leaves on adult plants are more or less swollen. Trisomic II results in a flower which is narrower and shorter than controls (Fig. 4.5).

Trisomic III. Only observation of the rosette (Fig. 4.4) permits screening of trisomic III in a mixed population with practically no risk of misidentification. The

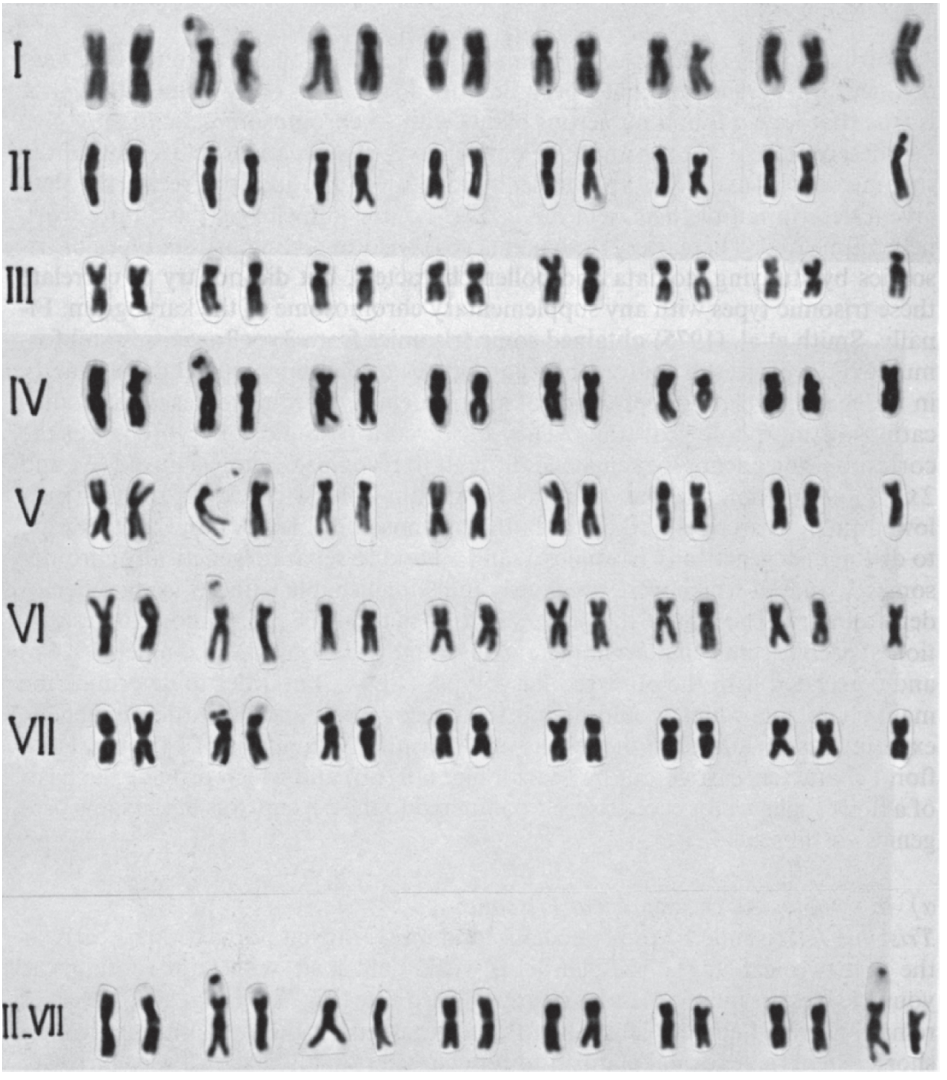


Fig. 4.3. Karyotypes of the seven primary trisomics and of a double (*II–VII*) primary trisomic of *Petunia*. The supplementary chromosomes are on the right. (After Maizonnier 1976 b)

rosette is flat with wide leaves which are ovoid and thick. Frequently, a light green zone lies on each side of the main nervation. The flower has a long tube and a wide and deformed corolla at the end of a flexuous peduncle which gives it a "pendula" aspect. Only the young stems are erect. It is almost impossible to mistake trisomic III for a control.

Trisomic IV. At the rosette stage (Fig. 4.4), the leaves of trisomic IV plants are more or less undulate and yellow-green but, during this stage, these trisomics remain difficult to recognize. On the other hand, floral deformations due to tri-

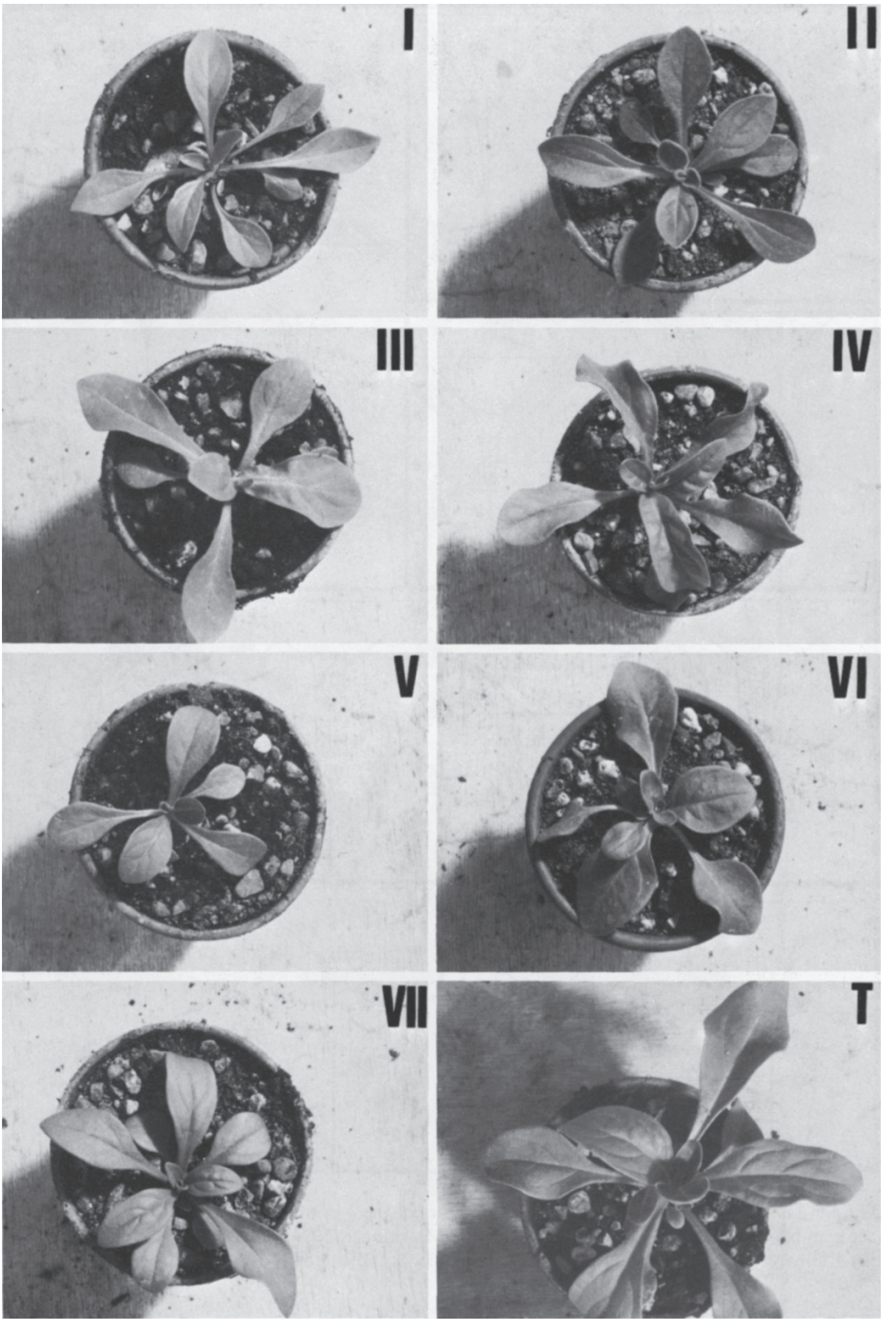


Fig. 4.4. Rosette stage of the 7 primary trisomics compared with a control (T) which belongs to the control population of trisomic VII. (Maizonnier 1976 b)

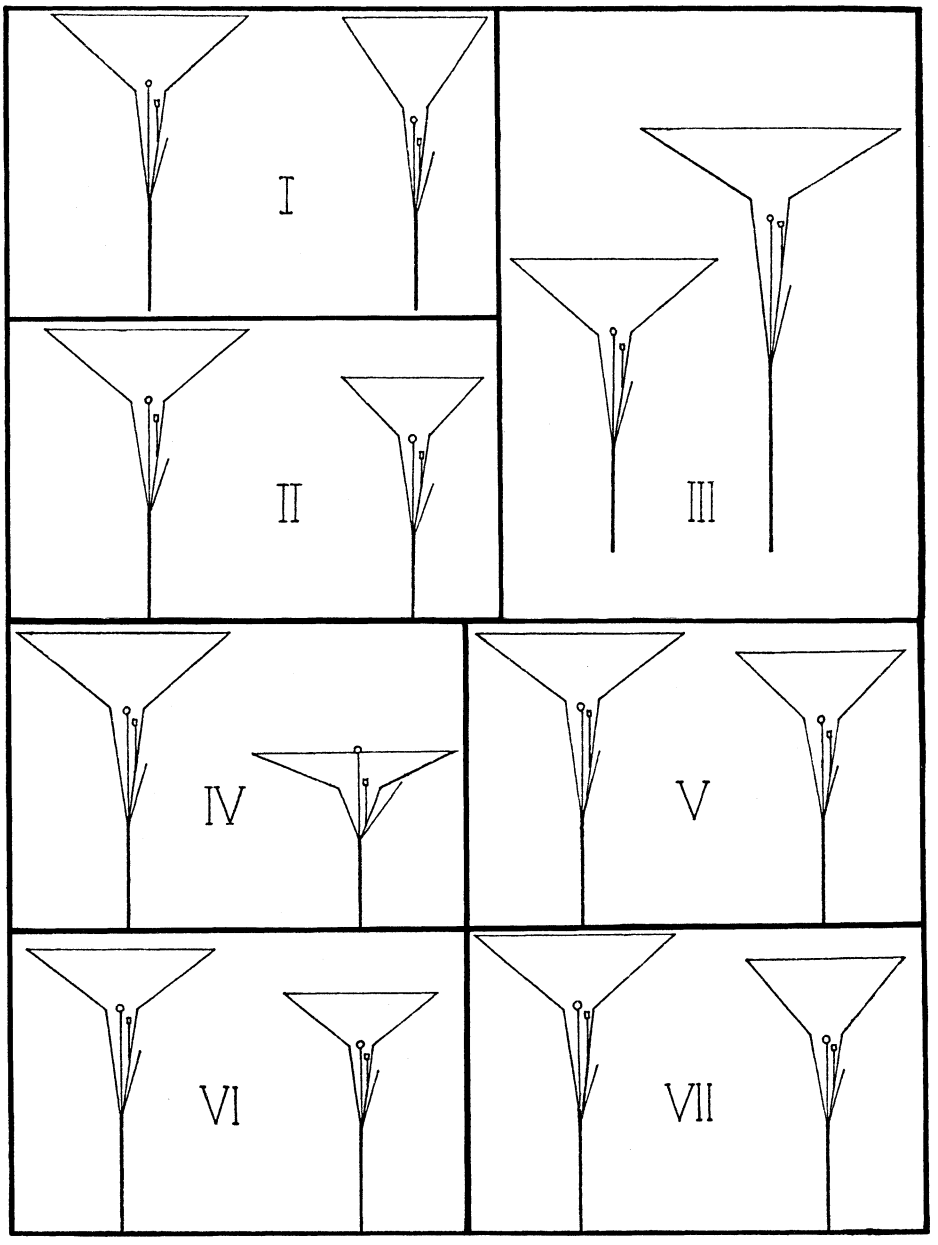


Fig. 4.5. Diagrammatic representation of the flowers of the 7 primary trisomics and their genetically comparable controls, established after the data of Maizonnier (1976b). The trisomic is on the right of the disomic control. A reduction coefficient of 0.7125 was applied to the data

somy IV are important though variable from one plant to another and from one flower to another on the same individual. The corolla may be crumpled with a more or less pronounced dialypetalization and presence of green zones, mainly at the level of principal nervations of petals. In these cases, stamens are often petaloid and the anthers sterile. Other flowers may be less affected by the presence of a supplementary chromosome IV: the corolla remains gamopetalous but with the margin turned down, giving the flower an umbrella shape.

The most consistent floral deformation is generally short flowers with a prominent stigma, (Fig. 4.5) and the presence of a crescent-shaped pad between the flower and the receptacle. This last anomaly causes inclination of the flower, with respect to the peduncle, and prevents the fading flower from falling during maturation of the capsule as is usually observed among disomic *Petunia* plants. In addition, trisomic IV individuals have a defective root system, without secondary roots at the level of the crown, which makes their cultivation rather difficult and frequently results in death.

Trisomic V. One can screen trisomic V as early as the two-leaf stage: plantlets are weak, leaves are rounded and light blue-green. The rosette (Fig. 4.4) remains small and the first flower opens at the end of a primary stem which is significantly shorter than in disomics. Flowering is also later; but flower characters are not extensively modified by the presence of a supplementary chromosome V (Fig. 4.5). The whole flower is smaller, the edge of petals turns up and, sometimes, the flower is inclinated as in trisomy IV.

Trisomic VI. The rosette (Fig. 4.4) of trisomic VI has rounded leaves, as in trisomic II, but in contrast to trisomic II the leaves are broad. The level of the first flower opening is significantly lower than for the control. To some extent, the flower of trisomic VI looks like that of trisomic II (Fig. 4.5). The foliage of trisomic VI plants is tinged pale green.

Trisomic VII. The rosette (Fig. 4.4) of trisomic VII is rather flat with long, narrow blue-green leaves. Flowering is delayed. The infundibular flower possesses a marked angle between the corolla tube and the so-called corolla. The diagram of Fig. 4.5 is therefore particularly suitable for the description of such a flower whereas it is only partially similar to the other trisomics.

β) Quantitative Analysis of Some Characters of Primary Trisomics

Some of the descriptive characters utilized to distinguish the different primary trisomics from their corresponding controls are not always specific of trisomic plants. For instance, rounded and crumpled leaves, chlorophyll deficiencies and flower deformations were also observed among apparent $2n = 14$ plants. Therefore, it seemed necessary to measure some characters in the populations of trisomics and their controls, that have the same genetic background in order to determine statistically the best means of recognizing each trisomic. For this study (Maizonnier 1976 b) the aim was to work on ideal populations of 30 trisomic and control individuals. As for certains trisomics the transmission rate is low, and it was necessary to select trisomics and controls among large populations of seed-

lings and rosettes on the basis of their phenotypes only. Afterwards, chromosome numbers were verified. Finally, except for the early flowering of trisomic III, it was possible to work with populations ranging from 25 to 32 plants. The 14 characters analyzed are as follows (floral characters were measured on the first flower):

1. Length/width of the fifth leaf of the rosette.
2. Height of opening measured from the crown to the base of the floral pedicel.
3. Length of floral pedicel.
4. Length of the large sepal.
5. Length of the corolla, measured in projection from the base of the tube to the distal end of corolla with a 5 mm approximation.
6. Large diameter of the open corolla.
7. Length/diameter of corolla.
8. Length of the large petal.
9. Height of corolla tube from the base to the inflexion point (beginning of the displayed part of corolla).
10. Diameter of corolla tube at the level of the inflexion point.
11. Total length of gynoecium, from the base of the ovary to the stigmatic plateau.
12. Total length of the shorter stamen.
13. Length of the free part of stamen pedicel (from corolla to anther) of the shorter stamen.
14. Earliness of flowering: number of days after sowing.

Statistical analyses of these different characters were made by comparing the means and variances of controls and trisomics in the different trisomic populations (Maizonnier 1976 b). The calculations (Table 4.1) were used to construct the diagrammatic flowers of Fig. 4.5.

γ) Some Other Features or Primary Trisomics

Maizonnier (1976 b) demonstrated that the transmission rate of the supplementary chromosome through the female gamete varied from one trisomic to another (3% to 30%), and for a given trisomic from one experiment to another (about 4% to 37% for trisomic IV). No transmission was observed when the seven trisomics were used as male parents (Maizonnier, unpublished data). In addition, *Petunia* is one of those species in which a trisomic for a given chromosome may yield in its progeny trisomics for other chromosomes (Maizonnier 1976 b) possibly through spontaneous meiotic nondisjunction for another pair of chromosomes independent of the presence of a triplicate chromosome.

c) Double Trisomics and Telotrisomics

Levan (1937), Rick (1971), and Smith et al. (1975) mentioned the occurrence of plants with 16 chromosomes when triploid plants were pollinated with pollen from a diploid. One could believe that these plants were tetrasomics ($2n+2$) but experiments reported by Maizonnier (1976 b and other unpublished data) sug-

Table 4.1. Analysis by t-test of 14 characters measured in the 7 trisomics and in their genetically comparable controls. (Established after the data of Maizonnier 1976 b)

Characters	Trisomics						
	I	II	III	IV	V	VI	VII
1 Length/width leave no. 5	+	−	−	=	−	−	−
2 Opening height of first flower	−	−	=	=	+	+	=
3 Flower stem length	−	−	+	=	=	=	=
4 Larger sepal length	+	−	+	−	−	=	=
5 Corolla length	+	−	+	−	−	−	−
6 Corolla larger diameter	−	−	+	−	−	−	−
7 Length/diameter of corolla	+	+	=	−	+	=	=
8 Lenght of larger petal	=	−	+	−	−	−	−
9 Corolla height at inflexion point	+	−	+	−	−	−	−
10 Corolla diameter at inflexion point	−	−	+	+	=	−	+
11 Total length of gynoecium	−	−	+	−	−	−	−
12 Total length of shorter stamen	−	−	+	−	−	−	−
13 Length of free part of shorter stamen pedicel	−	−	+	−	−	−	−
14 Flowering earliness (days after sowing)	=	+	+	−	=	+	+

+ = significantly higher value in trisomics.
− = significantly lower value in trisomics.
= = nonsignificant difference.

gested that they are most often doubled trisomics ($2n+1+1$). Such a karyotype is presented in Fig. 4.3. In his 1976 b report the author provided results of segregations for double trisomics II–VII and I–IV.

In telotrisomics only one chromosome arm is present as a supplementary chromosome. Maizonnier (1976 b) found 43 such plants in several progenies of trisomic plants. Wijsman et al. (1980) and Van den Berg and Wijsman (1981 a, b) also mentioned the occurrence of several telotrisomics. Some have already been identified and are involved presently in localization experiments (Maizonnier, unpublished data), and it seems reasonable to forsee a complete series of telotrisomics in the near future which would permit a more accurate localization of genes.

5 Genetics

A. CORNU [1]

5.1 Genetic Variability in *Petunia*

Due to its hybrid origin, genetic variability in *P. hybrida* is considerable. The parental species, *P. violacea* and *P. axillaris* both (Chap. 2), have variable genomes and their progeny thus inherite heterozygous genetic complexes. Such hybrids possess natural genetic variability and the germ plasm base for the induction of artificial mutants for genetic analysis.

Genes involved in floral pigmentation and growth habit were first detected. Presently, 32 loci controlling flower color are known (Chap. 6). Studies on growth habit, inheritance, and plant development (Bergounioux-Bunisset 1978; Brunaud et al. 1977) or seed germination (Jassey et al. 1979; see also Chap. 14) are more difficult due to their polygenic inheritance. Recently, Van den Berg and Wijsman (1980, 1981a) have detected genetic variability for some enzymes by electrophoretic analyses.

Occasionally, we have observed spontaneous mutations among F_2 populations cultivated for genetic studies or for detecting new phenotypes. However, mutagenic treatment strongly increases the usable mutation rate in *Petunia*. Until now, about 40 loci affecting chlorophyll synthesis or plant development have been detected in this manner. Mutagenic treatment has also yielded alleles, especially for previously known genes controlling anthocyanin pigmentation.

The existence of extranuclear genetic variability should also be mentioned. The study of organelle DNA structure is just beginning and will certainly allow to detect by analysis of restriction patterns, interspecific variability. On the other hand, it is possible to induce chlorophyll deficiencies controlled by extranuclear inheritance by mutagenic treatments (unpublished data). At present, about 100 loci are known in *Petunia*. A list of genes and nomenclature were recently proposed (Wiering et al. 1979 a). In Table 5.1 is a descriptive list of known loci, except those involved in flower pigmentation (Chap. 6), with the first known reference. More information and, possibly, seed samples are available from the Genetic Institute in Amsterdam (The Netherlands), the INRA plant breeding station in Dijon (France), and the department of Botany in the University of Nijmengen (The Netherlands).

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Table 5.1. Alphabetical List of *Petunia* Genes

List of nuclear genes for which a phenotypic description and genetic analysis has been carried out; excluding the flower color genes decribed in Chapter 6. The Gene symbol is written with a capital letter when the known alleles are partially or completely dominant to the wild type. The symbol is lower case when the described phenotype occurs only in the homozygous recessive. The reference given is that of the first description of the gene, when it is known; sometimes other references are given when they provide relevant information.

al – Albino seedling (Cornu unpublished). Seedlings lacking chlorophyll. Lethal. Several stocks produce albino mutants: allelism tests have not yet been conducted. Chromosome(s) unknown. See also Power et al. (1979): albino mutation carried by *P. inflata*

apt – Apetalous flower (Sink 1973). Allelic or identical to *gp*

bl – Blind (Wiering et al. 1979a). Flower limb very short, blocking the entrance of the tube. Viability and fertility normal. Chromosome IV

ch1 – Choripetalous-1 (Wiering et al. 1979a). Petals not fused, leafs acute, pistil reduced and split. Plants somewhat weak and sterile. Chromosome I

ch2 – Choripetalous-2 (Wiering et al. 1979a). Flower limb with strong incisions between petals. Expressivity variable. Viability and fertility normal. Chromosome unknown

ch3 – Choripetalous-3 (Wiering et al. 1979a). Petals not fused, small flowers, leaves narrow and acute, pistil reduced and split. Viability fair. Female fertility reduced. Chromosome III

co – Compact (Wiering et al. 1979a). Young plant with short internodes. Viability and fertility good. Chromosome unknown

Cp – Chlorophyllian petals (Cornu and Farcy unpubl.). Veins and margin of petals possess chlorophyll. Decreases anthocyanin synthesis. Viability and fertility normal at the heterozygous state. Chromosome unknown

cr – Crumpled (Wiering et al. 1979a). Flower limb crumpled. Viability fair. Female fertility reduced. Chromosome II

cu – Curled (Wiering and de Vlaming unpubl.). Curled leaves, plant and flower small. Viability and fertility reduced. Chromosome unknown

dg1 – Dark green-1 (Straub unpubl.). Leaves dark green, small. Plant small and somewhat weak. Flower pendulous. Fertility good. Chromosome VI

dg2 – Dark green-2 (Wiering et al. 1979a). Leaves dark green, lumpy. Viability and fertility normal. Chromosome unknown

dg3 – Dark green-3 (Wiering et al. 1979a). Leaves dark green, narrow. Plant small and sterile. Viability poor. Chromosome unknown

dg4 – Dark green-4 (Cornu unpubl.). Leaves dark green, round. Stem poorly branched. Fertility fair. Chromosome unknown

Do1 – Double-1 (Von Ubisch 1923, Scott 1937). Flower with increased number of petals and stamens. Often female-sterile. Chromosome VII. See also Natarella and Sink 1971

do2 – Double-2 (Wiering et al. 1979a). Flower parts increased in number and strongly reduced, flower head-shaped, female-sterile, anther sometimes fertile. Viability normal. Chromosome unknown

dw1 – Dwarf-1 (Bianchi et al. 1974, Wiering et al. 1979a). Plant with short internodes. Small, dark green leaves and small flowers. Viability reduced. Fertility good. Chromosome IV

dw2 – Dwarf-2 (Wiering et al. 1979a). Plant with short internodes and small leaves with necrotic spots. Viability poor. Fertility good. Chromosome VI

dw3 – Dwarf-3 (Wiering et al. 1979a). Small leaves; small flowers with deep incisions between the petals. Completely sterile. Viability reduced. Chromosome unknown

dw4 – Dwarf-4 (Wiering et al. 1979a). Plant small, leaves spoon-shaped, flowers small. Fertility fair. Chromosome VII

dw5 – Dwarf-5 (Wiering and de Vlaming unpubl.). Small plant. Small, somewhat curled leaves; small flowers. Viability and fertility reduced. Chromosome unknown

dw6 – Dwarf-6 (Wiering and de Vlaming unpubl.). Plant small with small leaves and small flowers. Viability poor. Chromosome unknown

dw7 – Dwarf-7 (Wiering and de Vlaming unpubl.). Plant small with short internodes, habit compact, small flowers. Viability and fertility reduced. Chromosome unknown

Table 5.1. (continued)

dw8 – Dwarf-8 (Wiering and de Vlaming unpubl.). Small plant with small leaves and small flowers. Viability and fertility reduced. Chromosome unknown

e – Male-sterile (Frankel 1971). Found in the offspring of a fertile symbiant graft with a cytoplasmic male sterile component. Chromosome unknown. See also Chapter 7

ea – Earshaped (Wiering et al. 1979a). Ear-shaped projections at outer surface of corolla between the petals. Expressivity variable. Male sterile. Viability good. Chromosome 1

fu – Fold-up (Wiering and de Vlaming unpubl.). Edge of leaf folds inwards. Viability and fertility reduced. Chromosome unknown

G – Grandiflora (Plickert 1936). Identical to *Un*

gp – Green petal (Wiering et al. 1979a). Petals with same shape and color as sepals. Viability and fertility good. Chromosome IV

gpi B – Glucose phosphate isomerase (van den Berg and Wijsman 1980). Enzyme variations detected by electrophoretic mobilities. Two alleles are known. Chromosome VII

lap B – Leucyl amino peptidase (van den Berg and Wijsman 1980). Enzyme variations detected by electrophoretic mobilities. Two alleles are known. Chromosome VII

le1 – Lethal-1 (Wiering et al. 1979a). Gametic or zygotic lethality. Chromosome 1

le2 – Lethal-2 (Wiering et al. 1979a). Homozygous lethal. Chromosome VI

le3 – Lethal-3 (Wiering et al. 1979a). Gametic or zygotic semilethality. Chromosome I

lg1 – Light green 1 (Wiering et al. 1979a). Plants with pale green leaves. Slow growth. Classification good at early stage. Normally fertile. Chromosome 1

lg2 – Light green 2 (Wiering et al. 1979a). Plant becoming diffuse pale green. Classification fair at emergence stage. Normally fertile. Chromosome II

lg3 – Light green 3 (Wiering et al. 1979a). Leaves pale green, concave. Development somewhat slow. Chromosome unknown

lg4 – Light green 4 (Wiering et al. 1979a). Leaves fading pale green, development very slow. Plant sometimes weak. Chromosome unknown

lg5 – Light green 5 (Wiering and de Vlaming unpubl.). Plant with pale green leaves. Vaibility and fertility normal. Chromosome unknown

ls – Long style (Cornu unpubl.). Style extends beyond the flower tube. Classification sometimes difficult. Fertility good. Chromosome unknown

lu1 – Luteus-1 (Maizonnier 1976b, Wiering et al. 1979a). Heterozygous with yellow or yellow-green leaves. Homozygous albino lethal. Several alleles are known. Fertility good. Classification good at seedling stage. Chromosome II

ms1 – Male-sterile 1 (Wiering et al. 1979a). Anthers empty. Classification sometimes difficult. Allelism with *ms1* and *ms2* reported by Wetzel (1954) and *e* reported by Frankel (1971) not known. Chromosome unknown

ms2 – Male-sterile 2 (Singh 1976). Anthers without pollen in heterozygous state; in a limited number of genotypic backgrounds. Chromosome unknown

nl – Narrow leaves (Wiering and de Vlaming unpubl.). Small plants with small narrow leaves. Viability and fertility reduced. Chromosome unknown

prx A – Peroxidase A (Van den Berg and Wijsman 1980). Enzyme variations detected by electrophoretic mobilities. Three alleles are known. Chromosome III

prx B – Peroxidase B (van den Berg and Wijsman 1981b). Enzyme variations detected by electrophoretic mobilities. Three alleles are known. Chromosome 1

px – Phoenix (Wiering and de Vlaming unpubl.). Flower crumpled with green nervation, flower tube with wrinkle. A complete new flower arises sometimes from the flower base. Expressivity variable. Viability and fertility good. Chromosome unknown

Rf1 – Restorer-1 (van Marrewijk 1969). Gene restoring fertility in cytoplasmic male-sterile plant. Classification fair at heterozygous state in several backgrounds. Chromosome unknown

rf2 – Restorer-2 (van Marrewijk 1969). Gene restoring fertility in cytoplasmic male sterile plant. Normally has no effect in heterozygous state. Chromosome VI

S – Self-incompatibility (Straub 1946). Responsible for the gametophytic self-incompatibility in *Petunia* species. Several alleles are known. For review of the problem see Linskens 1975. Chromosome unknown

Table 5.1. (continued)

sdh	– Shikimic acid dehydrogenase (van den Berg and Wijsman 1980). Enzyme variations detected by electrophoretic mobilities. Three alleles are known. Chromosome V
sp1	– Spotted-1 (Wiering et al. 1979a). Leaves with dark green spots. Viability and fertility normal. Chromosome V
sp2	– Spotted-2 (Wiering and de Vlaming unpubl.). Similar to *Sp1*. Chromosome unknown
st1	– Short-tube 1 (Wiering et al. 1979a). Flower flat with short tube leaves flat and round. Chromosome unknown
st2	– Short-tube 2 (Wiering et al. 1979a). Plant small, greenish yellow, flowers small. Chromosome unknown
tr	– Tree-like (Straub unpubl., Wiering et al. 1979a). Inflorescence strongly furcated ("tree-shaped"), leaves small, flowers sometimes reduced. Expressivity variable. Viability and fertility good. Chromosome unknown
Tu	– Trumpet (Wiering et al. 1979a). Flowers trumpet-shaped; leaves turn yellow-green at emergence stage. Homozygous *TuTu* complete sterile. Chromosome VI
Un	– Undulata (Bianchi 1959). Flower limb and calyx undulated, leaves yellowish green. Chromosome V. See also Reimann-Philipp 1962 and Smith et al. 1975
us	– Ustulata (Straub unpubl. and Wiering et al. 1979a). Leaves with necrotic spots. Viability and fertility good. Chromosome V
Vr	– Viridis (Cornu and Maizonnier 1983). Heterozygous with virescent leaves. Homozygous *VrVr* do not appear. Chromosome unknown
vs1	– Virescent seedling-1 (Wiering et al. 1979a). Leaves yellow becoming green. Often unstable, viability and fertility normal. Chromosome I
vs2	– Virescent seedling 2 (Wiering et al. 1979a). Young leaves yellowish becoming green. Viability and fertility normal. Chromosome III
vs3	– Virescent seedling 3 (Wiering et al. 1979a). Cotyledons downy white, young leaves with yellow base. Classification good at very early stage. Chromosome IV
vs4	– Virescent seedling 4 (Wiering et al. 1979a). Rosette with yellow heart. Classification fair at rosette stage. Chromosome I
vs5	– Virescent seedling 5 (Wiering et al. 1979a). Young leaves yellowish becoming green. Classification good at early stage (first leaf). Viability and fertility normal. Chromosome VII
wi	– Withered (Wiering and de Vlaming unpubl.). Tips of leaves withered. Viability reduced. Fertility good. Chromosome unknown
ws	– White stigma (Straub unpubl., Wiering et al. 1979a). Plants with yellow stem and white stigma. Leaves somewhat light green. Viability and fertility normal. Chromosome II
yg1	– Yellow green 1 (Cornu 1978, Wiering et al. 1979a). Leaves yellow-green (chlorotic-like). Classification fair at emergence stage. Viability and fertility normal. Chromosome V
yg2	– Yellow green 2 (Wiering and de Vlaming unpubl.). Plant small with leaves yellow-green. Viability poor. Chromosome unknown
yg3	– Yellow green 3 (Wiering and de Vlaming unpubl.). Entire plant or only the young leaves spotty chlorotic. Expressivity variable. Chromosome III

5.2 Gene Linkage and Mapping

Although numerous analysis have been performed to establish linkage relationships between genes of *Petunia*, few results have been published. The first available data concerned the main anthocyanin genes that control flower color, e.g., *An2, An4, Fl, Hf1, Po, Rt* (Chap. 6.1). It has readily been demonstrated that *An2, An4, Fl,* and *Hf1* segregate independently, while close linkage has been found to exist between *An2* and *Rt* and between *Po* and *Hf2* (Meynet et al. 1971; Dommer-

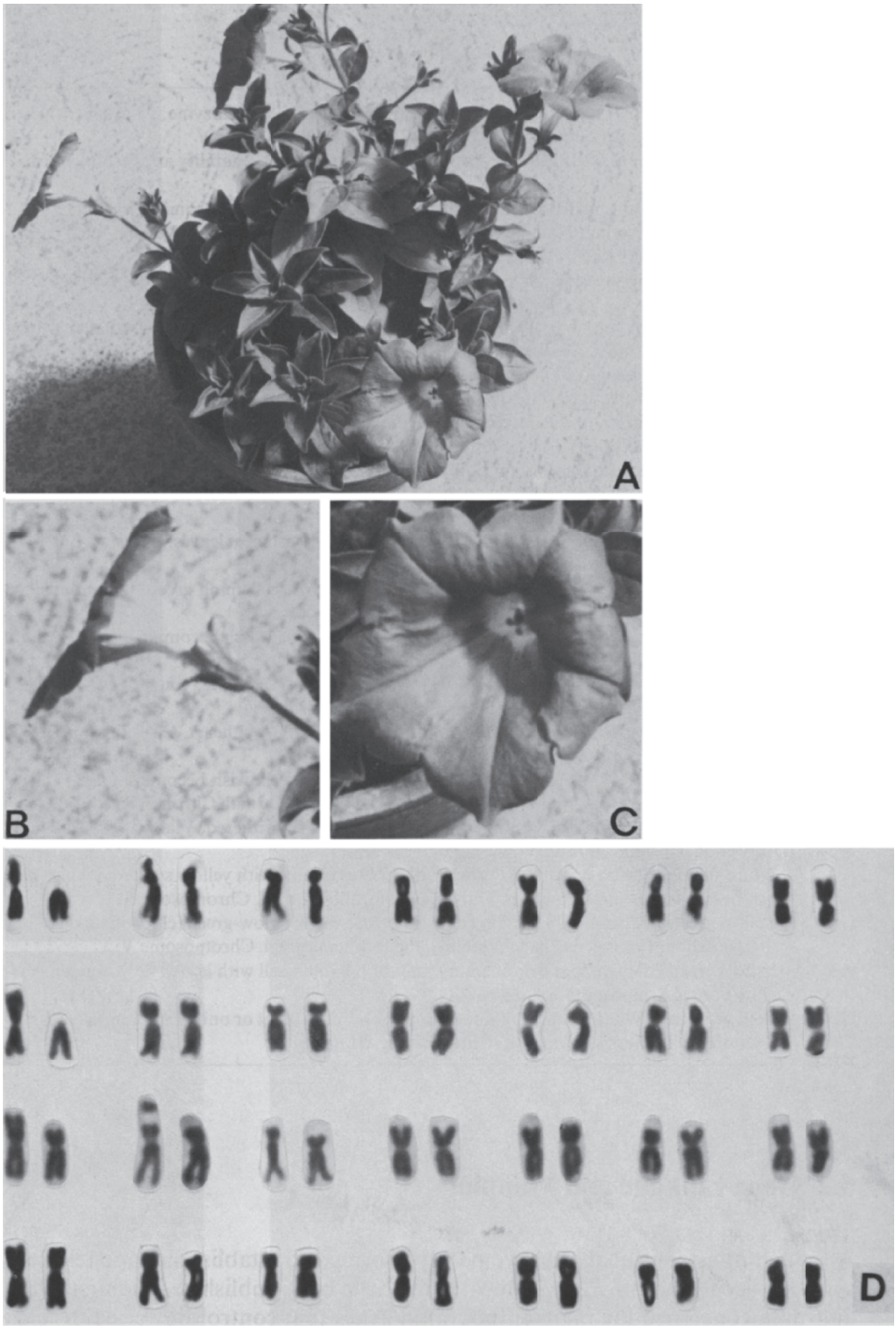


Fig. 5.1 A–D. Phenotypes and karyotypes of plants showing deletions of chromosome I and lacking *Hf1* marker. (After Maizonnier 1976 b). **A** Picture of a deleted plant. **B** Profile of a flower showing a short and light colored tube and a flat corolla. **C** Corolla with its light-colored tube. **D** Four karyotypes showing different deletions of whole or part of an arm of chromosome I

gues and Cornu 1974; Wiering and de Vlaming, personal communication). In connection with their studies on glycosylation and methylation genes, Wiering and de Vlaming (1973, 1977) observed independent inheritance for the following gene pairs: *Hf1/Gf, Hf1/Hf2, Hf1/Rt,* and *Mf1/Mf2* and linkage between *Gf/Hf2, Ht1/Mf1,* and *Ht2/Mf2*. Besides research on the *"grandiflora"* character, dependent on the dominant gene *Un* the independent inheritance of *Un* with regard to *Do* and *Gp* (Sink 1973) and to *Rt* (Smith et al. 1975) has been shown. These first results have led to the institution of "linkage groups heads", constituting leader genes, related to easily recognizable phenotypes, namely *Hf1, Fl, Hf2, Rt,* and *An4*. Later on, *Ht1* and *Bl* were chosen as representatives of the two remaining groups.

Various methods have been used by cytogeneticists to assign genes on chromosomes. For example, segregation analysis in progenies originating from trisomics identifies which chromosome carries a particular gene. Such results were first achieved by Smith et al. (1975) who assigned the locus *Un* to chromosome V, distinguished from chromosome VI by fluorescence and scanning reflectodensitometry. Thereafter, Maizonnier (1976 b), who described the whole set of primary trisomics of *Petunia* (Chap. 4.3), subsequently determined the chromosomal localization of the seven linkage groups (Maizonnier and Moessner 1979). The trisomic method was also used by Van den Berg and Wijsman (1981 b) to localize genes coding for several enzymes (see also Wijsman 1983).

More accurate assignment of chromosomes could be carried out by using induced chromosomal rearrangements (Maizonnier 1976). Analysis of a telocentric translocation (Maizonnier and Cornu 1971) presented the opportunity of assigning locus *Hf1* to the translocated arm of chromosome I. Further cytogenetic investigations on gamma induced deletions involving the loss of the dominant allele *Hf1* (Fig. 5.1), suggested that locus *Hf1* is very distal with regard to the centromere (Maizonnier 1976). In the same manner, locus *An2* could tentatively be located at the end of the short arm of chromosome VI (Maizonnier et al. in preparation).

Deletions involving a complete arm can be induced by γ-rays; this produces semimonosomic plants that have 13 chromosomes plus a telochromosome. This telo is sometimes sexually transmitted through female gametes carrying 7 chromosomes + telo, originating from meiotic nondisjunction (Cornu and Maizonnier 1979). It results in telosomic individuals (14 + telo) which permit gene assignment on each arm of the chromosomes. Such telotrisomics are now available for some chromosomes and they have already permitted the location of *Lg1* with *Hf1* on the same arm of chromosome I (Maizonnier, pers. commun.).

Using various sizes of satellites as chromosome markers, Maizonnier (1976 b) demonstrated that locus *lu1 (luteus 1)* was localized on chromosome II, close to the satellite region (Table 2). Although a chromosome marker was used in this experiment, recombination rates remained genetic distances only (not actually cytologic) and did not permit the assignment of *lu1* on the short arm (satellite arm) of chromosome II. In *Petunia*, meiotic recombination rates are quite variable and dependent on the genetic background. This was clearly shown by analyses of linkage data along chromosome V (Cornu et al. 1980). Thus, it would be better to dispose of physical data obtained by chromosome rearrangement.

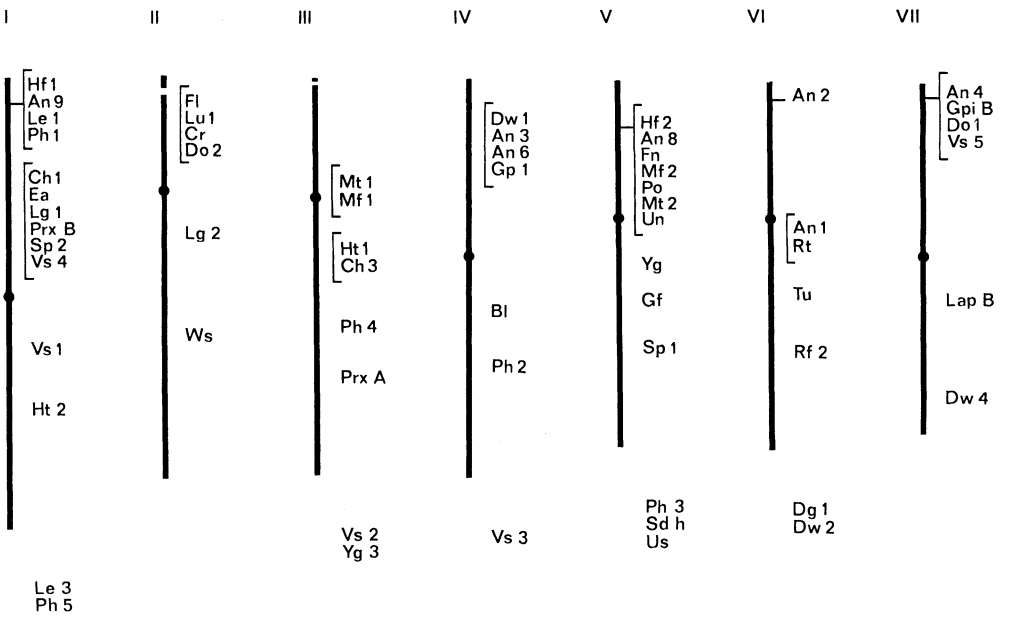


Fig. 5.2. Genic map of *Petunia* chromosomes. For details see text

Nevertheless, a genetic map of *Petunia* is now available (Fig. 5.2). About 50 loci are presently assigned to chromosomes, but generally without any knowledge of their position with respect to the centromere. The following comments can be made with regard to each chromosome:

Chromosome I. Both arms have the same size; one of them carries *Hf1* in a distal position (Fig. 5.1) and *Lg1* as previously mentioned. If we refer to the very low recombination rates observed between *Hf1* and the loci of *An9, Ch1, Ea, Le1, Ph1, Prx B,* and *Sp2*, we may reasonably believe that these loci are on the same arm as *Hf1*. On the other hand, it is not yet possible to determine whether or not *Vs1, Vs4,* and *Ht2* are carried by that arm. Concerning *Ph5*, it was assigned to chromosome I only by trisomic analysis (Maizonnier, pers. commun.).

Chromosome II. No recombination was found between *Fl–lu1, Fl–Cr, Fl–Do2*, and very few recombinants were obtained between the locus *Lu1* and the satellite area (see above and Table 5.2). These results suggest that the 4 loci are located more or less near the satellites, perhaps on the short arm. *Lg2* and *Ws* have high recombination rates with *Fl* and are possibly located on the long arm.

Chromosome III. Linkage relationships have been established between genes *Ch3, Ht1, Mf1, Mt1, Ph4, PrxA,* and *Yg3*. The assignment of loci *Ht1* and *Vs2* to chromosome III was made by trisomic analyses (Maizonnier and Moessner 1979 and unpublished data). Until now, no information about physical mapping is available for this chromosome.

Table 5.2. Genetic linkage between *lu*-1 and satellite area of chromosome II

Testcross[a] $\frac{lu\text{-}1\ BS}{lu\text{-}1^+\ SS} \times \frac{lu\text{-}1^+\ BS}{lu\text{-}1^+\ BS}$	Progenies[b]				Total
	Parental types		Recombinant types		
	Green BS/SS	Yellow BS/BS	Green BS/BS	Yellow BS/BS	
Experiment 1 with allele *lu*-1-2	302	199	0	0	501
Experiment 2 with allele *lu*-1-3	92	129	3	0	224

[a] BS = Big Satellite; SS = Small Satellite
[b] Genotypes *lu*-1^+/*lu*-1^+ give green plant; Genotypes *lu*-1/*lu*-1^+ give yellow plant (cf. Table 1)

Chromosome IV. Loci *An3, An6, Dw1,* and *Gp1* constitute a cluster in a chromosome area without exhibiting crossing over. High recombination rates between *Bl* and *Dw1* (about 27%) and *Ph2* and *Dw1* (about 27%) suggest that *Bl* and *Ph2* are distant from the cluster, perhaps on the other arm (Wiering and de Vlaming, unpublished data).

Chromosome V. The genetic map of this chromosome was recently published (Cornu et al. 1980). Loci *Hf2, An8, Po, Mf2, Mt2, Un* (*Hf2* being probably the more distal) can be gathered into a single cluster, but recombination rates within this group appear sometimes higher depending on the genetic background. However, deletion studies permit the assignment of *Hf2* and *An8* to the short arm. High recombination rates between *Gf*/*Hf2* and *Sp1*/*Hf2* suggest that *Gf* and *Sp1* may be located on the long arm.

Chromosome VI. Numerous cytogenetic analyses of various chromosome rearrangements involving locus *An2* (Maizonnier and Cornu 1979 and unpublished data) suggest that this locus is present at the end of the short arm. Furthermore, recent investigations with material containing a telochromosome VI (lacking the short arm) seem to indicate that loci *An1* and *Rt* would not be located on this arm in spite of the strong linkage with *An2* described by Cornu and Dommergues (1974).

Chromosome VII. Trisomic analyses assign *Dw4* and *An4* to this chromosome (Maizonnier and Moessner 1979). These two loci have a recombination rate of 40%; each one may be considered as a marker for each arm. *Do1, Gpi B,* and *Vs5,* which do not recombine with *An4,* can presently be assigned to the same arm as *An4.*

This genetic map, characterized by at least one nondeleterious and easily recognizable marker on each chromosome, is already useful. However, much remains to be done for the enrichment of this map, especially with markers suitable for in vitro conditions and for the establishment of a physical map.

5.3 Mutagenesis and its Applications

The various characters of the *Petunia* genus that facilitate studies in genetics and molecular biology have been reviewed by Bianchi and Dommergues (1979) and by Hanson (1980). For identical reasons, *Petunia* was chosen early to investigate the effects of mutagenic treatments (Cornu 1970 a, 1978). These characteristics include:

1. Low chromosome number ($x = 7$) and thus few linkage groups.
2. Ease of sexual reproduction. Crosses and selfs are easily performed. A single fruit contains hundreds of seeds. The large number of flowers per plant leads to a tremendous reproductive potential. The reproductive cycle is short, around 3 months, so that three to four generations can be obtained one year.
3. Asexual propagation can be achieved by traditional horticultural techniques (e.g., cutting, grafting) or by in vitro techniques (apex, foliar, and floral explants). In this way, nontransmissible mutations can easily be maintained.

Considering the ease of using *Petunia* material and with the help of marker genes scattered on every chromosome, numerous research studies could be conducted including the study and the development of new mutagenesis techniques, the analysis of genetic effects of mutagens, and the use of induced mutations for different purposes.

5.3.1 Study and Development of New Techniques for Mutagenic Treatments

The first pollen treatments with X-rays (Table 5.3) showed that *Petunia* pollen is extremely radio-resistant: pollen grains still germinate after receiving a 750 krad dose (Gilissen 1978). Research workers of the 1940's and 1950's were particularly interested in the effects of radiation on chromosomes (Rick 1943, Müller 1958). With the help of lines carrying marker genes involved in the anthocyanin syntheses, Dommergues and Cornu (1974) could measure directly on F1's the frequencies of induced mutations after pollen irradiations which correspond directly to loss or change of physiological functions as detected on the flower. This kind of experiment permits estimates on the effect of increasing doses of γ-rays on the decrease of seed-setting and the increase of induced mutations (Fig. 5.3) and also the determination of an optimal dose. The more efficient doses for obtaining genic or chromosomal mutations generally range from 10 to 20 krad. Pollen irradiation also has the advantage of producing heterozygous and genetically homogenous mutants which carry the mutation in the sporogenous tissues and, consequently, it may be transmissible to the next generation if the mutants are compatible with a normal development of micro- and macrospores (Cornu 1977).

Nonfertilized ovule treatment offers similar advantages but with a minor radioresistance. Efficient irradiation doses are about 1 krad (Dommergues and Cornu 1974). Ovule treatment after fertilization, i.e., zygote treatment, is a method which has been developed for *Petunia* (Table 5.3), however, it has been possible after the detailed study of proembryo development of the species (Val-

Table 5.3. Experiments with mutagenic treatments in *Petunia*

Treated material	Mutagenic treatment	Observed effects	References
Pollen	X-rays (10–100 krad)	Chromosomal rearrangements	Rick (1943)
Pollen	X-rays (10–20 krad)	Pollen sterilities	Welzel (1954)
Pollen	X-rays (10–30 krad)	Translocations	Müller (1958)
Pollen	γ-rays (5–30 krad)	Seed-setting and mutation frequencies	Dommergues and Cornu (1974)
Pollen	γ-rays (20 krad)	Genetic instabilities	Cornu (1977)
Pollen	γ-rays (15 krad)	Deletions	Cornu and Maizonnier (1979)
Pollen	X-rays (50–750 krad)	Pollen germination	Gilissen (1978)
Pollen	UV-irradiation (254 nm)	Pollen germination and DNA synthesis	Jackson and Linskens (1978)
Buds	γ-rays (2 krad)	Somatic mutations	Cornu (1970 a)
Seeds	γ-rays (2–8 krad), E.M.S. (2–5 g $\cdot$ l^{-1})	Germination, fertility and somatic mutations	Cornu (1970 b)
Ovules, zygotes	γ-rays (1 krad)	Seed-setting and somatic mutations	Dommergues and Cornu (1974)
Zygotes	γ-rays (1 krad)	Translocations	Maizonnier and Cornu (1971)
Zygotes	E.M.S. (2.5 g $\cdot$ l^{-1})	Seed-setting and somatic mutations	Dommergues and Cornu (1974)
Zygotes	γ-rays (0.5–1 krad) NG (1) (0.2 g $\cdot$ l^{-1}) Hydrazine (4 g $\cdot$ l^{-1})	Genetic instabilities	Cornu (1977)
Zygotes	γ-rays (0.75 krad) M.S.E. (2 g $\cdot$ l^{-1})	Mutations and deletions	Cornu and Maizonnier (1979)
Ovules	γ-rays (1 krad)	Occurrence of androgenetic haploids	Singh and Cornu (1976)
Cell suspension cultures	NG[a] (5–40 μg ml^{-1})	Drug resistance	Colijn et al. (1979 a)

[a] NG = N-methyl-N-nitro-N-nitrosoguanidine

lade 1970, Vallade and Cornu 1973). These works showed that the duration of the unicellular zygote stage, from fertilization to first division, is about 120 h at 20 °C. In addition, it was possible to measure rather accurately durations of the stages (G_1, S, and G_2) of the mitotic cycle of the zygote.

With this method, physical or chemical treatments could be applied at a precise stage (G_1, S, or G_2) of the zygotic cells. The method also allowed a very precise analysis of the effects of a mutagen and therefore, constituted a practical method for searching for mutations in other plant species in which the length of the zygotic stage and the number of ovules per flower are sufficient.

The experimental possibilities offered by in vitro culture of cells or tissues of *Petunia* lead to suitable mutagenic treatments. Binding (1974 a) pointed out the use of culturing haploid cells of *Petunia*, either from anther cultures or from spontaneous origin, and indicated various methods to select mutants. Mutagenic treatments may be applied before or during culture, and selection may be done during the monocellular stage or different pluricellular stages (undifferenciated or budding calli), according to the screening method utilized.

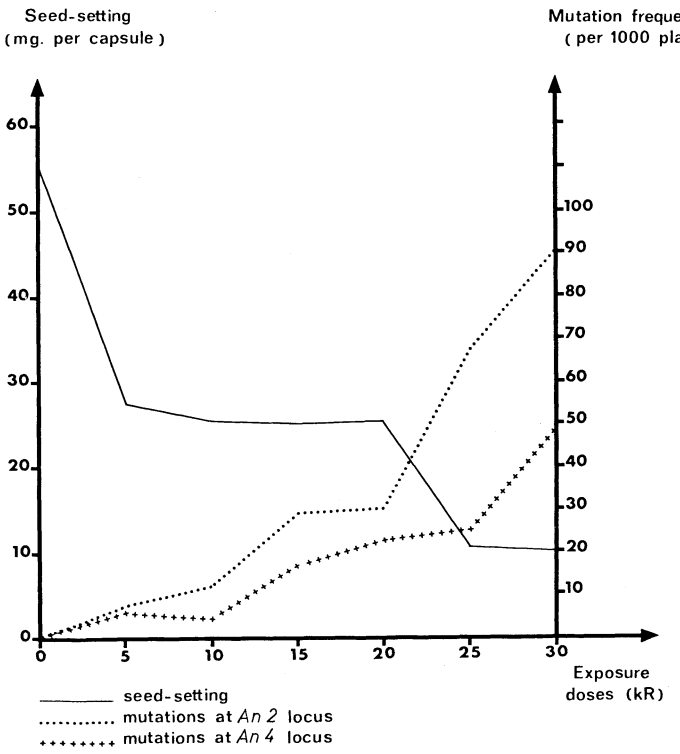


Fig. 5.3. Effects of Sg pollen irradiation with γ-rays on seed-setting and mutation frequencies in F1 hybrids between TL (*an2 an2 an4 an4*) line and Sg (*An2 An2 An4 An4*) line

Plantlet regeneration from explants provides a method for obtaining mutants. In *Petunia*, floral pedicels originating from γ-irradiated haploid plants were cultured (Cornu, unpublished data); the regenerated buds were often diploid. This method allows visual selection of diploid homozygous mutants. However, data presently available does not indicate that the mutagenic treatment is actually effective, as the spontaneous mutation frequency is relatively high in these explants. Colijn et al. (1979a) resolved this question by culturing diploid cells of *P. hybrida* with the chemical mutagen N-methyl-N-nitro-N-nitrosoguanidine (NG). Their mutagen criteria were the survival rate and the frequency of calli resistant to drugs. Their results demonstrated that NG at concentrations compatible with cell viability is a very effective mutagen. Plants can be obtained from mutant calli (Kool, pers. commun.); thus *Petunia* is suitable for genetic studies of biochemical mutants selected in vitro.

5.3.2 Genetic Effects of Mutagenic Treatments

Much work has been carried out concerning genetic effects of mutagenic treatments on microorganisms and some other special species such as *Neurospora cras-*

sa or *Drosophila melanogaster*. In higher plants, the species with which a study of mutagenic effects at the level of the gene and its function are scarce. In *Petunia*, the knowledge of anthocyanin gene and their chromosomal localization allowed a discrimination of different kinds of induced mutations.

The use of strongly linked genes clusters involved in anthocyanin synthesis and rather frequent in *Petunia* (Fig. 5.2) can be given as an example. Two lines with genotypes *AAbb* and *aaBB* are chosen and treatments are applied either on gametes or on heterozygous zygotes. If mutants for *A* and (or) *B* appear among the F1 progeny they are tested in three ways:

1. analysis of the progeny of the testcross with line *aabb*;
2. chromatographic analysis of pigments produced by the mutant which determine the phenotype accurately;
3. analysis of pollen fertility by a coloration technique.

The results of these three analyses allow discrimination between deletions or mutations (Table 5.4). In *Petunia*, a diploid species, the loss of a chromosome segment is incompatible with normal development of gametophytes which explains the absence of one class of phenotypes in the progeny of testcross and a semi-sterility of pollen. These mutants are called "monogametic mutants". This kind of experiment was carried out with gene markers of chromosome VI, *An2* and *Rt* (Cornu and Dommergues 1974) or chromosome V, *Po, Fn,* and *Hf2* (Cornu and Maizonnier 1979). In this way, it was shown that irradiation yielded many monogametic mutants and therefore, deletions especially in the area carrying *An2* locus (Table 5.5). On the other hand, MSE applied on zygotes induced less chromosome breaks and more true mutations than γ-rays. If analysis of progeny is performed up to the isolation of mutant lines (Table 5.5), it appears that the proportion of useful mutations is nearly similar irrespective of the kind of mutagenic treatment. One conclusion is that a treatment may be chosen in terms of pursued aims; thus, when the goal is to obtain a mutation corresponding to a change of function, it is better to use an alkylating drug, such as MSE, as evi-

Table 5.4. Genetic analysis of a B mutant [a] originating from a cross AA bb × aa BB [b]

Testcross progenies [c]	Gametic fertility %	Phenotype level (L)	Interpretation	
			Induced event	M1 Genotype
[Ab]+[ab] (digametic mutant)	Generally > 50	L = [b]	Nonfunctional mutation	$\frac{A\ b}{a\ b_1}$
		L = [b_1]+[b]	Functional mutation	$\frac{A\ b}{a\ b_1}$
[A b] (monogametic mutant)	Generally < 50	L = [b]	Deletion	$\frac{A\ b}{a}$

[a] Phenotype = [A b_1] instead of [A B]

[b] Loci A and B are strongly linked. Mutagenic treatment was applied on aa BB pollen or on $\frac{A\ b}{a\ B}$ zygote

[c] Progenies of the cross:mutant × aa bb

Table 5.5. A mutagenesis experiment on locus *An2* (chromosome VI) of *Petunia*. (After Cornu 1977)

Mutagenic treatment	M1 plants observed	Monogametic mutants	Digametic mutants	Homozygous isolated mutations	
				Number	Frequency $\times 10^{-4}$
γ-rays on microsporocytes	3500	0	2	2	5.7
γ-rays on pollen	11240	198	5	4	3.5
γ-rays on ovules	4170	12	0	0	<2.5
γ-rays on zygotes	24300	100	7	5	2.1
E.M.S. on zygotes	8000	8	5	1	1.2
Control	7750	1	0	0	<1.3

denced by other results concerning loci *Rt* and *Hf2* of *Petunia* (Cornu, unpublished data).

X-rays or γ-rays induce numerous chromosome breaks leading to various rearrangements (Sect. 4.2). The most frequent are terminal or intercalary deletions. Sometimes they can be observed directly on mitotic chromosomes; such is the case for chromosome I with marker *Hf1* (Maizonnier 1976b) and for chromosome VI with *An2* (Maizonnier et al., in preparation). These deficient chromosomes are not transmitted to the next generation except in addition to a complete set of seven chromosomes by means of meiotic nondisjunction (Cornu and Maizonnier 1979). Different types of X- or γ-induced translocations were also described in *Petunia* (Rick 1943; Müller 1958; Maizonnier and Cornu 1971; Maizonnier 1976b). However it is only after an accurate karyogram analysis (Maizonnier 1971) and the location of valuable genetic markers that the chromosome involved in such interchange could be determined. Some of them are transmissible and tolerate homozygosity.

Ring chromosomal changes were also found in *Petunia* after γ-ray treatment (Maizonnier 1976b; Maizonnier and Cornu 1979). This abnormality was observed for chromosome V, VI, and part of the VII revealed in each case by the instability of genetic markers. Depending upon the kind of mitotic anaphase, the ring chromosome may produce either ring chromosomes – of equal or different size – or rod chromosomes with deletions and duplications. Sexual transmission of a ring chromosome was possible only by gametes possessing the ring in addition to a complete haploid set.

5.3.3 Use of Induced Mutations

The use of mutagenesis in *Petunia* is very positive as more than 40 loci were discovered in this way. At present numerous mutations are used as genetic markers of chromosomes. Among them, chlorophyll mutations have an advantage as seedling markers and they are scattered on all the chromosomes. The more useful ones are *lg1* (chromosome I), *ws* (chromosome II), *vs2* (chromosome III), *vs3* (chromosome IV), *yg1* (chromosome V), *dg1* (chromosome VI), and *vs5*

(chromosome VII). Other mutations such as *lu1* (chromosome II) are particularly interesting because they permit marking in the hybrids.

Induced mutations may also be used as ontogenic markers. Cornu and Bugnon (1971) were able to study the functioning of flower meristem and the formation of the scorpioid inflorescence of *Petunia* by analyzing the size and location of mutated sectors after irradiation of the initial bud. Vallade and Cornu (1979) carried out a histocytological study of two mutants in which female sterility was correlated with a deficiency of flavonoid synthesis. They showed that sterility was due to a block in embryo development. In some cases, in vitro ovule culture of one mutant (with *ph5*) allowed the development of the embryo but not of the endosperm. These mutants bring precision to experiments in morphogenesis and the nutritional role of endosperm.

Induced mutations at the level of anthocyanin loci may yield new flower colors for plant breeding. New alleles induced at loci *Rt* and *An4* (Cornu, unpublished data) give rise to new anthocyanin equilibria which are transmissible to F1 hybrids. By means of mutagenesis, lines with an increased amount of pelargonidin, a pigment which is scarce in *Petunia*, were obtained (Cornu et al. 1974).

5.4 Genetic Instabilities

Genetic instabilities in *Petunia* are rather frequent. The most-documented cases concern floral pigmentation which was first studied by Malinowski (1935). Two types of genetic instabilities exist:

1. chromosomal changes whose somatic fate leads to an unequal distribution of genetic markers through breakage – fusion – bridge cycles (Maizonnier and Cornu 1971, 1979; Maizonnier 1976b);
2. a genic instability which generally gives rise to reversions to a normal activity of the gene. The first type is illustrated in Chap. 4; in the present chapter only genic instabilities will be discussed.

Petunia is particularly suitable for studies on instability phenomena because there is extensive information on the genetics of anthocyanin biosynthesis and linkage groups. Furthermore, plants can easily be regenerated from variant sectors cultured in vitro. Most investigations on instability concern anthocyanin loci, i.e., *An1* (Bianchi et al. 1978), *An2* and *Rt* (Cornu 1977); but an unstable dwarfing gene is also known (Bianchi et al. 1974), and, among the chlorophyll variegations observed in *Petunia*, many cases of nuclear unstable genes probably exist.

Genic instability in *Petunia* generally arises through the mutation of a dominant allele to a so-called unstable allele. The mutation may be spontaneous or induced. Instability for an anthocyanin gene is phenotypically characterized by frequent sectors which are more deeply colored than the background. These sectors are due to "reversions" of the mutated allele toward a new, generally stable, state. Different patterns of reversions, varying with the different unstable systems, are possible.

The properties of unstable alleles studied to date in *Petunia* can be summarized as follows:

a) Each unstable allele *a–x* obtained from a wild allele *A* is sexually transmissible. The two phenotypic expressions, i.e., different level of function and instability are jointly inherited.

b) Instability index, that is the frequency of reversions from a state *a–x* toward another state *a–n* may be done in two different ways. Bianchi et al. (1978) estimated the spot density per area unit on corolla strips observed under a microscope. Cornu (1977) was also able to evaluate, by means of microscopy, the cell number of each spot. He obtained a frequency pattern which allowed the comparison of reversion cell rates during cell cycles involved in petal morphogenesis. The results confirmed that reversion frequencies may be very different from one plant to another and also, for a given plant, according to the differentiation stage of the tissues. However, the genetic events which are at the origin of the new alleles *a–n* may occur in sporogenous tissues. Bianchi et al. (1978) found a positive correlation between spot density in mother plants and percentage of revertants in their progenies.

c) Unstable alleles express themselves in a heterozygote with the recessive allele *a* but not in the presence of the dominant allele *A*. They also express themselves in the haploid state (Straub 1973) and in the homozygote *a–x*/*a–x*. In the last case, the effects of both alleles are generally additive (Cornu unpublished data).

d) Genic instability yields new allelic series for the locus involved. In the case of anthocyanin loci, it produces a range of new colors. Stable *a–n* alleles obtained from instability have generally had an intermediate functioning between the recessive allele and the original wild allele (Bianchi et al. 1978, Farcy and Cornu 1979). Until now the observed differences between these alleles are only quantitative; however, since these genes have a regulation role for other genes involved in the same biosynthesis pathway, they are able to significantly modify the equilibria of endproducts (Farcy and Cornu 1979).

The similarities between the phenomena described in *Petunia*, maize, or snapdragon suggest that the genetic events yielding genetic instability in *Petunia* may be explained by transposable "control elements" (Mc Clintock 1965). These elements would be fragments of DNA, like episomes in bacteria, able to insert themselves into the chromosome and to modify the action of neighboring genes (Peterson 1970; Fincham 1973).

In *Petunia*, Bianchi et al. (1978) suggest that the *An1* locus consists of a structural gene responsible for the production of a specific enzyme and regulatory elements used for activating the structural gene when differentiation of epidermal cells occurs. Genic instability would be the result of mutation arising at the level of these regulatory elements and of subsequent repairs which could occur by amplification of the repetitive DNA, the main component of regulatory elements. Farcy and Cornu (1979) favor the possibility of insertion DNA-segments which would insert into or near the locus. The insertion would lead to a decrease of gene expression. Instability and, therefore, the occurrence of newstable alleles, would be due to occasional excision of insertion segments. On the basis of the available results, it is not possible to choose among the two hypotheses. Molecular biology techniques will likely be necessary to understand the genetic events responsible for such instabilities.

6 Inheritance and Biochemistry of Pigments

6.1 Genetics of Flower and Pollen Colors

H. WIERING [1] and P. DE VLAMING [2]

6.1.1 Introduction

Since the introduction of *Petunia axillaris* (Lam.) B.S.P. and *P. integrifolia* (Hook.) Sch. et Th. in Europe between 1823 and 1835, crossings have been made between the two species (Bailey 1896, Ferguson and Ottley 1932). The first results of these experiments, with regard to flower color, are pictured in 1837 by both Harrison and Hooker. It is impossible to explain those results in terms of genes, although the pictures allow us to attribute some presently known alleles to them. Also, the experiments of Naudin (1858, 1865) and Hoffmann (1869), done before the rediscovery of Mendelian laws, have only historical value.

The first attempt to analyze the inheritance of flower color was made by Westgate (1911). Further experiments were executed by Lotsy (1912), Malinowski (1914a, b), Malinowski and Sachs (1916), Rasmuson (1918), Ferguson and Ottley (1932), Ferguson (1934), Störmer and von Witsch (1938), Dale (1942), and Mather and Edwardes (1943). These authors explained the inheritance of flower and pollen color without examining the pigments involved. The translation of their results to presently used alleles remains very difficult.

A new period in flower color genetics in *Petunia* began with the introduction of paper chromatography in the analysis and identification of plant pigments. The first results using this method came from Kazuo (1952) and Werckmeister (1954), who published some paper chromatographical analysis of flower pigments.

From 1958 onward many papers have been published concerning genetics in *Petunia hybrida* Hort.; about 15 papers deal with the inheritance of flower color and the occurrence of flavonoid pigments in petals and pollen. Paris and Haney (1958), Paris and Goldsmith (1959), and Paris et al. (1959) described 13 genes responsible for corolla pigmentation. Müller (1958) mentioned a gene for pollen color. Mosig (1960), Meyer (1964), and Van Wyk (1964) together reported 12 genes controlling the synthesis of flavonoid compounds. Bianchi (1960) reported

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two genes regulating the nature of the anthocyanidins in the corolla. Meynet et al. (1971) found six genes for which the influence on the phenotypic floral color and also chemical activity was described. Some of these six genes had already been mentioned by Chenault et al. (1968), but not all of them were indicated by the same symbols. The work of Meynet et al., was a continuation of work done by Dommergues and Cornu (1974), Dommergues et al. (1973); they describe 14 genes involved in the formation of flower color in *Petunia*. Vallade and Cornu (1979), members of the Meynet et al. research group and Dommergues et al., established the existence of two new genes for flower color.

Hess (1969, 1970) also mentioned some genes involved in anthocyanin synthesis in *Petunia*. Some work on the flower color genetics of *Petunia* has been done by El-Gamassy et al. (1972) and Hussein and Misiha (1978).

Wiering (1974) has described 23 genes of which 19 regulate the nature and the distribution of flavonoid compounds and 4 affect flower color without changing the flavonoids. Some of these genes are discussed in detail by Wiering and de Vlaming (1973, 1977). The collaboration between the Genetical Institute of the University of Amsterdam and the Station d'Amélioration des Plantes, Centre de Recherches de Dijon has produced a list of genes found in *Petunia* (Wiering et al. 1979 a). In this list 32 genes are mentioned which affect flower pigments, flower color, and color of the pollen. A second paper by this group (Wiering et al. 1979 b) gives the identification tests necessary to correlate flower color genes found in the Dijon station with those of the Amsterdam institute.

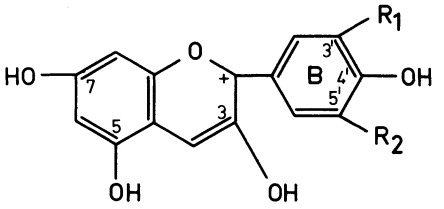
The papers of Wiering (1974) and Wiering et al. (1979 a) will be used as a basis for a detailed description of the genes involved in flavonoid synthesis and flower color in *Petunia hybrida*. The similarity between these genes and those published by other authors will also be surveyed briefly.

6.1.2 Chemical and Physical Basis of Flower Pigmentation

The first authors who analzyed flower pigments in *Petunia* were Willstätter and Burdick (1917). They found the diglucoside petunin and its aglycon petunidin. Beale et al. (1941) investigated the anthocyanins in *Petunia integrifolia*. Kazuo (1952) and Werckmeister (1954) published paper chromatographic results of their investigations on flower pigments in some *Petunia* cultivars. Birkofer and Kaiser (1962 a, b, 1963 a), and Birkofer et al. (1963 a, b, 1965 a, b) analyzed flavonoid compounds in *Petunia*; they were able to determine the chemical structure of different glycosides of flavonoids.

The color of the corolla in *Petunia* is chiefly caused by the presence of anthocyanin compounds dissolved in the vacuole of epidermal cells. Under the influence of flavonols, also present in the vacuole, the color may become modified (copigmentation). Carotenoids play only a minor role in determining ultimate floral color.

Anthocyanins consist of an aglycon – an anthocyanidin – linked with one or more sugar residues and occasionally with one or more acid groups. The most important anthocyanidins are shown in Fig. 6.1; they differ only in the degree of hy-



anthocyanidin	R_1	R_2	color *	HCC**
pelargonidin	H	H	red	ca. 22
cyanidin	OH	H	magenta	ca. 27
peonidin	OCH_3	H	magenta	ca. 27
delphinidin	OH	OH	purple	ca. 31
petunidin	OCH_3	OH	purple	ca. 31
malvidin	OCH_3	OCH_3	purple	ca. 31

Fig. 6.1. Survey of the principal anthocyanidins. *) In acid medium. **) Color according to the Horticultural Color Chart of the British Color Council

droxylation and methylation of the B-ring. All anthocyanidin pigments given here occur in *Petunia*, but pelargonidin is found only rarely and if so, it is present only in a very small quantity (see also Cornu et al. 1974).

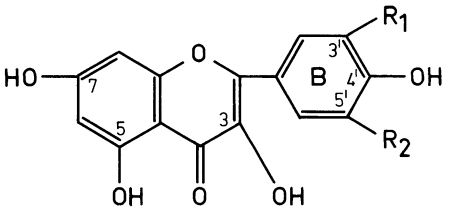
The most important glycosides of anthocyanins present in *Petunia* flowers are the 3-glucoside, the 3-rutinoside (=3-rhamnosyl-glucoside), and the 3-(p-cumaroyl)-rutinosido-5-glucoside of cyanidin and delphinidin and the 3-(p-cumaroyl)-rutinosido-5-glucoside of peonidin, petunidin, and malvidin.

The 3-(p-cumaroyl)-rutinosido-5-glucosides appear to occur in two forms which can be distinguished by their respective Rf values. Conceivably, the difference between these two acylated compounds is attributed to the position in which the acid residue is bound to the rutinose.

Some important differences in color between the various anthocyanidins found in *Petunia* appear to be caused by hydroxylation of position 5′, by the degree of glycosylation, and by acylation. Methylation of the B-ring has only a negligible influence, if any. If, as is often the case with the delphinidin-3-glucoside, most of the pigment is present as crystals, the color becomes lighter with a concomitant graying of the hue.

Flavonols occur in *Petunia hybrida* mainly as 3-diglucosides and 3-diglucosido-7-glucosides. Sometimes glucose is also found at position 4′ (Birkofer and Kaiser 1962a) or 3′ (van Wyk 1964). Birkofer and Kaiser (1962b) found kaempferol-diglucoside acylated with ferulic acid. Van Wijk (1964) reported the existence of flavonols acylated with benzoid acid; however, this observation has not been confirmed.

The most important aglycones of flavonols are shown in Fig. 6.2. Of the flavonols mentioned, myricetine is of fairly rare occurrence in *Petunia hybrida*; it is found only in some white flowering mutants. Flavonols are colorless to pale yellow and by themselves do not contribute much to the floral color. However, they



flavonol	R_1	R_2
kaempferol	H	H
quercetin	OH	H
myricetin	OH	OH

Fig. 6.2. Survey of the principal flavonols

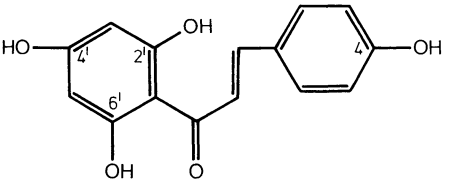


Fig. 6.3. Structure of *2',4',6',4* tetrahydroxychalcone

form complexes with the anthocyanins present, which results in a marked bluing of the anthocyanidin color (co-pigmentation). For actual floral color, the pH in the vacuoles of the epidermis cells also seems to be important: the higher the pH of homogenates of the flower limb, the bluer the color. Concomitant with this bluing a fading of color takes place.

In pollen of some inbred lines a yellow pigment can be found. This pigment has been identified by de Vlaming and Kho (1976) as 2',4',6',4-tetrahydroxychalcone (Fig. 6.3).

6.1.3 Material

Studies were started with inbred lines of commercially available cultivars and F_1-hybrids. By selection of the progenies obtained from crosses between these lines many recombinations of genes regulating floral pigmentation were obtained which eventually proved important for the genetical analyses.

6.1.4 Methods

Segregation of the various genes, to be enumerated in the following paragraphs, was studied in several thousand F_2 generations, backcrosses and additional inbred generations. Anthocyanidins and aglycones of flavonols present were identified by means of paper and cellulose thin-layer chromatography and determined semi-quantitatively. As solvents, Forestal's mixture (acetic acid: HCl 36%: water = 30:3:10) and iso-propanol-HCl (iso-propanol: 2 N HCl = 1:1), were employed. Anthocyanins were identified on silica gel G plates, using the solvent ethyl acetate: formic acid: water = 60:12:16. Spectrophotometry was also used. These methods have been described in great detail by Harborne (1958 a, b; see also Wiering and de Vlaming 1973). The pH was measured in homogenates of corolla limbs.

6.1.5 Nomenclature and Classification of the Genes

Most of the authors cited in the introduction used a single-letter code to indicate specific genes. Only Paris and Haney (1958) used a different system a one-, two- or three-letter code. Since most workers chose their code system independently, the same letter symbol was often used for different genes, especially for papers published before 1958.

In order to find a solution for these difficulties, and also to increase the number of possible code symbols to be used for the indication of color genes in *Petunia*, Wiering (1974) introduced a two-letter code, mostly followed by one figure.

In *Petunia* the following groups of floral color genes can be distinguished:

1. anthocyanin genes (symbol: *An*);
2. hydroxylation genes, composed of those controlling substitution at position 3′ (symbol: *Ht*), and those controlling substitution at positions 3′ and 5′ (symbol: *Hf*);
3. glycosilation genes, those regulating the addition of a rhamnosyl group at position 3 (symbol: *Rt*) and genes responsible for the introduction of a glucosyl group at position 5 (symbol: *Gf*);
4. methylation genes, those genes for substitution at position 3′ (symbol: *Mt*) and at positions 3′ and 5′ (symbol: *Mf*);
5. acylation genes (symbol: *Ac*);
6. flavonol genes (symbol: *Fl*);
7. genes responsible for a venation pattern on the corolla limb (symbols: *Ve* and *Fn*);
8. genes regulating the intensity of flower color (symbols: *In* and *Fa*);
9. genes regulating the pH of the vacuoles of the epidermis (symbol: *Ph*);
10. genes for pollen color (symbol: *Po*).

6.1.6 Floral Color Genes

Investigations have shown that phenotypic expression of some of the genes is restricted to sections of the corolla. The limb and the tube of the corolla must, therefore, be evaluated separately. Another distinction to be made is that between the color of the venation pattern and that of the interveinal tissue. If not indicated otherwise, the genes are expressed both in limb and tube as well as both on and between the veins, and they are also completely dominant.

6.1.6.1 Anthocyanin Genes

To date, at least eight separate genes have been found which determine whether or not anthocyanin synthesis can take place in one or more parts of the corolla. One of them is expressed only in the limb (*An2*); other genes only in the tube (*An4*, *An8*); however, most of the genes belonging to this group are also expressed both in the corolla limb and in the corolla tube. If the dominant allele of these genes

is present, normal synthesis of anthocyanins is possible in the limb and tube of the petals. As well, the synthesis of flavonols is normal in the limb and the tube.

The recessive genotypes for the *An*-genes can be described as follows:

an1an1: In this genotype there is hardly any anthocyanin synthesis; in *Hf1*-plants only weak anthocyanin venation can be found in the tube; the synthesis of the flavonols kaempferol and quercetin is normal; myricetin is never found. Bianchi et al. (1978) described several alleles of the gene *An1* which induce intermediate color intensities.

an2an2: Anthocyanin synthesis in the limb is highly inhibited; the limb is completely white or slightly suffused with anthocyanins or occasionally the anthocyanins occur in a venation pattern; the synthesis of kaempferol and quercetin is not inhibited; myricetin is found only in a few lines in small amounts. The color of the tube is not influenced by the gene *An2*. Several alleles of the gene *An2* are known with intermediate amounts of anthocyanin (Cornu 1977).

an3an3: Anthocyanin synthesis in the whole petal is strongly inhibited; the color is white or nearly white; flavonol synthesis also is strongly inhibited; myricetin is never found.

an3-1an3-1: A decreased synthesis of anthocyanins is found in the corolla tube; at the same time flavonol synthesis in the limb is reduced; this allele of *An3* was described by Wiering et al. (1979 a) as the recessive allele of the gene *An7*.

an4an4: Anthocyanin synthesis in the corolla tube is strongly inhibited; the tube is nearly white or provided with an anthocyanin containing venation pattern.

an5an5: This genotype was assigned to a completely white flowering line in which no flavonol synthesis could be detected; later on this type appears to be a variegation type in which the white color of the flower was the result of an extraordinary variegation.

Further studies with this line showed that most likely the white color was not induced by the homozygous recessive genotype of one gene, but that a number of genes was involved in the genesis of white color.

an6an6: Anthocyanin synthesis is fully inhibited; the synthesis of kaempferol and quercetin is normal; the particularity of this line is that in the limb of *Hf1*- and *Hf2*-genotypes, as also in the tube of *Hf1*-plants, a considerable amount of myricetin is synthesized; however, this amount is always lower than that of kaempferol and quercetin combined.

an7an7: An7 appears to be identical with *An3*.

an8an8: Synthesis of anthocyanins in the corolla tube is strongly reduced; no effect in the limb could be detected.

an9an9: Anthocyanin synthesis in the corolla of plants with this genotype is strongly reduced; the color is completely white if the hydroxylation genes *Hf1*, *Hf2*, and *Ht1* (*Ht2* not investigated) are homozygous recessive; if one of these

three genes is dominant, a slight synthesis of anthocyanins is possible; the synthesis of kaempferol and quercetin is normal; as in *an6an6*, this genotype has a considerable amount of myricetin if *Hf1* or *Hf2* are dominant; sometimes the amount of myricetin is greater than that of kaempferol and quercetin together.

All *An*-genes, mentioned above, show genetic complementation, i.e., crossing of any two lines homozygous recessive for two different *An*-genes gives plants that show normal anthocyanin and flavonol synthesis in the limb and the tube of the petals. The steps in pigment biosynthesis regulated by individual genes can be identified by examining the compounds accumulated since the gene of interest is homozygous recessive. In *an5an5*-plants the most primitive compound in flavonoid synthesis, i.e., p-coumaric-glucoside, is accumulated in the corolla limb. Therefore, this gene (or gene complex) is involved in one of the early steps of biosynthesis. This gene is followed by *An3*, since in *an3an3*-plants there is little or no synthesis of anthocyanins and flavonols. Next, the genes *An1* and *An2* may be expressed. It is impossible to ascertain the sequence of these two genes since in both genotypes homozygous recessive for *An1* or *An2* the synthesis of anthocyanins is reduced and that of kaempferol and quercetin is normal. The genes *An1* and *An2* are followed by *An6* and *An9*. Also, the mutual sequence of these genes is not clear. In both genotypes, *an6an6* and *an9an9*, there is practically no anthocyanin synthesis and both the synthesis of kaempferol and quercetin is normal; also both genotypes show considerable synthesis of myricetin. All genes mentioned above, with the exception of *An2*, are also active in the corolla tube. The sequence and phenotypes of the *An*-genes are summarized in Fig. 6.4.

The effect of the *An*-genes on anthocyanin synthesis in pollen is given under the heading genes for pollen color (Sect. 6.1.6.10).

Genes for limb	Phenotype	Genes for tube
	No anthocyanins No flavonols p-Coumaric-glycosides	
An5		*An5*
	No anthocyanins or anthocyanins in very small amounts No flavonols	
An3		*An3*
	No anthocyanins or anthocyanins in very small amounts or anthocyanins restricted to venation Kaempferol or quercetin	
An1, *An2*		*An1*, *An4*, *An8*
	No anthocyanins Kaempferol or quercetin in *Hf1*- and/or *Hf2*- plants: myricetin	
An6, *An9*		*An6*, *An9*
	Anthocyanins Kaempferol or quercetin	

Fig. 6.4. The *An*-genes

6.1.6.2 Hydroxylation Genes

a) Hydroxylation Genes for the 3′ and 5′ Positions of the B-ring

The most important gene is *Hf1*. If a dominant allele of this gene is present, the extract of the corolla limb yields, after hydrolysis, delphinidin, petunidin, or malvidin, only rarely together with a small quantity of cyanidin or peonidin. The extract of the corolla tube yields delphinidin or petunidin. *Hf1* has a third recessive allele *hf1-1* which is also dominant to *hf1*. Individuals possessing this allele instead of *Hf1* contain delphinidin or petunidin in the corolla tube; in the limb the effect of *hf1-1* is much weaker with only the synthesis of small quantities of delphinidin, petunidin, or malvidin being found.

In the corolla limb of individuals with the genotype *hf1hf1*, *hf1-1hf1*, or *hf1-1hf1-1*, the nature of anthocyanidins is controlled by the incompletely dominant gene *Hf2*. In the corolla tube this gene has no phenotypic expression. If the dominant allele is present at the *Hf2* locus, synthesis of delphinidin-, petunidin-, and malvidin-glycosides takes place in the limb, although in smaller quantities than in plants possessing the dominant allele of *Hf1*. The latter gene is apparently epistatic to *Hf2*.

Also, in the corolla limb of individuals which are homozygous recessive for both *Hf1*- and *Hf2*-genes the presence of small quantities of delphinidin, petunidin, and malvidin can be demonstrated, but these quantities are invariably much lower than in plants with the genotype *hf1-1hf1hf2hf2* or *hf1-1hf1-1 hf2hf2*.

b) Hydroxylation Genes for the 3′ Position of the B-ring

The genes *Ht1* and *Ht2* have a dual action. The dominant allele of these two genes stimulates the synthesis of cyanidin and peonidin, and, in addition, appears to regulate the degree of hydroxylation of the flavonols. In the corolla of plants which are homozygous recessive for these genes, kaempferol glycosides are found, but if one of these genes occurs in its dominant form, glycosides of quercetin are found instead. Both biosynthetic effects always occur simultaneously. Since the synthesis of cyanidin and peonidin by these genes is conceivably also caused by the hydroxylation of a precursor compound with a hydroxyl group at 4′ into one with hydroxyl groups at 3′ and 4′, both effects can be considered to be the result of the action of a single gene. As far as the biosynthesis of cyanidin and peonidin is concerned, *Hf1* is epistatic with respect to *Ht1* and *Ht2*. In individuals containing the dominant allele of *Hf2*, in which the colorless precursor is not totally used for the synthesis of delphinidin, petunidin, and malvidin, the genes *Ht1* and *Ht2* may give rise to the synthesis of cyanidin and peonidin. Of the two genes of this group, *Ht1* is phenotypically expressed in both the limb and the tube of the corolla; whereas, the action of *Ht2* is largely restricted to the tube and only appears in plants which are homozygous recessive for *Ht1*.

In Fig. 6.5 the biochemical effects of the hydroxylation genes *Hf1*, *Hf2*, and *Ht1* on floral pigmentation is shown diagrammatically. From the first genotype downwards an obvious increase of the amounts of delphinidin, petunidin, and malvidin with respect to cyanidin and peonidin can be noticed. The increase is as-

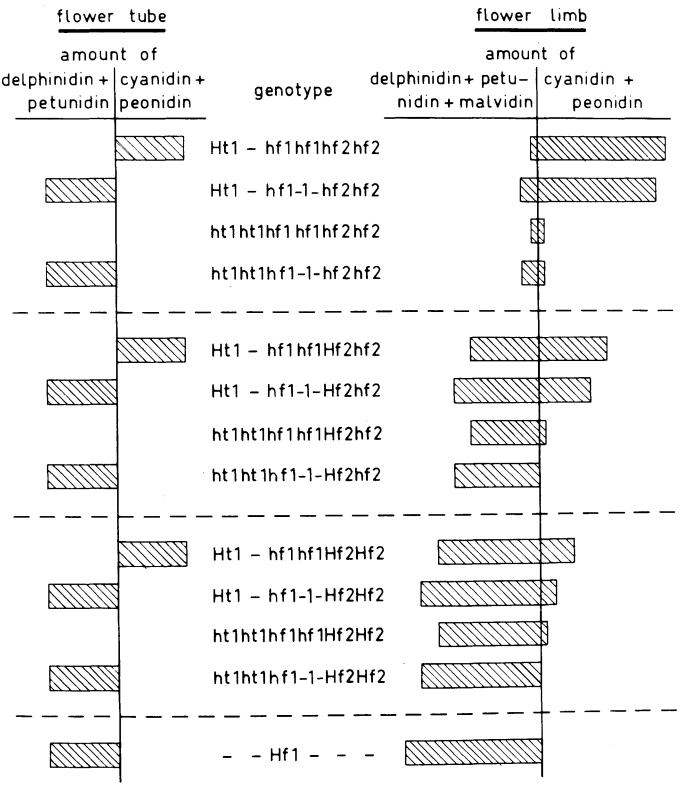


Fig. 6.5. Diagram showing the relative amounts of anthocyanidins in relation to hydroxylation genes. Genetical background *An-ht-2ht2flfl*

sociated with a bluing of the corolla. The diagram also clearly demonstrates that within the four principal groups of genes, the effect of the gene *Ht1* on anthocyanin synthesis (expressed in the intensity of color) decreases noticeably from the top to the bottom genotype.

6.1.6.3 Glycosylation Genes

The simplest glycosylation pattern of the anthocyanins is the 3-glucoside. If the dominant allele of the gene *Rt* is present, the anthocyanidins are mainly present as 3-rutinoside [=3-rhamnosyl (1 → 6) glucoside]. This change in glycosylation appears to have a slight effect on floral color. Generally speaking, the shade of the color in corollas with 3-rutinosides is a little bluer than in those with 3-glucosides. Cornu (pers. commun.) found a number of intermediate alleles of *Rt*. A considerable bluing of color occurs if the gene *Gf* is also present in its dominant form. In this case the anthocyanin pigment is almost completely present in the form of the acylated 3-rutinosido-5-glucoside, the acyl group usually being p-coumaric acid. So far it has not been ascertained whether this gene is responsible for

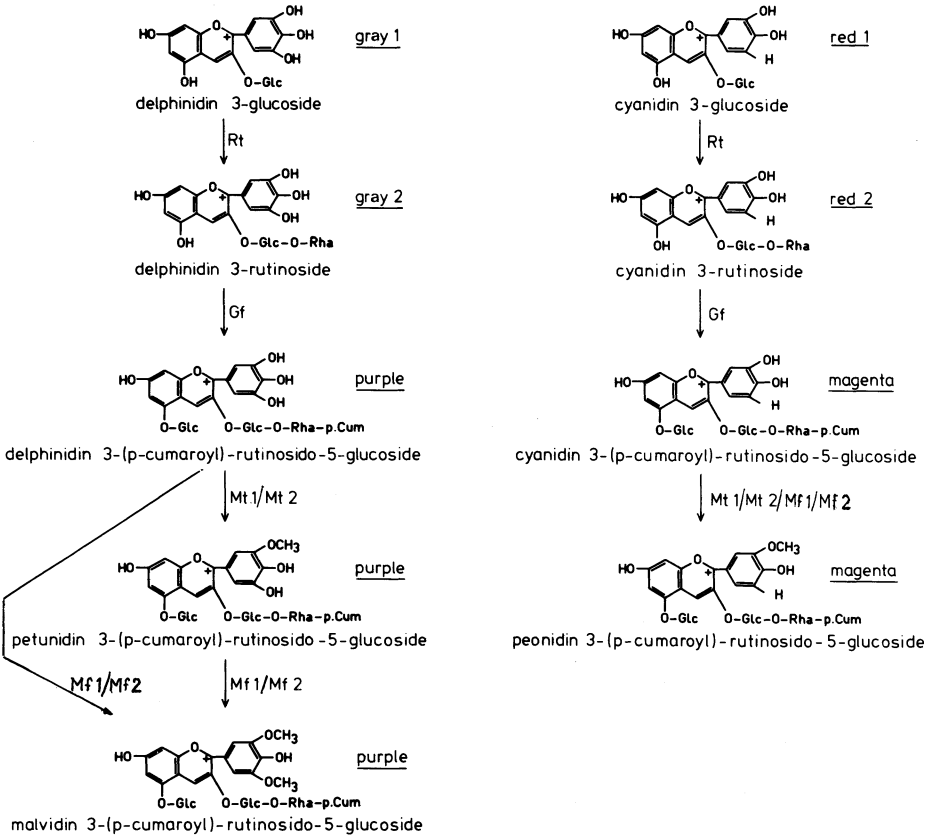


Fig. 6.6 Genes for glycosylation and methylation of the anthocyanins. The colors are restinted to the genetic background *An-flfl*. *Left* column: *Hf*1, *right* column: *hf*1*hf*1*hf*2*hf*2*Ht*1

the glycosylation at position 5, or if it controls the acylation of rutinose at position 3 (Wiering and de Vlaming 1973). In some phenotypes, in which the main anthocyanidin is cyanidin, next to the main pigment 3-glucoside, some 3-di-glucoside (3-sophoroside) is found. However, genetic control of the synthesis of 3-diglucoside could not be demonstrated.

The glycosylation genes are shown in Fig. 6.6. This diagram clearly shows that the sugar residues are coupled with the anthocyanidin molecule in a fixed sequence. It also indicates that methylation at position 3′ can only take place to an appreciable extent if the genes *Rt* and *Gf* are represented by their dominant alleles.

The flavonols occur mainly as 3-diglucosides and/or 3-diglucosido-7-glucosides. Van Wijk (1964) published some genes involved in the glycosylation of flavonols; the dominant allele of the gene *D*, which could be better renamed as *Gs*, introduces a glycosyl group at position 7.

6.1.6.4 Methylation Genes

Wiering (1974) described two duplicate genes *Mt1* and *Mt2* for the methylation at position 3′. If at least one of these two genes is represented by its dominant allele, the flower contains glycosides of peonidin instead of cyanidin, and of petunidin or malvidin instead of delphinidin. An other pair of duplicate methylation genes *Mf1* and *Mf2*, induce the substitution of a methyl group at position 5′. They can only express themselves if at least one of the genes *Mt1* or *Mt2* is represented by its dominant allele. Their action is restricted to the corolla limb. If only one of the genes *Mf1* or *Mf2* is present in the dominant form, the limb contains as its characteristic anthocyanin the 3-(p-cumaroyl)-rutinosido-5-glucoside of malvidin instead of the corresponding glycoside of petunidin. According to this theory the methylation of delphinidin to petunidin is a prerequisite for the methylation of petunidin to malvidin.

However, biochemical studies of the anthocyanin-methyltransferases revealed the existance of different methyltransferases, but the enzymes related to *Mt*-genes or *Mf*-genes were all capable of methylating derivatives of delphinidin to the corresponding malvidin-pigment (Jonsson et al. 1983 b). This makes it unlikely that the action of *Mt* is a prerequisite for the expression of *Mf*. Genetic studies have confirmed that the expression of *Mf2* is independent of *Mt2* (Jonsson et al. 1983 a). These experiments force a revision of the view of Wiering (1974): the duplicate genes *Mf1* and *Mf2* are responsible for the methylation of positions 3′ and 5′ of the anthocyanidin-molecule, while the gene *Mt2* introduces a methyl group only to position 3′ (Fig. 6.5). To explain the results of the experiments it seems not necessary to maintain the gene *Mt1*, which was very strongly linked to the gene *Mf1*.

Table 6.1 shows the effect of the various genes for hydroxylation, glycosilation and methylation of the flavonoids in the corolla in the presence of a given genotypical background. In order to simplify the survey, the gene *hf1-1* and also the genotypes heterozygous for *Hf2* are excluded. Table 6.1 shows that the genes *Hf1, Ht1, Rt*, and *Gf* can produce an almost continuous range of colours: from red (HCC about 21) to reddish purple (HCC about 729/1).

6.1.6.5 Acylation Genes

In *Petunia*, two different acylated anthocyanins occur. The first was identified by Birkofer et al. (1963 a, b, 1965 a) as 3-[4-(p-cumaroyl)-rhamnosyl (1 → 6)-glucosido]-5-glucoside. The second yields different Rf-values on paper and thin layer chromatograms. It is presumed that this difference must be attributed to a different chemical bond between the p-cumaric acid residue and the rutinosyl-group. It appears that the genetical factor regulating the second type of acylation is dominant over the first. The dominant allele of the responsible gene *Ac* causes a slight bluing. A gene *Ac* reported by van Wijk (1964), responsible for the acylation of flavonols with p-benzoic acid, remains obscure.

6.1.6.6 Flavonol Genes

As pointed out in the discussion of the anthocyanin genes, synthesis of flavonols can only occur if the genes *An5* and *An3* are represented by the dominant allele.

Table 6.1. Effect of genes for hydroxylation, glycosidation and methylation on flavonoids in flowers and on flower colour. Genetic background: *An-flflht2ht2Ph-* (*hf1-2* omitted)

Genotype[a]			Amount of characteristic anthocyanins in flower limb[b, c]	Flavonols in flower limb[b]	Anthocyanidins in flower tube	Flower color	H.C.C.
Ht1 – hf1hf1 hf2hf2	*rtrt* – –	– –	cyan3G + + +	Quercetin ±	Cyanidin	Red 1	621-021
	Rt – gfgf – –	– –	cyan3RG + + +	Quercetin ±	Cyanidin	Red 2	24/2
	Rt – Gf – mtmt	– –	cyan3AcRG5G + + +	Quercetin ±	Cyanidin	Magenta	627
	Rt – Gf – Mt –	*mfmf*	peon3AcRG5G + + +	Quercetin ±	Peonidin	Magenta	627
	Rt – Gf – –	*Mf –*	peon3AcRG5G + + +	Quercetin ±	Peonidin	Magenta	627
ht1ht1 hf1hf1 hf2hf2	*rtrt* – –	– –	cyan3G ±, del3G ±	Kaempferol ±	None	Very pale red	623/2
	Rt – gfgf – –	– –	cyan3RG ±, del3RG ±	Kaempferol ±	None	Very pale red	623/2
	Rt – Gf – mtmt	– –	cyan3AcRG5G ±, del3AcRG5G ±	Kaempferol ±	None	Very pale magenta	629/2
	Rt – Gf – Mt –	*mfmf*	peon3AcRG5G ±, pet3AcRG5G ±	Kaempferol ±	None	Very pale magenta	629/2
	Rt – Gf – –	*Mf –*	peon3AcRG5G ±, malv3AcRG5G ±	Kaempferol ±	None	Very pale magenta	629/2
Ht1 – hf1hf1 Hf2Hf2	*rtrt* – –	– –	cyan3G +, del3G + +	Quercetin ±	Cyanidin	Dull red 1	0022
	Rt – gfgf – –	– –	cyan3RG +, del3RG + +	Quercetin ±	Cyanidin	Dull red 2	025/1-027/1
	Rt – Gf – mtmt	– –	cyan3AcRG5G +, del3AcRG5G + +	Quercetin ±	Cyanidin	Bluish magenta 1	29
	Rt – Gf – Mt –	*mfmf*	peon3AcRG5G +, pet3AcRG5G + +	Quercetin ±	Peonidin	Bluish magenta 1	29
	Rt – Gf – –	*Mf –*	peon3AcRG5G +, malv3AcRG5G + +	Quercetin ±	Peonidin	Bluish magenta 1	29
ht1ht1 hf1hf1 Hf2Hf2	*rtrt* – –	– –	cyan3G ±, del3G + +	Kaempferol ±	None	Dull red 3	25/1
	Rt – gfgf – –	– –	cyan3RG ±, del3G + +	Kaempferol ±	None	Dull red 4	
	Rt – Gf – mtmt	– –	cyan3AcRG5G ±, del3AcRG5G + +	Kaempferol ±	None	Bluish magenta 2	30/1
	Rt – Gf – Mt –	*mfmf*	peon3AcRG5G ±, pet3AcRG5G + +	Kaempferol ±	None	Bluish magenta 2	30/1
	Rt – Gf – –	*Mf –*	peon3AcRG5G ±, malv3AcRG5G + +	Kaempferol ±	None	Bluish magenta 2	30/1
Ht1 – Hf1 –	*rtrt* – –	– –	del3G + + +	Quercetin ±	Delphinidin	Reddish gray 1	0023/1-025/2
	Rt – gfgf – –	– –	del3RG + + +	Quercetin ±	Delphinidin	Reddish gray 2	027/1
	Rt – Gf – mtmt	– –	del3AcRG5G + + +	Quercetin ±	Delphinidin	Reddish purple	729/1
	Rt – Gf – Mt –	*mfmf*	pet3AcRG5G + + +	Quercetin ±	Petunidin	Reddish purple	729/1
	Rt – Gf – –	*Mf –*	malv3AcRG5G + + +	Quercetin ±	Petunidin	Reddish purple	729/1
ht1ht1 Hf1 –	*rtrt* – –	– –	del3G + + +	Kaempferol ±	Delphinidin	Reddish gray 1	0023/1-025/1
	Rt – gfgf – –	– –	del3RG + + +	Kaempferol ±	Delphinidin	Reddish gray 2	027/1
	Rt – Gf – mtmt	– –	del3AcRG5G + + +	Kaempferol ±	Delphinidin	Reddish purple	729/1
	Rt – Gf – Mt –	*mfmf*	pet3AcRG5G + + +	Kaempferol ±	Petunidin	Reddish purple	729/1
	Rt – Gf – –	*Mf –*	malv3AcRG5G + + +	Kaempferol ±	Petunidin	Reddish purple	729/1

[a] *Mf –* means the dominant allele of *Mf1 or Mf2* or of both is present; *mfmf* means *Mf1* and *Mf2* both are homozygous recessive. *Mt* means the dominant allele of *Mt2* is present

[b] The notations + + +, + + and + indicate the relative amounts in which the compounds are present; ± means that only traces of the compound are present

[c] 3G = 3-glucoside, 3RG = 3-rutinoside, 3AcRG5G = acylated 3-rutinosido-5-glucoside

Table 6.2. Effect of alleles of *Fl* on amount of flavonols and on flower color. Genetic background: *An.Ht1-Gf-Ph-*

Genotype			Color group	*Fl-*		*fl-1fl-1* or *fl-1fl*		*flfl*	
				flavonols[a]	HCC	flavonols	HCC	flavonols	HCC
Hf1-	*--*	*Rt-*	Purple	++	735	±	729/1	±	729/1
Hf1-	*--*	*rtrt*	Gray	++	027/1	±	025/1	±	0023/1-025/2
hf1hf1	*Hf2Hf2*	*Rt-*	Bluish magenta	++	634	+	30/1	±	29
hf1hf1	*Hf2Hf2*	*rtrt*	Dull red	++	627/1	+	24/2	±	0022
hf1hf1	*hf2hf2*	*Rt-*	Magenta	++	629/1	++	629/1	±	627
hf1hf1	*hf2hf2*	*rtrt*	Red	++	23/2	++	23/2	±	621-021

[a] The notations ++ and + indicate the relative amount of flavonols in flower limb; ± means that only traces of flavonols are present

The synthesis of kaempferol and quercetin glycosides – the nature of the flavonol depends on the genes *Ht1* and *Ht2* – is to a large extent stimulated by the gene *Fl* for which three alleles are known. However, the synthesis of myricetin glycosides in plants homozygous recessive for the genes *An6* or *An9* is never stimulated by an allele of *Fl*. As indicated earlier, synthesis of myricetin occurs only in genotypes that are dominant for *Hf1* or *Hf2*.

In individuals with the dominant allele of *Fl*, synthesis of kaempferol and quercetin proceeds at the expense of the formation of the glycosides of both cyanidin and peonidin, and to a lesser extend those of delphinidin, petunidin, and malvidin. The allele *fl-1* is recessive with respect to *Fl*. In *fl-1fl-1-* and *fl-1fl*-genotypes, synthesis of kaempferol and quercetin competes only with that of cyanidin and peonidin glycosides. The allele *fl* is recessive with regard to the two previously mentioned alleles. In *flfl*-individuals only traces of kaempferol and quercetin glycosides occur.

The presence of kaempferol and quercetin has a dual effect on the color of flowers containing anthocyanin. In the first place, the diminished formation of anthocyanins already mentioned results in a lower intensity of the color. This dilution is not so extensive in flowers containing delphinidin, petunidin, or malvidin as it is in flowers containing cyanidin or peonidin. In flowers of the latter category, there is often a completely anthocyanin-free zone around the throat of the corolla. In the second place, the presence of appreciable quantities of these flavonols causes a bluing of the corolla color (co-pigmentation). Table 6.2 shows the effect of alleles of the gene *Fl* on floral color in several color groups.

6.1.6.7 Genes for a Venation Pattern on the Corolla

In white flowering *Petunia* sometimes a greenish or yellowish venation pattern is found; mostly it is accentuated by the presence of anthocyanins. So, a flower is formed with a more or less dark reticulation on a white or pale coloured background. The synthesis of anthocyanin in this venation only occurs in individuals which possess the dominant alleles of *An1, An3, An5, An6,* and *An9*. The venation can be assessed best in individuals which are homozygous recessive for *An2* (ac-

tive in the corolla limb) and *An4* (active in the tube). The color of the venation is decided by genes already discussed. Also, in full colored flowers a venation can be present. Often however, it is very difficult to analyze the type of venation in these flowers.

The incidence of venation in the corolla tube is controlled by the gene *Ve1*. Individuals possessing a dominant allele of this gene exhibit a close venation, whereas the homozygous recessive shows a less pronounced venation mainly consisting of parallel, longitudinal streaks. This venation pattern is especially clear in tubes without anthocyanin. The venation of the limb is regulated by three genes: *Ve2, Ve3,* and *Fn*. If the dominant allele of *Ve2* is present, limb venation is only very weakly developed; it does not extend beyond one-fourth of the distance from the corolla throat to the outer edge of the limb, measured along a midpetal vein. If *Ve2* is homozygous recessive, the pattern extends to about half or 3/4 of that distance. This degree of extension is dependent on the probably incomplete, dominant gene *Ve3*. There appears to be a marked correlation between the incidence of a stronger limb venation and the presence of a dominant allele of *An4*, which can possibly be attributed to a very strong linkage of the dominant *An4* allele and the recessive *Ve2* allele. Cornu et al. (1980) mentioned that the genes *An8* and *Ve1* could be allelic. Because more experiments have to be executed to determine this hypothesis, the two genes are considered nonallelic here.

The dominant allele of *Fn* introduces a thick venation in the corolla limb whereas the tube is brown from anthocyanin synthesis. In *fnfn*-genotypes in the limb a fine venation pattern is found whereas the tube is pale. Because the genes *Ve* are found in Amsterdam and *Fn* is found in Dijon and no crossing between the Amsterdam and Dijon genotypes has yet been done, it is impossible to state anything about an interaction between *Ve*-genes and *Fn*.

6.1.6.8 Color Intensity Genes

It became clear from our studies that an appreciable number of genes is involved in the establishment of the various degrees of color intensity distinguishable in *Petunia*. In this connection the role of the genes *Ht1, Ht2,* and *Fl* has already been pointed out. In addition, a presumably large number of genes have some influence on the intensity of pigmentation. The inquiry into the action of these intensity genes is rendered difficult by their possibly cumulative effect, so that in the progeny of crosses a gradual transition between individuals with pale flowers and specimens with deeply colored corollas can often be observed. Three genes belonging to this group are identified. The effect of the gene *In1* is uptil now, only known in red flowering plants. If the dominant allele is present, a paler color is found in the corolla limb as in *in1in1*-genotypes. So the effect of the gene *In2* is hitherto only known in purplish gray flowering plants. Here the dominant allele gives a higher intensity of the color of the corolla limb. In *in2in2*-genotypes a pale color is found. The gene *Fa* is related to a fading of the color of the corolla limb during flowering. In *Fa*-genotypes the intensity of the flower color is normal until shortly after the flower opens and fading begins. In magenta and purple flowering plants the corolla becomes nearly white. In red and gray flowering plants fading is not so strong. It seems that fading is restricted to *ph3ph3*- and *ph4ph4*-genotypes.

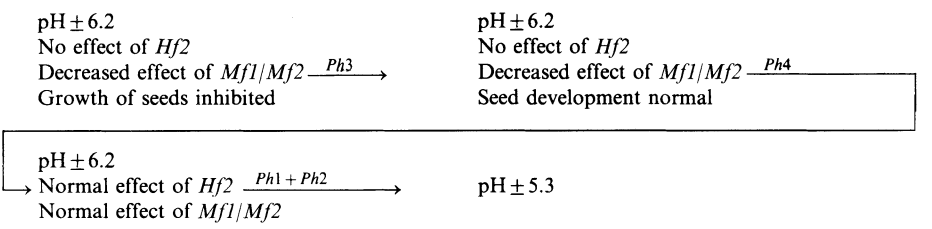


Fig. 6.7. Genes for pH

6.1.6.9 pH-Genes

The same anthocyanin may exhibit different hues owing to differences in the pH of the vacuole. Some idea of the acidity of the corolla can be obtained from pH-measurements of homogenates of the corolla limb. The values thus obtained appear to fall into one of two groups, viz., a group with a lower pH (ca. 5.3) or another with a higher value (pH ca. 6.2), which are correlated with a more reddish and a more bluish hue, respectively.

In *Petunia* at least four complementary genes play a role in determining the pH in the corolla limb (de Vlaming et al. 1983). These four can be arranged in a sequence in which the number of effects regulated by these genes decreases from left to right (Fig. 6.7). If the gene *Ph3* is represented by its homozygous recessive allele, three additional phenomena are observed apart from the raised pH value: the gene *Hf2* cannot be expressed; the synthesis of malvidin by the genes *Mf1* and *Mf2* is inhibited to some extent, and seed development ceases at an early stage. Therefore, plants with the *ph3ph3*-genotype exhibit female sterility. If the gene *Ph4* is homozygous recessive, the same effects can be observed as in *ph3ph3*-individuals with the exception of female sterility, i.e., their seed production is normal. The genes *Ph1* and *Ph2* appear only to regulate the pH. If gene *Fa* is dominant, in homozygous recessives for the genes *Ph3* or *Ph4* the color of the corolla fades appreciably upon aging (de Vlaming et al. 1982). Vallade and Cornu (1979) published the gene *Vt3*, which is also a pH gene. This gene is now named *Ph5*. According to Cornu (pers. commun.), in *ph5ph5* there is also female sterility and masking of the gene *Hf2*. Table 6.3 shows the ultimate color induced by various alleles of the flavonol and pH genes in the presence of prominent alleles of hydroxylation and glycosylation genes.

6.1.6.10 Pollen Color

The color of the pollen in *Petunia* ranges from white or yellow to blue or green (Ferguson 1934). The blue color is caused by the presence of delphinidin or petunidin glycosides and the yellow color by the presence of a chalcone (viz. 2′,4′,6′,4-tetrahydroxychalcone (de Vlaming and Kho 1976). Green-colored pollen is formed if anthocyanin is present together with the chalcone.

The synthesis of anthocyanin in pollen is brought about by genes discussed in preceding chapters; the presence of the dominant allele of the genes *An1, An3, An4, An5, An6, An9,* and *Hf1* (or the allele *hf1-1*) is needed for this synthesis. The

Table 6.3. Effect of the genes *Fl* (*fl-1* excl.) and *Ph* on flower color. Genetic background: *An-Ht1-Gf-*

Genotype			Color group	Color (HCC) in			
				Fl-Ph-[a] (middle type)	*Fl-phph* (bluish type)	*flflPh-* (reddish type)	*flflphph* (purplish type)
Hf1-	--	*Rt-*	Purple	735	738/1	729/1	733
Hf1-	--	*rtrt*	Gray	027/1	033	0023/1-025/2	033/3
hf1hf1	*Hf2Hf2*	*Rt-*	Bluish magenta	634	639	29	36/1
hf1hf1	*Hf2Hf2*	*rtrt*	Dull red	627/1	–	0022	–
hf1hf1	*hf2hf2*	*Rt-*	Magenta	629/1	640/2	627	32/1
hf1hf1	*hf2hf2*	*rtrt*	Red	23/2	437/1	621-021	0023/1

[a] *Ph-* means that the dominant allele of all pH-genes is present. *phph* means that at least one pH-gene is homozygous recessive

genes *Rt, Gf, Mt1,* and *Mt2* determine whether delphinidin glycosides or petunidin glycosides are formed. The genes *Mf* are not expressed in the pollen, so malvidin glycosides are never found. Also, the genes *Ht1* and *Ht2* do not have an effect in the pollen, so cyanidin and peonidin glycosides and quercetine are not found. Occassionally, some anthocyanin is synthesized in plants with the genotype *hf1hf1hf2hf2*; slightly more is found in *hf1hf1Hf2*-genotypes.

The yellow colored pigment 2′,4′,6′,4-tetrahydroxychalcone is found in pollen of plants homozygous recessive for the gene *Po*. In plants dominant for *Po*, the yellow pigment is not found, causing white pollen when no anthocyanin is present. The colors found in pollen are summarized in Fig. 6.8.

6.1.7 Relationships Between the Genes

Of the 32 above-mentioned genes influencing the coloration of the flower of *Petunia*, 4 regulate the pH measured in homogenates of the corolla limb, 4 decide the venation pattern on the corolla whereas the remaining 24 are all of importance in the synthesis of flavonoids in the flowers.

To bring these genes together in a single diagram, the following facts concluded from the previous chapters must be integrated:

1. In *an5an5-* and *an3an3*-plants no flavonol synthesis occurs.
2. Only in *an6an6* and *an9an9* plants does synthesis of myricetin occur; the synthesis only takes place in the presence of the dominant allele of *Hf1* or *Hf2*.

Genotype	Without anthocyanin	With anthocyanin
popo	Yellow	Green
Po-	White	Blue

Fig. 6.8. Color of pollen

3. The allele *Fl* stimulates the synthesis of kaempferol and quercetin, but not the synthesis of myricetin.
4. The substance $Z(OH)_3$ is not formed from $Z(OH)_2$ but directly from Z(OH); an important argument in favor of this assumption is that individuals possessing the dominant allele of *Hf1* can have phenotypes containing either kaempferol or quercetin.
5. As in plants homozygous recessive for *Ph3* or *Ph4*, the gene *Hf2* cannot be expressed in contrast to *Hf1*, the genes *Hf1* and *Hf2* regulate alternative pathways in the synthesis of $Z(OH)_3$ from Z(OH).
6. Methylation of anthocyanins takes place after hydroxylation and glycosylation. Kho et al. (1977) concluded from their experiments that in the synthesis of anthocyanins in *Petunia* the gene *Ht1* precedes the genes *An1* and *An2*.

All facts taken together result in the diagram given in Fig. 6.9. In this diagram only the aglycons of the anthocyanins and flavonols are given. The synthesis of the anthocyanidin-glycosides is given in Fig. 6.6. Some compounds mentioned in the diagram have been determined. Thus, compound A represents a p-coumaric acid derivative (Schram, pers. commun.). Compound B could be a flavanone or chalcone and Z(OH), $Z(OH)_2$, and $Z(OH)_3$ could represent glycosides of dihydrokaempferol, dihydroquercetin, and dihydromyricetin respectively (Kho et al. 1977). Although in *Petunia* the frequency of pelargonidin is relatively unimportant, this substance has been entered in the diagram, the supposition being that its synthetic pathway is blocked. Concurrently, the direct synthesis of myricetin from $Z(OH)_3$ also seemed blocked. However, in plants homozygous recessive for *An6* or *An9* the precursor *D* can be transformed into myricetin.

It is clear from the diagram that in two places there is competition between several genes for the same precursor. This applies to substance Z(OH) competed for by *Ht1, Ht2, Hf1, Hf2,* and *Fl*, and to $Z(OH)_2$ competed for by *Fl* and a complex of *An*-genes. The actual sequence of biochemical modification in a given case depends on the efficiency and catalytic activity of the enzymes induced by the competing genes.

6.1.8 Comparison of Genes Described by Different Authors

In Table 6.4 the genes described by other authors are compared with the genes described here. This comparison is based for the greater part on descriptions given in the literature. It appeared to be very difficult, sometimes nearly impossible, to compare genes for which the description is limited to flower color, with our mostly biochemically-defined genes. Therefore, the genes published before 1958 are omitted. We hope to give an explanation of these older genes in due time.

The identification of the genes recorded by Paris and Haney (1958), Paris and Goldsmith (1959), Paris et al. (1959) and by Mosig (1960) presents many problems. Some of the genes described by the latter were defined by Meyer (1964) and by van Wyk (1964), which enabled us to establish their identity. Sometimes it was possible to identify the genes of other authors using their material or closely related material (see notes with Table 6.4).

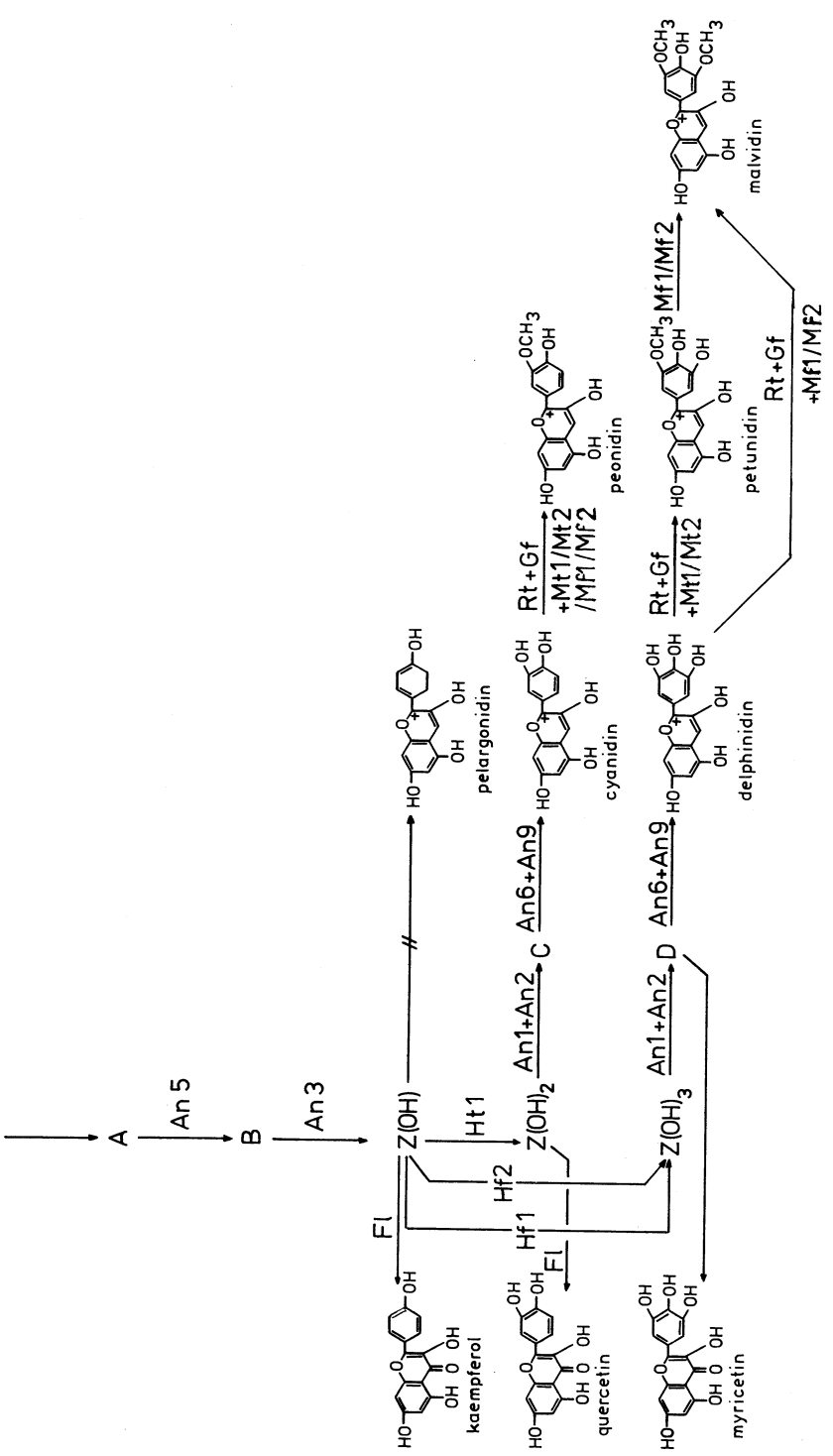


Fig. 6.9. Relation between 16 genes involved in the synthesis of flavoroids in the limb of *Petunia*

Table 6.4. Comparison of genes for flower color published since 1958

Gene code used in this monograph	Symbols used by: Paris and Haney (1958) Paris et al. (1959) Paris and Goldsmith (1959)	Müller (1958)	Bianchi (1960)	Mosig (1960)	Meyer (1964)	Van Wyk (1964)	Chenault et al. (1968)	Hess (1969, 1970)	Meynet et al. (1971)	Dommergues et al. (1973) Dommergues and Cornu (1974)	El-Gamassy et al. (1972)	Hussein and Misiha (1978)	Vallade and Cornu (1979)
An2	*Iv*+*W1*[b]			*A*			*A*	*A-1* or *A-2*	*A*	*A*[a]		*C*	
An4				*T* or *U*						P_3+P_4			
An5[d]	*W2*											*V*	
An6													*An6*
Ht1						*C*		*A-1* or *A-2*		*K*[a]			
Hf1	*Cl*+*R* part.		*O*		*M*		*D*?		P_1	P_1[a]			
Hf2	*Br*?				*L*		*C*		P_2	P_2[a]			
Rt	*S*+*R* part.		*M*		*F*		*B*		*R*	*R*[a]			
Gs						*D*							
Mf1					*K*					M_2[a]			
Fl				*B*		*B*			*B*	*B*[a]+*D*?			
Fn										*F*			
Ve3										*N*?			
In1	*In1*, *In2* or *In3*												
In2	*Dil*?												
Ph2	*B*1 or *B*2												
Ph5													*Vt3*
Po		*W*		*V* or *W*					*W*	*W*[a]			
Not identified				*C, D, E, F, G, H, K, L, M*		*Ac*				M_1	*P*		

[a] Based on identification tests (Wiering et al. 1979b)
[b] Identification of *W1* on account of our analysis of *Petunia parodii*
[c] Identification on account of analysis of material used by Hess
[d] Genetics unclear, see description 6.1.61

6.2 Biochemistry of Flavonoid Synthesis in *Petunia hybrida*

A. W. SCHRAM, L. M. V. JONSSON, and G. J. H. BENNINK [1]

6.2.1 Introduction

Anthocyanins are flower pigments accumulating in the vacuole of the epidermal cells of flowers. They have the C 15 skeleton which is characteristic of flavonoids in general.

The biosynthetic pathway of flavonoids has been thoroughly studied. The important contributions of Dr. Grisebach and Dr. Hahlbrock has to be acknowledged in this context. Results of their studies and of others have been described in excellent reviews (Hahlbrock and Grisebach 1975, 1979).

A schematic outline of the biosynthetic pathway of flavonoids is given in Fig. 6.10. The main precursor for all flavonoids is phenylalanine, which by the action of the enzyme *phenylalanine ammonia-lyase* is converted to cinnamic acid. This compound is further hydroxylated to *p*-coumaric acid. By condensation of *p*-coumaroyl-CoA with malonyl-CoA the C 15 skeleton of a chalcone is synthesized. Isomerization leads to a flavanone (naringenin, Fig. 6.11), which is hydroxylated to dihydrokaempferol, a dihydroflavonol. The colorless dihydroflavonols are the direct precursors of anthocyanins. Dihydrokaempferol, which has one hydroxyl group in the B-ring (Fig. 6.11) can in *Petunia hybrida* only rarely be converted into the corresponding anthocyanin pelargonidin (Cornu et al. 1974). Further hydroxylation of dihydrokaempferol to dihydroquercetin or dihydromyricetin with two or three hydroxylgroups in the B-ring, respectively, provides substrates for conversion to the anthocyanidins cyanidin and delphinidin. After synthesis of anthocyanidin several modifications of the molecule take place. The modification reactions occurring in *Petunia hybrida* are summarized in Fig. 6.6. After glucosylation at the 3-OH position of the anthocyanidin, a rhamnosylation of the glucose takes place. Further modifications involve an acylation with *p*-coumaric acid at the rhamnosyl group and a glucosylation at the 5-OH position. Methylation of the anthocyanin at the 3′-OH position and, in anthocyanins derived from delphinidin, at the 5′-OH position, are the final steps of these modification reactions.

Many genes controlling the biosynthesis of anthocyanins have been described. These genes are either involved in synthesis of the anthocyanin molecule or in reactions modifying this molecule. For a detailed description we refer to Chap. 6.1. The genes which are given particular attention in this chapter are summarized in Figs. 6.6 and 6.10.

In this chapter several aspects concerning the biochemistry of flavonoid biosynthesis are described with special reference to *Petunia hybrida*. We will also give attention to the current methodology in studying these aspects.

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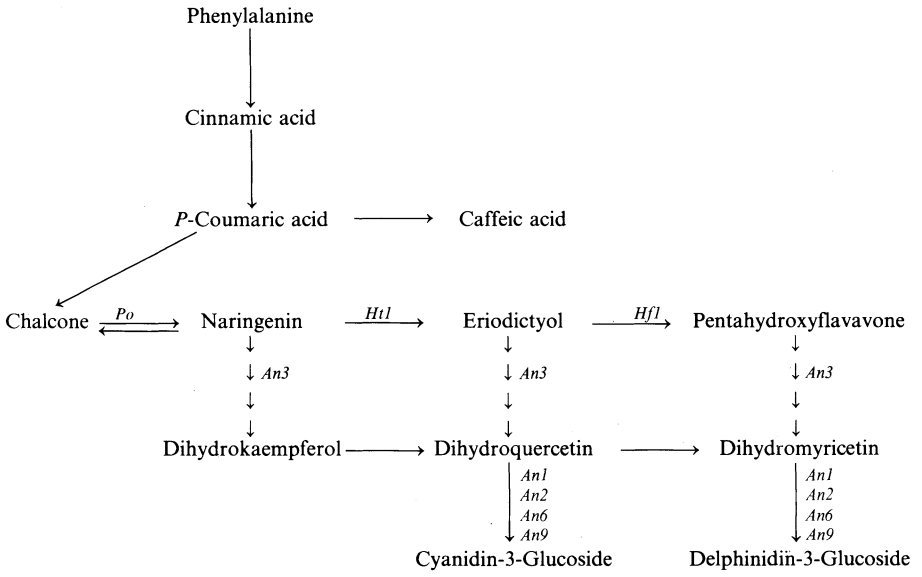
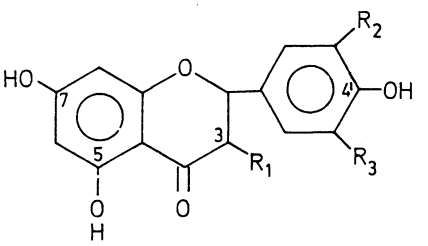


Fig. 6.10. Biosynthetic pathways of anthocyanins



Flavonoid	R_1	R_2	R_3
Naringenin	H	H	H
Eriodictyol	H	OH	H
Pentahydroxyflavanone	OH	OH	OH
Dihydrokaempferol	OH	H	H
Dihydroquercetin	OH	OH	H
Dihydromyricetin	OH	OH	OH

Fig. 6.11. Structure of flavanones and dihydroflavonols

6.2.2 Methodology

Isolation and Identification of Accumulated Precursors. When a gene involved in the biosynthesis of anthocyanins is homozygous recessive, usually the substrate of the biosynthetic reaction controlled by that gene will accumulate. The identification of accumulated precursors is therefore of great importance in the charac-

terization of the mutants. Several mutants of *Petunia hybrida* have been characterized in this way. In these experiments flower buds of mutants were extracted with acetone or methanol and the extracts were purified by means of column chromatography. The purified fractions were identified by thin layer chromatography (TLC) and by spectral analysis as described by Mabry et al. (1970). In this way an accumulation of naringenin in mutants homozygous recessive for the gene *An3* could be demonstrated (Tabak et al. 1978). Moreover, Kho et al. (1977) showed an accumulation of dihydroflavonols in mutants homozygous recessive for the genes *An1* or *An2*, whereas in mutants homozygous recessive for *"An5"* an accumulation of *p*-coumaric acid glucosides was demonstrated (Doodeman et al. 1982).

Complementation Experiments. A second approach to characterize mutants, used with *Petunia hybrida*, is the complementation technique as described by Kho et al. (1975). Methanolic extracts of flower buds of mutants homozygous recessive for one of the genes *An1, An2, An6,* or *An9* were evaporated and dissolved in a mineral medium containing 2% sucrose. By incubating limbs of flower buds of mutants of the *an3an3*-genotype in these media, anthocyanin synthesis in the limbs was obtained. In reverse experiments (flower buds from plants which were homozygous recessive for *An1* or *An2* were incubated with methanolic extracts from an *an3an3*-mutant) no anthocyanin was synthesized. In complementation experiments using mutants homozygous recessive for the genes *An1, An2, An6,* or *An9* as acceptor or as a source of substrate no combination resulted in anthocyanin synthesis. Based on these results the position of the genes *An3, An1, An2, An6,* and *An9* could be determined as shown in Fig. 6.10 (Kho et al. 1977, Gerats et al. 1982 a).

High Performance Liquid Chromatography. Cinnamic acids and flavonoids present in *Petunia hybrida* have been identified on TLC using various solvent systems (Kho et al. 1977, Tabak et al. 1978). Recent developments in chromatographic techniques made it possible to use high performance liquid chromatography (HPLC) to detect and identify these compounds. By extracting part of a flower in methanol-HCl (0.5%, v/v), within a few hours a sample can be prepared which allows a quantitative determination of flavanones, flavonols, dihydroflavonols, cinnamic acids, and anthocyanins (Schram et al. 1983). Furthermore, studies in vitro of the enzymes involved in biosynthesis of flavonoids are facilitated by the use of HPLC. The enzymatic incubation is terminated by adding chloroform-methanol (2:1, v/v) according to Folch (1957). Part of the upper phase of the partition, containing the compounds of interest, is injected into the chromatograph and quantitation of the enzymatic conversion is performed directly by measuring peak area.

6.2.3 Biosynthesis of Anthocyanins

Most of the steps in the biosynthesis of anthocyanins in *Petunia* have been established by tracer experiments, enzymological studies or genetic studies. The current information about the relation between enzymic activities and genes which

Table 6.5. Genes controlling the biosynthesis of anthocyanins in *Petunia hybrida*

Gene	Expression or supposed expression	Reference
"*An5*"	Chalcone synthase	Mol et al. (1983)
Po	Chalcone isomerase	Forkmann and Kuhn (1979), van Weely et al. (1983)
An3	Soluble hydroxylase	Forkmann et al. (1980)
Ht1	Microsomal hydroxylase	Fritsch and Grisebach (1975), Forkmann et al. (1984, pers. comm.)
Hf1	Microsomal hydroxylase	Fritsch and Grisebach (1975), Forkmann et al. (1984, pers. comm.)
An1, An2	Glucosyltransferase	Kho et al. (1978 a), Gerats et al. (1983)
An6[a]	–	Gerats et al. (1982 a)
An9[a]	–	Gerats et al. (1982 a)
Rt	Rhamnosyltransferase	Kamsteeg et al. (1980)
Gf	Acyltransferase	Jonsson et al. (1984)
Mt1, Mt2		
Mf1, Mf2	Methyltransferases	Jonsson et al. (1983 a)

[a] *An6* and *An9* are genes involved in the conversion of dihydroflavonols in anthocyanins

control a biosynthetic step is presented in Table 6.5. One has to keep in mind that some of these relations are derived from enzymological studies in other species and need confirmation in *P. hybrida*.

One enzyme, *p-coumarate:CoA ligase*, involved in very early steps of the flavonoid biosynthesis, has been extensively studied in enzyme extracts from leaves of *Petunia* (Ranjeva et al. 1975, 1976). The enzyme was partially purified and three isozymes were found. They showed differences in substrate specificity and sensitivity to inhibition by a number of phenolics. It was suggested that they are involved in different pathways of phenylpropanoid metabolism: biosynthesis of lignin, cinnamic esters, and flavonoids.

Tracer studies with radioactive substrates in flowers of *Petunia hybrida* have established that chalcones are natural intermediates in flavonoid biosynthesis (Endress 1972). However, for a long period of time enzymological studies gave no conclusive evidence whether a chalcone or a flavanone was the reaction product of condensation of *p*-coumaroyl-CoA with malonyl-CoA. It has now been demonstrated that the reaction product is a chalcone (Heller and Hahlbrock 1980). This reaction is catalyzed by the enzyme *chalcone synthase*. Thus a flavanone is the substrate for the enzymes catalyzing formation of dihydroflavonols, and the isomerization of chalcone to flavanone is an essential step in the biosynthesis of anthocyanins. In anthers of *Petunia hybrida*, the expression of the activity catalyzing this reaction is controlled by the gene *Po*, which was shown by the accumulation of chalcones in pollen of the *popo*-genotype (de Vlaming and Kho 1976). Anthers of this genotype also showed very low activity of *chalcone isomerase* (Forkmann and Kuhn 1979). However, in flower limbs of the *popo*-mutants there is no accumulation of chalcone and the isomerase activity has a wild-type level. Studies of the properties of *chalcone isomerase* indicate that the enzyme in anthers is different from the enzyme in flower limbs (van Weely et al. 1983).

Different *hydroxylation reactions* are involved in the conversion of flavanone via dihydrokaempferol to dihydroquercetin and dihydromyricetin, the substrates

for anthocyanin synthesis. In accordance with recent studies in *Haplopappus gracilis* (Fritsch and Grisebach 1975) and *Matthiola incana* (Forkmann et al. 1980) the 3-hydroxylation is believed to be a soluble enzyme and the hydroxylations at the 3′ and 5′ positions microsomal enzymes. The hydroxylation of the B-ring of dihydrokaempferol shows a complicated genetic control. The genes *Ht1* (and *Ht2*) regulate the hydroxylation of dihydrokaempferol to dihydroquercetin as shown by Tabak et al. (1978). In mutants which were homozygous recessive for the genes *Ht1*, *Hf1*, and *An1* an accumulation of dihydrokaempferol-glucosides was observed, whereas in mutants which were dominant for *Ht1* and homozygous recessive for *Hf1* and *An1* dihydroquercetin-glucosides accumulated. A similar effect of the gene *Hf1* was expected (see Fig. 6.10). However, in mutants homozygous recessive for *An1* and *Ht1* and dominant for *Hf1* no dihydromyricetin could be detected. This was explained as being caused by an inhibitory effect of hydroxylation towards dihydromyricetin by small amounts of dihydromyricetin or by a regulatory effect of *An1* on this hydroxylation. Since accumulation of dihydromyricetin was found in mutants which were dominant for *An1* and *Hf1* or *Hf2*, but unable to synthesize anthocyanins (*An6* was homozygous recessive), the latter explanation is the most probable (Tabak et al. 1981). It should be stressed that the genetic data imply that there exist at least two different 3′,5′-hydroxylating enzymes. One enzyme, related to *Ht1*, introduces one hydroxylgroup in the B-ring of the C 15 skeleton at the 3′ position and the other, related to *Hf1* introduces two hydroxylgroups, at both 3′ and 5′ positions. The hydroxylation of naringenin to eriodictyol is also controlled by *Ht1* (Tabak et al. 1978, Doodeman et al. 1982). In order to investigate at what level hydroxylation of the B-ring occurs (C 15 or C 9 level), the effect of dominant alleles of *Ht1* and *Hf1* on the hydroxylation of *p*-coumaric acid to caffeic acid has been investigated. Caffeic acid was found in flower buds of all investigated mutants of *Petunia hybrida* and the presence of this compound was not related to *Ht1* being dominant or homozygous recessive. These results confirm the notion that hydroxylation of the B-ring occurs after synthesis of the C 15 skeleton.

The mechanism of the *conversion of dihydroflavonols to anthocyanins* remains to be established. Dihydroflavonols accumulating in mutants which are unable to synthesize anthocyanins are glucosylated (at the 7-OH or the 4′-OH position) and the question arose whether this glucosylation is of any significance in the biosynthesis of anthocyanins. Feeding experiments using limbs of flower buds of anthocyanin-synthesizing mutants as acceptors and radioactively labeled malonic acid as substrate showed that *glucosylation of dihydroflavonols* occurs concomitant to the synthesis of anthocyanins (Tabak et al. 1981). Moreover, in the anthocyanin-synthesizing mutant R 27 dihydroquercetin 7-glucoside accumulates during flower bud development (Gerats et al. 1982 b). Thus it seems that glucosylation of dihydroflavonols is not the result of anthocyanin synthesis being blocked but a normally occurring event. This aspect was further investigated using flower buds of the mutant W 37 (*an3an3*). Limbs of flower buds were incubated in medium to which dihydroquercetin was added. Both anthocyanidin 3-glucosides and dihydroquercetin 7-glucoside were synthesized, their concentration increased with time of incubation for at least 48 h. In a parallel experiment, flower limbs were incubated with dihydroquercetin for 24 h and thereafter transferred to me-

dium without dihydroquercetin and incubated for another period of 24 h. No change in concentration of anthocyanin or dihydroquercetin 7-glucoside was observed after the second period of incubation. Obviously, the newly synthesized dihydroquercetin 7-glucoside could not act as a substrate for anthocyanin synthesis. Additional evidence of the inability of dihydroflavonol-glucosides to act as direct precursors for anthocyanin biosynthesis were obtained in complementation experiments with dihydroquercetin 7-glucoside and dihydroquercetin 4′-glucoside as substrates. Identification of the anthocyanins synthesized in the limbs as a result of feeding demonstrated that independently of the nature of the substrate (dihydroquercetin 7-glucoside or dihydroquercetin 4′-glucoside), cyanidin 3-glucosides were synthesized. Apparently, the dihydroquercetin-glucosides were first deglucosylated and the aglucon used as a substrate for the anthocyanin synthesis (Schram et al. 1981). This assumption was confirmed by studies in vitro on β-glucosidase activity in flower buds. These studies revealed the presence of an activity which was able to use dihydroquercetin 7-glucoside and dihydroquercetin 4′-glucoside as substrate. The enzyme had an acid pH-optimum and was a glycoprotein. More than 90% of the β-glucosidase activity was found in cell wall material, which explained the deglucosylation reactions occurring in the feeding experiments. Moreover, electrophoretic studies showed that in none of several white flowering mutants of *Petunia hybrida* investigated was the β-glucosidase activity affected, which could be expected if deglucosylation of dihydroflavonols is a necessary intermediate step of conversion to anthocyanins. We conclude that glucosylation of dihydroflavonols is not involved in anthocyanin biosynthesis (Schram et al. 1982).

Until now, four genes (*An1, An2, An6,* and *An9*) controlling the conversion of dihydroflavonols to anthocyanins have been identified. The genes *An1* and *An2* are involved in the expression of the enzyme activity which glucosylates anthocyanidin at the 3-OH position. In mutants which are homozygous recessive for *An1* or *An2* only 20% or less of the glucosyltransferase activity is present as related to other mutants (Kho et al. 1978a, Gerats et al. 1983). The remaining activity represents the same enzyme present in lesser amounts, as was shown by immunoprecipitation with antiserum directed against the glucosyltransferase (Jonsson, unpublished result).

The gene *An1* is of particular interest because of its instability in one mutant of *P. hybrida*. Series of different alleles of *An1* with increasing anthocyanin content have been described (Bianchi et al. 1978). Similar series of the gene *An2* are known (Farcy and Cornu 1979). It is interesting that the expression of *An2* in these alleles influences the expression of *Rt* and the methylating genes. In mutants with alleles of *An2* giving rise to low anthocyanin content mainly 3-glucosides of anthocyanidins are synthesized, whereas in mutants with higher anthocyanin content rhamnosylation and methylation occur. Based on these studies *An2* has been proposed to be a regulatory gene. Measurements of enzymic activity in these allelic series showed that both *An1* and *An2* control the 3-glucosyltransferase activity as well as anthocyanin-3′,5′methyltransferase activity (Gerats et al. 1984). The genes *An6* and *An9* might be involved in modification of the dihydroflavonol skeleton (removal of oxygen, introduction of the positive charge) or regulate transport of intermediates of anthocyanin synthesis in the cell. Substitutions of

anthocyanidins give rise to the anthocyanins normally present in flowers of *Petunia hybrida*. Some of these substitution reactions have been studied using protein extracts of flower buds. The activity of *UDP-glucose:Cyanidin 3-O-glucosyltransferase* was demonstrated and characterized by Kho et al. (1978 a). The enzyme glucosylates anthocyanidins as well as flavonols, is water-soluble and not bound to or enclosed by microsomal membranes (Jonsson, unpublished results).

The activity of an *UDP-glucose:anthocyanin 5-O-glucosyltransferase* was also demonstrated in vitro. This enzyme specifically glucosylates the rarely occurring substrate anthocyanidin 3-(*p*-coumaroyl)-rutinoside and is very specific with regard to the position to be glucosylated. The activity was very low in extracts from flower buds of the *an1an1*-genotype, but showed a normal level in extracts from the *gfgf*-genotype. This implies that the gene *Gf* probably controls the acylating step (Table 6.5) and illustrates the multiple effects of the gene *An1* (Jonsson et al. 1984). However, no relation was found between the gene *An2* and 5-glucosyltransferase activity.

A third modifying enzymic activity studied in vitro in *Petunia hybrida* is *SAM:anthocyanidin 3-(p-coumaroyl)-rutinoside-5-glucoside 3',5'-O-methyltransferase*. This enzyme activity appears to be very specific with regard to its substrate within the groups of anthocyanins. The methyltransferase has a high affinity (apparent $K_m \pm 20$ μM) for 3-(*p*-coumaroyl)-rutinoside-5-glucoside derivatives of anthocyanidins, but does not accept anthocyanidin aglycons or 3-glucoside and 3-rutinoside derivatives as substrate. Caffeic acid or *p*-coumaric acid are also not methylated by this enzyme (Jonsson et al. 1982). The very high substrate specificity of this methyltransferase is in good agreement with the scheme presented in Fig. 6.6, which shows that methylated anthocyanins accumulate only in mutants which are dominant for the genes *Rt* and *Gf*. A second interesting aspect is the involvement of two pairs of duplicate genes in anthocyanin methylation in *P. hybrida* (see Sect. 6.1.6.4). When one of the genes *Mt1* or *Mt2* is dominant, anthocyanins methylated at the 3'-OH position (peonidin or petunidin) accumulate, whereas methylation of the 5'-OH position (if present) occurs in flowers with one of the two genes *Mf1* or *Mf2* dominant. Each of these genes was shown to control a distinct methyltransferase. These isozymes were isolated and characterized and it appeared that the different degrees of methylation in vivo were related to different kinetic properties of the methyltransferases (Jonsson et al. 1983 b). There has been some controversy whether methylation of the B-ring of the anthocyanins occurs at the C 9 level or the C 15 level. Based on experiments with *Petunia*, Hess postulated the "cinnamic acid starter hypothesis", which implies that the nature of the B-ring is determined at the level of the cinnamic acids. The idea was supported by results from feeding experiments and studies of enzymic activity in an system in vitro (Hess 1964, 1966). More recent feeding experiments showed that methylated dihydroflavonols were converted into unmethylated anthocyanins, supporting the idea of methylation at the C 15 level (Kho et al. 1978 b). The demonstration of anthocyanin-methyltransferases gives clear-cut evidence for this idea.

Studies on methylating enzymes in soybean cell suspension cultures (Poulton et al. 1976), tulip anthers (Sütfeld and Wiermann 1978) and tobacco cell suspension cultures (Tsang and Ibrahim 1979) showed the existence of two methylating

enzymes in these systems: one which methylates cinnamic acids and might be related to lignification and another which methylates flavonoids. Possibly the enzymic activity studied by Hess was a cinnamic acid methyltransferase not related to flavonoid biosynthesis.

6.2.4 Turnover of Anthocyanins

Turnover of anthocyanins in *Petunia hybrida* was first studied by Steiner (1973). He concluded from tracer studies that these pigments are in active turnover during their accumulation period. A degradation concomitant to biosynthesis was postulated. However, contradictory results have been obtained in our laboratory (Schram et al. 1981, unpublished results). Only in mutants which are dominant for a particular gene called *Fa* ("fading") noticeable breakdown of anthocyanins was observed and only in open flowers. This was concluded from experiments using an inhibitor of biosynthesis, aminooxy acetic acid (AOA) (Amrhein et al. 1976). When limbs of flower buds are incubated in a mineral medium they synthesize anthocyanins. In the presence of AOA, this biosynthesis was completely inhibited. However, the anthocyanins already formed were not degraded; the anthocyanin concentration remained constant after 48 h of incubation. Only in limbs of mutants dominant for the gene *Fa* was degradation observed. Within 48 h the deeply colored flowers turned to nearly white flowers. This fading also occurs in vivo. The effect of the *Fa* is quite specific for the nature of the flavonoid. Flavonols or anthocyanidin 3-glucosides are not degraded and anthocyanidin 3-rutinosides slightly degraded, whereas flowers containing anthocyanidin 3-(*p*-coumaroyl)-rutinoside-5-glucosides completely fade within two to three days (de Vlaming et al. 1982). Moreover, fading occurs only in flowers that are homozygous recessive for certain pH-genes, which are correlated with an increased pH of the cell sap (de Vlaming et al. 1983). The mechanism underlying this specific pH-dependent degradation is not understood. As yet we have not been able to detect any anthocyanin-degrading enzyme in fading flowers. A further understanding of the *subcellular compartmentation of anthocyanin biosynthesis* and the role of the vacuole would be of great value in elucidating this degradation as well as the yet unexplained mechanisms of anthocyanin biosynthesis.

Subcellular compartmentation of the metabolism of phenolic compounds has been investigated in leaves of *Petunia hybrida* by Ranjeva and co-workers (1977), who used isolated chloroplasts to study the biosynthesis of chlorogenic acid and naringenin. They localized the enzymes chalcone ("flavanone") synthase and chalcone isomerase partly in the chloroplast, whereas these organelles were not able to activate the cinnamic acids to their CoA-esters. These data suggest that flavonoid biosynthesis may involve cooperation between different organelles.

It is now generally agreed that the vacuole is not involved in biosynthesis of anthocyanins. Hrazdina and co-workers (1978) studied enzymic activities in isolated vacuoles of *Hippeastrum* and *Tulipa* and showed that chalcone isomerase, chalcone synthase and anthocyanidin 3-glucosyltransferase were not present in the vacuolar fraction. Likewise, anthocyanin methylation, the very last step of an-

thocyanin modification in flowers of *Petunia hybrida*, is a non-vacuolar, "soluble" enzyme (Jonsson et al. 1983c). At present the mechanism of anthocyanin biosynthesis is far from understood. Important questions yet to be answered are where anthocyanin biosynthesis takes place, whether multienzyme complexes are involved and how the transport to the vacuole occurs.

7 Male Sterility in *Petunia*

S. IZHAR [1]

7.1 Origin and Types of Sterilities

Recently the topic of male sterility in plants was reviewed by Frankel and Galun (1977). In the present Chapter, the work on male sterility in *Petunia* will be discussed. Male sterility in *Petunia* is expressed as the lack of pollen grains in the anthers of otherwise normal flowers. The lack of pollen is a result of breakdown in microsporogenesis or gametogenesis; female fertility appears not to be affected.

Male sterility types in *Petunia* can be classified as being under nuclear or under cytoplasmic-nuclear control:

1. The first type of male sterility includes different male sterile phenotypes occurring spontaneously by selfing of plants from outcrossing populations. Many such cases have been described by *Petunia* breeders from different seed companies (pers. commun.). These phenotypes may be partially or fully male steriles. As an example, Izhar (1973) described the occurrence of cell budding and other abnormal divisions in young microspores of several highly inbred lines of *Petunia*. No viable pollen grains were produced as a result of these divisions. Other cases of abnormal development leading to partial sterility are those of multispores resulting from each pollen mother cell instead of the normal four in a tetrad (Izhar, unpublished).

Genetically, these sterilities may be caused by accumulation of recessive deleterious alleles as a result of homozygosity. An indication of that effect comes from the fact that fertility is restored immediately when these male steriles are crossed with normal lines. In some advanced, inbred lines, even female sterility can be observed. This type of sterility is not useful in practice for hybrid seed production because of its instability, and because of the female sterility and association with general weakness of the plant.

2. The second type of male sterility consists of genic male steriles (gms) in which certain defined alleles were recognized as controlling male sterility. The first case of monogenic male sterility was described by Frankel (1962, 1971). A male-sterile plant appeared in a F_2 population of plants derived from selfing of a fertile scion grafted on a cytoplasmic male sterile (cms) stock. This case will be discussed in detail later in this chapter.

Welzel (1954) reported on male sterility previously obtained by Straub in 1948 using X-rays. This sterility was found to be controlled by two pairs of recessive

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alleles, ms_1 and ms_2. A less defined case of sterility alleles in *Petunia* was reported by Singh (1974, 1975). This sterility mutant was obtained after mutagenic treatment with ethyl methane sulfonate or γ-irradiation. According to Singh (1975), genetic analysis did not reveal the number of genes involved.

Still another type of male sterility was reported by Levan (1942) who described a gene causing no release of microspores from tetrads in what is referred to as *P. nyctaginifolia*. Of the different cases described, Izhar (unpublished) made a test for allelism between the single recessive gene (Frankel 1971) and those genes reported by Singh (1974, 1975). Frankel's gene was found stable and independent of Singh's genes which could not be traced further after the first cross.

3. The third type of male sterility is cytoplasmic-nuclear. This sterility type will be referred to in this text as cytoplasmic male sterility (cms).

Sterility is conditioned by interaction between (S) plasmon and the proper nonrestorer alleles in the nuclear genome. Duvick (1959) described the attempts to obtain cms in *Petunia* by Everett and Gabelman as "Perhaps the best illustration of purposive plant breeding to produce cms". According to Everett (cited by Duvick 1959 and Frankel 1964), they used commercial varieties of *P. hybrida* as the male parent plant and one of the following species, *P. axillaris, P. integrifolia,* or *P. parodii* as female. After repeated backcrosses, cms was transferred to many lines. Apparently, a written record of the original crosses was either not made or lost. Thus, it is not known today which species contributed the (S) plasmon. Later, Bianchi (1963) obtained cms in *Petunia* by crossing a selection of *P. axillaris* as female plant with a line of *P. hybrida*. This work pointed toward *P. axillaris* cytoplasm as the origin for (S) plasmon. The presence of ample restorer genes in *P. axillaris* as reported by Izhar (1978), Izhar and Power (1979), and Izhar and Tabib (1980), also favors *P. axillaris* as the possible source of (S) plasmon. However, in contrast to Bianchi (1963), repeated backcrosses of a *P. hybrida* line on a line of *P. axillaris* as female did not produce cms (Izhar and Power 1979). It is thus possible that the species *P. axillaris* indeed consists of lines with normal plasmon and others with (S) plasmon.

Izhar and Frankel (1976) attempted to determine whether cms in *Petunia* is conditioned by a single (S) plasmon or whether there are more than one as in maize (Duvick 1965), or *Nicotiana* (Gerstel 1980). Comparative study of several cms sources obtained from different *Petunia* geneticists, using restorer genes strongly indicated that, unlike maize or *Nicotiana*, there is only a single (S) plasmon in *Petunia* (Izhar and Frankel 1976).

7.2 Cytological Aspects of Male Sterility

Cytological and anatomical descriptions of microsporogenesis and gametogenesis in male-sterile anthers were used by different investigators to determine the type of sterilities and possibly to obtain some clues on the mechanisms which cause this phenomenon (Laser and Lersten 1972). Characterization of male sterility in certain species according to the breakdown time of microsporogenesis or

gametogenesis using light microscopy is probably the most convenient way available to define types of sterilities. Such information may also be helpful in studying the genetic control of male sterility. Another important aspect concerning normal development as compared with abnormal development of pollen grains leading to sterility is the relationship between the sporogenic and the tapetal tissue. Welzel (1954) was the first to describe a case of postmeiotic breakdown in a genic male-sterile mutant in *Petunia*. Breakdown occurred during the stage of young microspores; the tapetal tissue was intact and of periplasmodial type secreting some greasy droplets into the anther locule.

Singh (1975) described abnormal tapetum development in *Petunia* in genic male sterile lines. The tapetum was of periplasmodial type and persisted longer in sterile anthers than in normal ones.

The first comprehensive account of the cytological changes of cms *Petunia* was given by Hamilton (1962, 1965) who showed clearly that in addition to the (S) plasmon, restorer genes expression is affected by temperature. Hamilton described his cms lines as quite stable, with breakdown at postmeiotic stages. However, one of the restorer lines (a cms line with nuclear restorers) which showed fertility at 21 °C, was completely sterile at 30 °C. Furthermore, Hamilton suggested that floral buds are sensitive to the influence of temperature at specific developmental stages (during meiosis). Hamilton (1965) described postmeiotic abnormalities apparently caused by events during meiosis: "Quintets" or "sextets" or microspores instead of the normal "tetrads" were caused, according to him, by "delayed" meiosis. He also reported "polyploidy" in meiocytes and microspores, and speculated that this polyploidy occurred during the last premeiotic mitosis as a result of the sensitivity of his restored cms line to high temperature (30 °C).

Izhar and Frankel (1971) described in detail two types of breakdown in the microsporogenesis and gametogenesis in *Petunia*. The normal development of the tapetal tissue in *Petunia* is described by these researchers as follows: "the tapetum in *Petunia* consists of one layer of elongated cells enveloping the meiocytes. The volume of the tapetal cells at prophase I is approximately that of the pmcs. At the tetrad stage, the tapetum cells attain their maximum volume, which is about twice that of the tetrads. Toward the end of the tetrad stage the tapetum cells are drastically reduced in size (apparently due to secretion) and remain so until maturation of the pollen grains."

Izhar and Frankel (1971) described, in comparison to the normal pattern of development, the "breakdown of tapetal cells corresponding to the breakdown of the meiocytes at prophase I"; this was in a cms line in which breakdown of the microsporogenesis occurred at early meiosis. In another plasmotype, described by Izhar and Frankel (1971), normal tetrads were produced at the end of meiosis "but there was no digestion of the callose envelope and consequently no release of microspores. At a stage in which mature pollen grains are achieved in the normal type, a complete destruction of the sporogenic tissue was observed in this gms type. The tapetum cells at the tetrad stage continued to enlarge and proliferate and eventually filled most of the anther locule." In that paper, Izhar and Frankel (1971) described the breakdown of the microsporogenesis in a gms line at the end of the tetrad stage.

It is interesting to note that the male sterility genes in *Petunia* show post-meiotic breakdown (Welzel 1954; Singh 1975; Izhar and Frankel 1971) while in cms with no restorers, the breakdown occurs at early meiosis (Izhar and Frankel 1971).

7.3 Genetics and Restorer Genetics of Cytoplasmic Male Sterility

The presence of male fertility restoration (mfr) alleles in *Petunia* was indicated in the work of several investigators in the past. However, this fact was not always recognized by the investigators themselves. The first indication of the presence of restorer alleles could be noted in the work of Everett and Gabelman who made the first crosses (cited by Duvick 1959) to obtain cms in *Petunia*. Everett and Gabelman reported that only certain segregants in the first generations carried mfr alleles but were discarded. Other indications of restorer alleles came from commercial breeders, who reported fertility restoration in cms of *Petunia* (Duvick 1959; Goldsmith 1968). Studies of the genetics and other aspects of fertility restoration started shortly after cms in *Petunia* became known about 1949 by Ferwerda and Lange in Holland (Ferwerda 1963), and later by van Marrewijk (1968, 1969), Hamilton (1962, 1965), and Edwardson and Warmke (1967) in the USA. Recently, Izhar and Frankel (1976) and Izhar (1977, 1978) published their further findings on the restorer genetics in *Petunia*.

A single dominant restorer allele was discovered by Edwardson and Warmke (1967) in a population of cms plants grown under low temperature conditions. When tested, this allele segregated 3 : 1 and fully restored fertility in cms plants. This gene was tested later by Izhar (1978), who confirmed the results of Edwardson and Warmke (1967). Van Marrewijk (1969) suggested that the monogenic restorer allele of Edwardson and Warmke restored fertility in a nonsensitive (S) plasmon or that additional criptomeric restorer alleles existed in their cms lines. Both explanations are not accepted in light of the results reported by Izhar (1978).

Van Marrewijk (1969) defined three different loci which restore fertility under different temperature regimes. A single dominant factor (Rf_1) restored fertility in plants grown under 12°–15 °C; however, Rf_1rf_1 was not completely fertile at higher temperatures (18°–21 °C). Only the homozygote $Rf_1Rf_1rf_2rf_2$ type was fully restored at 21 °C. An additional gene or complex of genes Rf_3 was needed to confer fertility to plants grown at temperatures over 24 °C. Van Marrewijk (1969) described an interaction among (S) plasmon, restorer alleles and temperature; similarly, Hamilton (1965) described an interaction among (S) plasmon, restorer alleles and temperature. Hamilton (1965) compared two restorer lines which were differentially effective, as pollinators, at 21° and 30 °C, but did not provide any genetic data concerning inheritance of the restorer genes.

A comprehensive study of the restoration gene systems in *Petunia* was conducted by Izhar (1977, 1978). Restorer genes, including the multigenic systems, were used to test the different sources of cytoplasms (Izhar and Frankel 1976).

In this study, it was shown that the multigenic system as well as the single dominant restorer allele can fully restore fertility in lines with (S) plasmon and no restorers. The basis for the genetic studies on the multigenic restorer system was the early cytological observations on variation in the time of breakdown in microsporogenesis of different cms types (Izhar and Frankel 1971). Selection for genetically stable lines with defined breakdown timing was carried out by Izhar (1977). In this selection pairs of isonuclear lines were obtained which include a cms line and its normal maintainer line carrying a number of mfr alleles. Following is the description of the stable cms types with the specific breakdown time (taken from Izhar 1977). These lines were isolated from the open-pollinated commercial variety Rosy Morn.

1. Rosy Morn (RM) cms-1. A prophase breakdown type, meiosis is initiated as in the normal type but development ceases at prophase I.
2. Rosy Morn (RM) cms-2. A tetrad breakdown type, meiocytes develop normally to the tetrad stage but no further development takes place.
3. Rosy Morn (RM) cms-3. A microspore breakdown type, microspores do not reach the first pollen mitosis.
4. Rosy Morn (RM) cms-4. A partial male-fertile, degeneration of certain portions of the microspores occurs at stages before or after first pollen mitosis.

In Table 7.1, Izhar presents data which describes the quantitative (additive) nature of the mfr alleles. The quantitative nature of the mfr alleles is also demonstrated by the interaction of the mfr alleles and environmental conditions. The data in Table 7.2 show that (S) plasmon is stable while the presence of mfr alleles conferred temperature sensitivity (see also Izhar 1975, 1977).

Genetic analysis of the mfr system (Izhar 1977, 1978) produced evidence that a minimum of three different loci is involved in restoring fertility. However, restoration is brought about by a series of multiple genes with no dominance or major effects. When enough of these alleles are accumulated, full fertility may be restored. Conversely, the presence of only some of the restorer alleles causes breakdown in microsporogenesis at different stages.

Table 7.1. Breakdown stages of microsporogenesis in F_1 of crosses between different RM cms types and the maintainer lines for other RM cms types. (Izhar 1977)

RM cms type × maintainer	Breakdown stage in F_1
RM cms-1 × maintainer of RM cms-2	Meiosis and tetrads
RM cms-1 × maintainer of RM cms-3	Tetrads and young microspores
RM cms-1 × maintainer of RM cms-4	Microspores
RM cms-2 × maintainer of RM cms-1	Meiosis and tetrads
RM cms-2 × maintainer of RM cms-3	Tetrads and young microspores
RM cms-2 × maintainer of RM cms-4	Microspores
RM cms-3 × maintainer of RM cms-1	Tetrads
RM cms-3 × maintainer of RM cms-2	Tetrads and young microspores
RM cms-3 × maintainer of RM cms-4	Microspores and some pollen
RM cms-4 × maintainer of RM cms-1	Tetrads and young microspores
RM cms-4 × maintainer of RM cms-2	Microspores
RM cms-4 × maintainer of RM cms-3	Microspores and some pollen

Table 7.2. The phenotypic expression of the different RM cms types under different environmental conditions. (Izhar 1977)

	Summer-like conditions	Winter-like conditions
RM cms-1	Prophase	Prophase
RM cms-2	Tetrads	Microspores
RM cms-3	Microspores	Partial fertile
RM cms-4	Partial fertile	Fertile

Izhar (1978) compared the different sources of the fertility restorers previously mentioned. This comparison also included tests for allelism and possible epistasis or complementation between restorer alleles from the different sources described above and from other sources. In this study, Izhar (1978) used the cms lines which were well defined with respect to their genetics, cytology, and interactions with temperature. The assumption was made that a common basis of cms lines is needed in order to compare the different restorers and to study their mode of inheritance and the interaction between them.

Some interesting conclusions were drawn from this study. When the restorer lines used by van Marrewijk (1969) were tested against the RM cms-1, Izhar (1978) did not observe sterility at low temperature as reported by van Marrewijk. The two restorer lines used by van Marrewijk were found to contain restorer alleles similar to those of Rosy Morn; no complementation was observed in crosses between the two groups. On the other hand, mfr alleles, not allelic to those of the Rosy Morn, were found in a cultivar called Red Satin and in a line of *P. axillaris* described also by Izhar and Power (1979).

On the basis of the studies described above, we may summarize that in *Petunia*, fertility restoration is conditioned by two genic systems. One is governed by a single dominant allele (Edwardson and Warmke 1967) and the other by a series of multiple genes of quantitative nature. The expression of the restorer alleles depends on the presence of (S) plasmon. Izhar and Frankel (1976) and Izhar (1977) showed that no pleiotropic effect could be attributed to the restorer alleles; however, there were some indications of a specific interaction between the mfr alleles in the haploid phase and the (S) plasmon. Restored plants with (S) plasmon gave a higher proportion than expected of gametes with restorer alleles than with no restorer alleles (Izhar 1978).

7.4 Physiological and Biochemical Aspects of Cytoplasmic Male Sterility

Early light microscope observations on microsporogenesis in cms anthers in *Petunia* showed the disappearance of callose from the meiocyte walls (Frankel et al. 1969). Callose deposition in the meiocyte walls appears to be a prerequisite for normal microsporogenesis (Eschrich 1964; Heslop-Harrison 1964). Prior to the end of the tetrad stage, callose is degraded by callase (1–3 β glucanase) and the

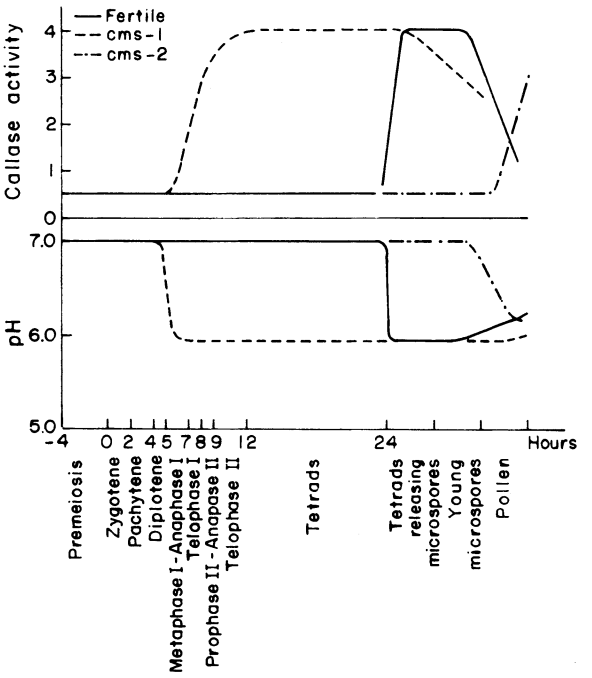


Fig. 7.1. The activity of callase and changes of pH in anthers of fertile and two types of cms during microsporogenesis. (After Izhar and Frankel 1971). The time scale of the meiotic states in hours is after Izhar and Frankel (1973 a)

four microspores of the tetrad are released into the anther locule (Eschrich 1964). Using several cms lines and a gms line of *Petunia* showing different times of breakdown, Izhar and Frankel (1971) showed that the phase-specific activity of callase varies in the different sterile types according to the breakdown time as observed by light microscope.

Figure 7.1 provides a description of callase activity in one normal and two cms types. In normal microsporogenesis, the activity rises prior to digestion of the tetrad walls by the enzyme. The activity of the enzyme is phase specific. In cms type-1, in which breakdown occurs at early prophase, the activity of the enzyme rises prior to the microscopically observed breakdown. In cms type-2, no activity of the enzyme is observed and no digestion of the callose walls in the tetrads takes place. These data demonstrate that precocious or delayed digestion of the callose wall as compared with the normal digestion time, is detrimental and may be directly associated with the breakdown in microsporogenesis and gametogenesis. In further studies, it was shown (Fig. 7.1) that prior to the rise in callase activity at the end of the tetrad stage in normal anthers, the pH within the anther locule decreases to less than pH 6. Change in the pH of normal anthers during microsporogenesis was observed in *Petunia* by Welzel (1954), who did not report any difference between normal and male sterile anthers. In cms type-2, lack of callase activity was associated with the pH remaining high, while in cms type-1 the pH dropped before callase activity, apparently during early meiosis. Thus, the data presented in Fig. 7.1 suggest that the activity of callase, which is pH-dependent, is associated with a specific pH-level, perhaps regulated by it or by a specific factor associated with it.

In further studies, Izhar and Frankel (1973) found changes in the profile of specific free amino acids between normal and male sterile anthers. At premeiosis the profiles of free amino acids were similar in the normal and the various cms types; deviation from the normal profile was apparent at an early meiotic stage in both cms type-1 and type-2. Specific changes were characteristic to cms type-1 and different from cms type-2. An early drastic rise in asparagine in cms type-1 may explain the early drop in pH in the anther locule (Izhar and Frankel 1973). Accumulation of glutamine in anthers of cms type-2 may explain the stability of the pH for a long period, in anthers of cms type-2. Titration of asparagine into normal anther homogenate caused a drop in the pH in the same way as changes in the anther pH in vivo occurred. On the other hand, titration of glutamine into anther homogenate did not cause a change in the pH (Izhar and Frankel 1973). These data strongly indicate the association between the balance of free amino acids, the pH changes and the enzyme activity in the anthers.

On the assumption that changes in the free amino acid profiles indicate deviation from normal development, it appears that the breakdown in cms anthers is initiated at early meiosis, regardless of whether the cytological expression of the breakdown is at early prophase, at the end of the tetrad stage, or later. In another study, Izhar (1975) showed that early meiosis is indeed the critical stage at which microsporogenesis in cms anthers is affected by temperature (35 °C day/15 °C night). In contrast to partial fertility in certain cms types under a 23 °C day/15° night regime, complete sterility resulted when buds at pre-meiosis of these types were exposed to high temperature. When buds of the same plants but of later developmental stages were exposed to the high temperatures (35 °C day/15° night) no effect was observed on the development of pollen in anthers. Thus, it seems that the premeiotic stage just preceding the onset of meiosis is the sensitive stage at which the genetic factor as well as the environmental factors (such as temperature) operate.

These data favor the idea that the role of the sporophytic (tapetum) tissue may be critical in controlling microsporogenesis and the breakdown mechanisms. Early breakdown as described seems to depend on the tapetum tissue secreting certain materials, including the enzyme callase. On the other hand, post-meiotic breakdown is associated with periplasmodial (nonsecreting) tapetum. However, the effect of temperature (Izhar 1975) on anthers at a certain stage does not resolve the question of whether the primary effect of temperatures is on the sporogenic tissue or the tapetum tissue. This is one of the critical questions which must be answered in order for us to understand the mechanism of male sterility.

Many studies dealing with possible mechanisms of male sterility in different species suggest that at a certain developmental point some sort of disharmony exists between the sporogenic tissue and the sporophytic tissue surrounding it. Whether it is the tapetum itself or other tissues of the sporophyte (Vasil 1967; Laser and Lersten 1972; Mascarenhas 1975; Singh 1975; Frankel and Galun 1977), the general tendency is to assume that the primary cause of the breakdown comes from the tapetum. Mascarenhas (1975), in a thorough review of microsporogenesis, concluded on the basis of callase activity in normal microsporogenesis (Mepham and Lane 1969; Stieglitz and Stern 1973) – "that the tapetum and other somatic tissues of the anther do control microspore development." Al-

though that is perhaps the obvious conclusion to draw, it is our opinion that it may be premature. It is highly possible that the primary event leading to the breakdown originates in the sporogenic tissue and only then are the observed reactions triggered in the tapetum. Additional attempts to study the ultrastructure of normal and cms microsporogenesis may yield information about the initial events leading to male sterility.

7.5 Asexual Transmission of Cytoplasmic Male Sterility

One of the reasons *Petunia* is becoming a model plant for somatic hybridization studies is the fact that cms was shown to be a very convenient extranuclear marker after it had been successfully used in asexual transmission via grafting (Frankel 1956; Edwardson and Corbet 1961; Bianchi 1963) and more recently by protoplast fusion (Izhar and Power 1979; Izhar and Tabib 1980).

7.5.1 Grafting Experiments

The original grafting experiment was carried out by Frankel (1956) with a fertile scion on a cms stock of *Petunia*. The author stated that the main reason for carrying out this experiment was that a "significant gap thus exists in our knowledge about hereditary elements of the cytoplasm." By selfing flowers of the fertile scion, Frankel (1956) obtained several cms segregants in the F_1. The cms progenies showed stable sterility which has been consistently cms until the present (over 40 generations). In further experiments, induction of nuclear-type male sterility exhibiting inheritance of a monogenetic recessive allele was demonstrated (Frankel 1962). Frankel (1971) proposed that the behavior of the cms factor in *Petunia* in the grafting experiments is best described by an analogy to the episomes in bacteria which can be inserted into the chromosome, "integrated state," or stay in the cytoplasm "autonomous state." Frankel proposed mutual exclusion of the "integrated state" by the "autonomous state" and vice versa. When crosses were made between two scions the "integrated state" prevailed. However, after the induction of cms in the F_1 segregants, it was not possible to restore fertility by the nuclear fertility allele (*E*).

Edwardson and Corbett (1961) also described a successful attempt to transmit cms in *Petunia* from cms stock to the progeny of the scion. Their results differ slightly from Frankel's in that they obtained only partially sterile plants in the F_1 and only in the F_2 were complete cms segregants obtained. These workers attributed the cms factor to a virus, although Edwardson subsequently (1968) negated this explanation. In another experiment, Corbett and Edwardson (1964) successfully transmitted cms in a sandwich graft consisting of *Petunia*-tobacco and *Petunia* scion.

Bianchi (1963) also demonstrated transmission of cms in *Petunia* by grafting. His results differed from those of Frankel, mainly by the presence of partially sterile plants in the F_1.

Some important aspects which were evident in all the grafting experiments described above were (a) the autonomy of the fertile scion itself which remained fertile throughout the life time of the graft combination; and (b) sterility in the F_1 was realized only when both the female and the male gametes came from grafted scions. Sterility was not realized in progenies when pollen for crosses with the grafted scion flowers was obtained from nongrafted flowers.

In addition to the successful experiments described above, some other authors reported failure in achieving transmission of cms by grafting in *Nicotiana* (Sand 1960).

Other successful experiments to transmit cms in *Petunia* were carried out by Hope (1979), who was able to realize graft-induced cms in the fertile scion. This finding is in contrast to the results reported by Frankel (1956, 1962, 1971), Edwardson and Corbett (1961) and Bianchi (1963). Hope's fertile line which served as scion in his graft was later used in somatic hybridization studies by Izhar (later in this chapter) and in grafting experiments. Izhar (unpublished data) did not observe sterility in the scion although cms was transmitted to the F_1 generation.

7.5.2 Protoplast Fusion

This technique was used to create heterozygosity in cytoplasmic factors (cms vs. normal) in order to study the nature and the inheritance of the extranuclear elements which cause male sterility.

In a first report, Izhar and Power (1979) described the transmission of cms in *Petunia* from the protoplasts of cms to protoplasts of a normal line by fusion. In this case, cms was unilaterally transferred into protoplasts of a line of *P. axillaris*. Because Izhar and Power (1979) were not aware of the presence of the restorer allele in the line of *P. axillaris* used, cms segregants were discovered only in the F_2 of plants regenerated from the fused protoplasts. In this case there was no indication of a possible heteroplasmic state regarding cms vs. normal elements in the somatic hybrid plants. A total of six plants out of 140 plants (Fig. 7.2) were cytoplasmic hybrids with (S) plasmon and the diploid genome of the other parental line 2785 (*P. axillaris*).

In the fusion experiments carried out by Izhar and Power (1979) on 28 January 1976 and on 6 February 1976, Izhar and Tabib (1980) reported additional types of cybrids. The data described in the above two papers are presented schematically in Fig. 7.2. Cybrids composed of either of the parental genomes (diploid) and a cytoplasm with mixed plasmons [(S) plasmon and (N) plasmon] were observed. The first one is a single heteroplasmic cybrid with the genome of the parental cms, line 2426. Cytoplasmic segregation of (S) plasmon and (N) plasmon was demonstrated by the two homoplasmic cms segregants, the 13 fertile homoplasmic fertile segregants, and the 14 fertile heteroplasmic segregants (Fig. 7.2). However, the cytoplasmic segregation of (S) plasmon and (N) plasmon was not complete after the first meiosis, and 14 plants continued to segregate even after the second meiotic cycle. This report is the first one, and so far the only one, to show the heteroplasmic state persisting for more than two meiotic cycles. These data indicate that cms in *Petunia* is different from that described by some inves-

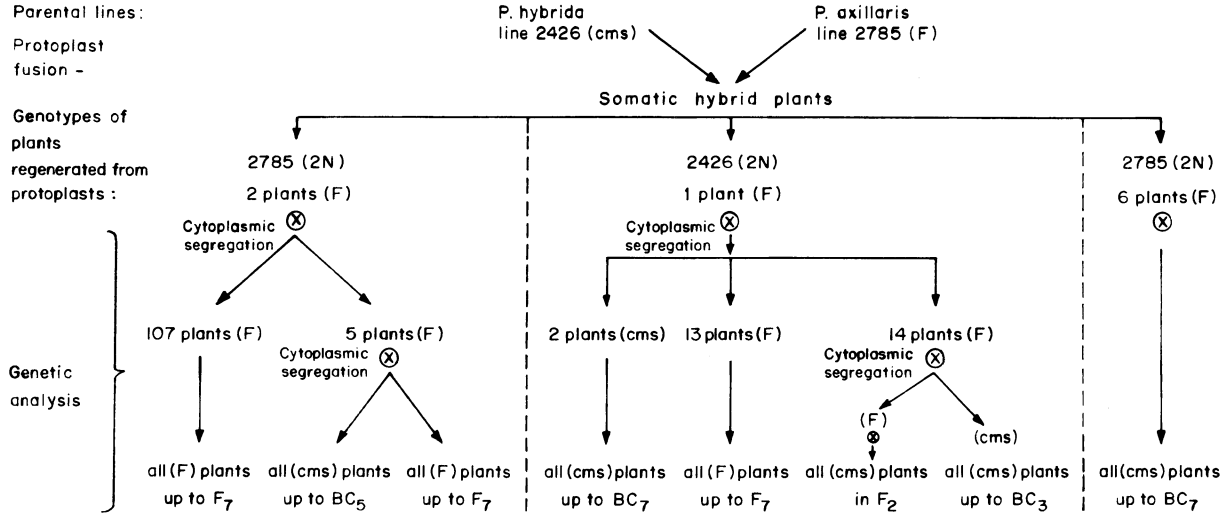


Fig. 7.2. Cybrids obtained by fusion of protoplasts of cms line 2426 *(P. hybrida)* and normal line 2785 (*P. axillaris*), based on data by Izhar and Power (1979) and Izhar and Tabib (1980)

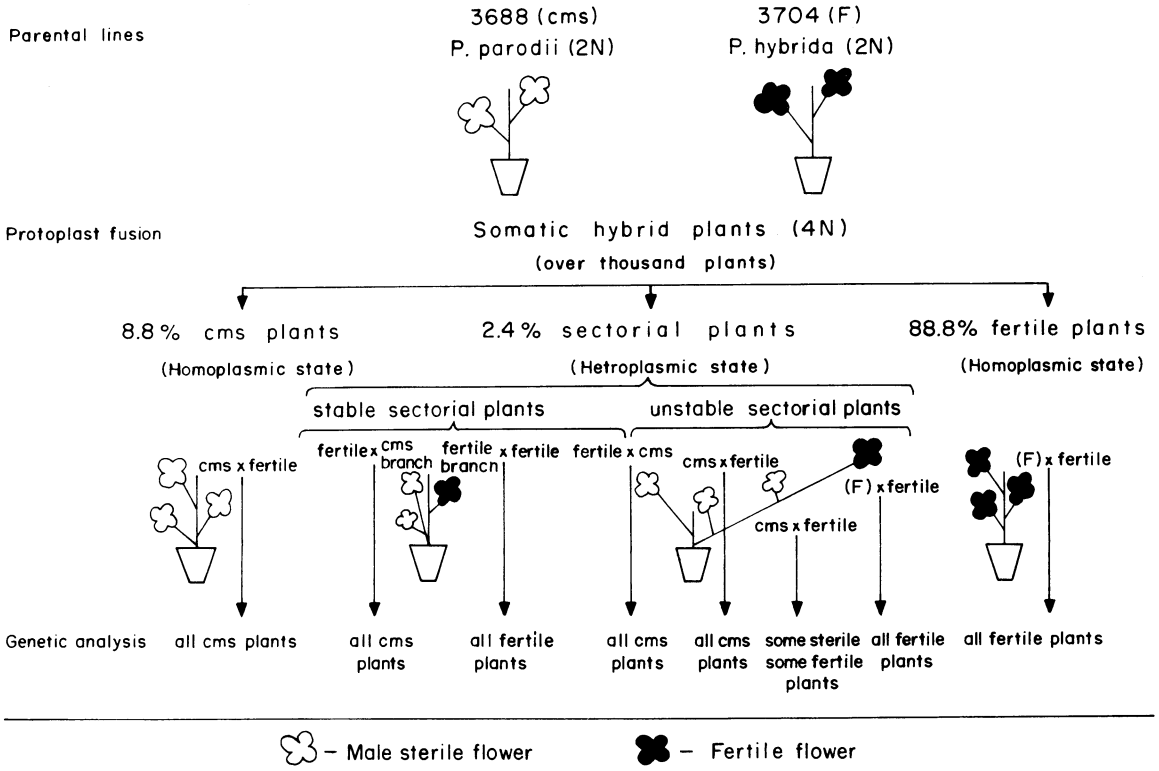


Fig. 7.3. Somatic hybrid plants obtained by fusion of protoplasts of line 3688 cms (*P. parodii*) and normal line 3704 (*P. hybrida*). The somatic hybrid plants are all tetraploids (*4N*). Based on data by Izhar et al. (1981)

tigators working with *Nicotiana* cms (Belliard et al. 1977; Gleba 1978; Zelcer et al. 1978; Aviv et al. 1980). In some of these publications cytoplasmic segregation of the heteroplastidal state was reported, but none of them reported cytoplasmic segregation of male fertility and male sterility.

In a recent experiment, Izhar et al. (1981) used a cms line with *P. parodii* nuclear background and a fertile line from *P. hybrida*. Several thousand somatic hybrid plants were obtained, of which 1 117 were analyzed thoroughly (Fig. 7.3). These somatic hybrids were all tetraploids (4 N) by chromosome counts and were morphologically similar to the tetraploid-plants obtained by colchicine treatment of the proper sexual hybrids.

Among the somatic hybrid plants analyzed, most were fully fertile (88.8%), some were totally male-sterile (8.8%) and 2.4% showed sterile and fertile flowers on different branches of the same plants or even on the same branch.

The fertile plants were tested genetically by selfing or testcrosses resulting in fully fertile populations. Similarly, genetic analysis was carried out on the cms somatic hybrids which yielded only male sterile progeny when crossed with pollen from fertile plants. The conclusion is that those plants were homoplasmic for fertility or sterility in spite of the fact that they developed from fused protoplasts in which the two plasmons were initially mixed during the fusion process.

The somatic hybrid plants of the third group were sectorial plants or cytoplasmic chimeras by their morphology. Cytoplasmic male-sterility in these mixed somatic hybrids prevailed in tissues where the heteroplasmic state existed; sterility was expressed at the phenotype level. In the mixed somatic hybrid plants, several types were found but only two are described here (Fig. 7.3). The first type of a mixed somatic hybrid was plants which carried two main sectors, one carrying male-fertile and the other male-sterile flowers. Characteristic to this type is the stability of the two sectors of the plant and their autonomy. Genetic analysis by crosses of these fertile or sterile branches yielded again only fertile or sterile progenies, respectively. In some branches of these somatic hybrids the phenotypic expression changed from sterile to fertile. While at the bases of the branches only male sterile progenies were observed, there were fertile segregants in the progenies of cms flowers which were situated in a transition state before the branch became homoplasmically fertile as judged by its phenotype and the proper test crosses.

7.6 The Genetic Nature of the Male Sterility Elements in *Petunia*

Some characteristics of cms in *Petunia* appear to be unique: (a) transmission of this trait via grafting (Frankel 1956, 1962; Edwardson and Corbett 1961; Bianchi 1963; Corbett and Edwardson 1964). Only recently a report on transmission of cms in alfalfa was published by Thompson and Axtell (1978). (b) The cytoplasmic elements inducing male sterility, here referred to as ste, were found to be of autonomous and particulate nature. This autonomy has been demonstrated by the fact that a heteroplasmic state exists in gametes and in somatic tissue. Izhar and

Tabib (1980) showed that ste can be combined with normal elements; they can coexist in single somatic cells (fused protoplasts) and in gametes. The mixed ste and normal elements later segregate following meiosis to stable cms and normal fertile lines. In a few cases the heteroplasmic state can persist for more than a single meiotic cycle (Fig. 7.2) and in some rare cases the heteroplasmic state can persist after three to four meiotic cycles (Izhar unpublished data). Furthermore, somatic segregation of ste and normal elements also occurred in somatic tissue (Fig. 7.3). (c) In heteroplasmons of mixed ste and normal elements no simple dominant-recessive relationship exists, the situation must apparently be more complex. In some cases the heteroplasmic state is expressed as male fertility (Izhar and Tabib 1980), but in other cases male sterility prevailed (Fig. 7.3).

The most peculiar characteristic of male sterility in *Petunia* is its existance as cms and also as a Mendelian monogenic recessive allele. According to Frankel, the gms (*ė*) had been induced in the nuclear genome of a fertile scion grafted on cms stock (Frankel 1962, 1971). This is a unique system which allows the study of genetic factors controlling male sterility in their alternative states; as cms, "autonomous state" and as genic male-sterile, the "integrated state" (Frankel 1971). There is no information available in higher plants about relationships between nuclear genes for male sterility and (S) plasmon. One important reason for this is that both types of sterility exist in very few species (Gottschalk and Kaul 1974).

In recent studies of the nuclear-cytoplasmic relations between the (S) plasmon and the gms (*ė*) allele, Evenor and Izhar (1984) were able to combine by sexual crosses and by using the restorer (*RF*) allele (Edwardson and Warmke 1967) the gms (*ė*) allele with the (S) plasmon showing that both the cytoplasmic and the nuclear elements can coexist in somatic cells and gametes. This finding is not consistant with the proposition of mutual exclusion between ste and (*ė*) as proposed by Frankel (1971) who did the original work at a time when the restorer allele (*Rf*) was not available in *Petunia*. Evenor and Izhar (1984) also observed that the gms (*ė*) allele when in homozygous conditions, conferred male sterility in the plasmotype consisting of, in addition to (*ė*), the (S) plasmon and the restorer allele (*Rf*). In other words, the plasmotype (S) *Rf Rf ė ė* is male sterile as compared to (S) *Rf Rf E ė* which is male fertile. It appears from the data obtained by Evenor and Izhar (1984) that the gms (*ė*) gene operates independently of the cytoplasmic ste elements.

Several possible explanations of the nature of the sterility determinants in *Petunia* have been proposed: Frankel (1971) suggested the analogy of the ste elements to episomes in bacteria. Edwardson and Corbett (1961) and Atanasoff (1964, 1971) favored the virus theory but later Edwardson (1968) negated this theory. The virus theory was not accepted by Frankel (1971). Hess (1973) suggested the possibility that "exosomes" can explain the behavior of the sterility determinants but supplied no evidence to support this theory.

Based on the data available today in *Petunia* and in other species, we can propose two models for which some evidence exists. (a) The first model suggests that the site of ste is in one of the cytoplasmic organelles either the mitochondria or the chloroplasts. The cytoplasmic segregation of stable male sterile and fertile lines from heteroplasmic plants occurred and is compatible with this model. The survival of ste and normal organelles in the cytoplasm may be random and inde-

pendent of any control mechanism or it may be under nuclear control. Between the organelles, some preliminary data obtained by restriction enzyme analysis of the DNA, indicates that the mitochondria are the more likely organelles than are the plastids to carry the genetic information for cms (Salts, Hanson, pers. commun.). Mitochondria are the probable organelles to code for cms also in *Nicotiana* and *Zea* (Gerstel 1980). The difficulties with a model which assumes a fixed site for ste elements in the organelle chromosome is that we have to assume very rapid and extensive movement of organelles in plants in order to explain the graft transmission of cms. The other difficulty with such a model is the appearance of the nuclear gms ($\dot{e}$) allele (Frankel 1962, 1971).

A second model assumes that ste is a plasmid-like particle which may be present in one or more of several states: It may be present free in the cytoplasm or under certain conditions (environmental or genetic consitution) may integrate into the organelles or the nuclear genomes. Such a model is compatible with the graft transmission phenomenon and the appearance of the gms ($\dot{e}$) allele in the grafting experiments. It is also compatible with the dilution phenomenon observed both in the F_1 of the grafting experiment (Frankel 1956; Edwardson and Corbett 1961; Bianchi 1963) and in the protoplast fusion experiments (see Figs. 7.2 and 7.3).

It is perhaps surprising that little is known about the mechanism of male sterility in higher plants although it is an important economic trait in several field crops. It is hoped now that with the help of somatic cell genetics, the genetical nature of the cytoplasmic genes causing sterility will be thoroughly studied and information will then be gathered about the phenomenon in higher plants. *Petunia*, for reasons that were mentioned, may be one of the better model plants in which male sterility can be studied.

Notes Added in Proofs

Since this manuscript has been written the author in collaboration with others found good evidence that the site of cms in *Petunia* is the mitochondrial DNA and not in the chloroplast DNA.

Some recent references which are specifically relevant to the genetical and biochemical nature of the cms in *Petunia*: Izhar et al., Molec. Gen. Genet. (1983) 190:468–474; Boeshore et al. (1983) 190:459–467; Boeshore et al., Plant Molec. Biology (in press).

8 Self-Incompatibility

P. D. ASCHER [1]

8.1 Introduction

Interactions between the pollen tube or pollen grain and the sporophytic tissue of the pistil control development of the male gametophyte in self-incompatible (SI) angiosperms. Matching specificities coded by alleles at the *S* (incompatibility) locus alter pollen germination or pollen tube growth such that fertilization does not occur. Numerous *S* alleles exist for most SI species and are identified by subscript numbers. An important feature of SI is that both male and female gametes are functional but inbreeding is prevented.

SI is widespread among angiosperms. Whitehouse (1950) proposed that the SI system was a primary characteristic of the first angiosperms. He reasoned that, since sporophytic tissue is necessary for regulation of pollen tube growth, SI would provide the selection pressures required for complete closing of the carple. Further, this unique control of outbreeding, without female gamete wastage, would maximize the recombinational features of sexual reproduction and result in the extreme diversity apparent in the earliest angiosperms.

8.1.1 Gametophytic SI

Although earlier workers observed SI in *Petunia*, Harland and Atteck (1933) first reported that pollen specificity, with respect to SI, was gametophytically controlled. Wergin (1936) confirmed this conclusion. Other attributes found in *Petunia*, which are considered typical of plants with gametophytic SI, include binucleate pollen with generative cell mitosis occurring after pollen tube penetration of the stigma (Brewbaker 1957) and expression of the SI reaction as differential rates of pollen tube growth in the pistil (Yasuda 1934; Bateman 1943; Straub 1946; Herrero and Dickinson 1980).

In the gametophytic SI system, a diploid plant heterozygous for *S* alleles expresses both *S* specificities in the pistil but meiosis results in a 1 : 1 segregation of *S* alleles in the pollen and, therefore, 2-pollen phenotypes. Complete diallel pollination of a progeny obtained by crossing two individuals without *S* alleles in common reveals four intra-incompatible but inter-compatible groups. Each group corresponds to a single, heterozygous *S* genotype. Parent – progeny pollinations are reciprocally compatible. Diallel pollination of a progeny generated

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from parents with an *S* allele in common produces only two classes, since pollen tubes carrying the *S* allele present in the pistil are incompatible. Both classes are reciprocally compatible with the female parent but one class is reciprocally compatible and the other reciprocally incompatible with the male parent.

8.1.2 Use of *Petunia* in SI Research

As noted by Linskens (1975), *Petunia* figures prominently in SI literature, perhaps because it is well suited for several experimental approaches. Unless freshly harvested, seeds germinate readily. Exposure to 100 ppm gibberellic acid (GA_3) on filter paper for 24 h before sowing results in rapid germination of freshly-harvested seeds (Dana 1982). Flowering can be expected 6–10 weeks from sowing. Multiplication and preservation of genotypes by asexual propagation from cuttings facilitates genetic studies involving replicated sibling diallels, backcrosses, and crosses of F_2 progeny with grandparents. Besides the ability to generate quantities of flowers of a given genotype at one time, the large size of floral organs, particularly the pistil, and an indeterminate flowering habit also make *Petunia* attractive for biochemical and physiological investigations of SI.

Another advantage is that *Petunia* plants homozygous for a single *S* allele can be obtained by bud self-pollination. Yasuda was probably the first to report circumvention of the SI reaction in *Petunia* by pollination of immature flowers. His review paper (Yasuda 1934) describes experiments, done in the 1920's, revealing that self-pollination of buds 2–4 days before anthesis resulted in seeds. *S*-allele homozygotes are useful in genetic studies as testers, allowing determination of *S*-genotypes in segregating progenies with the minimum number of pollinations. They are valuable for biochemical and physiological investigations because only one type of pollen is produced relative to incompatibility specificity.

The fact that *Petunia* was cultivated as an ornamental both simplified and complicated SI research. The availability of plant material from commercial sources was advantageous (Straub 1946, 1947; Takahashi 1972, Flaschenriem and Ascher 1978, 1980a) but the interspecific-hybrid nature of cultivated *Petunia* led to confusion regarding identity of research materials (Mather 1943). SI research with *Petunia* includes both studies designed to discover the genetic basis of the system by analysis of pollen tube growth and seed set through various generations, and gene-physiology studies directed toward defining the mechanism of the SI reaction through biochemical, physiological, and morphological analysis of pollen tubes and pistils.

8.2 Genetic Studies

From the first (Correns 1912), pseudo-self compatibility (PSC) complicated genetic analysis of SI in *Petunia*. PSC may be defined as genetic or environmental disruption of the SI system such that seeds result from incompatible pollinations. Progeny from these pollinations, however, retain some expression of SI in pollen, pistil, or both. Seed set may be low, amounting to a fraction of a percent of com-

patible (outcross) seed set in a fraction of self-pollinations, or high, when self-pollination regularly produces a full complement of seeds. PSC appears ubiquitous among SI angiosperms: it has been observed in all species subjected to serious SI studies, all too often confounding experiments designed to elucidate genetic control of pollen-pistil specificity. Bud pollination, pistil mutilation, chemical treatment, and environmental manipulation may be used to circumvent SI and must be considered PSC, as must those genotypes which express the phenotype without special treatment.

8.2.1 Polygenic PSC

With hindsight, it is not difficult to understand the problems generated by polygenic PSC in early studies. Workers attempting to describe genetic control of pollen-pistil specificity expected qualitative inheritance as reported for *Nicotiana* (East and Manglesdorf 1925). Development of compatibility patterns from sibling diallels was based on qualitative scoring of pollinations as compatible (+) or incompatible (−). With PSC, intermediate responses occurred and were difficult to classify. Harland and Atteck (1933) discarded families exhibiting excessive PSC and correctly scored low PSC as incompatible. The data fit patterns expected from a l-locus, gametophytic system. Low PSC was ascribed to modifying genes.

Wergin (1936) and Stout (1938), however, included highly PSC plants in their diallel analyses. Without realizing that PSC could be quantitatively inherited (Takahashi 1973), Wergin (1936) classified seed set as compatible when self-pollination always resulted in seeds, half compatible when seed set often occurred and capsules contained 1–150 seeds, and incompatible when self-pollination rarely produced seeds. With this classification, he noted an approximate 1:2:1 segregation in a sibling family. About a quarter of the plants were scored self-compatible, half were half-compatible, and the remaining fourth were incompatible. Wergin suggested that a locus independent of *S* with two co-dominant alleles controlled PSC. Plants homozygous for one allele at this PSC locus would be fully compatible; heterozygous plants would be half-compatible, and plants homozygous for the other allele would be SI. Although Wergin analyzed the data in classes, he reported seed numbers for pollinations in this sibling family. Continuous variation in number of seeds per pollination is apparent.

Stout (1938) also used a three-class designation, fully compatible, less than fully compatible, and incompatible and attempted to fit his data to an epistatic model involving one locus for incompatibility and another for compatibility. He proposed that the two loci interacted both qualitatively and quantitatively to produce pollen-pistil reactions. Considering compatibility patterns only in sibling diallels with low PSC, Wergin (1936) agreed with Harland and Atteck (1933) that gametophytic SI occurred in *Petunia*. Stout (1938) disagreed.

Another confirmation of gametophytic control of pollen specificity in SI *Petunia* came from Mather (1943) working with what he considered true *P. violacea*. He also reported differential expression of *S* alleles in the pistil of his *P. violacea* clone. A progeny obtained from PSC selfing of the clone was deficient in one class of *S* homozygotes while the other homozygous and the heterozygous

classes approached equality. The main objective of Mather's work, however, was to determine the inheritance of SI in F_1, F_2, and backcross progenies of interspecific hybrids between *P. violacea* and an apparently self compatible (SC) *P. axillaris*. Surprisingly, the interspecific F_1 segregated 1 SI: 1 SC. Mather reasoned that the SI : SC ratio reflected the segregation of *S* alleles from *P. violacea*, the allele expressed weakly in *P. violacea* being SC in the hybrid. To test this idea, he generated the interspecific hybrid using a *P. violacea* parent homozygous for this allele, and, as expected, all hybrids were SC.

Although Mather scored pollinations as successful, partially successful, or failures and did not report seed counts, PSC appeared somewhat increased in the SI plants of the interspecific F_1 and considerably higher in the F_2. Furthermore, expression of *S* alleles from *P. violacea* seemed changed in several F_2 plants. These plants were SI or weakly PSC and of the same *S* genotype as the parental *P. violacea* clone. However, pollinations of the F_2 plants with the parental clone succeeded. Mather concluded that expression of SI was under polygenic control, *S* operating as a switch gene. Full expression of SI would require an integrated polygenic complex, selected through evolution of the SI species to enhance *S* allele activity. Disruption of the complex would result in PSC. Therefore, increased PSC in the *P. axillaris* × *P. violacea* hybrid derivatives would arise from substitution of a part of the polygenic complex in *P. violacea* with ineffective genes from *P. axillaris*.

A general interpretation of Mather's conclusion regarding polygenic PSC has been that PSC in *Petunia hybrida*, and perhaps other plants as well, originated exclusively from interspecific hybridization, specifically, hybridization between SC and SI species. This interpretation, however, may not be proper. Although Mather (1943) took pains to verify the true species nature of his material and seemed convinced that *P. axillaris* was truly an SC species, Kojan (1950) and Bali (1971) report that *P. axillaris* (Lam.) B.S.P. is SI and bears the l-locus gametophytic system. Therefore, the cross giving rise to *P. hybrida* may have involved two SI species with PSC rather than an SI and an SC species. In addition, recent work with *Petunia* and previous work with another genus (Henny and Ascher 1976; Robacker and Ascher 1978) illustrate the selection of polygenic PSC from within a species.

The *Petunia* material was *P. violacea* P.I. 424655 collected in Argentina on February 2, 1978 by H.R. Winters of the USDA ARS Department of Plant Introduction. As reported by Mather (1943), we found low levels of PSC (Dana and Ascher, unpublished). Of ten plants, six set no seeds following self-pollinations but four did, exhibiting less than 10% PSC. Percent PSC was calculated by dividing the mean self-seed set by mean outcross (compatible) seed set as female and multiplying by 100. Self-pollination of one of the low PSC plants yielded 24 offspring with a progeny mean PSC of 1.5%. A plant from this progeny having 14.3% PSC produced a self-progeny with a mean PSC of 25.3% and four plants expressing more than 85% PSC. Self-pollination of the four highly PSC plants would have been scored successful by Mather. Striking inbreeding depression accompanied the two generations of selfing. This, taken with the small flower size, uniform magenta flower color, and unimproved plant habit, convinced us that we were working with a wild *Petunia*.

Expressing data as percent PSC is critical for this type of experiment. An axiom in SI literature has been the idea that low levels of PSC cannot be increased through selection and inbreeding. Studies giving rise to this belief used number of seeds per self-pollination as the selection criterion. When the number remained essentially the same through several generations of inbreeding, researchers concluded that selection was ineffective. Not apparent in these studies was the fact that number of seeds per compatible pollination may decrease drastically due to inbreeding depression. Therefore, number of seeds after self-pollination gave a false impression of gain from selection.

Takahashi (1973) first reported results of pollinations as a percent of outcross seed set in *Petunia*. He used mean seed set from a self or sibling cross pollination divided by mean compatible seed set and multiplied by 100 to analyze PSC in sibling diallels of *P. hybrida*. Seed set percentages fell into normal distributions with progeny means similar to parental means. Crosses between high and low PSC parents produced progenies with PSC distributions suggesting additive inheritance. By removing various lengths of the pistil from the stigma at various times after pollination, then determining seed set, Takahashi found that PSC pollen tubes grew slower than compatible pollen tubes. Slower growth of PSC pollen tubes was also reported by Bateman (1943) in hybrids between *P. axillaris* and *P. violacea*. Agreeing with Mather (1943), Takahashi (1973) concluded that polygenic PSC acted to decrease *S* allele activity rather than to alter *S* specificity.

8.2.2 Discriminating Styles and Pollen-Mediated PSC

"Unilateral pseudocompatibility" was the term coined by Takahashi (1972) to describe differences following reciprocal pollinations of plants bearing identical *S* alleles. The heterozygous class of a progeny obtained by bud self-pollination of a *P. hybrida* clone, heterozygous for *S*, produced no seeds when used to pollinate the parental clone but set seeds when pollinated by the parent. Mather (1943) may have witnessed the same phenomenon when he noted that F_2 *P. axillaris* × *P. violacea* hybrids, heterozygous for *S*, set seeds after pollinations with the grandparent *P. violacea*, heterozygous for the same *S* alleles. Takahashi (1972) attributed the unilateral response to diminished activity of *S* in the pistils of the progeny.

We began investigating the effects of PSC on the gametophytic SI system with a genus other than *Petunia*. First, we learned that PSC decreased pistil activity, as PSC plants produced pollen which was incompatible on pistils of SI plants with matching *S*-genotype (Henny and Ascher 1976). Second, we discovered that self-seed yield of PSC plants could differ significantly from incompatible-outcross seed set (Henny and Ascher 1977). Although these differences were not exclusive to parent-progeny pollinations, the phenomenon resembled "unilateral pseudocompatibility" (Takahashi 1972).

Later, we observed both of these responses in inbred *Petunia hybrida* lines derived from commercial F_1-hybrid cultivars. First, inbreds regularly yielding a full

complement of seeds following self pollination were incompatible as males on SI siblings (Flaschenriem and Ascher 1978), suggesting that PSC in *Petunia* also involved diminished pistil activity. Second, an I_7 plant of the genotype $S_{1.1}$ set no seed after self-pollination but produced varying numbers of seeds when pollinated with unrelated $S_{1.1}$ inbreds (Flaschenriem and Ascher 1979 a). Reciprocal pollinations failed. A progeny obtained by self-pollination after high-temperature treatment of the I_7 plant expressed "unilateral pseudocompatibility" with the parent, just as described by Takahashi (1972), and also discriminated among the unrelated $S_{1.1}$ inbred testers. Seed set on the I_7 plant and its I_8 appeared positively correlated with PSC levels of the pollinators, suggesting that the pollen exhibited a phenotype which could be measured by the discriminating styles (DS) of the I_7 and I_8 plants. We called the pollen phenotype pollen-mediated PSC (PMPSC). Since all of the plants in this study were homozygous for S_1, we had no idea whether the phenomenon was a function of the particular *S* allele, *S* allele homozygosity, or a general characteristic of PSC in *Petunia*.

Additional work (Dana 1982) revealed that *P. hybrida* plants homozygous for alleles other than S_1, as well as plants heterozygous for *S*, exhibited the DS-PMPSC response. We also found that PMPSC was not correlated with PSC level of the pollinator. Apparently, the relationship between PMPSC and the PSC level of the pollinator we observed in the earlier study (Flaschenriem and Ascher 1979 a) was a coincidence caused by choice of tester genotypes.

DS-PMPSC is an interaction between certain pollen tubes and pistils bearing matched *S* alleles which leads to seed set and, therefore, is an expression of PSC (Robacker and Ascher 1981, 1982; Dana 1982). DS plants respond to different incompatible pollinators by setting different amounts of seeds. Seed set may be higher, lower, or the same as self-seed set, depending on the PMPSC level of the male. If a PSC plant without DS is self- and incompatibly-cross-pollinated, seed sets from the various pollinations approach equality, that is, the style does not discriminate between pollinators. Ranking a group of plants by DS value involves calculating the mean and the variance about the mean seed set after self-pollination and cross-pollination with a group of incompatible males. High DS implies both high mean seed set and a large variance. PMPSC, on the other hand, describes the level of seed set by a given pollinator on a group of incompatible DS females. Like DS, PMPSC is independent of self-seed set (PSC) but unlike DS, high PMPSC simply refers to high mean seed set with an array of DS individuals.

Another way to visualize DS-PMPSC is to arrange DS plants as females with incompatible PMPSC plants as males in a matrix similar to that used to determine combining ability among inbred lines. Self-pollinations of females should be included. Seed yield, preferably as a percentage of compatible seed set, would appear in the cells of the matrix, DS would be calculated as the mean and variance around the mean seed yield of a particular female across all males (including self). PMPSC would be calculated as the mean seed yield of a particular male across all females. Relative DS values, then, would be akin to general combining ability as a female in incompatible crosses, while PMPSC levels would resemble general combining ability as a male in incompatible pollinations. While an interaction analogous to specific combining ability might be expected, we have not observed a low DS female and a low PMPSC male combining to produce high seed set.

Because the I_7 $S_{1.1}$ *Petunia* passed DS to its progeny in all crosses, we assumed that DS was simply inherited (Flaschenriem and Ascher 1979a). Now it appears that both DS and PMPSC are multigenically controlled, but not by the same genes (Robacker and Ascher 1981, 1982). Highly DS plants may exhibit no PMPSC and plants with high PMPSC may express no DS activity. Since DS and PMPSC segregate independently, and since both may occur in a single progeny, seed yields in a DS-PMPSC sibling diallel may suggest segregation of specificity genes in addition to *S*. Similarly, DS-PMPSC seed set from incompatible pollinations between parent and inbred progeny could be interpreted as the appearance of a new *S* specificity. Takahashi (1972) was actually attempting to generate new *S* alleles through inbreeding (Pandey 1970; Nettancourt et al. 1971) when he discovered "unilateral pseudocompatibility." After a study of DS-PMPSC in large progenies and through several generations (Robacker and Ascher 1981, 1982), we agree with Takahashi (1972) that DS-PMPSC involves change in *S* allele activity rather than specificity.

8.2.3 S_f-Genes

Not all PSC is polygenically controlled. Early SI researchers reported self-seed set which seemed conditioned by a factor segregating as a null-specificity allele at *S*. This allele was termed S_f (East 1929). Pistils heterozygous for S_f and a normal *S* allele expressed only the activity of the normal allele, pistils homozygous for S_f exhibited no *S* specificity, and pollen tubes bearing S_f were universal pollinators.

The first unequivocal report of S_f in *Petunia* was by Takahashi (1973). Bud self pollination of an apparently SC plant produced a progeny which segregated 1 $S_{7.7}$:2 $S_{7.f}$:1$S_{f.f}$. $S_{7.7}$ plants were SI while $S_{7.f}$ and $S_{f.f}$ plants produced 70%–100% of compatible seed set after selfing. In the sibling diallel, S_7 homozygotes were incompatible as males on $S_{7.f}$ plants but the reciprocal was compatible. Test pollinations of plants heterozygous for S_f and an allele other than S_7 indicated full expression of the normal allele.

Although Wergin (1936) concluded that a clone used in his studies was heterozygous for S_f, the data are not clear. Progenies from crosses between the S_f heterozygote and other SI plants, which should have segregated 1 SI:1 SC, exhibited excesses of plants classified SC. These excesses were probably due to polygenic PSC.

In theory, S_f was considered the equivalent of an *S* allele in SC species (East 1932). If *P. axillaris* was truly SC, Mather (1943) might have expected S_f alleles in his *P. axillaris* × *P. violacea* hybrids. Although he reported no specificity at *S* from *P. axillaris*, Mather did not designate alleles from *P. axillaris* S_f but, rather S_a. The cross exhibited unilateral interspecific incompatibility in that seeds resulted only when *P. axillaris* was pollinated with *P. violacea* and then in only a small percent of pollinations. Unilateral incompatibility also appeared in the F_1 and to a lesser degree in the F_2. Mather explained the unilateral response by proposing that pollen tubes carrying an *S* allele from *P. violacea* were not affected by alleles at *S* from *P. axillaris* (S_a) but pollen tubes carrying S_a were retarded in pistils with either of the *S* alleles from *P. violacea*. This general retardation of pol-

len tubes carrying an allele at *S* from *P. axillaris* did not fit the phenotypic description of an S_f allele.

The relationship between unilateral interspecific incompatibility and *S* alleles from an SC species implied in Mather's conclusion was taken by SI theorists as evidence that *S* governed interspecific pollen-pistil reactions as well as SI. This was unfortunate because Mather's data do not fit the theory. While the idea that S_a pollen tubes were retarded in pistils carrying an *S* allele from *P. violacea* explains the F_1 data, it does not hold with F_2 and backcross results. According to the theory, S_a pollen tubes would have been retarded in F_1 pistils so that no S_a homozygous F_2 plants occurred. Therefore, all 20 of the F_2 should have carried at least 1 *S* allele from *P. violacea*. However, pollinations with *P. axillaris* of 5 of 20 F_2 plants were scored successful. Furthermore, pollination with *P. axillaris* of all but 1 of 20 backcross plants (*P. axillaris* × F_1) were scored successful, although half should have carried an *S* allele from *P. violacea*. The number of exceptions, that is, plants with an *S* allele from *P. violacea* which accepted pollen from *P. axillaris*, suggests that interspecific incompatibility was segregating independently of *S*.

8.2.4 Pollen and Stylar-Activity Genes

Single genes blocking *S* activity in either pollen or pistil result in another form of PSC. Studies on the effects of irradiation on the SI system in several genera revealed a mutation which appeared to render pollen incapable of expressing *S* specificity (Lewis 1949). A plant with this "pollen-part" mutant was SC and a universal pollinator but expression of *S* alleles in the pistil was unchanged. Data from self and testcross progenies suggested the mutation occurred at *S* and led to development of the theory that *S* was a multipartite locus. The "stylar-part" mutation predicted by the model would render a plant SC with *S* activity expressed in the pollen but not the pistil.

Brewbaker and Shapiro (1959) irradiated both diploid and tetraploid *Petunia* plants in an attempt to induce mutations in *S*. Although the authors reported 36 "mutants", 12 of which behaved as "pollen-part" and 1 as a "stylar-part" mutant, mean seed yields from both treated and nontreated plants suggest polygenic PSC, perhaps DS-PMPSC. Later, Brewbaker and Natarajan (1960) subjected *Petunia* "pollen-part" mutants to cytogenetic analysis and found that each carried a centric fragment bearing an *S* allele. They concluded that competitive interaction between different *S* alleles in pollen carrying the centric fragment destroyed *S* activity (Stout and Chandler 1941, 1942), creating what appeared to be a "pollen-part" mutation.

Not all "pollen-part" mutants carry a centric fragment (Lewis 1961). We discovered a 100% PSC *P. hybrida* inbred which expressed *S*-allele activity in the pistil but not in the pollen (Dana 1982). Testcrosses demonstrated that the factor conferring loss of pollen activity was linked to S_2 and pollen tubes carrying it were competitive with compatible tubes in S_2 pistils. Cytogenetic analysis of root-tip mitosis revealed no centric fragment. Since the plant exhibited the phenotype de-

scribed by Lewis (1949) as a "pollen-part" mutation of *S*, we generated populations to test for recombination between S_2 and the pollen-inactivation factor.

Using a heterozygous plant bearing the S_2 with the linked pollen-inactivation factor and a normal S_3 as male to produce test progenies, we found 1 recombinant among 445 plants (Dana 1982). This suggested the pollen-inactivation factor was closely linked to but not inseparable from *S*. Quite different results occurred, however, when we used the plant carrying S_2 with the linked factor as female in generating test populations. Twenty-two of 218 plants were recombinants, exhibiting transfer of the pollen-inactivation factor from S_2 to S_3. Unfortunately, we were unable to separate the other class of recombinants, those carrying S_2 without the linked factor, from the parental class. We scored pollinations by capsule size rather than seed counts and, apparently, plants with the recombined S_2 were PSC rather than fully SI.

Without additional data, it is difficult to speculate as to the cause of the disparate recombinational frequencies realized by generating test progenies using the plant with the pollen-inactivation factor as male as opposed to female. It may be that recombination of other genes between the pollen-inactivation factor and *S* affect pollen tube metabolism such that pollen tubes carrying the recombined chromosome fail to fertilize due to gamete competition. If so, the single recombinant individual recovered when the plant with the pollen-inactivation factor was used as male could be a double crossover. In any case, the data suggest that the pollen-inactivation factor is not *S* and may not be closely linked to *S*.

A factor conferring the phenotype described by Lewis (1949) as a "stylar-part" mutation of *S* was found linked to S_1 in another inbred line of *P. Hybrida* (Flaschenriem and Ascher 1979 b). Crossing a 0% PSC $S_{3.3}$ inbred with the 100% PSC $S_{1.1}$ produced an F_1 progeny with 100% PSC. While backcross pollination of the F_1 with the $S_{1.1}$ parent resulted in full seed set, backcrossing with the $S_{3.3}$ parent yielded no seeds, suggesting that the factor affected only the *S* allele to which it was linked. Normal pollen activity with respect to *S* occurred in both the $S_{1.1}$ parent and the $S_{1.3}$ F_1.

The population resulting from crossing an F_1 plant with a 0% PSC, $S_{2.2}$ inbred and an F_2 population generated by selfing the same F_1 plant were analyzed for recombination between *S* and the pistil-inactivation factor. Although polygenic PSC occurred in the populations and made classification of some genotypes difficult or impossible, the factor responsible for loss of pistil activity appeared to be located some 28 map units from *S* (Flaschenriem and Ascher 1979 b).

Because the $S_{1.3}$, F_1 plant used in our earlier studies exhibited only 50% PSC (Flaschenriem and Ascher 1979 b), Dana (1982) chose two different 100% PSC siblings from the original F_1 and repeated the linkage tests. In an attempt to refine the tests by removing some of the effects of polygenic PSC, Dana adjusted seed set data for PMPSC and discovered that the linked factor accounted for only about 50% of the PSC. With the refinement, the distance between *S* and the pistil-inactivation factor appeared to be nearer to 20 map units.

Recombination between *S* and both pollen- and pistil-inactivation factors indicates that, at least in *Petunia*, the concept of a multipartite *S* gene consisting of tightly-linked specificity and activity components is incorrect.

8.3 Gene-Physiology Studies

With an understanding of the genetic basis for gametophytic SI, plant scientists became fascinated with the mechanism of the reaction. How could the pistil discriminate among a multitude of alleles at *S* and, subsequently, reject incompatible male gametophytes? This rejection and the many specificities, each recognized in the reaction, reminded researchers of the immune system in animals and, for several decades, the immune theory of SI dominated research. As early as 1947, however, Straub challenged the immune theory, pointing out that incompatibility specificities were present in the pistil before pollination and not generated as antibodies in response to the pollen antigens. Since then, many theories have been proposed to explain the SI reaction, a number of them developed from *Petunia* data (Straub 1947; Linskens 1962, 1965; Kroes 1973; van der Donk 1975).

8.3.1 Pollen Tubes

Knowledge of phenotypic expression of the reaction is fundamental to understanding the mechanism of SI. Sibling diallel crossing patterns which identify gametophytic control of pollen specificity depend on individual pollen tube responses. Siblings with an *S* allele in common are cross-compatible because only pollen tubes carrying the matched allele suffer an incompatibility reaction. Pollen tubes carrying the unmatched allele, although growing in intimate contact with incompatible tubes, escape the reaction and develop normally. Therefore, compatible and incompatible pollen tubes differ in phenotype.

8.3.1.1 Growth Rates

Yasuda (1934) reported differential growth rates for compatible and incompatible pollen tubes in *Petunia*; Straub (1946) confirmed these observations, noting that both types of tubes grew at the same velocity for up to 8 h after pollination at 24 °C and then accelerated to different velocities. At 12 h after pollination, incompatible tubes decreased in growth rate and continued at ever-decreasing velocities. Although compatible pollen tube velocity seemed to decrease slightly 12 h after pollination, tube tips reached the ovary at about 48 h after pollination. Herrero and Dickinson (1980, 1981) described the same pattern of pollen tube growth using *Petunia* flowers incubated at 25 °C. These observations reveal sigmoid growth patterns for both compatible and incompatible pollen tubes with an identical initial lag phase, different log-phase velocities, and different terminal-lag-phase velocities.

8.3.1.2 Morphology and Histochemistry

In an elegant study, Schlösser (1961) documented several differences between compatible and incompatible pollen tubes, most becoming apparent when the tubes entered the log phase of growth. More material was deposited on both the outer cellulosic and inner callosic walls so incompatible pollen tube walls were

thicker than those of compatible tubes, an observation also recorded by van der Pluijm and Linskens (1966). Agreeing with Linskens and Esser (1957), Schlösser (1961) reported that callose plugs, which were constructed beginning with the log phase of growth, occurred closer together in incompatible tubes. These plugs serve to restrict the cytoplasm to the tip region and wall off the remainder of the tube and pollen grain. In incompatible pollen tubes, Schlösser often noticed the vegetative nucleus and/or a substantial portion of cytoplasm trapped behind the callose plug. Trapped material seemed degenerative. Mitosis of the generative cell occurred later or not at all in incompatible tubes. Although tube tips were free of callose when either type of tube was growing, callose occluded the tips of incompatible pollen tubes when growth ceased.

Using histochemical techniques and radioactive tracers to illustrate pollen tube metabolism, Schlösser (1961) concluded that incompatible tubes surpassed compatible in uptake of metabolites beginning with the onset of the SI reaction. Acid phosphatase activity increased during the reaction and cytochrome oxidase activity was higher throughout the entire growth period of incompatible tubes. While starch content was low during the SI reaction, increased levels appeared later and, by 22 h after pollination, incompatible pollen tubes exhibited more starch than compatible.

Combining light and electron microscopy, Herrero and Dickinson (1981), confirmed Schlösser's observations. Compatible and incompatible pollen tubes exhibited the same fine structure while growing on the stigma and closely resembled *Petunia* pollen tubes growing in vitro (Sassen 1964; Kroh 1967). However, fine structure of both types changed when tube tips penetrated the style and began the log phase of growth. Although compatible and incompatible pollen tubes did not differ in structural components while growing in the style, incompatible tubes accumulated metabolic reserves and deposited carbohydrate wall materials at the same rate as compatible but elongated at only 65% of the velocity. Staining indicated increased levels of protein and carbohydrate in incompatible tubes 24 h after pollination. Both Schlösser (1961) and Herrero and Dickinson (1981) reported degeneration of the cytoplasm and vegetative nucleus in older incompatible pollen tubes. The latter also observed ruptured incompatible pollen tubes 24 h after pollination and some burst compatible tubes with degenerated cytoplasm near the ovary.

8.3.1.3 Physiology

Attempts to study the SI reaction with pollen tubes grown in vitro produced ambiguous results. Yasuda (1934) investigated the effects of stigmatic exudate and pistil extracts on pollen germination and pollen tube growth in sucrose solutions, reporting inhibition when *S*-genotype of pistil matched pollen genotype and stimulation when *S*-genotypes differed. Repeating the experiments with different *Petunia* genotypes, Straub (1946) found that all pistil extracts caused strong inhibition of pollen tubes in vitro, regardless of *S*-genotype. Yasuda (1934) did not report results of statistical analyses but rather visual comparisons of data from repeated experiments which revealed extreme variation in both pollen germina-

tion and pollen tube lengths. Today it is generally accepted that crude extracts of pistil tissue are detrimental to in vitro pollen tube growth.

Straub (1946, 1947) devised a semi-in vitro method for measuring the effect of the SI reaction on pollen tubes. The method involved cutting a pollinated pistil between the ovary and the front of advancing pollen tube tips and placing the cut end of the portion carrying the pollen tubes into a sucrose-boric acid solution. Number and length of pollen tubes growing into the solution from the stylar segment were recorded various times after cutting. Growth rate in the solution was fairly equal for compatible and incompatible tubes when cuts were made 4–6 mm from the stigma, but decreased rapidly for incompatible tubes as cuts were made increasingly more than 6 mm from the stigma. Incompatible tubes which grew through 15 mm of pistil were incapable of growing into the solution. Although compatible pollen tubes grew into the solution after passing through nearly the entire style, the longer they grew in the pistil, the less they were able to grow in the solution.

Using stylar grafts, Straub (1947) found that compatible pollen tubes were able to grow 1.5 times the length of the style. Grafting pistils of different genotypes revealed that some pollen tubes grew as compatible after passing through 10 mm of incompatible style, but the number was low. Reversing the graft such that pollen tubes began growth as compatible, Straub discovered that tubes grew significantly less into an incompatible pistil segment after having penetrated 20, as opposed to 4 mm of compatible pistil. These results, along with data from the semi-in vitro pollen tube experiments, led Straub to propose that pollen tube growth consumed a limiting, pollen-carried substance and that incompatible pollen tubes used more of the substance per unit length than compatible tubes.

8.3.2 Stigma and Style

Petunia bears a solid stigma-style with specialized transmitting tissue, sometimes called conductive tissue, through which pollen tubes grow to the ovary (Konar and Linskens 1966a). Maturation of the stigma, which involves cellular degeneration and the release of stigmatic exudate, occurs at anthesis and is independent of pollination (Konar and Linskens 1966a, b; Herrero and Dickinson 1979). Transmitting tissue is a cylindrical mass of thick-walled cells, rich in cellular organelles and metabolites, characterized by large, matrix-filled, intercellular spaces (van der Pluijm and Linskens 1966; Kroh 1973; Kroh and van Bakel 1973; Kroh and Helsper 1974; Sassen 1974; Herrero and Dickinson 1979). Pollen tubes do not penetrate cells of the transmitting tissue but, rather, proceed down the style through the intercellular spaces (Schlösser 1961; van der Pluijm and Linskens 1966). The stigma-style of *Petunia* is critical in nutritive support of the male gametophyte and is the site of the SI reaction.

8.3.2.1 Nutritive Support of Pollen Tube Growth

Although there is no direct evidence that the stigma plays a major role in pollen tube nutrition, the oily surface layer of stigmatic exudate, which is nonnutritive,

is subtended by an aqueous layer containing sugars and amino acids (Konar and Linskens 1966b). Pollen tubes growing on the stigma are more metabolically active than those growing in vitro (Kroh 1967). However, data from several lines of investigation point to nutritive support of pollen tubes as a function of transmitting tissue. Radioactive label incorporated into the transmitting tissue appears in the intercellular matrix (Kroh 1973; Kroh and van Bakel 1973) and pollen tubes take up radioactive substances from the pistil (Linskens and Esser 1959; Schlösser 1961). Cells of the transmitting tissue change in morphology after passage of a compatible pollen tube, becoming vacuolate with thin, peripheral cytoplasm containing few cellular organelles, while cells in contact with incompatible tubes retain the aspect of those of unpollinated pistils, rich in cellular organelles and reserves and unchanged in cytoplasm-to-vacuole ratio (Herrero and Dickinson 1979).

8.3.2.2 Localization of the SI Reaction

SI researchers often terminate experiments dealing with pollen tube growth when compatible tubes approach the ovary. In *Petunia*, as in other genera, incompatible pollen tubes are in the terminal lag phase at this time, growing at ever-decreasing velocities. Without measurement at regular intervals for some time after compatible tubes reach the ovary, incompatible pollen tubes may appear to have ceased elongation. This and the repeatibility of pollen tube growth experiments when based on a limited number of genotypes and done at a constant temperature, gave rise to the idea that incompatible pollen tube growth was inhibited at a characteristic place in the style. Straub (1946) tested this idea by pollinating the cut end of styles amputated at the neck of the ovary. The pollen tubes grew toward the stigma at normal rates and compatible and incompatible tubes exhibited the normal difference in velocity. In other words, the SI reaction occurred normally although the pollen tubes were growing backwards. With stylar grafts, Straub (1947) added more evidence that all portions of the *Petunia* pistil support the SI reaction.

8.3.3 Pollen-Pistil Interactions

Because *Petunia* has a solid style and pollen tubes cannot be removed for separate analysis, biochemical investigations of the SI reaction must be conducted on combined pollen-tube and stylar tissue. The general format for these experiments is a comparison of self- and cross-pollinated pistils with nonpollinated pistils, the idea being that the contribution of the pollen can be documented from the nonpollinated pistil and differences between self- and cross-pollinated pistils will reflect the SI reaction. Several difficulties are inherent in this design. For one, techniques are sufficiently refined to illustrate biochemical differences in pollen genotypes. Therefore, using a single style genotype but varying pollen genotypes to achieve compatible and incompatible situations poses the possibility of confounding the contribution of pollen genotype with the SI reaction. A more important complication, however, is the fact that *Petunia* responds to pollination with

a mobilization of floral metabolites leading to a floral-wilting response. Therefore, biochemical investigators must attempt to separate pollination response from the SI reaction.

8.3.3.1 Pollination Response

Petunia exhibits accelerated floral senescence in response to pollination. A small experiment carried out in our laboratory illustrated this fact (Flaschenriem and Ascher unpublished). Having a number of plants of an inbred *P. hybrida* clone, we began emasculating flowers 1 day before anthesis. On the day of anthesis, we compatibly cross pollinated an emasculated flower, self-pollinated a second, and reserved a third as a nonpollinated control. We continued this daily until the control flower from the first day senesced completely, then harvested all flowers and arranged them into a matrix with days from anthesis on the Y-axis and treatment on the X-axis. The result was a graphic demonstration that compatibly cross-pollinated flowers wilted in 2–3 days, self-pollinated flowers faded in 3–5 days, and nonpollinated flowers wilted 8–9 days after anthesis.

The difference in timing of floral senescence following self- as opposed to cross-pollination was noted by Linskens (1975) who remarked that "It is a general observation that self-pollinated flowers fade away later than cross-pollinated ones." Using radioactive labels, Linskens (1974, 1975) demonstrated a general movement of metabolites from anthers, corolla, and calyx into the pistil after compatible pollination. Nonpollinated flowers remained in a steady state. At 10 h after self-pollination, the influx of metabolites into the pistil from other floral organs seemed more pronounced than after cross-pollination but, by 18 h after self-pollination, translocation reversed and metabolites moved out of the flower.

Deurenberg (1977) extracted polysomes from *Petunia* ovaries and translated them in *Xenopus laevis* egg cells, separating the products by gel electrophoresis. By 6 h after pollination, long before pollen tubes penetrated the style, ovaries from pollinated pistils produced protein patterns different from those of ovaries from nonpollinated pistils. By 12 h after pollination, protein patterns also differed depending on whether the pistils had been self- or cross-pollinated. Later samples indicated that ovaries of self-pollinated pistils reverted to the metabolic state of nonpollinated controls. Deurenberg concluded that two subsequent signals passed to the ovary after pollination. The first, occurring very soon after pollination, activated the ovary, while the second, which was estimated to occur at about 4 h after pollination, induced continued ovary development when compatible pollen tubes entered the style. Implied in Deurenberg's conclusion is the idea that ovary de-activation in self-pollinated pistils resulted from lack of the second signal.

Herrero and Dickinson (1979) reported an activation of transmitting tissue near the stigma. Cells in the "neck" of the style were spherical and had wall ridges which fit indentations in walls of adjacent cells. Within 30 min of pollination, numbers of polyribosomes increased and many single-membrane-bound cytoplasmic inclusions became associated with the plasma membrane, forming embayments. The fibrillar matrix of the embayments merged with the cell wall. Activity at the plasma membrane ceased at about 4 h after pollination, before pollen

tube tips reached the "neck" of the style. Clearly, pollination induces metabolic activation in the *Petunia* flower in locations remote from the position of pollen grains or tubes.

Attempting to learn more about control of floral wilting, Gilissen (1976) removed the stigma or the stigma and the upper half of the style from nonpollinated *Petunia* flowers and discovered that the amputations induced floral wilting typical of that observed after compatible pollination. Removing the whole style at the top of the ovary caused the flower to wilt faster than the control but more slowly than the other treatments.

In another experiment, Gilissen (1977) found that delayed floral senescence from self- as opposed to cross-pollination did not extend to incompatible cross-pollinations. Wilting began at the same time after incompatible cross-pollinations as after compatible cross-pollinations, although the rate was slower. Age of plant modified both the timing and the rate of wilting. Further, Gilissen (1976, 1977) reported that pollination of *Petunia* with *Nicotiana* induced a strong wilting reaction, notwithstanding the fact that *Nicotiana* pollen tubes were retarded in the top quarter of the style by an interspecific incompatibility reaction. Pollination of *Petunia* with other genera resulted in pollen germination but tubes were unable to penetrate the stigmatic surface. These pollinations had no significant effect on floral senescence. Likewise, self, cross, and intergeneric pollinations with X-ray-killed pollen resulted in floral senescence typical of nonpollinated flowers.

Differences in mobilization, translocation, and floral-wilting patterns in compatibly and incompatibly pollinated *Petunia* flowers have been interpreted as evidence that signals sent through the system at pollination contain information concerning recognition of incompatibility alleles (Linskens 1974, 1975; van der Donk 1975; Gilissen 1976, 1977; Deurenberg 1977; Herrero and Dickinson 1979). Taken together, the data surely indicate that pollination activates the system before pollen tubes penetrate the stigma. However, it seems clear that this first signal is independent of compatibility status of pollen tubes growing on the stigma (Deurenberg 1977; Herrero and Dickinson 1979).

The second signal, transmitted beginning 4–6 h after pollination (Deurenberg 1977) produces differences in floral metabolism, depending on the type of pollination. However, these differences could be explained without invoking recognition of *S* alleles by the style. At 4–6 h after pollination, both compatible and incompatible pollen tubes begin the log phase of growth (Straub 1946; Herrero and Dickinson 1980, 1981) and penetrate the "neck" of the activated style where they incorporate the secretion of the "neck" cells (Herrero and Dickinson, 1979). Removal of metabolites from the transmitting tissue in the "neck" of the style by a large number of exponentially growing pollen tubes would set up a strong sink, a sink which would signal the rest of the flower, mobilizing it as a source. In response to the signal, metabolites move into the pistil (Linskens 1974, 1975) and the ovary continues the activation process (Deurenberg 1977).

Having penetrated the "neck" of the style, individual incompatible pollen tubes begin entering the terminal-lag phase of growth, exhibiting evidence of a disturbed carbohydrate metabolism (Schlösser 1961; Herrero and Dickinson 1980, 1981). Over time, more and more incompatible tubes enter the terminal-lag phase so that fewer tubes continue log-phase growth (Straub 1947; Herrero and

Dickinson 1980), and sink strength dwindles. Metabolites such as starch begin accumulating in the style (Schlösser 1961; Herrero and Dickinson 1979) and floral metabolism changes. The second signal, then, would be a source-sink reaction with compatible pollen tubes maintaining strong sink capacity and incompatible tubes gradually losing sink strength.

Several observations from Gilissen (1977) suggest source-sink control of floral metabolism. A minimum of about 800 viable pollen grains was necessary to induce floral wilting, indicating that quantity of pollen tubes entering the style is important. Differences in rate of floral wilting after various incompatible pollinations would reflect different sink strengths of pollen genotypes, that is, differences in times that individual pollen tubes enter the terminal-lag phase of growth. Failure to initiate a wilting response by intergeneric pollen which germinated but did not penetrate the stigma would occur because a sink was not created.

8.3.3.2 Physiology and Biochemistry of Pollinated Pistils

Beginning in the 1950's, Linskens (1955) documented several differences in compatibly- and incompatibly-pollinated *P. hybrida* pistils. Extracts taken within 25 h of either type of pollination revealed large reductions in free sugars when compared to extracts from virgin pistils. Linskens concluded that both types of pollen tubes received sustenance from the pistil. Respiration, measured with a Warburg apparatus, was 10% greater for the first 12 h after self-pollination. This was statistically significant from 3 to 11 h after pollination. Respiration quotients (R_q) were less than 1 for the first 3 h after pollination, then rapidly increased to values larger than 1 from 5 to 12 h after pollination. After 14 h following pollination, the R_q remained at 1, as did the R_q for nonpollinated pistils. Linskens interpreted these results as indicating that germination and early growth involved conversion of pollen reserves from oils to carbohydrates and a carbohydrate metabolism but, after 4 h, pollen tubes switched to an energy source richer in oxygen than carbohydrates.

Paper electrophoresis of pistil extracts produced two peaks, X and Y, typical of self-pollinated pistils, and a third peak, Z, characteristic of cross-pollinated pistils, none of which were present in extracts from nonpollinated styles (Linskens 1955). Elution of the peaks from the electrophoresis paper followed by acid hydrolysis and paper chromatography indicated that all three fractions were glycoproteins with large amounts of carbohydrate. Labeling the pistil with radioactive leucine before pollination, Linskens (1958) found incorporation of label into all three peaks. However, only fractions X and Y exhibited label when pistils were given radioactive glucose before pollination. Attempts to label pollen were less successful (Linskens 1959) but some radioactivity was found in the Z fraction when pollen had been labelled with glucose.

Since electrophoretic data suggested significant differences in protein, Linskens and Tupy (1966) investigated amino-acid pools after both self- and cross-pollination of cut flowers. Protein content of nonpollinated pistils increased gradually during the 24 h of the experiment. A slight decrease in protein content was observed following cross-pollination but a sharp decrease occurred during the first 12–18 h after self-pollination. Protein content of self-pollinated pistils be-

gan increasing 20 h after pollination. Increase in the free amino acid pool did not follow decrease in protein content, leading the authors to conclude that protein was hydrolyzed and the amino acids used as an energy source. This idea reflects the observation that respiration quotients larger than 1 occurred in pollinated pistils from 5 to 12 h after pollination (Linskens 1955).

Roggen (1967) undertook a rather ambitious study of enzyme activity in cross-pollinated pistils. Acid phosphatase activity decreased markedly after pollination but the other enzymes tested increased. Particularly large increases were observed in enzymes important in carbohydrate metabolism. Paying special attention to an enzyme important in amino-acid metabolism, glutamate dehydrogenase (GDH), Roggen discovered that the enzyme from the pistil was activated and changed in conformation by pollen tube growth. Mixing pollen and pistil extracts resulted in activation of GDH deamination. It appears, therefore, that compatible pollination of the *Petunia* pistil affects both carbohydrate and amino acid metabolisms.

Using radioactive amino acids, Roggen (1967) discovered that, after compatible pollination, two difficult to metabolize amino acids, threonine and leucine, were incorporated into protein at levels 10% greater than in nonpollinated pistils. Direct measurement also revealed a 10% increase in protein content, suggesting de novo protein synthesis in compatibly-pollinated pistils.

Van der Donk (1974a) approached analysis of SI in *Petunia* by looking at RNA synthesis after self- or cross-pollination of pistils held in radioactive orotic acid. Specific incorporation was significantly higher in pollinated pistils. Values corrected for size of the free-nucleotide pool indicated that RNA synthesis was highest in cross-pollinated pistils. In self-pollinated pistils, newly synthesized RNA was mainly of a molecular weight typical of ribosomal RNA, while, in cross-pollinated pistils, newly synthesized RNA had molecular weights between those of 18 and 5 *S* ribosomal RNA. Van der Donk concluded that self- and cross-pollinated pistils differed in quality as well as quantity of RNA synthesis.

Pulse-label experiments with orotic acid also revealed different patterns for self- and cross-pollinated pistils (van der Donk 1974b). Free-nucleotide pools increased shortly after pollination, first for cross-, then, 3 h later, for self-pollinated pistils. Although the increase in free-nucleotide pools with a concomitant decrease in specific activity could signal degradation of RNA in the pistil, van der Donk suggested that transport from other flower parts (Linskens 1974, 1975) could explain the results. Relative RNA synthesis, calculated as the ratio of specific incorporation in pollinated pistils to specific incorporation in nonpollinated pistils, also differed after self- and cross-pollination. RNA synthesis reached a first maximum about 3 h and a second about 12 h after self-pollination. Only 1 maximum appeared in cross-pollinated pistils, this at 6 h after pollination. In vitro translation of polyribosomes extracted from pollinated pistils produced protein patterns which followed RNA-synthesis patterns with about a 3-h delay. Van der Donk interpreted the remarkable similarity between RNA and protein synthesis as evidence that some of the RNA synthesized in pollinated pistils possessed messenger activity.

Working from the premise that the SI reaction affected pollen tube wall membranes, Delbart et al. (1980a–c) analyzed glycosphingolipids, antigenic molecules

involved in membranes which could mediate cell recognition. They found quantitative differences in fatty acids and long-chain bases when pollen and nonpollinated *Petunia* styles were studied separately. Pollination changed glycolipid metabolism within 8 h but, by 24 h after pollination, pistils returned to a slightly modified version of the original metabolism. Changes in glycolipid metabolism were extremely complex and difficult to interpret.

A conclusion common to biochemical investigation of SI in *Petunia* is that compatibly and incompatibly pollinated pistils exhibit profound metabolic differences. These differences extend from RNA synthesis through protein synthesis and enzyme activation to aspects of general metabolism such as respiration and uptake of stylar metabolites. However, the central issues of SI, the bases for pollen-pistil recognition and the means of subsequent control of pollen tube growth, remain unknown.

8.4 Miscellaneous Experiments

Certain experimental approaches taken with *Petunia*, although not always producing conclusive data of themselves, offer potential for future research. Similarly, certain intriguing preliminary observations have not been followed by investigation. In some cases, newer techniques applied to an older idea would yield interesting data. In other instances, the critical experiments have not been done.

An example would be the use of immunological techniques to investigate SI. Linskens (1960) began this work, proving that antisera raised against either pollen or pistil extracts precipitated antigens from extracts of either pistil or pollen of the same plant. This experiment was directed by the immune theory of SI, which was prevalent at the time, but current use of immunology for biochemical investigations suggests that relevant information would be gained from applying these techniques again. The use of immuno-electrophoresis to characterize *Petunia* pollen and pistils grown under different environments (van Herpen 1981) seems a good beginning.

Another example comes from an experiment by van der Donk (1975) involving application of proteins extracted from pollinated pistils to fresh pistils before pollination. Significant retardation or enhancement of pollen tube lengths, depending on whether genotype of extract source matched or differed from pollen genotype, was reported. These results resemble data reported by Yasuda (1934) from in vitro pollen germination experiments. In addition, application of polypeptides, translated in *Xenopus* eggs from polyribosomes taken from pistils, induced synthesis of new proteins in the treated styles. An obvious interpretation is that the proteins applied to the stigma caused an incompatibility reaction. However, this may not be the case. It would be interesting to know whether activation of stylar "neck" cells (Herrero and Dickinson 1979) or the ovary (Deurenberg 1977) was induced by application of protein to the pistil. This information would allow separation of the pollination response from the SI reaction and facilitate interpretation of the data.

Considerable observational data but little critical experimentation describes our knowledge of the genetic basis for environmental modification of SI. For example, we know that temperature and light intensity strongly influence PSC in *Petunia* (Linskens 1975; Flaschenriem and Ascher 1980 a, b; van Herpen and Linskens 1981). Although it is apparent that plants differ and that the phenomena have a genetic foundation, we know little of the inheritance of environmental sensitivity or of its relationship with regularly-expressed PSC. A related area needing study is the biochemical analysis of PSC. Using environmentally-sensitive genotypes should simplify this work. A single genotype of pollen and pistil could be analyzed, comparing pollinated pistils incubated in an environment producing incompatible tube growth with those held in an environment resulting in compatible pollen tube growth. It could be that important information concerning both recognition and control of pollen tube growth by SI will arise from study of PSC.

9 Cell, Tissue, and Organ Culture in *Petunia**

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9.1 Introduction

The application of tissue culture techniques to *Petunia* started in the late 1960's with the work of Binding (1971), Binding et al. (1970) and others. The main objective of this early work was to study aspects of morphogenesis and organogenesis from organs or unorganized callus tissue. It was generally found, as will be described here, that normal plants could be regenerated from explants, but that the methodology involved needs further improvement. These early studies contributed much to the development of protocols for the culture of whole plants from cells or protoplasts which is available in many *Petunia* species and lines (Power et al. 1976).

This review includes work done on several forms of *Petunia* cultures. We have not dealt with the early stages of protoplast development or with single cells (Chaps. 10 and 11). The role of certain growth regulators as major factors in the in vitro morphogenetic processes is discussed. We describe the development of colonies and calli from protoplasts and their differentiation to whole plants. A lot of work has been done, attempting to regenerate shoot-buds from stem, leaf and other explants efficiently. The primary goal was to clone certain *Petunia* lines in vitro for horticultural uses. Another aspect of this in vitro culture work in *Petunia* is floral bud culture for the purpose of studying developmental processes in the early stages of bud development, fertilization, and incompatibility.

9.2 Growth Regulators as the Major Factor Determining Developmental Patterns in *Petunia* Tissue Culture

9.2.1 The Effects of Growth Regulators on Growth of Cells and Small Colonies

The growth of *Petunia* cells and tissues in vitro was found to be significantly affected by the addition of specific growth regulators to the medium. The growth of protoplasts and colonies in liquid or in semisolid media of a wide range of *Petunia* species and lines require both auxins and cytokinins for sustained growth (Frearson et al. 1973; Izhar and Power 1977; Ettinger-Paltin 1981). Although this

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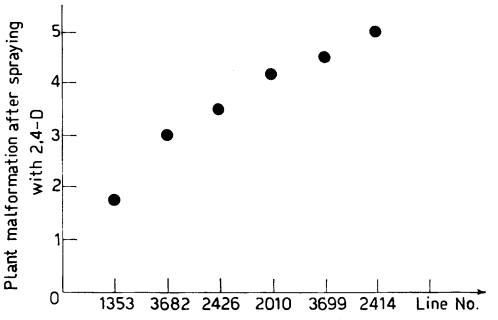


Fig. 9.1. The relationship between the requirements of 2,4-D by protoplasts and the median of the whole plant to a 2,4-D spray (after Ettinger-Paltin 1981). Ranking of lines according to the requirement of their protoplasts to 2,4-D

requirement exists for cells and small colonies, it is not true for suspension cell cultures and for the sustained growth of friable callus (Izhar, unpublished data; Hanson, Sink, pers. commun.). However, there is always a need for the external addition of auxin in other forms of culture in any case. Izhar and Power (1977) showed that protoplasts of some homozygous *Petunia* lines require a specific auxins for their cell and small colony development. For example, certain lines require 2,4-D as auxin while other lines require NAA in the presence of BA as cytokinin. In some cases certain lines require both auxins for early colony development (Hayward and Power 1975; Izhar, unpublished data).

The necessity for growth regulators was found to be genetically controlled; hybrids of a parent which requires NAA and another parent requiring 2,4-D could use either of the auxins (Izhar and Power 1977). The ability of the hybrid to grow on either auxin was lost by a single backcross of the hybrid to one of the parents. This specific requirement for auxins by cells and colonies served as the basis for a selection procedure in somatic hybridization in *Petunia* (Power et al. 1976; Izhar and Power 1979; Izhar and Tabib 1980; Izhar et al. 1983). The use of growth regulators as a selection tool in *Petunia* was improved by Ettinger-Paltin (1981). In this later work an interesting correlation between the reaction of whole plants of certain lines to foliar spray with 2,4-D and the requirement of protoplasts and small colonies of these lines to 2,4-D was observed. Figure 9.1 shows that lines which require 2,4-D for their development in tissue culture are more sensitive as plants to spray application of 2,4-D. It was possible to predict the reaction of whole plants of certain lines to 2,4-D based on their culture requirement of 2,4-D as compared to NAA. Moreover, it was also possible to determine the preferred auxin for protoplasts and colony development based on the reaction of the whole plant to 2,4-D spray. Spray of the whole plants with NAA did not produce effects similar to 2,4-D. Differential requirements or sensitivity of *Petunia* lines to BA have also been observed Izhar and Power (1977) and by Skvirsky et al. (1980). Ettinger-Paltin (1981) showed a correlation between the level of BA and the growth capability of certain *Petunia* lines. Furthermore, Ettinger-Paltin showed that in some cases the optimal level of BA varies according to the amount of external auxin (2,4-D or NAA).

In Table 9.1 data are presented on the conditions used for inducing differentiation of shoots from calli developed in culture. Rooting was not reported as a problem; 0.1 mg/l NAA was effective in most cases reported. It is generally ac-

Table 9.1. Combinations of growth regulators (mg/l) for differentiation of shoots from calli developed from protoplasts. The basal medium is after Murashige and Skoog (1962). The number in table are mg/l

Species	Growth regulator		Reference
P. hybrida cv. comanche	1.0	Zeatin	Power et al. (1976)
P. hybrida other lines	1.0	Zeatin	Izhar and Tabib (1980)
P. hybrida × *P. parodii*	2.0	1AA + 1.0 BA	Power et al. (1976)
	1.0–3.0	1AA + 1.0–2.0 Zeatin	Ettinger-Paltin (1981)
P. axillaris	0.06	NAA + 0.5 BA	Power et al. (1976)
P. violaceae	1.0	Zeatin	Power et al. (1976)
	or 1.0	BA	Power et al. (1976)
P. inflata	0.05	Kinetin	Power et al. (1976)
	1.0	Zeatin	Power et al. (1976)
P. parodii	4.0	1AA + 2.56 Kinetin	Hayward and Power (1975)
P. parviflora	2.0	1AA + 1.0 BA	Sink and Power (1977)
	1.0	Zeatin	Sink and Power (1977)

cepted that cytokinins (BA, zeatin, and kinetin) are necessary for differentiation. However, addition of low levels of IAA or NAA often stimulate shoot differentiation (Izhar unpublished data). There are some distinct differences in the requirements of growth regulators for differentiation from leaf or stem explants and those required by calli produced from protoplasts. For example, the data presented by Rao et al. (1973 a) regarding differentiation of shoot buds from leaf explants show some basic differences, especially in the formation of somatic embryos, as a response to 2,4-D. In contrast, the effects of 2,4-D on calli produced from single cells is usually friable callus with no signs of differentiation of buds or embryos (Ettinger-Paltin 1981).

9.2.2 The Effects of Growth Regulators on Morphogenesis of Different Explants in Vitro

Rao et al. (1973 a) has presented some basic data regarding the specific effects of some growth regulators on leaf and stem cultures of *P. inflata* and two cultivars of *P. hybrida* (Table 9.2). IAA caused limited callus formation and root initiation, while NAA was highly effective in inducing abundant roots. 2,4-D promoted callus growth which eventually produced some embryos and subsequently, developed into plantlets. BA, kinetin, and zeatin induced direct bud development. Mixtures of 2,4-D and cytokinins produced adverse effects; both callus and embryo formation were inhibited as compared to the effect of 2,4-D alone. On the other hand, 2,4-D inhibited the typical induction of buds by the cytokinins. The addition of cytokinins inhibited the formation of roots. We have recently tested the effect of gibberellin on shoot organogenesis. The addition of gibberellic acid had no effect on the differentiation from callus in *Petunia*. However, a marked elongation of differentiated shoots was observed in cases where growth had been very slow or even arrested.

Table 9.2. Morphogenetic effects of growth regulators on *in vitro*-grown *Petunia* explants. Basic media were MS according to Murashige and Skoog (1962), mMS: modified MS medium, Heller according to Heller (1953), Nitsch according to Nitsch and Nitsch (1965), *LS* according to Linsmair and Skoog (1965). The growth regulators were added in mg/l. The main characteristics of the tissue development are mentioned

Species	Source of explant	Basic medium	BA	Cytokinins		Auxins			Development to	References
				Zeatin	Kinetin	2,4-D	IAA	NAA		
	Flower buds sections	MS[a] – Heller[b]	2					1	Plantlets (in 2–3w)	Pelletier and Delise (1969)
P. hybrida	Leaf segment	MS + Nitsch	–	–	0.4	–	–	–	Hard green cal. + few buds	
	Leaf segment	MS + Nitsch	–	–	0.4	–	1	–	Hard green cal. + buds	
	Leaf segment	MS + Nitsch	–	–	0.8	–	1	–	Nodular hard green cal. + few buds	
P. inflata *P. hybrida*	Stem and leaf explant	MS + Nitsch	–	–	–	–	–	–	Nodular cal. (2w) Buds (4w)	Rao et al. (1973a) and Handro et al. (1972)
		MS + Nitsch	–	–	–	–	1	–	Small cal. + root initiation (2w) Nodular cal. + roots (4w)	
		MS + Nitsch	–	–	–	–	–	1	Roots	
		MS + Nitsch	–	–	–	1	–	–	Soft friable cal. + roots (2w) Embryos in cal. to plantlets (4w)	
		MS + Nitsch	0.2	–	–	–	1	–	Small nodular cal. + buds (2w) Plantlets from buds (4w)	
		MS + Nitsch	0.2	–	–	–	–	1	Cal. + root initiation (2w) Compact cal. + roots (4w)	
		MS + Nitsch	0.2	–	–	–	1	–	Compact cal. + roots (2w) Compact cal. + embryos (4w)	
		MS + Nitsch	0.2	–	–	–	–	–	Nodular cal. + bud initiation (2w) Vigorous buds to plantlets (4w)	
		MS + Nitsch	–	0.1	–	–	–	–	Nodular cal. (2w) Buds to plantlets (4w)	
		MS + Nitsch	–	–	0.1	–	–	–	Limited nodular cal. (2w) Retarded buds to plantlets (4w)	
P. inflata *P. hybrida*	Stem + leaf explant	MS	–	–	–	–	–	–	Negligible growth (2w) Limited cal. growth (4w)	Rao et al. (1973b)
		MS	0.2	–	–	–	–	–	Compact cal. (2w) Buds + plantlets (4w)	
		MS	–	–	–	1	–	–	Soft and friable cal. (2w) Embryos + plantlets (4w)	
		MS	0.2	–	–	1	–	–	Compact cal. (2w) Cal. + embryos (4w)	

P. hybrida double petunia	Leaf pieces shoots.	LS	0.2	–	–	–	–	–	Shoots (4w)	Daykin et al. (1976)
		LS	–	–	–	–	–	1	Large white cal.	
	Cut ends of shoots	LS	–	–	–	–	–	1	Roots	
	Shoots + cut ends of shoots	LS	–	–	–	–	–	–	Rooted very slowly or not at all	
P. hybrida	Apical meristem	MS[a] + Nitsch[b]	–	–	–	–	–	–	Little cal.	Sharma and Mitra (1976)
	Apical meristem	MS + Nitsch	–	–	–	–	–	–	Hard green cal. very little	
	Apical meristem	MS + Nitsch	0.2	–	–	–	–	–	Small cal. hard green + few buds	
	Apical meristem	MS + Nitsch	0.5	–	–	–	–	–	Cal. + buds	
	Apical meristem	MS + Nitsch	0.2	–	–	–	1	–	Cal. + buds to shoots	
	Apical meristem	MS + Nitsch	0.5	–	–	–	1	–	Good cal. + many buds to shoots	
	Apical meristem	MS + Nitsch	–	–	0.4	–	–	–	Little cal. hard green + few buds	
	Apical meristem	MS + Nitsch	–	–	0.4	–	1	–	Cal. hard green + few buds	
	Apical meristem	MS + Nitsch	–	–	0.8	–	1	–	Cal. hard green + few buds	
	Leaf segment	MS + Nitsch	–	–	–	–	–	–	–	
	Leaf segment	MS + Nitsch	–	–	–	–	1	–	Cal. hard green	
	Leaf segment	MS + Nitsch	0.2	–	–	–	–	–	Buds from nodular cal.	
	Leaf segment	MS + Nitsch	0.5	–	–	–	–	–	Buds from hard green cal.	
	Leaf segment	MS + Nitsch	0.2	–	–	–	1	–	Buds to shoots from nodular cal.	
	Leaf segment	MS + Nitsch	0.5	–	–	–	1	–	Many buds from nodular cal.	
P. hybrida	Leaf	mMS	0.2	–	–	–	–	1	Shoots	
									Cal. + shoots	Gavinlertvatana et al. (1980)
P. hybrida	Leaf	LS	0.2	–	–	–	–	–	Shoots	Read et al. (1979)
P. hybrida	Callus	MS	–	–	9	–	9	–	Shoots	Bincliny et al. (1971)
P. inflata R. Fries	Leaf + stem	mMS	0.2	–	–	1	–	–	Embryos + shoots nodular cal.	Handro et al. (1972)
P. hybrida	Root meristem	MS	0–0.5	–	–	–	–	0.25–2	Roots	Colijn et al. (1979b)
	Root meristem	MS	0.25–2	–	–	–	–	0.05–0.5	Shoots	
P. hybrida	Leaf	mMS	200–800[c]	–	–	–	–	0.05–0.2	Shoots	Economou and Read (1982)
P. hybrida	Leaf	MS	–	–	–	–	–	–	Shoot formation	Economou and Read (1980)
		MS	0.2	–	–	–	–	–	Shoots	
		MS	0.4	–	–	–	–	–	Shoots	
		MS	0.8	–	–	–	–	–	Many shoots	

[a] macroelements; [b] microelements; [c] pretreatment by dipping; w = weeks; Cal. = Callus or calli.

An interesting observation was made by Gavinlertvatana et al. (1980). Using leaf discs of *Petunia* they have shown that NAA (1.0 mg/l) promotes callus and root formation and simultaneously induces a high level of ethylene, while kinetin, (0.2 mg/l) which stimulates shoot formation, induces a much lower level of ethylene.

In a series of papers, P. Read and co-workers at the University of Minnesota have described the effects of varieties (genotype) and different environmental conditions on growth in leaf explants of *Petunia*. The role of BA as a necessary hormone for shoot differentiation was confirmed by Economou and Read (1980). Later, Economou and Read (1982) tested the role of NAA in two *Petunia* cultivars. They used leaf explants from Sugar Daddy and Sugar Plum, which were pretreated by dipping in 0, 200, 400, 800 mg/l BA. Leaf discs from the pretreated leaves were later placed on cytokinin-free MS medium with 0, 0.05, 0.1, 0.2 mg/ NAA (Table 9.2). The lower concentrations of 0.05 and 0.1 were effective in promoting the number as well as quality of shoots in Sugar Daddy while affecting the quality of shoots in Sugar Plum but not the number per explant. Large differences in shoot production in vitro among several *Petunia* hybrids cultivars were observed by Read et al. (1979); Red Satin exhibited very poor shoot formation in vitro, while Blue Picotee produced many shoots.

In order to understand the inheritance of requirements to growth regulators, Hanson and Read (1981) attempted to correlate the tissue culturability of certain lines with the whole plant performance in several F_1 *Petunia* hybrids and their inbred parents. The F_1 hybrids were more vigorous than their inbred parents, both in vitro and in the field. However, no correlation was found between the performance of certain parental lines and their F_1 hybrid. The authors concluded that the cultivar differences underscored the importance of cultivar selection in tissue culture research. They also proposed that this plant material which showed different responses to external growth regulators may be very useful for studies of endogenous plant growth regulator-genetic interactions.

9.3 In Vitro Propagation of *Petunia* Plantlets

Rapid propagation of *Petunia* plantlets may be useful for the maintainance of different *Petunia* lines; it may give basic clues to the differentiation of buds from callus. Daykin et al. (1976) suggested the use of the in vitro rapid propagation technique for cloning of double *Petunia* parental lines. The double *Petunia* clones are pistile sterile (female sterile) and are used in hybrid seed production as pollinators on single-flowered lines as the female parent. Micropropagation of the pollen line enables maintainance of disease-free plants (mainly free of viruses). Rapid propagation of single-flowered lines of *Petunia* was suggested earlier by Rao et al. (1973 a, b). For the double *Petunia*, fully expanded leaves of young plants were chosen. Explants from the leaves were taken after surface sterilization of the leaves. The leaf pieces were placed in Petri dishes on LS medium (Linsmair and Skoog 1965) with the addition of 0.2 mg/l BA and incubated under continuous low light (about 1 000 lx) at 25 °C. Transfers to fresh medium were made every

2 weeks. Shoots were produced after 4 weeks from the "shiny green nodules" developed on the little callus which developed around the edges of the leaf explants. The shoots were cut and placed on agar with LS medium with 1.0 mg/l NAA on which roots appeared after a few days. Daykin et al. (1976) claim that the plants developed by the micropropagation technique were identical to the plants of the original double *Petunia* clone.

Pelletier and Delise (1969) used floral buds for the rapid propagation of plantlets of *Petunia pendula.* Floral buds were excised 1.0 cm from the flower and placed on agar medium containing half concentrations of the macroelements according to Murashige and Skoog and half the concentration of the microelements according to Heller with 2.0 mg/l BA and 1.0 mg/l NAA. After 2–3 weeks approximately 100 plantlets per bud had formed. No data are supplied with respect to uniformity and "true to type" state of this procedure. There was no description indicating whether the plantlets were differentiated directly or via callus culture (Table 9.2).

Sharma and Mitra (1976) described an in vitro procedure for mass propagation of *Petunia hybrida* plantlets. Shoot apical meristems (3–4 mm) are cultured *in vitro* on a modified MS medium with 1.0 mg/l IAA and 0.5 mg/l. The apical meristem produces large masses of callus with considerable numbers of shoots within 30 days. About 2000 flowering plants can be produced in 6 months from a single apical meristem. The plants produced were "true to type" to the mother plant in size, shape, and flower color. Leaf explants were also utilized for the same purpose, but the efficiency of this tissue was less than that of the apical meristem. The plants produced from the leaf explants were also less vigorous than those of the apical meristem; the optimal combination of growth hormones for the leaf explants was the same as above (see Table 9.2).

Handro et al. (1972) used leaf and stem explants of *Petunia hybrida* and *P. inflata* for induction of shoots. Macroelements were according to MS and microelements according to Nitsch and Nitsch (1965). It was possible to obtain callus, roots, shoot buds, or somatic embryos *in vitro* according to the growth regulators supplied. Addition of 2,4-D to the medium promoted the production of somatic embryos directly from the explants or via friable intermediary callus. These embryos were similar to zygotic embryos. It was also possible to obtain embryos on calli transferred to liquid culture.

According to microscopical observations, the roots and shoots are formed from subepidermal meristematic tissue which is found in the periphery of the callus (Handro et al. 1973). Embryos are produced from meristematic tissue or from cambium tissue mainly in stem explants. Handro et al. (1972) describe the effect of certain growth hormones on the direction of differentiation e.g. cytokinin clearly induces the production of shoot buds while 2,4-D induces the production of somatic embryos and NAA induces root production (see Table 9.2).

Economou and Read (1980) used *Petunia hybrida* leaf explants to produce shoots by varying the concentration, exposure time, and application method of BA. The authors used 4 × 18 mm strips (cross sections with the midrib) from recently expanded leaves. They pretreated the whole leaves and the strips before placing them on basic medium (MS basic with myo-inositol thiamine-HCl and 2.0% sucrose) in BA solutions 0, 200, 400, and 800 mg/l. Alternatively, BA was

added to the agar medium (in concentrations as follows: 0, 0.1, 0.2, 0.4 mg/l. Both modes of application were effective, dipping giving better results than the addition of BA to the medium. The authors observed varietal differences. Blue Magic responded similarly to dipping or to the presence of BA in the medium while Blue Picotee responded better to dipping in number and quality of the shoots formed.

To summarize, there is enough evidence to show that cloning of *Petunia* lines for horticultural use is possible, although the stability and "true to type" nature of the plant material produced still requires further confirmation.

9.4 Floral Bud Culture for Studying Gametogenesis and Microsporogenesis

The in vitro culture of floral buds could be used as a tool for studying different morphogenetic aspects of floral bud development under defined conditions, to study problems of incompatibility between pollen and stigma, and, mainly, for biochemical investigation of metabolic pathways.

Izhar and Frankel (1973) established a procedure for floral bud culture in vitro the main goal being the study of gametogenesis and sporogenesis. For this purpose floral buds at different developmental stages were excised, the buds were surface sterilized, their precise developmental stage was determined on one single anther, and then the buds were placed on agar-medium in a Petri dish.

The main objective was to find conditions to support the normal growth of the male gametophyte from premeiotic stages. A relatively simple medium composed of agar and 3.0% sucrose was found to be sufficient to support this growth. Without sucrose the development of the premeiotic buds did not occur, while buds excised at later developmental stages developed well on medium containing agar only. In their study (Izhar and Frankel 1973) the investigators did not find any advantage for the development of floral buds in vitro by the addition of growth regulators such as IAA, NAA, kinetin, and gibberellin.

Using this simple in vitro culture procedure, Izhar and Frankel (1973) compared the duration of meiosis in the anthers in vivo and in vitro and found it very similar. They were able to define the duration of each meiotic stage and found that the whole meiotic process lasts about 12 h. A comparison of buds from normal lines with buds from different male sterile lines showed that in vitro development of buds from the different sources correspond to their development in vivo. Thus, the in vitro procedure described provided a good tool for the study of gametogenesis and microsporogenesis. Izhar and Frankel (1973) also studied the effect of temperature on the development of floral buds in vitro. It was shown that the growth of excised buds under low temperature regimes (20° day and 15 °C night) was arrested and that this arrest was partially reversible when the buds were transferred to higher temperatures (35° day and 15 °C night).

9.5 In Vitro Ovule, Placenta, and Embryo Culture for the Study of Fertilization and Incompatibility

In vitro techniques have been used to study incompatibility and pollination in *Petunia*. When compared with floral bud culture described previously, the flower bud organs necessary for studying pollination may be at an advanced developmental stage, e.g., the male gametophyte past meiosis (see Izhar and Frankel 1973). The in vitro methodology for the different floral bud organ cultures and their applications was recently described by Rangaswamy (1977). *Petunia* was used mainly for the study of self-incompatibility. In vitro pollination and fertilization enable the use of ovule and "placental pollination", thus, overcoming the pollen grain-stigma incompatible interaction. In *Petunia axillaris* Rangaswamy and Shivanna (1967) successfully used the "placental pollination" method to overcome self-incompatibility. In placental culture the whole mass of ovules of an ovary was cultured.

The ovules were intact and connected by the placental tissue. This, apparently, helps to prevent the mechanical damage done by cutting the placenta. The pollen grains are applied directly on the ovule surface and they germinate in 2–3 h after application and in 24 h the pollen tubes are observed inside the ovules. Mature seeds were collected after 24 days which is the same time needed for obtaining mature seed in vivo. Overcoming self-incompatibility using a test-tube pollination technique was also described by Niimi (1973, 1974). He obtained several seedlings of *Petunia hybrida* from a self-incompatible genotype. Higuchi (1969), (quoted by Niimi 1976), also developed a technique of "stylar pollination", whereby intact stigmas are dusted with pollen grains and then the styles are excised and placed directly on the in vitro cultured ovules attached to the plancenta. Niimi (1976) compared stylar pollination with placental pollination in which pollen grains are applied directly to the cultured ovules. The stylar pollination was found more efficient than the placental pollination. These results are similar to those of Wagner and Hess (1973).

In vitro pollination was used in further studies (Rangaswamy and Shivanna, 1967) to study different aspects of pollination and by Wagner and Hess (1973), to study the relative fertilization competence of pollen grains deposited on the stigma as compared with pollen deposited directly on the intact ovules of the same cultured bud. They found that pollen grains deposited on the stigma have a significant advantage when compared with pollen grains applied directly to the ovules. Rangaswamy (1977) suggests the use of in vitro culture of placenta for obtaining haploid plants; this technique was used successfully by Wagner and Hess (1973) to obtain haploid plants in *Mimulus*. In further studies Niimi (1973, 1974) tried to improve the seed yield of test-tube-fertilized ovules grown in vitro on half a placenta. Niimi used the basic Nitsch (1951) medium modified by addition of KNO_3 and NH_4NO_3. The effect of iron, boron, sugars, and Fe-EDTA was determined. Sucrose interacted positively with boron and iron while fructose was found not useful. Interestingly, the ovules cultured after fertilization did not require growth hormones. The addition of Fe-EDTA was essential but addition

of casamino acids was not effective. Also, lower sucrose concentration was better for the normal development of ovules.

In summary, *Petunia* seems to be a good system in which different developmental aspects in the flower and fertilization can be studied using in vitro culture techniques.

9.6 Cell Suspension Culture

The establishment of cell suspension cultures of *Petunia* is achieved with relative ease. Initiation of such cultures can be done by inoculation of flasks with friable callus or with other sources of explants. The flasks are then placed on an orbital shaker at about 80–100 rpm. Depending upon the growth regulators added and the genetic line, cell clusters of different sizes are observed. For most purposes small clusters or, ideally, "single cell suspension culture" are preferred; however, this has not been achieved so far in higher plant cultures. In some cases suspension cultures of *Petunia* have been obtained in which the majority of the aggregates contained 1–3 cells. The basic medium used for many of the cell suspensions reported was MS with 2,4-D at 0.2–2.0 mg/l as the sole growth regulator. The use of cell suspension culture in *Petunia* was mainly as a source for protoplasts or for the isolation of mutants (Colijn et al. 1979a; Shneyour et al. 1983). Lifshitz et al. (1982) used *Petunia* cell suspensions for protoplast isolation from which circular mitochondrial DNA molecules were obtained. Boeshore et al. (1983) also used cell suspension culture of *Petunia* for mitochondrial DNA isolation from many lines and somatic hybrids. In several cases (M.R. Hanson pers. commun., and Izhar, unpublished data) it has been possible to regenerate whole plants from cell suspension cultures of *Petunia*. It was interesting to observe that no change in chromosome numbers was detected in these cases.

9.7 Induction and Isolation of Mutants

The isolation of biochemically-defined mutants by in vitro plant systems is a promising research field and has attracted several laboratories to design specific selection schemes for a number of useful mutant traits (for review see Maliga 1980). Surprisingly, *Petunia* in vitro systems have been almost completely neglected in the past, in spite of the many advantages exhibited by this genus, with respect to the feasibility of isolation and genetic analyses of putative mutants. Haploid ($n=7$) *Petunia hybrida* explants have been exposed to 0.5 mg/ml streptomycin in MS medium. Following 6–7 weeks of incubation, proliferating clumps of cells were observed and resistant calli developed. No data were reported on the frequency of appearance of resistant calli, but cytological examinations determined that resistant cells contained 7, 14, or 28 chromosomes (Binding et al. 1970).

The isolation of mutants is performed by exposing a large cell population to selection pressure, which causes arrest of cell division, or cell death in the wild-

type genotype. Additionally, physical or chemical mutagenic treatments are sometimes utilized in order to increase the frequency of mutant cells that can be recovered in such an experiment. Both aspects, the exposure of cells to selective pressure and the inclusion of mutagenic treatments, require an accurate follow-up of the dynamics of growth in the treated cell population in order to optimize conditions for selection of mutants. The lethality of mutagens or selective treatments in *Petunia* systems was monitored by two different approaches:

1. Differential staining of viable or dead cells. The compound fluorescein diacetate is specifically modified by viable plant cells into a fluorescence-emitting molecule (Widholm 1972) and has been reported to be a reliable probe for estimating the "survival" fraction of *Petunia* cells (Colijn et al. 1979 b; Shneyour et al. 1983).
   On the other hand, dyes like phenosafranine (Colijn et al. 1979 b) and erythrosin B (Sareen et al. 1982) stain dead cells differentially, and have been used for the estimation of killing effect of mutagens in *Petunia* cell systems.
2. Plating of treated cells. This is undoubtedly the most reliable technique, since it measures cell viability under the same experimental conditions that are eventually used for isolation of mutants. However, it is a lengthy and more tedious procedure. Under many circumstances, the differential staining techniques can furnish an adequate and fast estimate of the effect of mutagens or selective treatments on plant cells.

A rather elaborate method for determining the effect of different mutagens on leaf pieces of *Petunia* has been proposed (Sareen et al. 1982). Following exposure of leaf tissue to mutagenic compounds, protoplasts are isolated and yield of protoplast formation is determined. The capacity of cells to plasmolyze and from protoplasts is in good correlation with conventional criteria for cell viability, e.g., exclusion of vital dyes (Sareen et al. 1982).

A detailed quantitative study has been performed by Colijn et al. (1979 b) on mutation frequencies in *Petunia* cell suspensions exposed to increasing amounts of mutagenic compounds. Diploid *P. hybrida* cells grown in MS medium supplemented with 1 mg/ml 2,4-D were exposed for 4 h to different mutagens. The killing effect was ascertained 2 days later by differential staining with phenosafranine. While ethyl methanesulfonate, nitrosoguanidine (NG), and hydroxylamine affected cell survival markedly, no substantial killing effect was obtained when acridine orange, ethidium bromide, or ethyl methanesulfonate were utilized. NG was arbitrarly chosen for further experiments.

Since a positive selection scheme was sought, 29 drugs were tested for growth inhibition of *Petunia* cells. From among 10 outstandingly active drugs, $HgCl_2$ and DL-6-fluorotryptophan (6 FT) were choosen as probes for detection of resistant mutants. Following an expression time of 4 days, normal or NG-mutagenized cells were plated under selective conditions, and putative mutants were scored 4–6 weeks later. The highest mutation frequencies were obtained with NG concentrations that had no visible effect on cell survival. The highest mutation frequency reported for drug resistance was 1×10^{-5} (for $HgCl_2$) or 2×10^{-6} (for 6 FT). 80% of the $HgCl_2$-resistant colonies and virtually all the 6 FT-resistant colonies expressed this phenotype even after 35 generations under selective conditions. Moreover, all these colonies still exhibited the resistance phenotype in a

stable way, even after 35 generations in medium devoid of drugs (Colijn et al. 1979 b).

The isolation of mutant cells requires good plating ability of the single cells. Given the current limitations for growth of plant cells at low plating densities, techniques had to be developed for rescue of mutant colonies appearing at low frequencies within a nongrowing population of wild-type cells.

An efficient and simple technique for this purpose has been recently established for *Petunia* in vitro systems (Shneyour et al. 1983). Cells or protoplasts at low densities are plated on a cellophane membrane, which was placed above a "feeder layer" containing logarithmic-phase cells of a *Petunia hybrida* cell suspension in an appropriate solidified medium.

This technique allows for efficient plating of *Petunia* cells or protoplasts, even at concentrations as low as 500 cells/ml.

A whole range of spontaneous putative mutants of *Petunia hybrida* have been isolated with the aid of the above-mentioned technique, as described in the following:

1. Stable resistance to 6-azauridine (at 5×10^{-3} M) appearing at frequencies of 5×10^{-6}.
2. Resistance to methotrexate (MTX). Stable mutants isolated under drug concentrations of 5×10^{-7} M appeared at frequencies of 1×10^{-6}. Additionally (R. Barg unpublished results), cell suspension resistant to up to 750 μM MTX were established, through a stepwise selection scheme of increasing MTX concentrations. Upon transfer to nonselective conditions, some of the "stepwise-mutants" exhibited a stable resistance to the drug, while others proved to be unstable.
3. Stable resistance to the antibiotic G-418 (at 10 μg/ml), appearing at frequencies of 2×10^{-6}.
4. Stable resistance to allyl alcohol (10–10′ exposure to a concentration of 10^{-2} M), appearing at frequencies of 2.5×10^{-6}.
   This drug is known to kill selectively cells with normal alcohol-dehydrogenase activity.

Interestingly, all the mutants described above were identified within a specific isolate of *Petunia hybrida* line 3 704 (S. Izhar), while no mutants could yet be recovered from a different isolate of the same genotype or from *P. parodii* cell culture. The basis of this differential genetic behavior is not clear, but it strongly pleads for the use of as many isolates (genotypes) as possible when mutants are sought in a particular cell culture system.

Unfortunately, the "high mutant-yielding" isolate has apparently lost any plant regeneration capabilities. Experiments are currently performed in order to transfer the mutant trait to regenerating genotypes via somatic hybridization.

To summarize, the use of different in vitro culture forms of *Petunia* has become routine with few technical difficulties. It is perhaps for this reason that many aspects of tissue culture conditions were not studied in depth. An ideal genotype which regenerates easily and with great efficiency is not available in *Petunia*. The intensive use of *Petunia* in somatic hybridization studies and genetic engineering also require a better understanding of the processes of single cell development into plants.

10 Protoplast Regeneration

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10.1 Introduction

Isolated protoplasts of higher plants have fascinated biologists since the end of the last century (Klercker 1892). The main purpose of these early experiments was to study the physical properties of the cytoplasm and the plasmalemma. Presently, interest is focused on aspects of cell metabolism, development, and genetics.

As demonstrated in this monograph, the genus *Petunia* – and *Petunia hybrida* in particular – is an excellent model taxon for basic investigations on plant physiology and genetics. Consequently, plant regeneration of *Petunia* protoplasts was attempted as soon as it had been established in a higher plant (tobacco, Takebe et al. 1971). First reports on callus formation from *Petunia* protoplasts appeared in 1972 (Hess and Potrykus, Potrykus and Durand). Plant regeneration was achieved one year later (Donn et al. 1973, Durand et al. 1973, Frearson et al. 1973). *Petunia hybrida* was the third plant species after tobacco and carrot (Grambow et al. 1972) in which plants have been grown from isolated protoplasts of spermatophytes. *Petunia* was, furthermore, the first genus in which monohaploids were regenerated from protoplasts (Binding 1974a, b). Experiments with isolated protoplasts of *Petunia* were not only interesting with respect to this species; elaborated protocols were useful models for investigations on many other plants and provided basic information on regeneration and genetic modification. Furthermore, *Petunia* protoplasts appeared to be appropriate for co-culture with protoplasts of a remote species (*Vicia faba* Binding and Nehls 1978) to obtain callus formation of this species.

The procedures and observations described in the following section are based mainly on the paper by Binding (1974b) and more recent unpublished observations in our laboratory, but also on the pioneering investigations of Durand et al. (1973) and Frearson et al. (1973) and several others.

10.2 Protoplast Isolation

10.2.1 Viability

Petunia protoplasts have been isolated from several types of tissue grown under various conditions more or less successfully. Depending on the experimental de-

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mands, a number of methods have been proposed for the estimation of viability. The easiest and, therefore, most commonly used criterium is the integrity of the plasmalemma as indicated by osmotic stability or by impermeability to certain dyes. Furthermore, several biochemical potencies have been evaluated with isolated protoplasts. All these tests rely on selected physical or physiological properties of a living cell but do not necessarily indicate the complete functional integrity of the isolated structure. Preference must therefore be given to a more complex reaction, relative to the regeneration capacity, provided that high plating efficiencies are routinely obtained. As this was established to exceed 0.8 in *Petunia* it had been proposed to index routinely the viability of *Petunia* protoplasts by their mitosis capability.

10.2.2 Protoplast Donor Material

Most cases aiming at plant regeneration from isolated protoplasts require genetic stability. As a lot of mutations occur in tissue cultures of higher plants, special care is needed in this regard for protoplast isolation. First of all, cytogenetic stability of the donor cell material is required. It has been shown that leaf mesophyll as well as shoot cultures meet this demand.

Yields of viable protoplasts depend largely on the physical and physiological properties of the plant material provided by the developmental stage of the cells and by environmental conditions.

Mesophyll tissue is an excellent source of protoplasts for *Petunia* as it is for many other plant species belonging to Solanaceae family. However, green leaves are very sensitive to light. In experiments with haploid plants from greenhouse culture as well as haploid shoot in in vitro culture, an optimum light intensity was observed at about 7–8 klx at a photoperiod of 16 h. On the other hand, the leaves for preparation are best harvested at the end of the dark phase. The influence of temperature on haploid shoot cultures was investigated. Yields of protoplasts increased by increasing temperatures up to 28 °C. Apparently, other culture conditions of the plant material are rather critical for the success of protoplast isolation (e.g., supply of water and nutrients). This is indicated by comparing greenhouse grown plants with shoot cultures. Reproducibly high yields were obtained from shoot cultures whereas numbers of protoplasts and degree of broken ones varied largely from experiment to experiment when leaves of plants were used. Shoot cultures have routinely been grown on agar media with nutrients B 5 (Gamborg et al. 1968) or MS (Murashige and Skoog 1962) with cytokinin (kinetin or 6-BA; 2.5 to 5 μM) but no auxin. Detailed experiments aiming at the optimization of the nutrient constitution with respect to protoplast yield have not been carried out to our knowledge.

Age of leaves from the greenhouse as well as time since the last transfer of shoot cultures does not seem to play a decisive role. However, old plant material should be avoided.

Shoot tips including leaf primordia also proved well suited for obtaining preparations of viable protoplasts. Compared with mesophyll, they are less sensitive

to environmental conditions based on the result of studies using greenhouse material (Binding unpublished).

Protoplasts have been isolated from several other tissues and organs and they subsequently exhibited different capabilities of regeneration. For instance, Potrykus isolated protoplasts from ovules (1971) and petals (Potrykus 1973) for experiments on induction of fusion, Hoffmann and Hess (1973) from petals for studies on DNA uptake; liberation of protoplasts and subprotoplasts from *Petunia* pollen has been reported by Bajaj and Davey (1974); plants have been grown from petals (Binding unpubl.) and callus (Binding 1974c; Vasil and Vasil 1974).

Various species and genotypes of *Petunia* have been used for protoplast experiments. No significant differences in the yields of isolated protoplasts between different species have been observed (Power et al. 1976). Comparative studies on protoplast regeneration have been performed with different genotypes, species, and hybrids in regard to protoplast technology, taxonomic relationships, and somatic hybridization (Frearson et al. 1973; Power et al. 1976; Izhar and Power 1977). Callus formation has been established for a number of chlorophyll deficient mutant strains (e.g., Binding 1974d; Cocking et al. 1977; Power et al. 1979, 1980; Binding et al. 1982). In the last case shoots were regenerated. Light sensitive, chlorophyll deficient variants are grown at dim light of about 1 000 lx to obtain sufficient yields of protoplasts.

10.2.3 Decontamination of the Plant Material

Plant material not grown in closed vessels is inherently contaminated by microorganisms. Surface sterilization is managed by commonly established methods (see Chap. 9). It is usually sufficient with organs of young plants grown from seeds and juvenile parts of older plants. Durand et al. (1973) observed that flowering plants had been contaminated systemically so that decontamination was not possible. The same is often true with plants grown from cuttings. Antibiotics were added during subsequent treatments to prevent proliferation of the microorganisms (Power et al. 1976).

In vitro cultures are normally axenic. In case of shoot culture contamination, it is advisable to grow plants from them in soil first and then, again, initiate shoot cultures.

10.2.4 Protoplast Isolation

The various established protocols of plant protoplast isolation have been either developed for *Petunia*, adapted to this species or utilized for it. It would be too lengthy to discuss the advantages and fields of application of all these procedures here. Some commonly applied steps are included in Table 10.1. For details see reviews on protoplast culture (e.g., Vasil and Vasil 1980; Maheshwari 1984 in prep.).

Table 10.1. Commonly used protoplast Isolation Procedures

I *Preparation of the plant material*
a) Peel off the lower epiderm: leaves
b) Slice carefully with a razor blade into pieces 1–2 mm in 0.5 *M* mannitol or in enzyme solution: various types of tissues
c) Sieve and centrifuge: cell cultures, squashed anthers

II *Solutions for the digestion of cell walls*
1. Pectinase (e.g., 0.3% Macerozyme)
2. Cellulase (e.g., 1 to 3% Meicellase or Cellulase Onozuka R10; Driselase is powerful but often harmful; contains pectinase)
3. Osmotic stabilizer (0.5 *M* mannitol, sorbitol, or sucrose)
4. Calcium ions [5 m*M* $CaCl_2$ or $Ca(NO_3)_2$; stabilizes plasmalemma]
5. Other ions, occasionally
6. Dextran sulfate (0.5 to 2%; increases the yield slightly)
7. pH 5.4 to 6.2

III *Incubation*
1. About 10 mg plant material per ml solution
2. Weak vacuum (1 min; for infiltration of sliced material)
3. Vessels:
   a) Petri dishes (for 10 cm∅: 10–12 ml suspension)
   b) several higher vessels (for agitated incubation)
4. Temperature: 28 °C (25–35 °C)
5. Illumination: 1–2 klx by white fluorescent light
6. Agitation: 50–100 reciprocal strikes per min (accelerates the reaction; sometimes harmful; generally not needed)
7. Duration: 5 h (1–12 h)

IV *Separation of tissue from the isolated protoplasts*
a) Remove undigested parts of peeled leaves with forceps
b) Sieve through nylon or steel sieves (pore sizes 40–100 μm depending on the size of the protoplasts)

V *Removal of debris and dissolved substances*
A. Centrifuge: 5 min at 50–100 × g
B. Resuspend:
   a) in sucrose (for floating of vacuolated protoplasts)
   b) in mannitol (0.5 *M*; with 5 m*M* Ca^{2+}) or media (for sedimentation)
   c) in NaCl (0.25 m*M*; with 10% of the ions of culture media) or diluted sea water (0.5 osmol) (for sedimentation, especially of protoplasts with large vacuoles)
C. Centrifuge (ready for plating; however commonly repeated up to 5 times)

The qualities of the resulting protoplast preparations depend mainly on the nature of the donor tissue. When considerable debris is evident it is advisable to improve the culture conditions of the plant material; modification of the isolation procedures has only a limited effect on higher degrees of viable protoplasts. *Petunia hybrida* is one of the species in which multinucleate protoplasts were found rather frequently (up to 20%) apparently formed by spontanous fusion (Withers and Cocking 1972). The degree can be reduced by plasmolysing the donor tissue prior to the incubation (Frearson et al. 1973; Binding 1974b). A small amount of subprotoplasts is found in mesophyll protoplast preparations (Binding 1979) derived mainly from plasmolysis of spongy parenchyma cells. They have not been found in apical preparations.

10.3 Protoplast Culture

A proposed protocol is shown in Table 10.2. The nutrient solutions most frequently used for *Petunia* protoplasts are original and modified media FPC (Frearson et al. 1973), V-47 (Binding 1974b, developed from the DPD-medium of Durand et al. 1973), but also in the original DPD medium (Table 10.3), in KM 8p (developed for *Vicia hajastana* by Kao and Michayluk 1975), V-KM (introduced for *Solanum dulcamara* by Binding and Nehls 1977), modified MS medium (Power et al. 1976), and several other media (most recently: Zhang 1982). Adequate hormone combinations are: 2.5 μM 6-BA and 6 μM NAA (V47) or 5 μM 6-BA and 9 μM NAA (FPC) or 2.5 μM 6-BA, 5 μM NAA, and 0.5 μM 2,4-D (V-KM). Naked protoplasts are often adversely affected by agar. It is, therefore, better to grow them initially in liquid media. Favorable plating densities range between 7×10^3 and 2×10^5 ml^{-1} depending on the protoplast type and culture techniques. The cell suspensions are layered on the bottom of Petri dishes (0.7 ml in dishes about 35 mm in diameter, 1.8 ml in those of 5 mm and 6 ml in those of 10 mm in diameter). Culture in droplets of 40 to 100 µl have also been used. Embedding in agarose media promoted regeneration (Binding, unpubl.).

Appropriate light conditions are continuous illumination by cool white fluorescent light. Favorable temperatures are 25° to 28 °C.

Protoplasts may be embedded into agar as soon as rigid cell walls are regenerated. Following the procedure that has been developed for tobacco cell culture

Table 10.2. A proposed protocol for *Petunia* protoplast regeneration

I Initial conditions	
Culture media	FPC, DPD, V-47, V-KM (preferred in our laboratory), MS with mannitol; liquid
Plating density	5×10^4 cells/ml
Culture vessel	Petri dish (35 mm∅: 0.7 ml; 50 mm∅: 1.8 ml)
Illumination	2 klx cool white fluorescent light; continuously
Temperature	28 °C
II Subcultures	
3. day	Dilute by 0.7 (1.8 ml) liquid medium; in 2 dishes
6. day	Dilute in the same way but use medium with 0.25% agar
9. day	Dilute by same media, 1.1 (2.5) ml, each; transfer into Petri dishes of 5 mm (10 mm)∅
12. day	Layer on top of agar (protoplast culture medium but with 0.8% agar); spread to obtain about 20 colonies cm^{-2}
30. to 40. day	Transfer onto low osmotic media (e.g., B5C for *P. hybrida*) for shoot formation when colonies are 1–2 mm large
III Shoot formation	
7 days	at about 1 klx, 16 h day
continued	at about 3 klx, 16 h day
IV Root formation	
	Cut shoots of about 1 cm length (remove callus at base); use media B5 or MS with out growth hormones or with IAA at an initial concentration fo 20 μM

Table 10.3. Selected protoplast culture media for *Petunia*

	FPC	DPD	V-47		FPC	DPD	V-47
Macroelements (m*M*)				Phytohormones (μ*M*)			
Mg^{2+}	2.7	1.2	4.0	6-BA	5	2	2
Ca^{2+}	4.0	3.9	5.0	NAA	9	–	6
K^{+}	7.8	15.2	15.0	2,4-D	–	6	–
NH_4^{+}	5.2	3.4	3.5	Vitamins (m*M*)			
SO^{2-}	2.7	1.2	4.0	Thiamine · HCl	1	4	4
NO_3^{-}	10.3	18.0	18.0	Nicotinic acid	40	32	32
Cl^{-}	7.9	7.8	10.0	Pyridoxin	2.4	3.4	3.4
$H_2PO_4^{-}$	2.6	0.6	0.5	Biotin	0.2	0.16	0.16
Trace elements (μ*M*)				Folic acid	1.1	0.9	0.9
Mn^{2+}	50	23	23	Further organic ingredients (m*M*)			
Zn^{2+}	15	5	5	Mannitol	710/375	300	500
J^{+}	30	15	15	Sucrose	30	50	50
MoO_4^{2-}	0.5	0.4	0.4	Meso-inositol	0.55	0.55	0.55
Cu^{2+}	0.1	0.06	0.06	Glycine	0.013	0.018	0.018
Co^{2+}	0.05	0.04	0.04	pH	5.8	5.8	5.8
Fe^{2+}	50	100	100				
EDTA	50	100	100				

FPC (Frearson et al. 1973, values calculated from w/v; Binding 1974 b) DPD (Durand et al. 1973, values calculated from w/v; Binding 1974 b) V-47 (Binding 1974 b, modified from DPD)

(Bergmann 1960) and which has been applied to tobacco protoplast culture by Nagata and Takebe (1971), liquid suspensions are mixed with molten agar media at 45 °C. This result in a semi-solid media allowing the fixation of the cell clones to follow up single regenerants. If this is not planned, lower agar concentrations are used. Media with not more than 0.25% agar can be mixed with the cell suspensions at room temperature and the resulting culture can easily be diluted at daily intervals. The dilutions must be repeated every three days and the suspensions overlaid on agar media at about the 12 th day.

10.4 Protoplast Regeneration

Protoplast regeneration has been followed in several species and genotypes of *Petunia*. The developmental patterns are so congruent that no special reference will be given to certain provenances of the protoplasts.

10.4.1 Cell Wall Formation

Cell wall regeneration starts soon after plating. The cell wall is strong enough to influence the formation of the cell shapes after 24 h as is indicated by the occurance of egg-shaped cells. The cell walls are directly visible 8 h later after plasmolysis (Binding 1974 b). However, transfer to low osmotic media is not tolerated before the 10 th day of culture or before the 5th day in agarose media.

10.4.2 Cell Differentiation and Division

The development of the cells particularly depends on the differentiation state of the donor cells and on the culture media. Chlorophyll is reduced in mesophyll protoplasts within the first day of culture. This process is slowed by raising the plating densities (5×10^4 ml and more). Pigmentation in the vacuoles of corolla-derived protoplasts is carried over in some cell divisions. Cytoplasmic strands are formed in vacuolated cells; cell size increases, especially in media with low complexity of organic compounds. In the rich media KM 8 p and V-KM, vacuolated cells show an increase of cytoplasm, as well, but frequently do not enlarge. In cells from apical meristems, vigorously growing calli, and cell cultures, often no changes are visible prior to mitotic activities.

Cell divisions set in after 30 h in protoplasts of shoot tips and shoot cultures. A cell cycle, then, lasts about 20 h. Protoplasts from leaves of greenhouse plants started division after 5 days of culture (Durand et al. 1973; Frearson et al. 1973). Under optimal conditions, plating efficiencies of more than 0.9 can be obtained in shoot tip protoplasts.

Asynchrony of mitosis in daughter cells (Fig. 10.1.1) seems to be the rule. Synchronous divisions in cell clusters (Fig. 10.1.5) have rarely been found. The daughter cells may be equal in size (Fig. 10.1.1) or more or less different. Divisions of cells with large vacuoles (e.g., mesophyll-derived cells) frequently result in a large cell and a small meristematic one, the latter of which gives rise to the regenerant (Fig. 10.1.4). This was observed more frequently in rich medium V-KM than in V-47. Unequal divisions occur also in spontaneous fusion bodies; the small daughter cell, often containing only the nucleus of the original ploidy level, divides predominantly.

Incomplete divisions (Fig. 10.1.2) favoring the formation of polyploid regenerants (Fig. 10.1.5) must be kept as rare as possible by applying favorable growth conditions. For instance, cells as shown in Fig. 10.1.2 have been found less frequently in the rich medium V-KM than in V-47; cultures kept at appropriate cell densities by stepwise dilutions were superior to prolonged cultures at higher densities; less polyploid cells were found at reduced levels of 2,4-D. It can be assumed that not only polyploidization but the formation of aneuploid cells too is reduced under optimal culture conditions.

10.4.3 Organogenesis

Shoot formation occurs rather consistently in regenerated small callus of *Petunia hybrida* (strain T 2 × T 1; Fig. 10.1.6) upon transfer onto medium B5C (B5 medium with 5% coconut endosperm and 2.5 μM 6-BA). Shoot primordia can be detected after one week. However, it is important that good growth conditions have been provided during the preceding steps and that transfer is carried out early, that is, within the second month after protoplast isolation. The capability of shoot initiation declines significantly with the age of the clones; reactivation has not been possible in a number of investigations. Other culture media (MS:

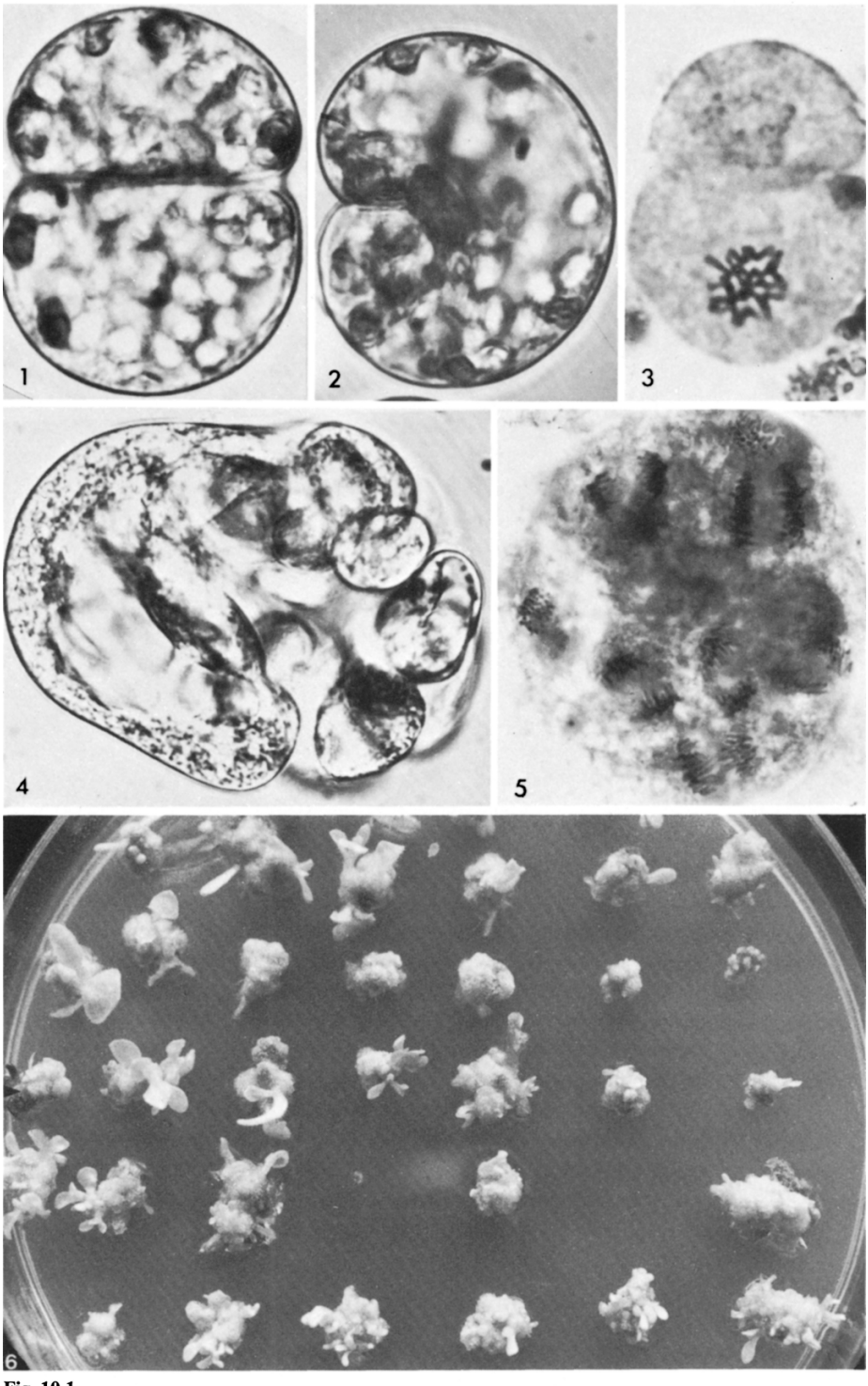


Fig. 10.1

Murashige and Skoog 1962, NT: Nagata and Takebe 1971) have been used for shoot induction in different species (Power et al. 1976; Donn et al. 1973).

Root formation is easily induced by transferring callus or shoots onto media free of hormones or by adding an auxin. Plants survive the transfer to planting medium especially when the roots have grown to a length of about 5 mm.

10.4.4 Properties of Regenerated Shoots and Plants

Plants have been regenerated from isolated protoplasts of the *Petunia* species *axillaris* (Power et al. 1976), *hybrida* (Durand et al. 1973; Frearson et al. 1973, and others), *inflata* (Power et al. 1976), *parodii* (Hayward and Power 1975), *parviflora* (Sink and Power 1977), and *violacea* (Power et al. 1976).

Young plants in the greenhouse often show morphological abnormalities such as irregular leaves, short internodes, and multiple shoot formation. These characteristics usually disappear during further development.

Cytological investigations of regenerated plants revealed the recovery of plants with conserved chromosome numbers. This has been reported for haploid *Petunia hybrida* (Binding 1974 b), for *P. parodii* (Hayword and Power 1975) and for *P. parviflora* (Sink and Power 1977). However, polyploid and mixoploid regenerants were also found. They were derived either from mutant donor cells, from spontaneous fusion bodies or from irregular mitoses during regeneration. Attempts have been made to calculate the degrees of spontaneous fusion products between protoplasts of different layers of the shoot apex in the regenerated shoot population by using periclinal chimeras containing mutant white plastids. No mosaic was found in nearly 500 clones (Fig. 10.1.6; Binding et al. 1982).

10.5 Conclusions

Isolated plant protoplasts are usually described as naked single cells which are exceptionally suited for investigations on developmental physiology, clonal propagation, and genetic modification. Commonly, several demands must be met in a protoplast system utilized for these purposes. Protoplasts should be genetically well defined by genetic stability of the donor material; they should represent the internal contents of single cells; regenerated plants should exhibit a conserved genetic constitution when compared with the original isolated protoplast if not a desired genetic modification has been introduced; plant regeneration should be reliably established. As illustrated in this chapter, all these problems have been in-

Fig. 10.1. Protoplast regeneration in *Petunia hybrida*: *1* Regular product of first mitosis. *2* Incompletely divided cell probably giving rise to a polyploid regenerant. *3* Mitotic asynchrony in sister cells. *4* Cell cluster demonstrating unequal development of the sister cells of the first mitosis. *5* Synchronous mitoses in a protoplast regenerant of a diploid shoot. The telophases show tetraploid condition. *6* Adventitious shoots formed within 14 days on medium B5C. Protoplasts were isolated from chimeric shoot apices with L 1 and L 2 containing white plastids. *Arrow* marks a green regenerant

vestigated thoroughly in *Petunia* and the technology is so far developed that most of the critical factors are under sufficient control: Mesophyll and shoot apices are quite stable in cytology; fractionation of the protoplasts by plasmolysis has not been observed in meristematic tissue; spontaneous fusion is reduced by preplasmolysis – the best plasmolytica for this purpose must be evaluated; spontaneous fusion bodies are not followed by equal high frequencies of polyploid regenerants as a result of unequal cell divisions – the phenomenon should be further studied in chimeric systems; cytogenetic stability during cell proliferation is quite easily obtained by fast regeneration under appropriate conditions – direct embryoid formation from isolated protoplasts would improve this factor; shoots and plants are easily regenerated in at least some of the *Petunia* genotypes, in others the technology must be improved.

The preference of *Petunia* as a model genus and the well established protoplast technology are two prime reasons for the utilization of *Petunia* protoplasts in a number of investigations which are treated in other chapters of this monograph.

11 Protoplast Fusion

K. C. SINK[1]

11.1 Cell Hybrids

The purpose of this section is to relate and summarize the observations of those protoplast fusion experiments in which one partner was a *Petunia* species and hybrid cells or their derived calli were produced, but hybrid plants were not regenerated (Table 11.1). Those fusion experiments involving *Petunia* where somatic hybrid plants were successfully regenerated are discussed in Sect. 11.2.

The first instance of such hybrid cells involving *Petunia* was protoplast fusion of *P. hybrida* + crown-gall-infected callus of *Parthenocissus tricuspidata* as reported by Power et al. (1975). Selection of heterokaryons was based on a two-step selection scheme theoretically dependent on the presence of both genomes for sustained division in culture. Cytological analysis of callus cells passing both selection criteria indicated the presence of only *Parthenocissus* chromosomes. However, such selected calli consistently expressed the isoperoxidase banding patterns typical of the expected summation of the pattern for both parental species. The additional bands were not induced in *Parthenocissus* by varying the cultural conditions, nor were they due to reversion or their inherent tumorous state. The loss of *Petunia* chromosomes was possibly due to premature chromosome condensation (PCC) by the *Petunia* interphase nucleus precociously entering mitosis. Thus, normal chromosome condensation and dissolution of the nuclear membrane of the mitotic *Parthenocissus* cells were not coordinated and the *Petunia* chromosomes were preferentially lost. It could not be determined whether the peroxidase isozyme patterns were a reflection of a possible hybrid nature of the cells or the integration of *Petunia* nuclear DNA into the *Parthenocissus* genome. Furthermore, after about one year, some of the *Petunia* isoperoxidases were lost from the callus cultures.

Binding (1976) fused protoplasts of *Nicotiana tabacum* Xanthi with those of *P. hybrida* (Table 11.1). By differential plastid pigmentation, he could determine that 10% of the protoplast population was heteroplasmic. However, after 8 days in culture only 0.5%–1.0% of the cells contained both plastids. The nuclei were not differentiated by carbol fusion stain. Finally, of 100 5 day old regenerates, no heterokaryons for the plastids were observed. Binding (1976) proposed that plastid segregation occurred because mixing was only partial in the fused cells and genome-plastid incompatibility existed. In the same report, Binding (1976) related

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Table 11.1. Somatic cell hybrids created by using *Petunia* as one of the fusion partners

Species	Time in culture	Chromosomes lost	References
Parthenocissus tricuspidata + *Petunia hybrida*	1 year	*Petunia*	Power et al. (1975)
Nicotiana tabacum + *Petunia hybrida*	5 days	–	Binding (1976)
Lycopersicon esculentum + *Petunia hybrida*	5 days	*Lycopersicon*	Binding (1976)
Daucus carota + *Petunia hybrida*	7 days	–	Reinert and Gosch (1976)
Datura innoxia + *Petunia hybrida*	30 days	–	Schieder (1977)
Vicia faba + *Petunia hybrida*	50 days	One or the other	Binding and Nehls (1978)
Atropa belladonna + *Petunia hybrida*	4 weeks	Mostly *Atropa*	Gosch and Reinert 1976 (1978)
Nicotiana tabacum + *Petunia hybrida*	Plants were obtained	*Nicotiana*	Xianghui et al. (1982)

attempts to fuse *Petunia* protoplasts with tomato subprotoplasts. Mitosis was initiated 24 h after fusion with up to 10 tomato chromosomes involved. However, all 300 regenerates examined after 5 days in culture had only the *Petunia* karyotype; the tomato interphase nucleus was not observed in multicellular regenerates. Since tomato subprotoplasts were used for fusion, Binding (1976) hypothesized that incompatibility in this system was apparently due to the interaction of the tomato nucleus with the *Petunia* protoplast.

Reinert and Gosch conducted protoplast fusion experiments between *Daucus carota* + *Petunia hybrida* (1976) and between *Atropa belladonna* + *P. hybrida* (Gosch and Reinert 1976, 1978). Heterokaryons of the former two species entered first division in 2–3 days. About 20% of the original heterokaryons sustained division during the first week in culture to form colonies of more than 15 cells. Cytological analysis of the regenerated colonies was not undertaken to determine the fate of the parental chromosomes. The primary intent of the study was to use the differential color of the parental protoplasts as markers to follow the early cell divisions of the heterokaryons. The fusion products between *P. hybrida* ($2n = 14$) + *Atropa belladonna* ($2n = 72$) were more definitively analyzed.

These two species were chosen because of their contrasting number and size of chromosomes, and both were used as isolated mesophyll protoplasts. Cytological examination was made through the first 4 weeks of culture. Mitosis of the heterokaryons began 5 days after the start of culture. It was synchronous even when the nuclei were unfused and such division patterns led to the formation of chimeras. Other heterokaryocytes were observed with fused nuclei in a common metaphase. From the fourth to the ninth day of culture about 5% of the metaphases were hybrid. The authors felt that the very high mitotic synchrony was especially remarkable since *Petunia* and *Atropa* have completely different time modes of entering cell division in culture. A factor contributing to the high rate of synchrony may have been the culture medium which was favorable for division of both species. Of plants regenerated from these fusion experiments, 235 were *P. hybrida,* 5 were *A. belladonna* (4*n*), and 33 shoots were phenotypically neither parental species. Thus, the results at the whole plant level are of interest in that these two species appeared to exhibit chromosome compatibility as evidenced by synchronous mitosis in the early hybrid cell divisions. However, during the latter period of culture the chromosomes of either parent, but predominantly *A. belladonna,* were lost from some cells and the remaining ones were incapable of shoot regeneration.

Schieder (1977) fused albino protoplasts of an X-irradiated mutant line of *P. hybrida* with a chlorophyll-deficient line of *Datura innoxia.* Following fusion, up to 3% hybrid protoplasts were observed but the green calli expected by complementation of the parental mutants was not observed. Schieder (1977) believed the failure to hybridize was due to a lack of complementation to chlorophyll biosynthesis; perhaps more specifically due to a loss of one or more sister chromatids carrying the locus (loci) required.

Binding and Nehls (1978) conducted a protoplast fusion experiment with *Vicia faba* and *P. hybrida.* As was the case with the *P. hybrida* + *A. belladonna* species, these two species differ markedly in chromosome morphology and staining. Thus, heterokaryons were readily identified by differential staining characteristics of the nuclei in aceto carmine. After 50 days of culture only three hybrid tissues were identified. These three callus cultures contained nuclei or chromosomes primarily of one or the other species.

Most recently, Xianghui et al. (1982) fused *Nicotiana tabacum* with *P. hybrida.* The B653 tumor line of *N. tabacum* was infected with *Agrobacterium tumefaciens* and thus characterized by phytohormone independence, inability to regenerate shoots and containing, from t-DNA, the genes for lysopine dehydrogenase (LpDH). LpDH is the enzyme responsible for the synthesis of the amino acid octopine which can be used as a genetic marker in transformation experiments. In contrast, the *P. hybrida* line W43 used was auxotrophic and possessed good shoot regeneration capacity. A two-step selection system was used to recover transformed cells that both maintained division to the green callus stage and regenerated. The plants recovered were all *P. hybrida* types but 21 of the 25 contained variable levels of LpDH. Six of these plants had significant LpDH activity in their leaf extracts in repeated tests. Cytological examination of root tips of some of the plants with lower LpDH activity indicated most were aneuploid (*P. hybrida* $2n = 28$) 23, 25, 32, 38, 40, and higher. It would appear that by a mechanism as yet

not well understood, some *P. hybrida* regenerates acquired LpDH activity. It has not been determined whether the transformation occurred by insertion of a segment of *N. tabacum* chromosome carrying the tDNA into the *Petunia* genome or by a transposon mode in the early cell hybrids. Furthermore, no cytological studies were conducted on single fusion products to elucidate the degree of chromosome incompatibility relative to survival of calli through the progressive selection steps and the concomitant activity of LpDH.

The above studies indicate the general consequences of cell-to-cell fusion using *Petunia* as one species. Incompatibility of these cell hybrids was usually expressed as partial or complete loss of one or the other chromosome complement. This loss seems to occur irrespective of apparent, intial mitotic synchrony between the parental species as in the *Petunia* + *Daucus* cell hybrids. Thus, chromosome losses occur due to nonsynchrony, perhaps by chromosome imbalance, but also in seemingly synchronous combinations for reasons that are not well understood. A major limitation of these early studies in analyzing the cytological behavior of these cell hybrids was that populations including fusion products and unfused cells had to be sampled. The more recent technique of culturing single fusion products like the *Arabidopsis* + *Brassica* hybrids (Gleba and Hoffmann 1978) enables cytological examination of heterokaryons at successive time intervals during the formation of colonies and callus stages. Thus, while some of the early hybrids could probably not be expected to succeed based on taxonomic considerations, the newer techniques should provide a better understanding of why certain desired combinations such as *Petunia* + *Nicotiana* have not succeeded, and may lead to methods to overcome such apparent incompatibilities at the somatic cell level.

11.2 Somatic Hybrid Plants

The first somatic hybrid *Petunia* plants were the result of protoplast fusion experiments between *Petunia parodii* ($2n = 14$) and *P. hybrida* Comanche ($2n = 14$), which are sexually compatible, as reported by Power et al. 1976a. The protocol for media and environmental requirements for sustained division of protoplast-derived cells and for plant regeneration were established prior to using *P. parodii,* a wild species, in fusion experiments (Hayward and Power 1975). Also, limited division of *P. parodii* to the 50-cell stage in the culture medium as used for hybrid calli selection was observed during earlier experiments on the regeneration of several species of *Petunia* (Power et al. 1976b). This same paper also defined the protoplast culture and regeneration conditions for *P. hybrida* Comanche. Both species had relatively high plating efficiencies of mesophyll protoplasts which were fused by polyethylene glycol (MW 6000) and plated in a selective medium. The selection system was based on limited cell division of one parent, *P. parodii,* and growth inhibition of Comanche by actinomycin-D, with complementation to sustained division and resistance to the drug exhibited by the hybrid cells. The 4% fusion rate observed eventually led to the identification of ten calli of separate or-

igin of which eight regenerated shoots. The overall frequency of somatic hybrid plant formation, although variable, was one in $1–8 \times 10^5$ protoplasts, not accounting for the loss of protoplasts at various experimental steps. The resultant somatic hybrid plants were purple-flowered and had a chromosome range from 24 to the expected 28. The somatic hybrids were found to have vegetative and floral morphologies parallel to tetraploid plants of the sexual cross. In addition, 20% of the gels had a faint isoperoxidase band peculiar to the tetraploid sexual hybrid in addition to those expected from both parents. The large and small subunits of fraction-I protein were idential for both parental species and could not, therefore, be used to discern differences in the behavior of these polypeptides in the somatic hybrid plants.

Somtic hybrid plants between these two *Petunia* species were also obtained by the use of selection systems based on differential growth of the two parental and hybrid protoplasts observed in two defined media (Power et al. 1977). The success of these two selection systems was based primarily on the tolerance of the somatic hybrid cells to 2,4-D and 6-benzylamino-purine (BAP) used in the culture medium. When actinomycin D was added to the 2,4-D (5.0 mg/l) and BAP (0.5 mg/l) medium, a higher frequency of somatic hybrid recovery was obtained than in the original (Power et al. 1976a) medium using naphthalene acetic acid and BAP as growth regulators.

One of the first instances where a chlorophyll-deficient mutant was used in a selection system for recovery of somatic hybrid plants was the same *P. parodii* + *P. hybrida* combination (Cocking et al. 1977). Sexually transmitted albino seedlings were obtained by selfing a spontaneous, mutant albino shoot of an otherwise normal *P. hybrida* Comanche plant. Protoplasts were isolated from albino suspension cultures of the former species and from leaf cells of *P. parodii*. Fusion was induced with 15% polyethylene glycol and the 1% induced heterokaryons were readily identified by the combination of clear cytoplasm and refractile bodies of *P. hybrida* with green chloroplasts of *P. parodii*.

The selection of somatic hybrid calli was based on limited division of *P. parodii* to the 50-cell stage, and continuous division of albino *P. hybrida* to yield colorless calli. The hybrid callus was competent for chlorophyll synthesis and for sustained division and thus visually identified among the colorless calli of *P. hybrida* in the culture dishes. Three of 35 selected green calli regenerated shoots of which two had $2n = 28$ and one $2n - 2 = 26$ chromosome numbers. The somatic hybrid plants were equivalent in morphological features to the somatic hybrids of previous experiments involving these same two species (Power et al. 1976a; Power et al. 1977).

The subsequent report on somatic plant hybrids in *Petunia* was that of Power et al. (1979) for the combination of *P. parodii* ($2n = 14$) + *P. inflata* ($2n = 14$). These two *Petunia* species are unilateral sexually incompatible with bud-pollination of *P. parodii* at a specific style length (2.3 cm) by *P. inflata* needed to obtain hybrid seed (Sink et al. 1978). Mesophyll protoplasts of *P. parodii* were fused with albino protoplasts of *P. inflata* using the high pH and calcium method (Keller and Melchers 1973). The selection system for hybrid calli was identical to that used for *P. parodii* + *P. hybrida* as previously mentioned. From two experiments, 8 of 29 and 10 of 50 selected calli regenerated shoots. The plants were confirmed

as hybrids by their vegetative and floral morphology and chromosome number to the expected $2n = 28$ with some aneuploid and euploid variants. Most of the somatic hybrid plants were self-fertile and the offspring thus derived segregated for parental characters. The somatic hybrids were relatively easy to achieve as indicated by a recover rate of 1 hybrid per 8.3×10^4 protoplasts. The significance of obtaining these somatic hybrids was that two species that had prezygotic barriers to sexual reproduction were successfully combined through protoplast fusion techniques.

The most recent report on *Petunia* somatic hybrid plants was by Power et al. (1980) involving two species that are sexually incompatible. *Petunia parodii* and *P. parviflora* ($2n = 18$) were selected in an attempt to affect morphological change in ornamental petunias by the transfer of the different growth habit of the former species to *P. parodii*. Wild-type leaf protoplasts of *P. parodii* were fused with those of a nuclear inherited albino mutant of *P. parviflora*. The regeneration protocol for both parental species was already known (Hayward and Power 1975; Sink and Power 1977). The experimental design and selection system for hybrid calli were the same as used for *P. parodii* + *P. inflata* (Power et al. 1979).

Thus, 30 green calli were visually identified 30 days after fusion. During subsequent culture on solid medium, 24 of those calli reverted to a near colorless appearance, became slow-growing and did not regenerate shoots. Three of the remaining green calli produced shoots. From one callus 50 plants were obtained; all of those examined had 31 chromosomes in contrast to the expected $2n = 32$. The other two shoot-regenerating calli gave rise to plants with 40 and 36 chromosomes. Those with 40 had a hybrid phenotype; whereas, the ones with 36 resembled *P. parviflora*. The recovery rate for these plants was 1 for every 2×10^7 protoplasts used in fusion. Furthermore, the somatic hybrid had 36% pollen fertility based on acetocarmine staining. The frequency of these somatic hybrids was about 250 times lower than that for the previously mentioned sexually and unilateral cross-incompatible *Petunia* species combinations. This lower recovery rate reflects the difficulty in obtaining only a few heterokaryons between sexually incompatible *Petunia* species that are capable of sustained division and plant regeneration without undergoing chromosomal elimination or otherwise expressing some type of incompatibility barrier at the cellular level.

Fraction-1 protein analysis to provide information on gene expression for RuBP Case in the *P. parodii* + *P. parviflora* somatic hybrids was possible since the two parental species differed in the small subunit (SS) polypeptide patterns (Gatenby and Cocking 1977) and differential staining intensity of the large subunit (LS) (Kumar et al. 1981). The 31 chromosome somatic hybrid plant was found to have all 5 SS units from both species but only LS band A from *P. parodii* (Kumar et al. 1981). The presence of the 5 SS units confirmed the presence of the nuclear genes from both species for this trait in the somatic hybrid plants. The presence and expression of only the *P. parodii* LS in the somatic hybrid plant is consistent with the finding of others (Kung et al. 1975; Melchers et al. 1978) that only the chloroplast DNA of one species is usually found in somatic hybrids and is probably not due, as Kumar et al. (1982) suggest, to the fact that *P. parodii* was the wild-type with competent chloroplasts as opposed to those of the nuclear albino of *P. parvilora*.

12 Anther and Pollen Culture

M. R. HANSON[1]

In this chapter, the published reports of anther and microspore culture of *Petunia* will be reviewed in comparison with culture of related Solanaceous genera. The reasons for the type and frequency of microspore development in *Petunia* will be explored. Factors which should be considered in attempting anther and microspore culture of *Petunia* will be examined.

12.1 Frequency of Success Compared to Culture of Other Solanaceous Genera

Like many other members of the Solanaceae, *Petunia* cells and tissue have been amenable to standard in vitro culture techniques. Unfortunately, anthers and microspores of *Petunia* are exceptions to this generalization. Relatively low frequencies of plantlet development from cultured anthers of various *Petunia* genotypes have been reported (Table 12.1), compared to related Solanaceous genera such as *Nicotiana, Datura, Atropa, Hyoscyamus,* and *Solanum* (Nitsch and Nitsch 1969; Zenkteler 1971; Naraynaswamy and Chandy 1971; Engvild et al. 1972; Dunwell and Sunderland 1973; Raghavan 1977; Rashid and Street 1973; Tomes and Collins 1976; Wernicke and Kohlenbach 1977; Sopory and Rogan 1976; Heberle and Reinert 1977; Sunderland et al. 1979). Not only are the number of anthers reported to respond to culture often lower than 2% in *Petunia,* but also only one to four plantlets developed from the cultured *Petunia* anthers. This is unlike the situation in other Solanaceae, in which many plantlets often develop from the same anther. For example, in *N. tabacum* Wisconsin 38, anthers containing 25–30 plantlets are not uncommon (Martineau et al. 1981).

On the other hand, anthers of specific *Petunia* genotypes will produce plantlets in culture at rates as high as 10% (Table 12.1). Thus, anther and microspore culture of *Petunia* has been much more successful than in certain genera. Furthermore, in contrast to the low plantlet development frequencies, high frequencies of callus and root formation can be achieved with anthers from a number of *Petunia* genotypes (Table 12.1). The block in *Petunia* anther culture may, therefore, be in obtaining embryogenesis from microspores rather than in causing the switch from reproductive differentiation to cell division. However, some of the callus resulting from *Petunia* anthers may be of somatic rather than of microspore

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Table 12.1. Callus and plantlets from *Petunia* anthers and microspores in culture

Line	% Anthers forming callus	% Anthers forming plantlets directly	References
P. axillaris × *P. hybrida* Rose du Ciel	1–2%	0.67–1.2%	Raquin and Pilet (1972), Raquin (1973)
Petunia (unidentified)	Callus formed, % not reported	0	Iyer and Raina (1972)
P. hybrida (unidentified)	0.9–19.6	Plantlets formed, % not reported	Sopory and Maheshwari (1973)
P. axillaris (Botanical Garden, Copenhagen)	Callus formed, % not reported	1.1	Engvild (1973)
P. hybrida Cyandintyp	Not reported	0.6	Wagner and Hess (1974)
P. hybrida 34d10	Not reported	0.4	
P. hybrida Cyandintyp × 34d10	Not reported	0.85–1.2	
P. hybrida Rose du Ciel Cascade	20[a] (Plantlets regenerated)	0	Sangwan and Norreel (1975)
P. hybrida (unidentified)	Callus formed, % not reported	1.0–2.0	Ganugapati et al. (1976)
P. hybrida (unidentified)	0.2–7.9 (Callus formed embryoids)	4.4–10.1	Malhotra and Maheshwari (1977)
AxAC-14, AxAC-2 (Rose du Ciel × *P. axillaris* progeny from backcross to *P. axillaris*	85	4.0	Mitchell et al. (1980)
Rose du Ciel × *P. axillaris* selected backcross progeny	49–92	0.5–1.5	Martineau et al. (1981)

[a] Ranges reflect the minima and maxima resulting from different experiments

origin. A microspore origin for *Petunia* anther callus has been demonstrated only in a few instances (Mitchell et al. 1980, see Sect. 12.5).

In comparison to other Solanaceae, the genus *Petunia* does not have any obvious general characteristics which can explain the observed difficulty in achieving high rates of embryogenesis from microspores. The *Petunia* anther is not an inhospitable location for embryogenesis of all microspores; *N. tabacum* plantlets will develop if tobacco microspores are inserted into *Petunia* anthers (Pelletier and Ilami 1972; Berville et al. 1971). However, *Petunia* microspores injected into tobacco anthers did not form embryos (Pelletier 1973).

The rate of abnormal pollen development among the Solanaceae may also be a relevant factor to examine in comparing anther development frequencies. Dunwell (1978) pointed out that abnormal pollen development occurs not only in vitro, leading to callus and/or embryo formation, but also in vivo in many species of the Solanaceae and Graminae, the two families in which the highest rates of microspore embryogenesis have been obtained. If abnormal pollen development in vivo is important in order for a species to exhibit microspore division in vitro, then *Petunia hybrida* would be expected to be more amenable to microspore culture. Budding and fission in microspores of certain lines, abnormally large mega-

pollen, and unreduced pollen has been reported in *Petunia hybrida* (Izhar 1973; Maizonnier 1974, 1976a, b).

Perhaps formation of abnormal pollen by a plant species in vivo is negatively correlated with the species' rate of microspore division in vitro. The amount of abnormal pollen increases as *N. tabacum* plants age, and embryogenesis from microspores is also reduced in anthers from older plants (Reinert and Bajaj 1977).

12.2 Effect of Genotype

Only certain genotypes of anther donor *Petunia* plants have produced plantlets from cultured anthers (Table 12.1). As unsuccessful experiments to culture anthers often remain unpublished, the number of *Petunia* genotypes which have been cultured fruitlessly remains a matter of speculation. Genotypic differences in anther culture results among lines of *Solanum tuberosum* (Foroughi-Wehr et al. 1977; Sopory and Rogan 1976) and between species of *Nicotiana* (Tomes and Collins 1976) have been noted.

The genotypes of *Petunia* used in reported anther culture experiments include inbred lines, F1 hybrids, backcross progeny, and unspecified genotypes which may be highly heterozygous. Generally, heterozygous material appears to be more suitable as a source of anthers for culture. The F1 hybrid cultured by Wagner and Hess (1974) produced plantlets at approximately double the rate of the two parental lines. Six F1 hybrids tested by Mitchell et al. (1980), which were derived from five inbred lines, produced callus from anthers at substantially higher frequencies than the parental inbred lines (Table 12.2; Fig. 12.1).

Table 12.2. Effect of genotype of anther donor plant on frequency and type of development in culture. (Data from Mitchell et al. 1980)

Petunia line	Number of anthers cultured	% Development
I. Callus formation		
Yellow 47	1 295	0.1
Blue 147	813	8.2
Yellow × Blue	180	20.6
P. axillaris	142	91.5
Rose du Ciel	295	23.7
P. axillaris × Rose du Ciel	408	82.0
II. Plantlet formation		
Rose du Ciel	295	0.3
P. axillaris	142	0
Rose × *P. axillaris*	303	0
P. axillaris × Rose	555	0.7
Individuals from *P. axillaris* × (*P. axillaris* × Rose)	339	4–6

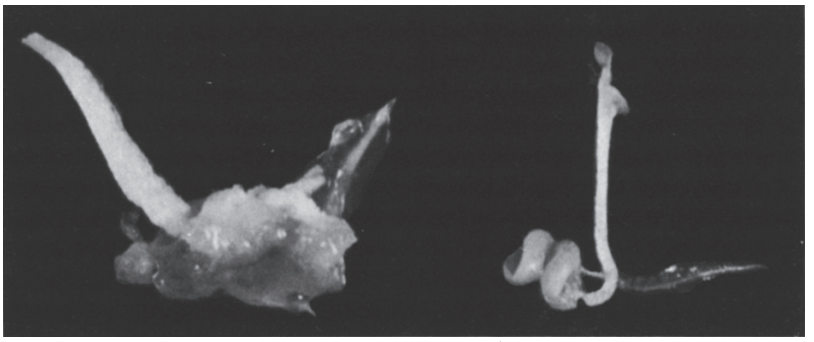


Fig. 12.1. *Petunia* plantlets emerging from anthers in culture. Plantlets emerging from anthers which also have produced callus are often misshapen

Lines of *Petunia* referred to as *P. axillaris* and *P. hybrida* Rose du Ciel have been the plant material utilized in several reports of successful *Petunia* anther culture (Raquin and Pilet 1972; Sangwan and Norreel 1975; Engvild 1973; Mitchell et al. 1980; Martineau et al. 1981). However, depending on the source of seed, the *P. axillaris* and Rose du Ciel lines may differ betwen laboratories thus causing differences in experimental results. For example, mitoses occurs in the 0.6–0.8 cm long *P. axillaris* buds of Engvild (1973) but in 1.4–1.8 cm long buds of the *P. axillaris* line used by Mitchell et al. (1980); plants of these two *P. axillaris* lines also have different morphologies. Only 2.4% of the cultured anthers of Heavenly Rose variety from Gurney Seed Co. (South Dakota, USA) developed callus, while 23.7% produced callus from anthers of Rose du Ciel derived from seed from France (Stokes Seed Co.) (Mitchell et al. 1980). Furthermore, microspores cultured from anthers of these two Rose du Ciel lines never divided under conditions devised by Sangwan and Norreel (1975) for microspore culture of *P. hybrida* Rose du Ciel (Mitchell, Hanson, Skvirsky, Ausubel, unpublished).

Most reports of *Petunia* anther culture have dealt with only a limited number of genotypes but these results are impossible to compare due to different culture conditions. However, genotype as a factor affecting anther development in culture was specifically investigated by Mitchell et al. (1980). Genotype of the anther donor plant was observed to affect profoundly the frequency of formation of callus, roots, and plantlets from anthers on a particular medium (see Table 12.2 for selected examples). By testing progeny of backcrosses between *P. hybrida* Rose du Ciel and *P. axillaris,* individual plants could be identified with enhanced plantlet development frequency (>4%) from anthers cultured on a defined medium (Mitchell et al. 1980). The two parental genotypes of these hybrid progeny exhibit high frequency of callus formation from anthers (Table 12.2) and also regenerate readily from protoplasts (Skvirsky, Hanson, Smith, Ausubel, unpublished; Power et al. 1976). Hybridizing *Petunia* lines which exhibit high division rates and morphogenesis from vegetative tissue may be a reasonable strategy for increasing the number of genotypes represented by anther-derived plants.

Another method to increase the number of genotypes from which anther-derived plantlets can be obtained would be to regenerate shoots from callus arising

from anthers by transfer to a suitable medium. However, while anther-derived *Petunia* callus has been induced to form shoots in some cases (Table 12.1), in other cases such callus was observed to be particularly refractive to induction of shoot formation (Mitchell et al. 1980). Regeneration of shoots from roots arising from *Petunia* anthers and from anther callus may be a better method for obtaining plants from nonembryogenic anthers. Colijn et al. (1979 b) have recently published methods for inducing shoots from roots of *Petunia*.

Why should one particular anther donor plant produce plantlets at a higher rate than another of a different genotype? Many factors and their combinations may comprise genotypic differences in microspore development rates on a particular medium. For example, the physiological conditions of the anther donor plant affect anther development (see Sect. 12.4). Certain genotypes may be able to produce androgenic microspores under suboptimal environmental conditions. If this is true, Mitchell et al. (1980) may have actually selected for capability to produce androgenic embryos in the range of plant environments in their greenhouse. Testing several genotypes under different defined environmental conditions can settle this question.

An alternative explantation for the genotypic effect is that the genotype of the diploid anther tissue affects the development of the enclosed *Petunia* microspores during culture. Both inhibitory and stimulatory effects of anther somatic tissue have been reported in other Solanaceae (Pelletier and Ilami 1972; Nitsch 1977; Horner and Street 1978). Pollen transfer experiments of the type performed by Pelletier and Ilami (1972), in which microspores from one genotype are placed inside anthers of another genotype, can test this hypothesis in *Petunia*. The genotype of the *Petunia* anther donor is already known to affect development of inserted tobacco microspores. When tobacco microspores were inserted into anthers from a cytoplasmic male-sterile *Petunia,* tobacco plantlets appeared from 16% of the anthers, versus 3.5% of male-fertile *Petunia* anthers containing inserted tobacco microspores (Berville et al. 1971).

Perhaps the genotype of the pollen determines whether embryogenesis or cell division will occur. According to this hypothesis, only microspores with a particular chromosome complement are induced to divide, and among these, only particular genotypes will be directed toward embryo formation. Differences in morphogenetic response of vegetative tissue, depending on the genotype, has been demonstrated in *Petunia* (Skvirksy et al. 1982; Power et al. 1976). A more frequent presence of particular genotypes among androgenic plants derived from heterozygous donor *Petunia* plants would be evidence for this hypothesis. In the experiments of Mitchell et al. (1980), no one particular genotype of anther-derived plant was obviously favored; phenotypes of the anther-derived plants differed with respect to a number of flower color characteristics. However, a larger sample of anther-derived plants and genetically marked anther donor plants are necessary to test this possibility.

Another genetic factor in anther development could be the line-specific tendency for microspores to undergo budding and fission in *Petunia* (Izhar 1973) or formation of megapollen (Maizonnier 1976 b). Tests to find whether abnormal pollen in vivo is correlated positively or negatively with a species' anther development or rate could be carried out in *Petunia*.

12.3 Effect of Microspore Stage, Media, and Incubation Conditions

12.3.1 Microspore Stage

Petunia microspores of particular developmental stage should be selected in order to obtain optimum division rates, as observed by Nitsch and Nitsch (1969) for *N. tabacum*. *Petunia* microspores at the uninucleate (Wagner and Hess 1974; Swamy and Chacko 1973; Ganugapati et al 1976), first mitosis (Raquin and Pilet 1972; Sangwan and Norreel 1975), and binucleate (Engvild 1973) stages have all been reported to have the highest rate of development. Genotypic differences may be responsible for this disparity in results. Initial experiments with a genotype not previously cultured should therefore be performed with microspores of all three stages.

For rigorous evaluation of which anther age is best for culture, one anther from each bud should be examined microscopically before culturing the remaining four anthers. Furthermore, the degree of synchrony between anthers of the same bud and within one anther must be determined. Mitchell et al. (1980) observed that *Petunia* anthers from buds of a particular size could contain either predominantly uninucleate, dividing, or binucleate microspores. Morphological appearance of the bud (total length or sepal/petal ratio) may not always be a reliable guide to microspore developmental stage, perhaps varying with environmental conditions, plant age, or genotype.

In *Nicotiana,* Sunderland et al. (1974) found that the correlation between corolla length and microspore stage did not apply except under standardized growth conditions. Tomes and Collins (1976) also reported that bud morphology could not be used to determine microspore age in greenhouse-grown *Nicotinana* plants.

The stage of microspore development may be more critical for subsequent division of isolated microspores and for embryo formation from anthers than for anther callus formation. Both Swamy and Chacko (1973) and Mitchell et al. (1980) obtained callus from anthers of all bud stages cultured. However, Sangwan and Norrell (1975) found that divisions occurred only in microspores cultured around the time of first mitosis, and Engvild (1973) observed that culture of binucleate microspores was critical for plantlet formation. Precise determination of the pathway of division and embryogenesis in *Petunia* anthers is feasible only if microspore development is a frequent event. Unfortunately, most cultured *Petunia* anthers produced plantlets at rates less than 2%. However, microspore development leading to callus formation could be feasibly studied in the genotypes reported to produce callus at frequencies greater than 80% (Mitchell et al. 1980), provided that most of this callus is microspore-derived.

12.3.2 Media and Incubation Conditions

Unlike many Solanaceous relatives (Sunderland and Dunwell 1977; Raghavan 1976), *Petunia* falls in the class of species usually requiring growth regulators in

anther culture. The principal media used in successful culture of *Petunia* anthers are listed in Table 12.3. The basal medium used most frequently was developed by Nitsch (1969) for *Nicotiana,* and contains approximately one-half the major salts and increased vitamins and microelements relative to Murashige and Skoog (1962) medium. Either an auxin, a cytokinin, or coconut milk, or combinations among these three growth regulators, have been important in achieving anther development (Table 12.3). No generalization about content of *Petunia* anther culture media can be made; diverse levels and auxin-cytokinin ratios have been successful with different genotypes. A number of authors who worked on *Petunia* anther culture comment than many media were tried without success.

As in other species (reviewed in Sunderland and Dunwell 1977; Duncan and Heberle 1976; Sunderland 1979), cold treatment of buds prior to anther culture was found to be beneficial (Malhotra and Maheshwari 1977; Mitchell et al. 1980). Incubating buds at 6 °C for 48 h prior to culture doubled the number of anthers producing embryos directly without prior callus formation on a coconut milk medium (Malhotra and Maheshwari 1977).

The growth regulator content of *Petunia* anther culture medium clearly affects whether callus or plantlets, or both, develop from anthers (Table 12.3). Insufficient numbers of anther-derived plantlets have been obtained to determine conclusively whether the growth regulator content of the media also affects the ploidy of microspore-derived plants.

For anther culture of *Petunia,* the standard techniques have been followed: surface-sterilization of buds with or without prior cold treatment, dissection of anthers, and incubation of anthers on agar medium. Except for the one report of successful microspore culture, in all reports listed in Table 12.3, anthers were cultured on agar medium and were illuminated at temperatures between 25 °C and 30 °C for 4–12 weeks.

Sangwan and Norreel (1975) were able to culture microspores of *P. hybrida* Rose du Ciel and Cascade in liquid medium. Optimal callus formation, and, eventually, shoots, could be obtained from microspores cultured in medium containing 1.0 mg/l BA and 0.1 mg/l NAA (Sangwan and Norreel 1975). The 0.1 mg/l BA - 1.0 mg/l NAA and 10 mg/l BA - 0.1 mg/l NAA media developed by Sangwan and Norreel (1975) were also used successfully by Mitchell et al. (1980) for anther culture.

For microspore culture, fine details of technique may be important (see also Sect. 12.4). Sangwan-Norreel (1977) reported that such steps as centrifugation time and velocity in preparing *Datura* microspores for culture could profoundly affect their division rate. In culturing *Petunia* microspores, Sangwan and Norreel (1975) homogenized anthers, washed the microspores by three centrifugations at 120 g for 5 min, and cultured the microspores of two anthers in 1 ml of medium. If the centrifugation was too short, the microspores were contaminated with anther pieces, and if too long, the microspores were injured (B. S. Sangwan-Norreel pers. commun.). Temperatures lower than 28 °C and light stronger than 1 000 lx were unfavorable to microspore development (B. S. Sangwan-Norreel pers. commun.).

The use of nurse tissues for *Petunia* microspore culture has not been reported. *Petunia* callus can serve as nurse tissue for development of *N. tabacum* micro-

Table 12.3. Principal media for induction of microspore division and development in *Petunia*

Basal medium	Hormones and special components	Result
I. Raquin and Pilet (1972), Raquin (1973)		
Nitsch (1969)	1.0 mg l^{-1} BA,[a] 0.1 mg l^{-1} NAA	Callus and plantlets
	0.2 mg l^{-1} BA, 0.1 mg l^{-1} NAA	Callus
	no hormones	2 plantlets/300 anthers
II. Envild (1973)		
MS and Nitsch (1969) combination	2 mg l^{-1} kinetin	Callus and triploid plantlets
III. Sopory and Maheshwari (1973)		
Nitsch (1969)	15% coconut milk	Callus and plantlets
	2.0 mg l^{-1} kinetin, 5.0 mg l^{-1} NAA	Optimum callus formation
	0.2 mg l^{-1} kinetin	Plantlets
	0.02 mg l^{-1} kinetin	Plantlets
IV. Swamy and Chacko (1973)		
Nitsch (1969)	0.5 mg l^{-1} zeatin, 5.0 mg l^{-1} GAA	Callus
	0.1 mg l^{-1} zeatin, 1.0 mg l^{-1} NAA	Callus
	0.5 mg l^{-1} kinetin, 1.0 mg l^{-1} NAA	Callus and roots
	2.0 mg l^{-1} BA	Plantlets
V. Wagner and Hess (1974)		
Nitsch (1969)	2 mg l^{-1} BA, 0.5 mg l^{-1} kinetin, 0.5 mg l^{-1} NAA, 15 mg l^{-1} PFP	1 haploid plantlet
	10 unspecified combinations of 0–2.0 mg l^{-1} BA, 0.1–1.0 mg l^{-1} kinetin, 0–0.5 mg l^{-1} NAA	Diploid and triploid plantlets
VI. Sangwan and Norreel (1975)		
MS and Nitsch (1969) combination	0.1 mg l^{-1} NAA, 1.0 mg l^{-1} BA	Optimum callus formation
	0.1 mg l^{-1} NAA, 0.1 mg l^{-1} BA	Callus
	0.5 mg l^{-1} NAA, 0.5 mg l^{-1} BA	Callus
	1.0 mg l^{-1} NAA, 0.1 mg l^{-1} BA	Callus
	1.0 mg l^{-1} NAA, 1.0 mg l^{-1} BA	Callus
VII. Ganugapati et al. (1976)		
Nitsch (1969)	20% CM, 2 mg l^{-1} adenine	Triploid plantlets
	1.0 mg l^{-1} BA	Diploid plantlets
	2.0 mg l^{-1} NAA, 0.1 or 1.0 mg l^{-1} zeatin	Callus and misshapen plantlets
	1.0 mg l^{-1} 2,4-D	Callus
	5.0 mg l^{-1} BA	Callus, shoots upon subculture
	5–10 mg l^{-1} IBA, 0.5–1 mg l^{-1} BA	Green callus with shoot buds
VIII. Malhotra and Maheshwari (1977)		
Nitsch (1969)	15% coconut milk	Plantlets
	0.2 mg/l kinetin	Plantlets and callus differentiating embryos
IX. Mitchell et al. (1980), Martineau et al. (1981)		
MS and Nitsch (1969)	1.0 mg l^{-1} NAA, 0.1 mg l^{-1} BA	Callus, roots, plantlets
	0.1 mg l^{-1} BA, 1.0 mg l^{-1} NAA	Callus and plantlets

[a] Abbreviations: BA (benzyladenine), NAA (α-naphthalene acetic acid), MS (Murashige and Skoog, 1962 medium), CM (coconut milk), PFP (para-fluorophenylalanine), 2,4-D (2,4-dichlorophenoxy acetic acid), IBA (indole butyric acid)

spores (Pelletier und Durran 1972). However, only callus tissue derived from *Petunia* petals, and not from stem or sepal, permitted development of *N. tabacum* microspores (Pelletier and Durran 1972). Pelletier and Ferault (1976) later found that the *Petunia* petal-derived callus contained a few *Petunia* embryos, while the leaf callus did not, and suggested that embryogenic callus is required as nurse tissue for microspore embryogenesis.

12.4 Characteristics of Cells and Plants Produced from *Petunia* Anthers

Anther and pollen culture of *Petunia* has produced more diploids and tetraploids than haploids (Table 12.4). A survey of cultured plant species reveals that haploid production from anthers is actually the exception rather than the rule. While haploid anther-derived plants are most common in *N. tabacum,* diploids, triploids, and tetraploids are common in other genera, including *Datura, Atropa,* and *Solanum* (reviewed in Vasil and Nitsch 1975; Sunderland and Dunwell 1977).

In other Solanceous genera, the microspore-derived embryo has been shown to develop following (1) repeated division of the germ cell, (2) repeated division of the vegetative cell, (3) equal division of the vegetative and generative cell and (4) fusion of the vegetative and generative cell. Either haploids, diploids, or triploids can be derived by these events. The origin of triploids, in *Petunia* and other species, has not been determined conclusively, but could result from such diverse events as a triple nuclear fusion, or endomitosis in a haploid cell followed by fusion with a haploid cell. Engvild (1973) and Raquin (1973) both pointed out that triploids could arise from fusion of the vegetative nucleus with a generative nu-

Table 12.4. Ploidy Frequency in plants from anther and microspore cultures of *Petunia*

Cultivar or individual anther donor	Frequency				
	Haploid	Diploid	Triploid	Tetraploid	Reference
P. axillaris × Rose du Ciel	2	3	45	2	Raquin and Pilet (1972), Raquin (1973)
Rose du Ciel	–	–	4	1	
P. axillaris	–	–	20	–	Engvild (1973)
P. hybrida (Cyandintyp × 34d10)	–	7	6	–	Wagner and Hess (1974)
Cyandintyp	–	6	6	–	
34d10	–	4	4	–	
Rose du Ciel	3	7	20	–	Sangwan and Norreel (1975)
P. hybrida (unidentified)	–	[a]	[a]	1	Ganugapati et al. (1976)
Rose du Ciel and *P. axillaris* hybrid backcrosses	2	17	12	4	Mitchell et al. (1980)

[a] Exact number not reported

cleus which has completed DNA synthesis prior to the second pollen mitosis. Raquin's (1973) observation of a triploid metaphase in a dividing microspore indicates early establishment of triploidy.

The presence of diploid and tetraploid plants in populations regenerated from anther-derived callus is not unexpected. Diploids and tetraploids arise at high frequencies from plants regenerated from tissue explants and protoplasts of haploid *Petunia* (Maizonnier 1976a; Ausubel et al. 1980; Hanson, unpublished).

In *Petunia*, anther-derived plants have been obtained through three routes – direct embryogenesis, callus formation followed by embryogenesis, and regeneration of shoots from callus. Perhaps the developmental pathway followed by a *Petunia* microspore depends on such factors as genotype, microspore stage at time of culture, or composition of culture medium. In *Datura,* ploidy level of anther-derived plants increases with increase in microspore stage at time of culture (Sunderland et al. 1974; Engvild et al. 1972).

The growth regulators necessary for *Petunia* anther culture medium can be suspected as a possible cause of the higher ploidies (diploid and triploid) of microspore-derived *Petunia* plants, in comparison to the haploid *N. tabacum* plants obtained on growth regular-free medium (Nitsch and Nitsch 1969). However, *Datura* anthers produce nonhaploids in growth regulator-free medium (Engvild et al. 1972), and haploid *N. tabacum* plants appear from anthers on both growth regulator-free and medium containing growth regulator (Martineau et al. 1981). Furthermore, Raquin (1973) obtained triploid plants from *Petunia* anthers on growth regulator-free medium. Thus, the ploidy of resultant plants may reflect the preferred developmental pathway of the induced microspores, which may or may not be affected by the growth regulator content of the medium.

Division of the anther wall, connective tissue, and the filament is an undesirable possible effect of incorporating growth regulators in anther culture medium. For example, embryoids were induced from somatic, not vegetative tissues of *Atropa belladonna* anthers on medium which contained a growth regulator (Rashid and Street 1973). Because growth regulators must be used at present in culturing *Petunia* anthers, the tissue origin of plantlets and any callus which forms should be determined.

Tissue origin of plantlets and callus from anthers can be established through microscopic observation, ploidy determination, and genotype evaluation. Haploid and triploid chromosome numbers indicate a microspore origin, while diploid and tetraploid cells could arise from either microspores or anther somatic tissue. The origin of nonhaploid anther-derived plants can be determined if heterozygous *Petunia* lines are used as anther donors. If the genotype of an anther-derived plant differs from that of the anther donor plant, a microspore origin is implicated. A difference in genotype between the plants derived from the same anther also indicates that all the plants did not arise from anther somatic tissue. Raquin and Pilet (1972), Wagner and Hess (1973), and Mitchell et al. (1980) assigned a microspore origin to anther-derived *Petunia* plants by comparing their flower color to that of the anther donor plant. Also, ploidy of anther-derived cells and roots, and flower color of plants regenerated from anther callus indicated that at least some of the callus derived from anthers cultured by Mitchell et al. (1980) was of microspore origin.

Ploidy of anther-derived plants can be determined either by root tip cell-staining techniques (Marks 1973; Mitchell et al. 1980) or by a chloroplast/guard cell pair counting method (Butterfass 1973; Maizonnier 1976a; Mitchell et al. 1980). Chloroplasts per guard cell pair typically number 6–14 in haploids, 14–21 in diploids, 21–30 in triploids, and 28–43 in tetraploids, subject to some genotypic and environmental variation (Mitchell et al. 1980). Examining both roots and shoots is advisable in order to identify root/shoot chimeras of different ploidies, which have been reported from *Petunia* (Engvild 1973) and from *Nicotiana* (Sunderland 1971) anther culture.

Homozygous diploids from *Petunia* haploid plants can be quickly derived by taking advantage of chromosome doubling during regeneration of *Petunia* tissue explants. Diploids appear frequently among shoots regenerated from pedicels (Maizonnier 1976a; Pellitier and Delise 1969) or stem (Ausubel et al. 1980), or leaf strip culture of haploid *Petunia* (Hanson, unpublished). By culturing leaf strips of an anther-derived haploid on MS medium containing 0.1 mg l^{-1} NAA and 1.0 mg l^{-1} BA, diploid *Petunia* plants ("Mitchell") with high fertility were obtained (Ausubel et al. 1980).

12.5 Effect of Plant Age and Environmental Conditions

A decrease in frequencies of plantlet development from *Petunia* anthers has been observed by Mitchell, Martineau, and Hanson (unpublished). Such a decrease in anther development frequencies during the flowering season has also been observed in *N. tabacum* (Anagnostakis 1974; Reinert and Bajaj 1977) and in *N. paniculata* (Tomes and Collins 1976). However, no difference in anther development frequency as plants aged could be detected in *N. rustica* (Tomes and Collins 1976).

In *Petunia,* experiments specifically designed to test the effect of environmental conditions of the donor plant on growth response of anthers in culture have not been reported. However, in other members of the Solanaceae, such experiments indicate a critical importance of the growth conditions of the anther donor plants. In *N. knightiana,* twice the number of anthers developed embryos when plants are grown at 20 °C than at 14 °C (Sunderland and Dunwell 1977). Five times as many plantlets were produced from *N. tabacum* anthers when plants were given 8 h rather than 16 h of light (Sunderland and Dunwell 1977). *N. tabacum* cv. Badischer Burley plants given additional salt during growth produced plants from microspores at much higher rates (Heberle and Reinert 1977). Seasonal variations in anther growth response in culture have been reported in *Solanum tuberosum* (Dunwell and Sunderland 1973).

Another modification to the donor plant's growth condition that can affect anther culture is disconnection from the root system and nutrient feeding. When Sunderland and Roberts (in Sunderland and Dunwell 1977) placed flowering stems of *N. sylvestris* in nutrient solutions prior to anther culture, the number of anthers developing plantlets increased relative to controls.

A further method to modify the growth condition of *Petunia* flowering stems prior to culture would be to graft different *Petunia* genotypes in combinations with other Solanaceous pecies. An intriguing question is wether grafting of *Petunia* on species such as *N. tabacum,* which have high anther development rates, will affect culture of the *Petunia* anthers. The effect of a *Petunia* graft on anther culture of a *Nicotiana* stock or scion would also be interesting to determine.

The environmental conditions of the anther donor plants used in the pioneering microspore culture work of Sangwan and Norreel (1975) may be critical for obtaining microspore culture division, and should be considered when attempting to repeat or extend this successful microspore culture work. Using greenhouse-grown plants and the culture medium and techniques described by Sangwan and Norreel (1975), Mitchell, Skvirksy, Hanson, and Ausubel (unpublished) were unable to induce division in microspores of 3000 anthers of *P. hybrida* Rose du Ciel or three other lines. In contrast, Sangwan and Norreel (1975) reported that one plant developed from the pollen of eight Rose du Ciel anthers. The plants used by Sangwan and Norreel (1975) were grown in the Phytotron at Gif on vermiculite, watered daily with Nitsch's solution, and maintained at 24° during the 16-h day and 18 °C at night (B. S. Sangwan-Norreel, pers. commun.). Illumination was 19000 lx total, of which 36 lx was from incandescent lights and the remainder fluorescent (B. S. Sangwan-Norreel pers. commun.). Nitsch's (1965) solution contains the following salts, in mg/l: $Ca(NO_3)_2 \cdot 4H_2O$: 959, KNO_3: 411, $(NH_4)_2SO_4$: 137, $MgSO_4 \cdot 7H_2O$: 518, KH_2PO_4: 137, EDTA-NaFe·$2H_2O$: 41, H_2BO_2: 3, KCl: 2.7, $MnSO_4 \cdot H_2O$: 1.7, $ZnSO_4$: 0.27, $(NH_4)_6Mo_7O_4 \cdot 4H_2O$: 0.27, $CuSO_4 \cdot 5H_2O$: 0.13.

12.6 Practical Considerations

The primary reasons for performing anther and pollen culture are (1) to isolate haploids, (2) to produce homozygous diploids, and (3) to study the process by which reproductive cells are switched to vegetative development. The low yields of haploids from *Petunia* anther culture suggests that the traditional genetic methods described by Maizonner (Chap. 13) for producing haploids are better approaches than tissue culture at present, if a haploid from a particular genotype is desired and greenhouse space is not limiting. Furthermore, studying the process of embryogenesis from microspores is not practical in most of the *Petunia* lines previously examined, because of the low frequency of development. However, this type of study is feasible using the genotypes selected by Mitchell et al. (1980) and those used by Malhotra and Maheshwari (1977) in which plantlet development frequency/anther ranges from 5%–15%. Furthermore, additional experiments on anther and pollen culture of *Petunia* will be extremely useful if the factors resulting in low anther response can be determined and overcome and microspore culture extended to additional genotypes. Techniques for achieving higher embryogenesis rates from *Petunia* microspores may also be applicable to the many other species exhibiting low rates of development in culture.

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13 Haploidy

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13.1 Origin of Haploids

Although it was possible to obtain haploids of *Petunia* (with $n = 7$ chromosomes, Fig. 13.1) from anther cultures (Raquin and Pilet 1972; Raquin 1973; Mitchell et al. 1980, see also Sect. 12), or even from pollen cultures (Sangwan-Norreel 1975), it was only at low frequencies when compared to the other Solanaceous genera in which similar attempts were carried out. The best means to obtain *Petunia* haploids is still the natural way as used by Straub (1973), Maizonnier (1973), and Singh and Cornu (1976). The method consists of taking lines with genetic markers and making hybrids between these lines. Each plant in the progeny which exhibits the phenotype of one of the parent lines instead of the hybrid phenotype may be a potential haploid. Such plants appeared with a frequency of at least 10^{-4} in the experiments of above mentioned authors, but sometimes with a genotypic influence of the pollen parent. This natural method presents three advantages: (a) natural haploids may have either a gynogenetic or an androgenetic origin that is detectable with genetic markers and that have similar frequencies (Maizonnier 1973), (b) seedling markers enable an early screening among very large populations grown in a small area and, (c) a given genome can be introduced directly into a new cytoplasm (Singh and Cornu 1976). A rather complete review of available *Petunia* haploids, with their main characteristics, was made by the different laboratories in the first issue of the Plant Molecular Biology Newsletter (1980).

13.2 Cytology of Haploids

As expected, haploid cells are smaller than corresponding cells in diploids but cell size is not sufficient for screening $2n$ and n plants without risk. On the other hand, Maizonnier (1976 b) showed that chloroplast number in guard cells of stomata provides a very efficient means of screening the two ploidy levels.

Concerning meiosis, Sen (1969) gave a rapid analysis. He observed a nonspecific pairing between nonhomologous chromosomes during pachytene, bivalents, and trivalents at diakinesis and metaphase I and short segments of synaptinemal complexes. Sen (1969) also mentioned a splitting of univalents in 8% of the PMCs.

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A more complete study of meiosis concerning several haploid plants was made by Maizonnier (1974 and 1976 b). Generally, the seven chromosomes yield univalents (Fig. 13.2), but sometimes, associations may occur forming bivalents (Fig. 13.3 and 13.4) and even chains of three (Fig. 13.4) to six chromosomes. If in certain cases one suspects that heterologous associations involving several chromosomes are actually due to segmental homologies, some bivalents seem to present undisputable chiasmata (Fig. 13.4). Most of the PMCs show an unequal anaphase I distribution yielding to unbalanced microspores (Fig. 13.6). However, in a proportion of meiocytes, univalents split (Fig. 13.5) instead of distributing, and gave rise to dyads with two viable pollen grains. In some cases, after splitting, the resulting 14 chromosomes do not migrate to the pole; they yield a restitution nucleus (Fig. 13.6) and, finally, a diploid spore. The same phenomena, – at least production of egg cells with 7 chromosomes – must take place during megasporogenesis as evidenced by the diploid progeny obtained from selfed haploids (Maizonnier 1976 b). Thus, contrary to most of the haploids in other species, *Petunia* haploids are not fully sterile and can yield as much as 30% stainable pollen (Fig. 13.7).

13.3 Autodiploid Plants and Lines

Generally, in crop plants, haploids yield pure lines which are very useful for genetics and plant breeding work. In *Petunia*, Maizonnier (1973) demonstrated that haploids do not always have the same fate. Some of them double spontaneously and were difficult to preserve in a haploid form. Others were doubled artificially by means of colchicine – DMSO treatments or in vitro cultures of pedicels (Pelletier and Delise 1969). Some haploids remained despite attempted doubling treatments and finally died. Thus, not all haploid plants appear to have the same doubling ability. Maizonnier (1973) could obtain an autodiploid line by direct selfing of a haploid plant.

Three cases of spontaneous mutations concerning flower color or chlorophyll deficiencies appeared in the collection of haploids preserved in Dijon (PMB News

Fig. 13.1. The seven chromosomes of a haploid plant during mitosis

Fig. 13.2. Meiotic metaphase I with 7 univalents

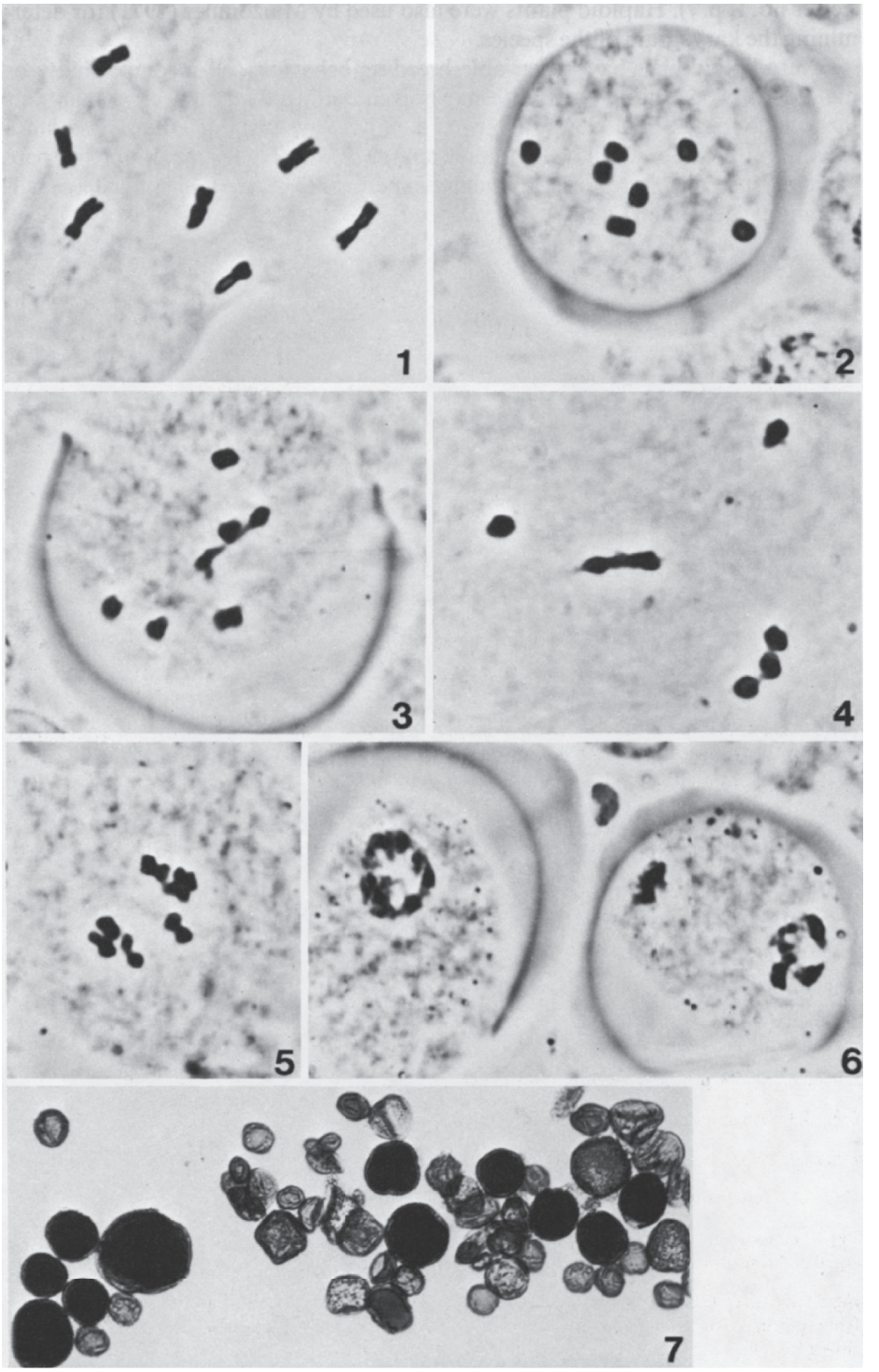
Fig. 13.3. Five univalents and a bivalent in a microsporocyte

Fig. 13.4. Two univalents, a bivalent and a chain of three

Fig. 13.5. Univalents split instead of dividing

Fig. 13.6. Two kinds of meiotic end products: on the right, two unbalanced nuclei originating from unequal distribution of chromosomes during anaphase I; on the left, a restitution nucleus

Fig. 13.7. Pollen population from a haploid plant. Dark pollen represents haploid pollen originating from splitting of univalents, and doubled pollen corresponding to restitution nuclei. Poorly colored and shrivelled pollen correspond to aborted unbalanced microspores


1
2
3
4
5
6
7

Letter no. 1, p. 7). Haploid plants were also used by Maizonnier (1971) for determining the karyogram of the species.

Autodiploid lines have a variable breeding behavior: self-incompatibility or male sterility of autodiploid plants may prevent selfing. Thus, among 40 haploids mentioned in the PMB News Letter no. 1, only 15 gave usable autodiploid lines. In addition, sometimes these lines have a poor or variable fertility. Such lines are currently used, as pure lines, for many experiments concerning the genetics of *Petunia* carried out in Dijon.

14 Molecular Biology*

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Members of the genus *Petunia* have long been utilized as experimental subjects to investigate molecular aspects of biological phenomena, particularly pigmentation (Chap. 6) and self-incompatibility (Chap. 8). More recently, studies have been begun on nuclear gene and genome structure, organelle gene and genome structure, and transformation of *Petunia* cells with exogenous DNA. For this work, the wealth of information concerning cytology (Chap. 4) genetics (Chap. 5) and tissue, protoplast culture, and fusion (Chaps. 9–11) will be invaluable. In this Chapter, information concerning the nuclear genome will be reviewed, surveys made of the nucleic acid-mediated diseases, characterized proteins, and transformation studies, and analyses of the organelle genome presented.

14.1 Nuclear Genome and Nuclear Gene Mutations

The relatively small nuclear genome size of *P. hybrida,* 1.6 picograms of DNA (Bennett and Smith 1976) is not surprising, considering the small size of the seven chromosomes present in haploid cells (Chap. 13). Although not among the very lowest, the genome size of *Petunia* is in the lower fifth of the 753 species surveyed by Bennett and Smith (1976) (Table 14.1). Small genome sizes are advantageous in attempts to identify recombinant clones of unique genes. Clone libraries of *Petunia* nuclear DNA are currently being constructed in several laboratories (F. M. Ausubel, J. Bedbrook, R. Meagher, personal communications).

The ability to assay and purify enzymes can aid in identifying DNA fragments coding for the protein. Information concerning *Petunia* proteins which have been characterized to some degree is summarized in Table 14.2.

Cloned DNA fragments coding for enzymes will be useful tools to establish whether and, if so, how exogenous DNA can be taken up, integrated, and expressed in higher plant cells. *Petunia* has been the object of more reported attempts for DNA uptake and transformation than any other plant species (Table 14.3). Most of these studies have suffered from one or more of three problems: (1) the lack of defined sequences to supply as exogenous DNA, (2) no selection method for transformants, or (3) no method for detecting presence or integration of exogenously supplied sequences at the molecular level.

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Table 14.1. Nuclear DNA content of *Petunia hybrida* compared to other plant species. (Data taken from Bennett and Smith 1976; Narajan and Rees 1974)

Plant species	Picograms DNA			
	1C	2C	3C	4C
Arabidopsis thaliana	0.2	0.5	0.7	0.9
Lycopersicon esculentum[a]	1.0	2.0	3.1	4.1
Petunia hybrida[a]	1.6	3.1	4.7	6.2
Solanum tuberosum[a]	2.1	4.2	6.3	8.4
Datura innoxia[a]	2.1	4.3	6.4	8.6
Nicotiana tabacum[a]	3.9	7.8	11.6	15.5
Hordeum vulgare	5.5	11.1	16.6	22.2
Secale cereale	8.8	16.6	25.4	33.1
Triticum aestivum	17.3	34.6	51.9	69.3

[a] Members of Solanaceae

Table 14.2. *Petunia* proteins studied

Enzyme	Comments	Reference
Phenylalanine ammonia-lyase	Located in chloroplast	Ranjeva et al. (1977 a)
Cinnamate-4-hydroxylase	Absent in chloroplast	
Cinnamate-2-hydroxylase	Located in chloroplast	
Hydroxycinnamoyl-CoA-quinate-hydroxycinnamoyl transferase	Located in chloroplast	Ranjeva et al. (1977 b)
Flavanone-synthase	Located in chloroplast	
ONPG-hydrolyzing activity	Does not hydrolyze lactose	Malhotra et al. (1979), Komp and Hess (1977)
Chalcone isomerase	Present in anthers in genotypes with white or blue pollen (*Po*) Absent in *po*/*po* genotypes in pollen but present in corolla	Forkmann and Kuhn (1979)
Ferredoxin	Two different components present; tryptic digests differ between *P. inflata* and *P. axillaris*	Huisman et al. (1978)
Peroxidases Glucose phosphate isomerase	Genes for 5 enzymes mapped by Mendelian segregation of isozymes	Van den Berg and Wijsman (1980)

Much progress toward establishing techniques for higher plant transformation has recently been made by using *Agrobacterium* Ti-plasmid DNA, with which the above three problems are avoided. In addition to the Ti-plasmid, future experiments to transform *Petunia* cells are likely to involve cloned genes which can correct a deficiency or confer resistance to an inhibitor to a defined *Petunia* genotype.

Table 14.3. Experiments concerning DNA uptake and/or transformation of cells or nuclei of *Petunia*

Type of study	Reference
Examination of plants for anthocyanin synthesis after incubation of seedling and zygotes of a white-flowering genotype with DNA from a cyanidin type. Similar experiments with leaf shape	Hess (1969 a, b, 1970 a, b, 1972, 1973)
Analysis of a dwarf mutant which is a phenocopy of a plant described by Hess (1972) as a transformant	Bianchi et al. (1974)
A discussion of the sectoring which would be expected to appear following transformation of seedlings	Bianchi and Walet-Foederer (1974)
Measurement of double-labeled cyanidin type *Petunia* DNA associated with *Petunia* nuclei following incubation of DNA with line 34d10 protoplasts; procedure for purification of nuclei from small amounts of material	Hoffman (1973)
Measurement of transcription of bacterial DNA by nuclei incubated with linear and supercoiled Col E_1 plasmid DNA	Blaschek and Hess (1977)
Measurement of radioactive *E. coli* DNA associated with *Petunia* nuclei following incubation of DNA with isolated nuclei	Liebke et al. (1977)
Measurement of radioactive *E. coli* DNA associated with *Petunia* nuclei following incubation of DNA with protoplasts	Liebke and Hess (1977)
Measurement of radioactive *E. coli* DNA associated chromatin following incubation of DNA with *Petunia* nuclei; procedure for purification of nuclei	Blaschek (1979)
Incubation of protoplasts with λ phages under various conditions, examination for uptake by electron microscopy and by assay for plaque-forming units	Jenne and Hess (1980)
Selection for hormone-auxotrophic colonies following incubation of *Petunia* protoplasts with Ti-plasmid DNA	Davey et al. (1980)
Incubation of Ti plasmid DNA under various conditions with pollen, which is then used to pollinate a defined *Petunia* genotype. Progeny examined for abnormalities or tumors	Jackson et al. (1980)

For construction of mutants for transformation experiments, both traditional and tissue culture methods of mutant selection are well advanced in *Petunia* (Chap. 5, this Vol.; Colijn et al. 1979 b; Binding 1972; Binding et al. 1970). The availability of haploid and highly fertile diploid *Petunia* plants, such as the Mitchell *Petunia,* will facilitate traditional and in vitro mutant isolation experiments. The Mitchell line of *Petunia* has been adopted by a number of laboratories for molecular biological experiments and for development of the relevant cell and tissue culture technologies (Plant Molecular Biology Newsletter, 1980).

Although additional types of mutants will be needed for genetic engineering and genome structure analyses, many highly interesting mutants have been isolated and some mapped to individual chromosomes (Chap. 5, this Vol.; Wiering et al. 1979). The naturally occurring variability of genes in different Petunia *species* should also not be ignored as fruitful material for molecular approaches to understanding gene structure and evolution, particularly since 6 of the 14-

chromosome *Petunia* species can be sexually or somatically hybridized (Chaps. 2 and 11). For example, van den Berg and Wijsman (1980) mapped the structural genes for five enzymes by observing segregation of electrophoretic variation in crosses of wild and cultivated genotypes.

Of the *Petunia* mutants in existence, those affecting flower color are presently the best understood at a biochemical level (Chap. 6). Other types of mutations presently available include chlorophyll deficiencies (see Chap. 14.2.1.1), abnormal floral morphologies, and aberrant growth habits (Wiering et al. 1979).

Variegated flower mutants resulting from instabilities in genes affecting pigmentation form a particularly intriguing class of mutations. Two different unstable systems are under intensive study, and the existence of regulatory elements analogous to those in maize has been postulated (Bianchi et al. 1978; Cornu 1977; Chap. 5). Unfortunately, the products of the *An1*- and *An2*-genes affected by instability mutations have not yet been characterized. Determination of the gene products of an unstable locus will be important to permit molecular cloning of the DNA at and surrounding such a locus. If a transposable controlling element is causing the *An1* and *An2* instabilities, then it may be possible to select for transpositions into genes with known products. Such mutations in known genes would be extremely valuable material for analyses of gene structure and control of gene expression.

14.2 Nucleic Acid-Mediated Diseases

14.2.1 Viruses

Viruses of higher plants have been coming under increasing study, as potential vehicles for genetic engineering and in order to understand nucleic acid processing and the molecular basis of viral disease. *Petunia* is susceptible to many different plant viruses (Table 14.4; Chap. 16). In fact, *P. hybrida* is often used as an assay species to test extracts of other plants for presence of virus (Gibbs and Harrison 1976).

Most plant viruses which have been examined contain RNA as the genomic material. However, the caulomoviruses (carnation etched ring virus, cauliflower mosaic virus, dahlia mosaic virus, mirabilis virus, and strawberry vein banding virus) are DNA viruses (Shepherd 1972). Whether or not strains of cauliflower mosaic virus and dahlia mosaic virus can infect *Petunia* has not been reported, but these viruses have been reported to infect other Solanaceae (Shepherd 1972; Brunt 1971). Ultrastructural similaries between the caulomoviruses and the petunia vein-clearing virus described by Lesemann and Casper (1973) suggest that this virus should be investigated further as a possible DNA virus.

14.2.2 Viroids

Viroids are infectious naked nucleic acid species of low molecular weight (approximately 10^5 m.w.). Six different plant diseases are currently known to be viroid-

induced (Diener 1979). How viroids replicate and cause diseases are unanswered intriguing questions.

Petunia has been reported sensitive to two viroids, potato spindle tuber viroid (PSTV) and citrus exocortis viroid (CEV) (Diener and Hadidi 1977; Fazio et al. 1977; Singh and Clark 1973; Semancik and Weathers 1972; Sing 1973). Two strains of PSTV are known; Singh (1973) reported that the severe strain produced symptoms in *P. hybrida,* but the mild strain did not result in visible infection.

PSTV is a RNA of molecular weight 1.3×10^5 m.w., infectious in tomato both as a linear or as a circular molecule (Diener 1979). Although CEV and PSTV have been reported to induce similar responses in *Petunia* (Semancik and Weathers 1972; Singh and Clark 1973), that they are discrete entities is established by the smaller size of CEV (1.2×10^5 m.w.), CEV's narrower host range, and differences in base composition (Diener and Hadidi 1977; Diener 1979).

A possible origin of PSTV from host nucleic acids has been suggested by the finding of sequences complementary to PSTV in uninfected plants of three Solanaceous species (Hadidi et al. 1976). Similar tests of viroid homology to the *Petunia* genome have not been reported. *Petunia* may be a useful host for further studies of these interesting nucleic acids.

14.2.3 Ti-Plasmid

The crown gall disease is caused by Ti-plasmid-containing strains of *Agrobacterium.* Ti-plasmid DNA sequences are present in bacteria-free tumor tissue (Matthysse 1977). Like *Agrobacterium*-induced tumor cells of other plant species, *Petunia* crown-gall tumor tissue, unlike normal cells in culture, are hormone-autotrophic and synthesize unusual opines (Davey et al. 1980).

Table 14.4. Some virus diseases of *Petunia*

Virus	Reference
Arabis mosaic	Daft and Okasanya (1973), Gerola et al. (1966), Fritzsche (1975)
Alfalfa mosaic	Conti et al. (1972)
Broad bean wilt (petunia ringspot)	Gracia and Feldman (1976), Doel (1976)
Citrus ringspot	Desjardins et al. (1969)
Chrysanthemum mild mottle	Tochihara (1970)
Cucumber mosaic	Mishra and Srivastava (1971)
Grapevine fanleaf	Saric and Wrischer (1975)
Petunia mosaic	Jaijada et al. (1979)
Petunia mottle	Naqvi et al. (1978)
Petunia vein-clearing	Lesemann and Casper (1973)
Potato virus X, Y	Shimomura and Ohashi (1971), Brunt et al. (1971)
Raspberry ringspot	Harrison et al. (1972, 1974)
Tobacco mosaic	Takahishi et al. (1970)
Tobacco rattle	Ghabrial and Lister (1973)
Tomato bushy stunt (petunia asteroid mosaic)	Campbell et al. (1975)
Yellow ringspot	Fritzsche and Thiele (1974)

Common laboratory strains of *Agrobacterium* will induce tumors on mechanically or ether-wounded haploid, diploid, triploid, or tetraploid *P. hybrida* (Jackson et al. 1980; Hanson unpublished). The phenotype of the tumors induced by different bacterial strains on the same *Petunia* genotype varies, as does tumor phenotype resulting from infection of different *Petunia* genotypes with the same bacterial strain (Fig. 14.1).

The TI plasmid is considered a potential vehicle for genetic engineering (Chilton et al. 1980; Schell et al. 1979; Thomashaw et al. 1980), and has been used in two types of transformation experiments with *Petunia*. However, only normal plants were obtained from seed derived from fertilization with *Petunia* pollen that had been incubated under various conditions with Ti-plasmid DNA or *A. tumefaciens* (Jackson et al. 1980). Attempts to transform *Petunia* protoplasts with naked Ti-plasmid DNA were more successful; hormone autotrophic octopine-synthesizing callus colonies were obtained (Davey et al. 1980). Further experiments with *Petunia* may establish the molecular basis for Ti-plasmid-induced tumorgenesis and whether Ti-plasmid-based vehicles are indeed suitable for genetic engineering.

14.3 Organelles

14.3.1 Nuclear Genome Effects on *Petunia* Chloroplasts

14.3.1.1 Nuclear Mutations Affecting Chlorophyll Content

Both the organelle and nuclear genomes specify chloroplast components (Krik and Tilney-Bassett 1978). Nuclear gene mutations can reveal much about nuclear control of chloroplast structure and function. Over 20 independently isolated nuclear-coded mutants affecting chlorophyll content have been isolated in *Petunia* (Wiering et al. 1979; Power et al. 1979, 1980; Izhar et al. 1979). Ten of these mutations are known to be at different loci on five different chromosomes (Wiering et al. 1979). The mutants range from plants with leaves abnormally dark green, pale green, dark green spotted, or yellow, to those with seedling leaves yellow which then become green. Most such mutations analyzed were found to be recessive; one is homozygous lethal with yellow leaves in the heterozygotes (Wiering et al. 1979). One nuclear-coded chlorophyll mutant has been used as a genetic marker for selection of somatic hybrids (Chap. 11; Power et al. 1979, 1980).

Most of these nuclear-coded chlorophyll mutant await biochemical and ultrastructural characterization. One recessive nuclear mutation in *P. hybrida* resulting in chlorophyll deficiency at normal light intensities, has been examined for chlorophyll and ribulose-biphosphate carboxylase content and organelle ultrastructure (Izhar et al. 1979). Although leaves were green and contained 0.71 mg chlorophyll g^{-1} fresh weight when plants were grown under low (700 lx) light intensity, leaves were yellow (0.18 mg chlorophyll g^{-1} fresh weight) when grown at 11 000 lx. Yellow leaves contained approximately 35% more RuBCase than green leaves and both chloroplast and mitochondrial membranes appeared abnormal. Grana differentiation was also disturbed in the *P. hybrida* variegated mutant



Fig. 14.1. Difference in morphology of crown-gall tumors induced by *Agrobacterium tumefaciens* B6 on two different inbred *Petunia* lines (Scarlet 124 and B147 from Burpee Seed. Co.). *Arrows* point to tumors present 4 weeks after inoculation of the stem with B6. S124 tumors are typically large and white, while B147 tumors are small and necrotic. (Hanson unpublished)

studied by Muszynski (1975), and no normal thylakoid membranes were observed in a *P. inflata* albino mutant (L. Schnabelrauch and K. C. Sink personal communication).

Another type of nuclear mutation affecting chloroplasts is the chloroplast mutator described by Potrykus (1970). This nuclear mutation, obtained from X-rayed *P. hybrida,* was found to affect the frequency of appearance and stability of cytoplasmically inherited albino mutations. Important facets of nuclear control over chloroplast functions may be revealed when the molecular basis of this mutation is understood.

14.3.1.2 Effect of Nuclear DNA Content on Chloroplast Number

An increase in number of chloroplasts per cell with increasing ploidy is a general rule of plant cell biology (Butterfass 1980). *Petunia* is no exception; the number of chloroplasts per guard cell pair is a measure sufficiently reliable for ploidy determination (Maizonnier 1976; Mitchell et al. 1980; Fig. 2).

The number of chloroplasts per guard cell pair can vary in the same *Petunia* line grown in different environmental conditions. However, between different lines of the same ploidy grown under the same conditions, consistent differences in average number of chloroplasts per guard cell pair can be observed (Fig. 14.2). Whether these differences are nuclear-specified and/or chloroplast-specified is unknown. Analysis of a set of isonuclear lines carrying different cytoplasms could answer this question.

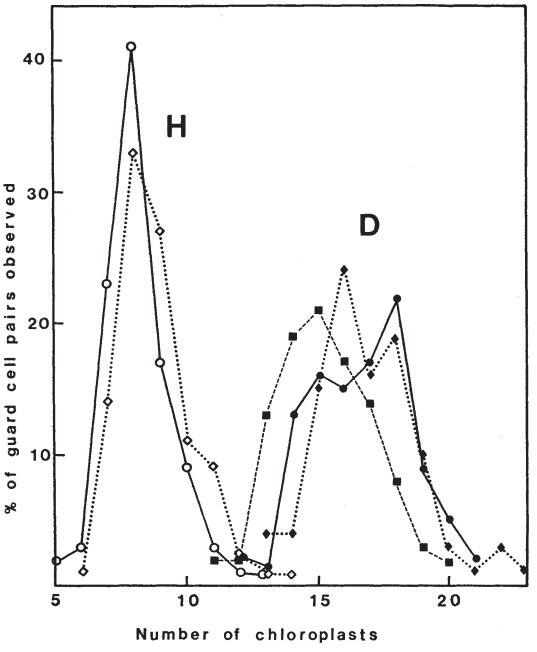


Fig. 14.2. Numer of chloroplasts per guard cell pair in two haploid (*H*) and three diploid (*D*) *Petunia* genotypes. (Data courtesy A. Mitchell)

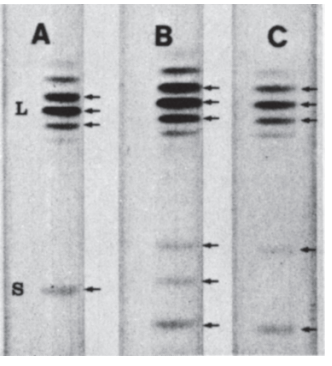


Fig. 14.3 A–C. *Petunia* RuBPCase large (*L*) and small (*S*) subunits after electrofocusing with 8 *M* urea. **A** 30 µg of *P. parviflora* RuBPCase. **B** 30 µg of *P. parviflora* RuBPCase and 30 µg of *P. parodii* RuBPCase. **C** 30 µg of *P. parodii* RuBPCase. Not visible are two minor small subunit polypeptides present below the *arrowed* subunit in *P. parviflora* slot. (Courtesy of A. A. Gatenby, see also Gatenby 1977)

14.3.1.3 Nuclear-Specified Chloroplast-Proteins

Crosses involving *Nicotiana* species with differences in electrophoretic mobility of the small subunit of RuBPCase have revealed that this subunit is nuclear-coded (Wildman 1979). The isoelectric points of the two small subunit polypeptides of *P. axillaris*, *P. hybrida*, and *P. inflata*, *P. violaceae*, and *P. parodii* are all identical (Gatenby and Cocking 1977). *P. parviflora* exhibits one major and two minor small subunit polypeptides upon isofocusing, none of which have isoelectric points identical to the other species' small subunits (Fig. 14.3).

In several plant species, the site of synthesis of the small subunit of RuBPCase has been determined to be on ribosomes outside of the chloroplast (Gray and Kekwick 1974; Chua and Schmidt 1979). Whether this is true of all nuclear-coded chloroplast proteins is unknown. There is yet no evidence that nuclear-transcribed mRNA enters the chloroplasts to be translated on chloroplast ribosomes.

Can nucleic acid enter the chloroplast from without? RNA and DNA viruses may provide tools to investigate this tantalizing question. There is a report that the tobacco mosaic (RNA) virus can be present in *Petunia* chloroplasts (Takahishi et al. 1970). Whether and, if so, how viruses enter organelles merits further investigation. This question is important not only for understanding nucleo-cytoplasmic interactions, but also has bearing on attempts to transform organelles with foreign or engineered genes.

Since viruses often induce chlorophyll deficiencies, studies of the molecular mechanisms of viral disease induction may also provide information on the chloroplast's dependence on other cellular components. Viral-induced chloroplast ultrastructural changes can mimic those of nuclear and cytoplasmic mutants (Harrison et al. 1974).

14.3.2 Chloroplast Genome

Chloroplast DNA (cpDNA) from higher plants exists as circular DNA molecules ranging from 85–103 $\times$ 10^6 m.w. In the last few years much information has been obtained on the physical structure, coding capacity, nature, and function of gene products, genetic organization, and regulation of expression of cpDNA of a number of plant species (for reviews see: Bedbrook and Kolodner 1979; Ellis and Barraclough 1978; Kung 1977).

In this section the phenotypic markers, isolation, characterization, and expression of *Petunia hybrida* chloroplast DNA will be discussed.

14.3.2.1 Phenotypes Controlled by the Chloroplast Genome

All chloroplast genomes examined code for chloroplast rRNAs, tRNAs, and the large subunit of ribulose biphosphate carboxylase-oxygenase (RuBPCase). In addition, chloroplast genomes are known to specify reaction to tentoxin, resistance to chloroplast protein synthesis inhibitors, chlorophyll deficiency, and loss of photosynthetic capacity (Gillham 1978; Davidson et al. 1978; Kirk and Tilney-Bassett 1978; Durbin and Uchytil 1977 b).

In *Petunia*, chloroplast genome-specified chlorophyll deficiency is a useful genetic marker. Maternal inheritance of the chloroplast genome has been inferred from the results of reciprocal crosses involving variegated mutants (Table 14.5). Cytoplasmically inherited chlorophyll deficient mutants of *P. inflata, P. violaceae, P. hybrida, P. axillaris,* and *P. parodii* have been isolated (Cocking et al. 1977; A. Cornu, K.C. Sink personal communications; Muszynski 1964, 1967, 1975; Potrykus 1973; Power et al. 1979, 1980). Chloroplast-coded albino mutants of *P. hybrida* and *P. inflata* can be maintained as shoot or suspension cultures and

Table 14.5. Maternal inheritance of two independently isolated chlorophyll mutations in *P. violaceae*

Cross	Progeny		References
	No. albino	No. green	
♀ Albino × ♂ Green	531	0	Pandey and Blaydes (1957)
♀ Green × ♂ Albino	0	1,024	
♀ Albino × ♂ Green	153	0	Terao (1929)
♀ Green × ♂ Albino	0	110	

Table 14.6. *Petunia* lines sensitive to tentoxin at 10 or 20 μg ml^{-1}. Seeds were germinated on filter paper moistened with a tentoxin solution. Seven days after germination, those plantlets with yellow leaves were scored as sensitive, those with green leaves as resistant

Species or line	Phenotype
P. parodii (from K. C. Sink)	S
P. violaceae (from S. Izhar)	S
P. axillaris (from K. C. Sink)	S
P. parviflora (from K. C. Sink)	S
P. hybrida cv. Blue Bedder	S
Petunia line Mitchell	S
P. inflata (from K. C. Sink)	S
Lycopersicon esculentum cv. Rutgers	R
Nicotiana tabacum cv. Wisconsin 38	R

have been used in protoplast fusions leading to regeneration of somatic hybrid plants (Cocking et al. 1977; Power et al. 1979, 1980).

Reaction to tentoxin, an inhibitor of chloroplast ATPase, is also a phenotypic marker for the chloroplast genome in certain genera such as *Nicotiana* (Durbin and Uchytil 1977a, b; Steele et al. 1976). Unfortunately, to date all *Petunia* cytoplasmas tested have been found to be sensitive to tentoxin (Table 14.6). As new collections of *Petunia* species are made and tested for tentoxin resistance, however, a source of resistance in the genus may be discovered.

Cell cultures of *Petunia* have provided two cell lines which may have originated through mutation in the chloroplast genome. Binding (1972) isolated eight lines resistant to the chloroplast ribosome inhibitor streptomycin from callus cultures of haploid *P. hybrida*. Following chemical mutagenesis (Colijn et al. 1980), a *P. hybrida* suspension culture with altered greening (see Sect. 14.3.2.5.2) was recently isolated. Whether such lines with altered chloroplast phenotypes represent variants, nuclear or chloroplast mutations requires genetic analysis through sexual or somatic hybridization.

A molecular chloroplast genotype marker useful in *Nicotiana* is the isoelectric point of RuBPCase large subunits. Unlike *Nicotiana,* no interspecific diversity in

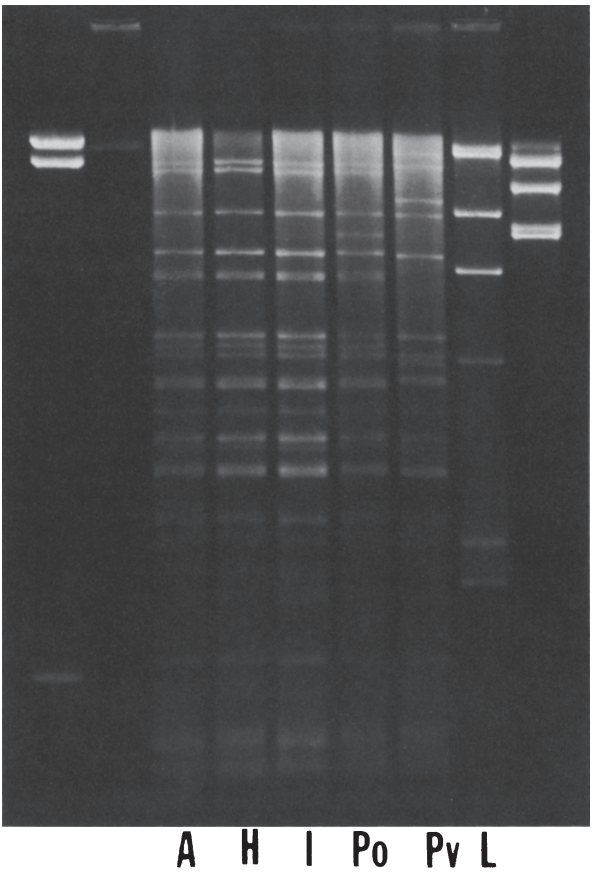


Fig. 14.4. Chloroplast DNA of *P. axillaris* (*A*), *P. hybrida* (*H*), *P. inflata* (*I*), *P. parodii* (*Po*), and *P. parviflora* (*Pv*) visualized on agarose gel after restriction with BAM HI. DNA prepared by crude purification method described in text (R. de-Lorimier, M.R. Hanson, H.J. Smith, F.M. Ausubel unpublished). (*L*) phage lambda DNA fragment generated by restriction with HINDIII

mobility of the large subunit of *Petunia* RuBPCase has been detected (Gatenby and Cocking 1977; L. Mets personal communication). Three large subunit polypeptides are resolved in isoelectric focusing of *P. axillaris, P. hybrida, P. inflata, P. parodii, P. violaceae,* and *P. parviflora.* All species' large subunit polypeptides exhibit identical isofocusing patterns (Fig. 14.3). Furthermore, immunodiffusion comparison on all of the species against anti-tobacco RuBPCase indicate complete homology (Gatenby 1978 personal communication).

Another molecular phenotype which can distinguish different chloroplast genotypes is DNA restriction fragment sizes, reflected in fragment banding patterns on agarose gels. Chloroplast DNA of *P. parodii* and *P. parviflora* can be distinguished from *P. hybrida, P. inflata,* and *P. axillaris* following restriction with Bam Hl (R. DeLorimier, E. Clark, H. J. Smith, M. R. Hanson, F. M. Ausubel, unpublished). *P. parviflora,* which is the most distant taxonomically from the other species (Chap. 2), also exhibits the greatest divergence in chloroplast DNA restriction sites according to analysis with several different enzymes (Fig. 14.4).

14.3.2.2 Isolation of Chloroplast DNA

Methods described for the isolation of chloroplast DNA from spinach and pea (Herrmann et al. 1975; Kolodner and Tewari 1975) have usually not been suitable for the isolation of DNA from *Petunia hybrida* chloroplasts. The DNAse treatment, used in these methods to remove contaminating nuclear DNA from the chloroplast preparation, often causes degradation of *Petunia* cpDNA. When this happens, the yield of undegraded cpDNA is very low. Therefore, isolation procedures especially adapted for *Petunia* chloroplasts should be used.

Bovenberg and Kool (1981) developed a method which gives high yields of pure circular cpDNA molecules that can be digested with restriction endonucleases. For a typical isolation, about 400 g of leaves from 2–5 month-old *Petunia* plants are homogenized in 2 l of buffer (0.05*M* Tris-HCl pH 8.0, 0.35*M* sucrose, 7 m*M* EDTA, 5 m*M* 2-mercaptoethanol). Chloroplasts are isolated from the homogenate by filtration and centrifugation and directly lysed by the addition of sodium sarkosyl. DNA is purified from the lysate by CsCl-ethidium bromide centrifugation. Under ultraviolet light two fluorescent DNA bands are visible in the gradients. DNA is recovered from these bands by dialysis and precipitation. The procedure yields about 250 μg of lower and upper band DNA. Electron microscopic analysis of the lower band indicates that this band represents covalently closed circular cpDNA. Restriction enzyme analysis of this DNA does not show any contamination with mtDNA, which has a quite different restriction profile (Bovenberg and Kool 1980). The cpDNA from the upper band in the CsCl gradient is also predominantly high molecular weight DNA, and is usually also suitable for restriction endonuclease mapping work, but may contain some contaminating nuclear DNA, resulting in a slight smear when the DNA is analyzed by electrophoresis on agarose gels.

The method described by Bovenberg and Kool (1980) also yields cpDNA from *Petunia hybrida* cell suspension cultures that have been growing in the laboratory for 4 years. The fragments obtained upon digestion of this DNA with several restriction endonucleases did not differ from the fragments obtained upon digestion of cpDNA isolated from leaf chloroplasts (Kool, unpublished results). This implies that major genetic alterations in the cpDNA did not occur during this in vitro culture period of *Petunia* cells.

Another *Petunia* chloroplast DNA purification method, which gives cruder cpDNA, can be used for rapid screening of genotypes from relatively small amounts of tissue (R. deLorimier, M. R. Hanson, H. J. Smith, F. M. Ausubel, unpublished). This method, modified from that of Whitfield et al. (1978), is based on the centrifugal purification of intact chloroplasts by divalent cation-mediated chloroplast aggregation. Growth conditions of plants can affect quality of preparations; young rapidly growing tissue with low starch content gives best results. Leaves are homogenized in buffer A (0.33 *M* sorbitol, 1 m*M* $MgCl_2$, 1 m*M* $MnCl_2$, 50 m*M* Tricine, 4 m*M* mercaptoethanol, 2 m*M* Na_2EDTA, pH 8) with a Waring blender. The homogenate is centrifuged at 4 °C at 2000 *g* for 40 s, with hand braking. The pellet is resuspended in the same buffer and the rapid centrifugation repeated. Although the resulting pellet usually contains over 90% intact chloroplasts as judged by phase microscopy, DNAse treatment cannot be used,

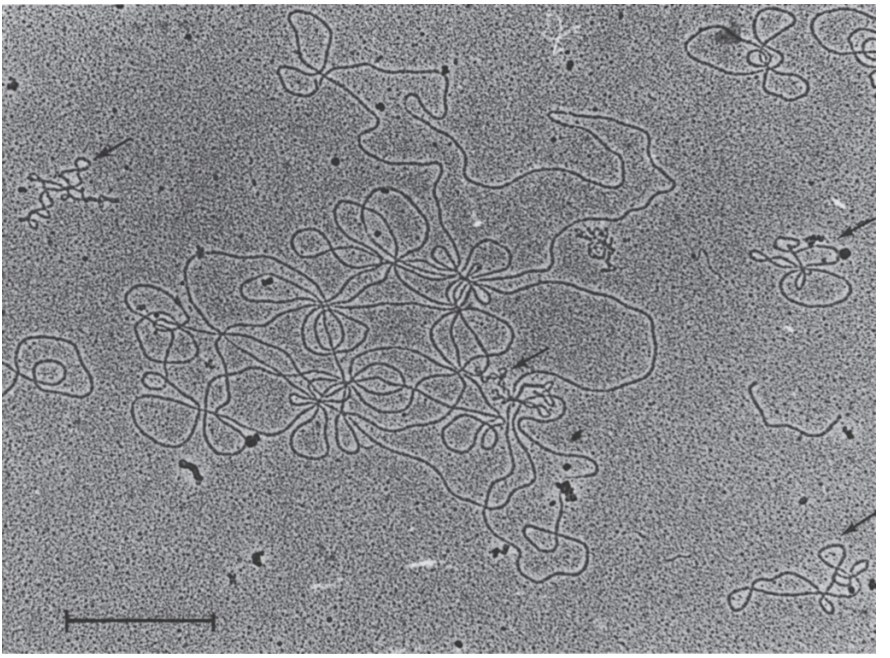


Fig. 14.5. Electron micrograph of *Petunia hybrida* chloroplast DNA. *Arrow* indicates Clo DF13 plasmid DNA (9600 bp), added as marker. *Bar* represents 1 μm. (Bovenberg and Kool 1980)

or no cpDNA will be obtained. These chloroplasts are instead washed in buffer B (buffer A minus divalent cations) by centrifugation for 2 min at 3000 g. Chloroplast preparâtions from certain genotypes are improved by a 30%–60% sucrose gradient in Buffer B. Chloroplasts are then lysed in 2% Sarkosyl, 100 μg/ml proteinase K, 20 m*M* Tris, 50 m*M* EDTA, pH 8 for 30 min at 25 °C. Following two phenol and two chloroform extractions, DNA is precipitated overnight in sodium acetate-ethanol. The cpDNA obtained is contaminated with nuclear DNA, but suitable restriction enzymes will give clear cpDNA bandings patterns on agarose gels (Fig. 14.4). Chloroplast DNA so prepared can also be cloned into plasmid vehicle pBR322 (M. R. Hanson, H. J. Smith, R. Riedel, F. M. Ausubel, unpublished).

14.3.2.3 Physical Properties of Chloroplast DNA

Electron microscopic analysis of *Petunia* cpDNA prepared by Bovenberg and Kool (1980) showed that these molecules were circular and had a homogeneous contour length (Fig. 14.5). The contour length was determined by using the bacterial plasmid Clo DF13, which was added during mounting, as length standard. The size of this plasmid was previously determined at 9600 bp (van den Elzen et al. 1980). The ratio of the length of cpDNA to Clo DF13 DNA was 15.1 – (±0.7) which corresponds to a molecular weight of 96.0 (±4.5) × 10^6 m.w. This value

is in good agreement with the estimated value of 101.2 ($\pm$0.5) $\times$ 10^6 daltons, that was obtained from restriction endonuclease analysis of cpDNA.

An analysis of *Petunia* cpDNA by analytical CsCl gradients revealed the presence of only one type of DNA at a buoyant density of 1.6987 ($\pm$0.0005) g cm^{-3}, which corresponds with a GC-content of 39.3 ($\pm$0.05)%. Nuclear and mitochondrial DNA of petunia have buoyant densities of respectively 1.6960 and 1.1066 g cm^{-3} (Bovenberg and Kool 1980). The buoyant density of *Petunia* cpDNA agrees with the buoyant density of cpDNA from other plants that are in the region of 1.694–1.698 g cm^{-3} (Kolodner and Tewari 1975; Bedbrook and Kolodner 1979).

14.3.2.4 Construction of SAL I, BGL I, and HPA I Restriction Endonuclease Map of cpDNA

The chloroplast of *Petunia hybrida* contains circular DNA molecules of molecular weight 101.2 $\times$ 10^6 m.w. The size of this chloroplast genome is large enough to code for at least 150–200 protein species of average size. Studies with isolated *Petunia* chloroplasts indicate that transcription and translation of at least part of the chloroplast genome occurs in isolated chloroplasts (Colijn and Kool 1980). However, for a full understanding of the coding properties and genetic organization of the cpDNA, the physical mapping of genes and the study of the expression of cloned cpDNA in in vitro systems will be essential. For these reasons Bovenberg and Kool (1980) constructed a physical map of the cleavage sites of the restriction endonucleases Sal I, Bgl I, and Hpa I on *Petunia hybrida* cpDNA. The construction of the physical map involved the following steps: analysis of cpDNA with various restriction endonucleases to select potentially useful enzymes, ^{32}P-

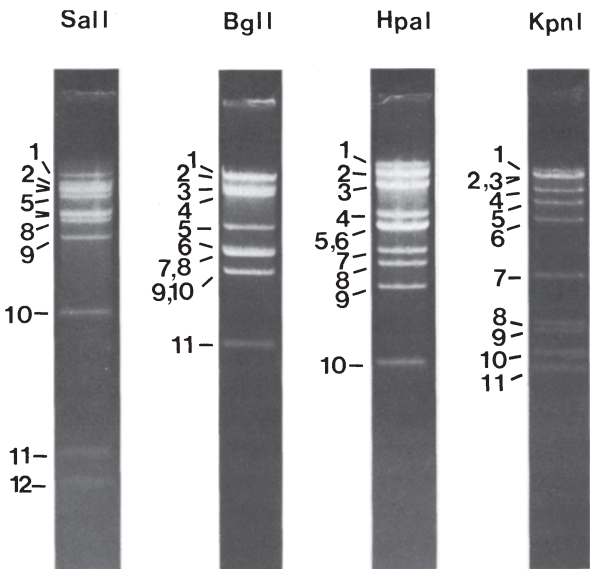


Fig. 14.6. Agarose gel electrophoresis of *Petunia* chloroplast DNA digested with restriction endonucleases. Slot *1*, Sal I digest; slot *2*, Bgl I; slot *3*, Hpa I; slot *4*, Kpn I. The fragments S13, K12, and K13 are not visible on this photograph. (Bovenberg and Kool 1980)

labeling of cpDNA fragments by nick-translation, extraction of ^{32}P-labeled cpDNA from low-melting-temperature agarose gels and redigestion with a second restriction endonuclease.

a) Restriction Endonuclease Analysis of cpDNA

The simplest way to construct a physical map of cpDNA is to use enzymes that cut the DNA infrequently and thus produce a small number of DNA fragments. In addition, the mol. wt. of the DNA fragments produced upon double digestion by two enzymes should add up exactly to the total molecular weight of the cpDNA.

On the basis of the low fragment number and the close similarity of the sum of mol. wts. of DNA fragments produced, the enzymes Sal I, Bgl I, and Hpa I were chosen by Bovenberg and Kool (1980) for physical mapping of the cpDNA (Fig. 14.6). The molecular weights of the DNA fragments resulting from digestion of cpDNA with these enzymes are listed in Table 14.7.

Table 14.7. Chloroplast DNA-fragments resulting from digestion with various restriction endonucleases. (Bovenberg and Kool 1980)

Fragment no.	Sal I	Bgl I	Hpa I	Sal I/Bgl I	Sal I/Hpa I
1	17.6	20.1	29.3	12.4	14.3
2	14.3	18.7	19.5	9.9	8.2
3	12.4	14.4	14.0	9.8	8.0
4	11.9	13.2	8.2	8.2	7.3
5	10.7	7.3	7.2[a]	7.3	7.2 (2×)
6	8.2	5.5	7.2[a]	6.1	6.7
7	8.0	5.3[a]	5.3	5.8	5.3
8	7.3	5.3[a]	4.65	5.3 (2×)	4.45
9	5.7	4.4[a]	3.7	4.8	4.3
10	2.85	4.4[a]	2.2	4.6 (2×)	4.05
11	0.95	2.55		2.85	3.9
12	0.85			2.5 (2×)	3.7
13	0.45			1.9 (2×)	3.3
14				1.45	2.85
15				0.95	2.45
16				0.9	2.2
17				0.8	1.35
18				0.65	1.15 (2×)
19				0.45	0.95
20				0.2	0.8
21					0.45
Sum of Mol. wts.	101.2	101.15	101.25	101.15	101.25

Molecular weights of the fragments are given in megadaltons. The numbers given in brackets refer to multiple copies of a fragment

[a] Bgl I fragments B7 and B8, B9 and B10 and Hpa I fragments H5 and H6 are identical

Table 14.8. Relationship between primary restriction endonuclease fragments and double digest fragments of *Petunia* cpDNA. (Bovenberg and Kool 1980)

Sal I/Bgl I double digest fragments	Primary Sal I and Bgl I fragments from which derived		Sal I/Hpa I double digest fragments	Primary Sal I and Hpa I fragments from which derived	
SB1	S2	B4	SH1	S2	H2
SB2	S5	B1	SH2	S6	H1
SB3	S1	B3	SH3	S7	H1
SB4	S6	B2	SH4	S8	H1
SB5	S4	B1	SH5 (2×)	S1, S3	H5, H6
SB6	S7	B5	SH6	S5	H3
SB7	S8	B2	SH7	S1	H7
SB8 (2×)[a]	S1, S3	B7, B8	SH8	S4	H3
SB9	S9	B2	SH9	S3	H4
SB10 (2×)	S3, S4	B3, B6	SH10	S5	H2
SB11	S10	B1	SH11	S1	H4
SB12 (2×)	S1, S3	B9, B10	SH12	S4	H9
SB13 (2×)	S2, S7	B9, B10	SH13	S9	H8
SB14	S8	B11	SH14	S10	H3
SB15	S11	B5	SH15	S9	H1
SB16	S9	B6	SH16	S4	H10
SB17	S5	B4	SH17	S4	H8
SB18	S12	B11	SH18 (2×)	S1, S3	H1, H2
SB19	S13	B11	SH19	S11	H1
SB20	S12	B5	SH20	S12	H1
			SH21	S13	H1

[a] The number given in brackets refers to multiple copies of a fragment. Molecular weights of the fragments are given in Table 14.1

b) Physical Mapping of Cleavage Sites

Ordering of the Sal I, Bgl I, and Hpa I restriction endonuclease fragments of cpDNA on a cleavage site physical map was obtained by determining the cleavage sites of the enzymes Bgl I and Hpa I relative to those of Sal I. For this purpose the individual Sal I fragments were isolated and redigested with Bgl I or Hpa I and or vice versa. The isolation of DNA fragments from the gels was facilitated by the use of low-gelling-temperature (LGT) agarose (Herrmann et al. 1980). The results of these experiments are summarized in Table 14.8. From the common double digest fragments, the overlapping between the primary Sal I, Bgl I, and Hpa I fragments could be assigned. This allowed the determination of the order of most of the DNA fragments. The order of the fragments Sal I-1 and 3 and Hpa I–9 and 10 could not be determined unambigously from the data in Table 14.8 and was therefore further checked by reciprocal digestion with Kpn I.

The resulting Sal I/Bgl I/Hpa I restriction map of petunia cpDNA is shown in Fig. 14.7. The map indicates that petunia cpDNA contains duplicated regions that are in an inverted orientation (indicated by thick lines). The inverted repeated regions have a minimum size of 15 kbp but may actually extend into the adjacent parts of the cpDNA molecule. Inverted repeat regions have also been found in cpDNA of spinach, maize and *Chlamydomonas*, where the size of one copy of the repeat is 19.5–22.5 kbp (Bedbrook and Kolodner 1979).

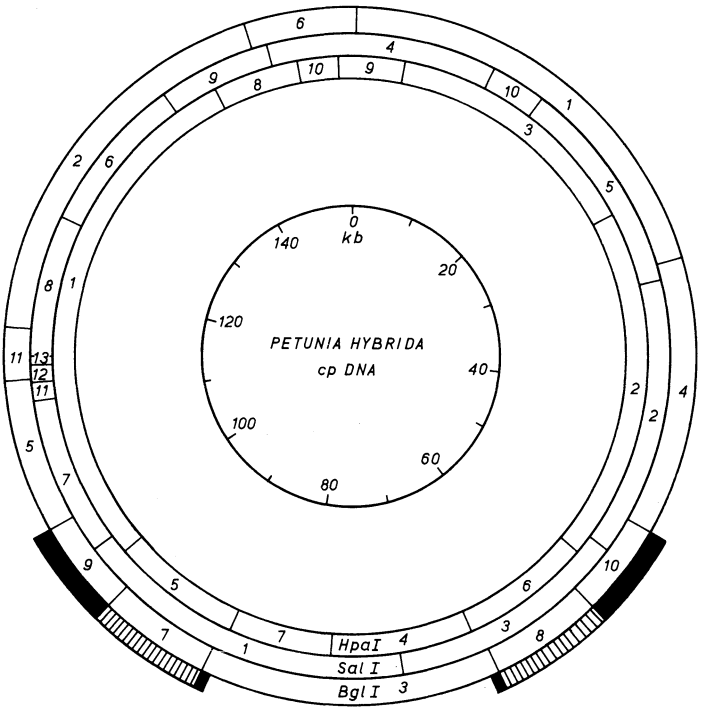


Fig. 14.7. Physical map of restriction endonuclease fragments of *Petunia* cpDNA, showing the Sal I, Bgl I, and Hpa I cleavage sites. Sizes of the numbered fragments are listed in Table 14.1. *Thick lines* indicate the positions of the inverted repeat regions. (Bovenberg and Kool 1980)

c) Mapping of Chloroplast rRNA Genes

Chloroplasts of higher plants contain ribosomes of a similar size to ribosomes of procaryotes (Lyttleton 1962). The major RNAs in the chloroplast ribosomes are 23S, 16S, and 5S. RNA-DNA hybridization experiments between chloroplast rRNA and restriction endonuclease fragments of ctDNA indicate that the cpDNA contains sequences that are complementary to these rRNA's. In the case of *Zea mays, Spinacia oleracea*, and *Chlamydomonas reinhardii* it was shown that two sets of rRNA genes are present on each cDNA molecule, located in the inverted repeat regions of the cpDNA (Bedbrook et al. 1977; Rochaix and Malnoë 1978; Herrmann et al. 1976).

Schwarz and Kössel (1979) showed that the nucleotide sequence of the 16S RNA gene is strikingly similar for *Zea mays* chloroplasts and *E. coli*. In view of this similarity of rDNA in *E. coli* and chloroplasts, Bovenberg and Kool (1980) used ^{32}P-rRNA from *E. coli* as a probe for hybridization with restriction endonuclease fragments of petunia cpDNA to obtain information on the location of the chloroplast rRNA genes. Figure 14.8 shows that *E. coli* rRNA specifically hybridizes with Sal I fragments S1 and S3, Bgl I fragments B7 and B8 and Hpa I fragments H5 and H6. All these fragments are part of the inverted repeat sequences of cpDNA (Fig. 14.7). The hybridization data indicate a sequence homology between Petunia ctDNA and *E. coli* rDNA. They also strongly suggest that the chloroplast rRNA genes are located within these fragments. Since no hybridization was observed with fragments B3 and B9/10, the maximum sizes of the

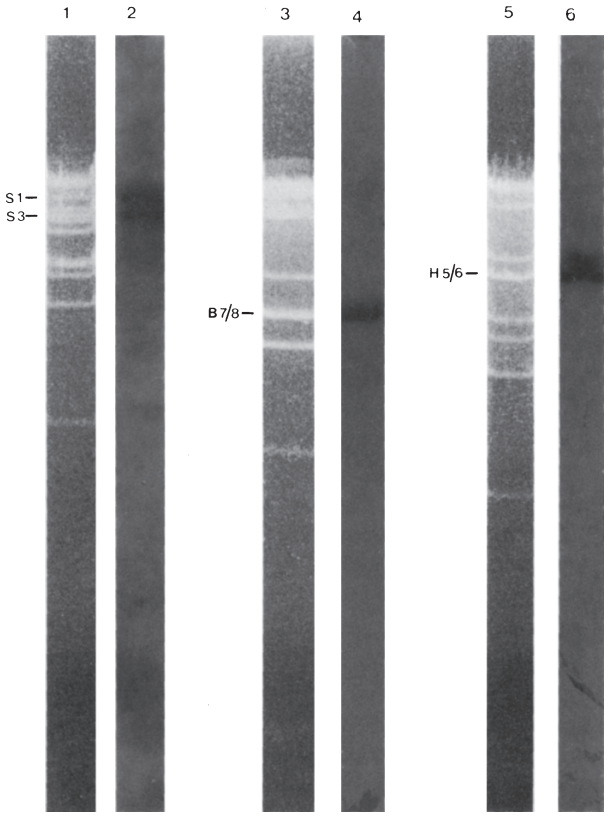


Fig. 14.8. Hybridization of ^{32}P-labeled rRNA from *E. coli* to restriction endonuclease fragments of *Petunia* cpDNA. Lane *1* and *2* Sal I fragments; *3* and *4* Bgl I fragments; *5* and *6* Hpa I fragments. *1, 3,* and *5* are photographs of ethidium bromide stained gels; *2, 4,* and *6* are autoradiographs of the transferred fragments on DBM-paper to which ^{32}P-rRNA was hybridized. (Bovenberg and Kool 1980)

cpDNA regions that could be occupied by these rRNA genes are the 8 kbp dashed regions in the inverted repeats (Fig. 14.7).

14.3.2.5 Expression of the Chloroplast Genome in Isolated Chloroplasts

Isolated chloroplasts from a number of plant species can use light or added ATP to drive incorporation of labeled amino acids into protein. Analysis of the products of this protein synthesis on SDS polyacrylamide gels reveals the presence of up to 90 discrete, labeled polypeptide species. Control experiments have eliminated the possibility that cells or organelles other than chloroplasts are responsible for the observed protein synthesis (Ellis 1976, 1977). Therefore, the isolated chloroplast system represents a powerful tool for the study of the expression of the chloroplast genome. This system has been used to identify the chloroplast as the site of synthesis of a number of chloroplast polypeptides such as: large subunit of ribulose-1,5-bisphosphate carboxylase, elongation factors T and G, α, β, and ε subunit of Coupling Factor I, Peak D or the 32000 dalton thylakoid polypeptide, and the cytochromes f and b_{559} (Ellis and Barraclough 1978; Silverthorne and Ellis 1980).

Recently Colijn and Kool (1980) developed conditions for the isolation of *Petunia hybrida* chloroplasts that are able to use either light or an ATP-generating systems as sole energy source for in vitro RNA and protein synthesis.

a) Light-Driven RNA and Protein Synthesis

Colijn and Kool (1980) observed that the ability of isolated petunia chloroplasts to perform RNA and protein synthesis depended very strongly on the medium used for the isolation of chloroplasts. *Petunia* chloroplasts isolated in the sucrose medium, used by Hartley and Ellis (1973) for the isolation of pea chloroplasts, are not able to incorporate labeled amino acids into protein. Similar results were obtained by Bottomley et al. (1974) for spinach chloroplasts. Protein synthesis in petunia chloroplasts occurs equally well when chloroplasts are isolated in either the sorbitol-containing medium described by Morgenthaler et al. (1974) or the medium used by Bottomley et al. (1974).

Chloroplasts of *Petunia* can use light as the energy source for the incorporation of ^{3}H-uridine into acid-insoluble material. When incubation of the chloroplasts is performed in the dark or in the presence of actinomycin D, this incorporation is reduced to about 40%. When chloroplasts are ruptured after 40 min incubation with ^{3}H-uridine and then incubated with RNAse the amount of acid-insoluble material decreases to the same level as obtained in the presence of actinomycin D (Colijn and Kool 1980). These results indicate that at least 60% of the incorporation of ^{3}H-uridine represents light-dependent RNA synthesis in chloroplasts.

A time course of the incorporation of ^{3}H-amino acids into hot acid-insoluble material in isolated chloroplasts is shown in Fig. 14.9. When chloroplasts are incubated in the dark or in the presence of DCMU, an inhibitor of photophos-

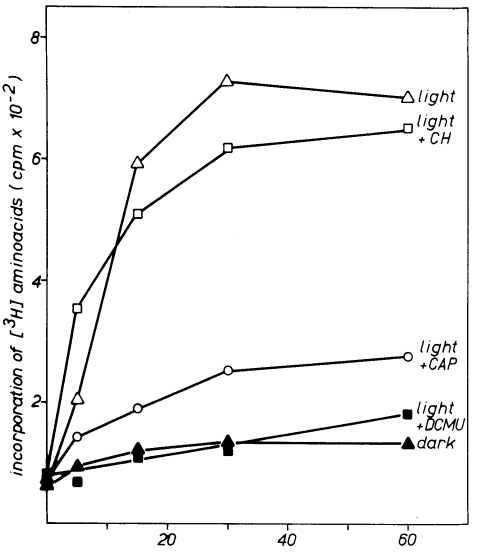


Fig. 14.9. Light-driven protein synthesis by isolated chloroplasts from *Petunia hybrida* (Roter Vogel, PW5201). Incorporation of amino acids by chloroplasts, incubated in 0.33 *M* sorbitol, 0.05 *M* Tricine-KOH pH 8.3). ^{3}H-amino acids (S.A. 50 mC/milliatom C) were added at a concentration of 50 μCi/ml. When indicated, the following inhibitors were added: chloramphenicol (100 μg/ml), cycloheximide (25 μg/ml), and DCMU (1 μ*M*). (Colijn and Kool 1980)

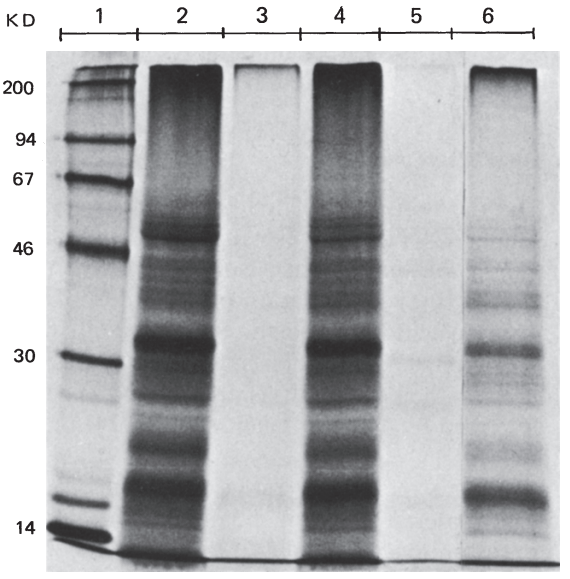


Fig. 14.10. Sodium-dodecyl sulphate polyacrylamide gel electrophoresis of proteins synthesized in isolated *Petunia* chloroplasts. Chloroplasts were incubated in the presence of ^{35}S-methionine (S.A. 600 Ci/mmol) at a concentration of 250 μCi/ml. The figure shows an autoradiograph of the gel containing ^{14}C-marker proteins (slot *1*), proteins synthesized in light-incubated chloroplasts (slot *2*), proteins synthesized in the presence of: chloramphenicol (slot *3*), RNAse (30 μg/ml) (slot *4*), or DCMU (slot *5*), and proteins synthesized in chloroplasts incubated in the dark (slot *6*). (Colijn and Kool 1980)

phorylation, the level of incorporation is strongly reduced. This strong dependence of incorporation on light excludes the possibility that it is the result of contaminating mitochondria or micro-organisms. Addition of cycloheximide did not inhibit protein synthesis which excludes the possibility that the observed incorporation of radioactivity occurs on 80S cytoplasmic ribosomes. When chloramphenicol, an inhibitor of protein synthesis on 70S ribosomes, was added, a strong inhibition of protein synthesis was observed. These results prove that the observed incorporation of ^{3}H-amino acids represents light-driven protein synthesis in intact chloroplasts.

For the analysis of the products of light-driven protein synthesis in *Petunia* chloroplasts, the chloroplasts were incubated with ^{35}S-methionine, fractionated and examined on SDS-polyacrylamide gels. The autoradiograph of such a gel is shown in Fig. 14.10. When incubated in the light, *Petunia* chloroplasts synthesize at least 25 discrete polypeptide species (slot 2). In the dark the same polypeptide species are synthesized, however at a much lower level (slot 6). This suggests that some ATP, required for in vitro protein synthesis, is present in these chloroplasts at the time of isolation. When chloroplasts are incubated with chloramphenicol or DCMU no distinct polypeptide species are synthesized (slot 3 and 5).

The gel-pattern and mol. wt. of the major polypeptide species synthesized in isolated *Petunia* chloroplasts show great similarity with the polypeptides synthesized in isolated pea and maize chloroplasts (Silverthorne and Ellis 1980; Grebanier et al. 1979). On the basis of this similarity Colijn and Kool (1980) tentatively identified some of the radioactive polypeptides (Fig. 14.11). The mol. wts. (about 55000 and 52000) and relative amounts of the polypeptides P1 and P2 make them good candidates for the α and β subunits of chloroplast coupling factor I. Polypeptide P4, molecular weight about 32000 m.w., is the most dominant radioac-

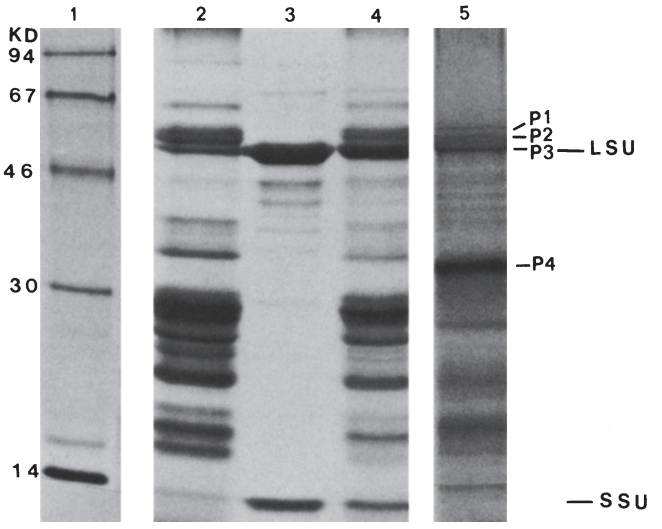


Fig. 14.11. Preliminary identification of some of the polypeptides synthesized in isolated *Petunia* chloroplasts. Labeled and unlabeled chloroplast polypeptides were separated on 12.5% acrylamide gels containing SDS. Lane *1–4* Coomassie blue staining of: marker proteins (lane *1*), thylakoid fraction of chloroplasts (lane *2*), partially purified *Petunia* ribulose-1,5-bisphosphate carboxylase (RuBPCase) (lane *3*), total chloroplast polypeptides (lane *4*). Lane 5 represents an autoradiograph of ^{35}S-methionine labeled polypeptides, synthesized in isolated chloroplast. L.S.U. and S.S.U. denote the large and small subunit of the RuBPCase. (Colijn and Kool 1980)

tive thylakoid polypeptide synthesized in isolated *Petunia* chloroplasts and therefore shows great resemblance with the peak D thylakoid polypeptide from pea (Silverthorne and Ellis 1980) and the P 32000 thylakoid polypeptide from *Spirodela* (Edelman and Raisfeld 1980) which were recently identified as being, most probably, the DCMU-binding protein component of the light-harvesting pigment-protein complex (Trebst personal communication).

Polypeptide P3, molecular weight about 50000, co-migrates with the large subunit of purified *Petunia* RuBPCase (lane 3) which is the most abundant protein in *Petunia* leaf cells and chloroplasts (lane 4). The protein is soluble and therefore not present in thylakoid preparations (lane 2). This is in agreement with the observation that the radioactive polypeptide P3 is released in the supernatant after centrifugation of osmotically ruptured chloroplasts (Colijn and Kool 1980). Therefore, these results strongly suggest that polypeptide P3 is the large subunit of RuBPCase.

b) ATP-Driven Protein Synthesis in Chloroplasts from Leaves and Cell Cultures

Plant cell cultures present valuable tools for the study of chloroplast biogenesis. In cell cultures of tobacco (Seyer et al. 1975) and *Petunia* (Colijn et al. 1979a), cytokinin can be used to induce developmental processes that lead to the transition of white plastids into chloroplasts. The use of mutant cell lines with an al-

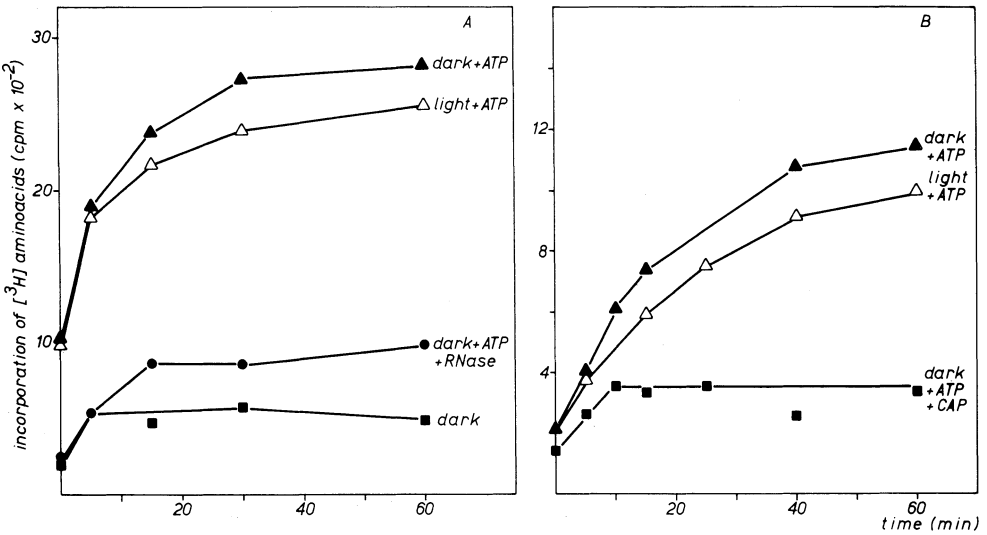


Fig. 14.12 A, B. ATP-driven protein synthesis in isolated chloroplasts from *Petunia* leaves and cell suspension cultures. Chloroplasts were incubated with ^{3}H-amino acids in the following buffer: 50 m*M* Tricine/KOH pH 8.3, 10 m*M* $MgCl_2$, 14 m*M* 2-mercaptoethanol, 80 m*M* NH_4Cl, 2 m*M* ATP, 0.2 m*M* GTP, 10 m*M* creatine phosphate and creatine phosphokinase (200 µg/ml). Panel *A* ATP-driven protein synthesis in chloroplasts isolated from *Petunia* leaves. When indicated, RNAse was added at a concentration of 30 µg/ml. Panel *B* ATP-driven protein synthesis in chloroplasts isolated from the green cell suspension culture AK-2401. (Colijn and Kool 1980)

tered chloroplast development can be extremely useful. For this purpose, Colijn et al. (1979 b) developed an effective chemical mutagenesis procedure for *Petunia hybrida* suspension cultures. As a result, they were able to isolate various mutants with an altered pattern of chloroplast biogenesis, such as mutants that are no longer able to develop chloroplasts or mutants that are always green, even without the addition of cytokinin.

For many analyses of polypeptide patterns in developing mutant and normal plastids, the light-driven chloroplast system can not be used. In many of these mutant plastids, but also in certain development stages of normal plastids, photophosphorylation will not take place. Therefore the necessary energy for protein synthesis in these plastids has to be added in an other way. Bottomley et al. (1974) showed that exogenously supplied ATP can penetrate into isolated spinach chloroplasts when their outer membranes were ruptured by subjecting them to osmotic shock. Under such conditions these chloroplasts are able to perform ATP-driven protein synthesis in the dark. Colijn and Kool (1980) showed that isolated *Petunia* chloroplasts, when osmotically shocked, can also use an ATP-generating system as energy source for in vitro protein synthesis, both in the light and in the dark (Fig. 14.12 A). The incorporation of ^{3}H-amino acids could be inhibited by the addition of chloramphenicol but not by cycloheximide, indicating that this protein synthesis occurs on 70S ribosomes. When ATP was omitted, the shocked chloroplasts were not able to perform protein synthesis either in the light or in

the dark. This result indicates that the shocked chloroplasts can no longer use light as energy source for protein synthesis.

Similar results were obtained with chloroplasts isolated from cultured *Petunia* cells. Chloroplasts were isolated from exponentially growing cells from the green, liquid suspension culture AK-2401, osmotically shocked and incubated in the presence of ATP. Figure 14.12 B shows that these chloroplasts can incorporate labelled amino acids into protein, both in the light and dark. However, incubation of chloroplasts in the dark gave consistently somewhat higher incorporation than in the light. A difference between the level of ATP-driven protein synthesis in the light and dark was also observed in chloroplasts of pea (Ellis 1977) and spinach (Bottomley et al. 1974). The significance of this difference is not clear. Analysis of the products of ATP-driven protein synthesis on sodium dodecyl sulphate-polyacrylamide gels shows essentially the same polypeptides as synthesized in the light-driven system, except that there is less radioactivity present in major thylakoid polypeptide P4.

The results mentioned above clearly indicate that the occurrance of photophosphorylation in plastids is not a prerequisite for studying polypeptide synthesis in chloroplasts. The ability of osmotically ruptured *Petunia* plastids to use added ATP as energy source makes it possible to study expression of the *Petunia* chloroplast genome at various, photosynthetically inactive, developmental stages of the chloroplast.

14.3.2.6 Expression of Cloned cpDNA

In addition to the study of transcription and translation in ATP-driven and light-driven chloroplast systems, expression of chloroplast genetic information can also be studied in various other in vitro systems. Cell-free translation of chloroplast mRNA's in mRNA-dependent reticulocyte lysates has been used to study differential gene expression at the mRNA level (Link et al. 1978; Silverthorne and Ellis 1980). Bottomley and Whitfeld (1979) have described the coupled transcription and translation of spinach chloroplast DNA in a cell-free *E. coli* system. One of the major products formed was identified as the large subunit of the RuBP-Case.

Kool and Glas (unpublished results) have examined the possibility of studying gene expression of cpDNA in *E. coli* minicells. Minicells are the result of aberrant cell divisions and contain little, if any, chromosomal DNA. However they were found to carry plasmid DNA when plasmids were present in the minicell-producing parental cells. In minicells only plasmid DNA-specific expression occurs (Kool et al. 1974, 1975). Sal I and Bam HI restriction endonuclease fragments of *Petunia hybrida* cpDNA were cloned into the tetracycline-resistance gene of plasmid pBR 322. The resulting recombinant DNA molecules were transferred to the *E. coli* minicell-producing strain P678-54 by transformation. Minicells were purified from clones containing recombinant plasmids with various chloroplast DNA fragments. In these minicells de novo RNA and protein synthesis occurred. Analysis of this RNA and protein on polyacrylamide gels revealed the presence of additional RNA and polypeptide species that are not synthesized in pBR 322-harboring minicells. These results indicate that expression of cloned chloroplast

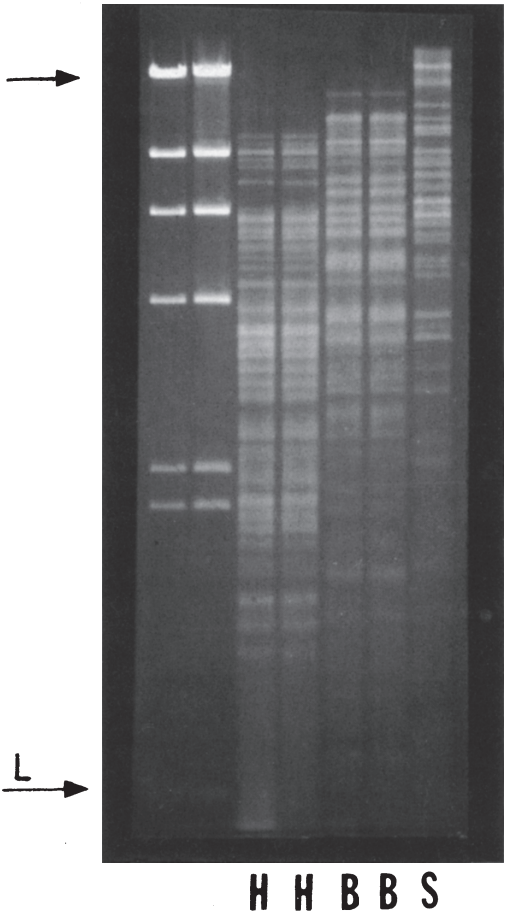


Fig. 14.13. Mitochondrial DNA prepared from *Petunia* suspension cultures (Boeshore and Hanson unpublished). MtDNA digested with HIND III (*H*), BAM HI (*B*), or SAL I (*S*). (*L*) positions of 23 kb and 0.49 kb lambda DNA fragments generated by restriction with HIND III

DNA occurs in *E. coli* minicells. Experiments are now in progress in A. Kool's laboratory to determine whether this expression of cloned chloroplast DNA in *E. coli* minicells represents faithful transcription and translation of the chloroplast DNA.

14.3.2.7 Future Prospects

Identified products coded for by the chloroplast genome still represent only a small portion of the coding capacity. Approaches such as expression of cloned cpDNA in minicells and in vitro transcription-translation of cpDNA will be necessary to identify the remaining polypeptides coded for by cpDNA. However, the function of such polypeptides must be determined before a true understanding of the informational content of cpDNA can be attained.

Since the chloroplast is composed of both nuclear and chloroplast-coded proteins, how these two genomes coordinate their activities is an important question. Combined molecular, biochemical, and genetic approaches using mutants in

chloroplast development and purified nuclear and chloroplast genes may reveal nucleo-cytoplasmic regulatory mechanisms.

14.3.3 Mitochondrial Genome

Study of plant mitochondrial DNA (mtDNA) is presently in an early stage compared to cpDNA. One factor contributing to the lack of knowledge of plant mtDNA is the difficulty in isolating mitochondria in good yield, particularly from green tissue. Generally, technical aspects of mitochondrial isolation must be tailored to the particular genotype and tissue source (Truelove and Davis 1977; Jackson and Moore 1980). *Petunia* is no exception to this rule. Mitochondrial DNA isolation procedures for tobacco and pea leaves (Kolodner and Tewari 1972; Vedel and Quetier 1978) give extremely poor yields of *Petunia* mtDNA in comparison to yield of tobacco mtDNA (Boeshore and Hanson, Bovenberg and Kool unpublished).

Another factor in the slower progress in analyzing plant mitochondrial genomes is the apparent heterogeneity in mtDNA. A number of different size classes of circular and linear mtDNA molecules have been observed in several plant species (Quetier and Vedel 1980; Synenki et al. 1978; Kolodner and Tewari 1972). Also, the sum of plant mtDNA restriction fragment mol. wts. is larger than the mol. wt. of the large circular molecules observed (Vedel and Quetier 1978; Levings and Pring 1979). The complex restriction patterns typical of plant mtDNA are observed with *Petunia* mtDNA (Fig. 14.13); Boeshore and Hanson (unpubl.); Bovenberg and Kool (unpubl.).

Key areas for future work concerning *Petunia* mtDNA are (a) the identification of the molecular basis of the discrepancy between plant mtDNA restriction fragment sums and individual molecule sizes, (b) the informational content of mtDNA, including whether mtDNA specifies cytoplasmic male sterility (Chap. 7), and (c) replication, transmission, and evolution of the mitochondrial genome.

Literatur review for this article was completed in October, 1980.

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15 Plant Breeding

L. EWART[1]

15.1 Introduction

The primary purpose of *Petunia* plant breeding is to develop cultivars that are of high quality in relation to cost and ease of production, and to satisfy the needs of the producer, plant grower, and consumer. If this is to be attained, the *Petunia* breeder must have the skill and knowledge to set up the procedures for the assemblage of germplasm, and subsequent hybridization, isolation, maintenance, evaluation, multiplication, and distribution. The breeding program, therefore, must be carefully and properly planned with a set of breeding objectives.

15.2 Hybridization at the Species Level

References are lacking as to the hybridization at the *Petunia* species level except during the early introduction of some of the species into cultivation, by researchers studying the *Petunia* self-incompatibility system and those trying to sort out the species which are thought to be the ancestors of todays *Petunia hybrida* Vilm.

The future value of breeding at the species level may be with an in depth study of their gene pool for classification for possible resistance to some of the diseases and insects that beset many of todays *Petunia* cultivars.

15.3 Hybridization within *P. hybrida*

15.3.1 Early Morphological and Phenotype Types

By the late 1800's much of the genetic variability we know today was available (Bailey 1906; Henderson 1890; Weddle 1976), but how to control and use the material was a challenge.

Variability was unlocked quickly after the early interspecific crosses were first made. As early as 1838 a variety called *Petunia vittata* was introduced (Bailey 1906); this variety was described as being faintly striped with the stripes originat-

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ing in the throat of the flower and extending outward as do most of the presently existing striped cultivars. In 1844 the variety *Petunia Nixenii* was introduced (Bailey 1906); this was a striped type in which the stripes originated at the border of the flower similar to the cultivars Blue and Cherry Frost and Blue Picotee.

According to Henderson (1890), Isaac Buchanan of New York is given credit for having first hybridized *P. nyctaginiflora (P. axillaris)* and *P. violacea* in the United States. His first effort was crowned with the most complete success and was offered in seedmen's catalogs as Buchanan's Hybrids.

Later the Fringed *Petunia* was developed in the U.S. From Henderson's line drawing the Fringed *Petunia* appears to be the same as our present-day grandiflora, variegated *Petunia*.

German breeders succeeded in getting a double-flowered variety from the fringed type (Henderson 1890), and if any particular color was desired, cuttings would have to be taken from the desired plant. Francis (1915), however, states that the first double *Petunia* appeared in a private garden in France in 1855.

The grandiflora had made its appearance by 1881 (Ball 1976) and could be found as a segregate in Burpee's Defiance strain (Bailey 1906). Published record as to how the grandiflora first appeared is lacking.

In 1892 26 new varieties were introduced in the U.S. (Bailey 1906). *Petunia hybrida grandiflora superbissima* hort, was first encountered in Erfurt, Germany in 1872 (Chlebowski 1964), and again a second time in Ventura, California in 1888. This form was found to be tetraploid which arose from a genome mutation (Chlebowski 1964). One plant appeared in the cultures of Mrs. T. Gould (Bailey 1910) from which Mrs T. Shepherd developed the California Giant strain, the leading *Petunia* types until the 1930's (Weedle 1976).

In the 1920's and 1930's breeders discovered the efficacy of inbreeding and individual plant selection, and separate color-type cultivars began to appear first in the multiflora type (Weddle 1976).

15.3.2 Pollination Equipment and Procedures

Just about all plant breeding activities for *Petunia* revolves around the pollination mechanisms involved for the development of the phenotypes desired. The identity of inbred lines and parental material must be kept constant by proper maintenance of the original genotype. The maintenance and multiplication, therefore, largely depends on the proper procedures for the modes of pollination within the population.

The equipment necessary for making *Petunia* pollinations for inbred development or testcrosses is not elaborate (Fig. 15.1). A greenhouse structure built to exclude insects and benched properly to facilitate the growing and handling of plants during pollination is also necessary.

Petunia pollination is a highly labor-intensive procedure, and a well thought out program is necessary. The planned self- or cross-pollinations must be determined and charted before any actual work begins. Under controlled conditions, selected plants for selfing can have their open flowers brushed with an artist's brush and marked in some fashion to denote what has taken place (Fig. 15.2 A).



Fig. 15.1. Equipment and material utilized in *Petunia* pollination

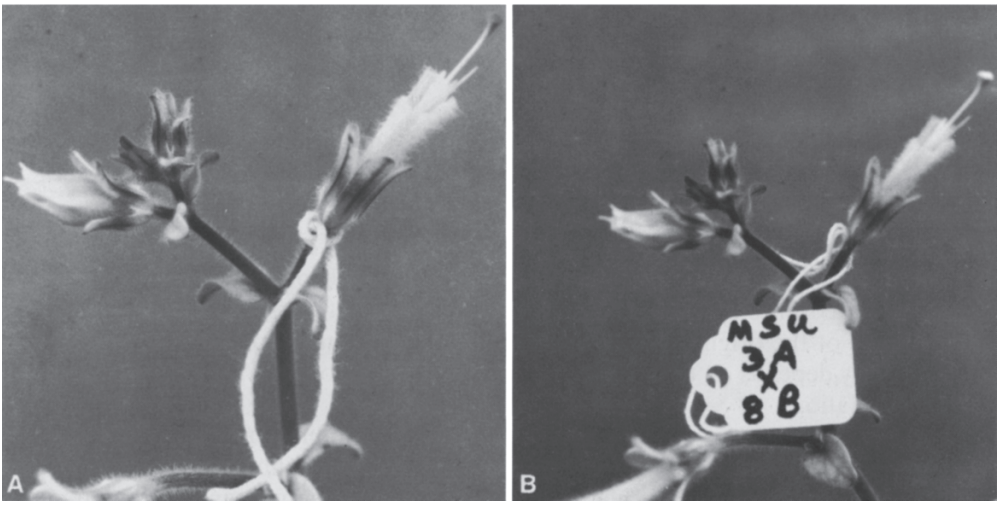


Fig. 15.2 A, B. Methods that can be used for marking pollinated *Petunia* flowers. **A** Selfed flower designated by white string. **B** Cross-pollinated flower designated by white tag marked with the involved parents



Fig. 15.3. Emasculated *Petunia* flower bud

If cross-pollinations are to be made, unopened flower buds must be emasculated on the selected female parents. The writer has found that splitting the flower bud lengthwise with the sharp point of a pair of tweezers and removing the top half of the corolla and the unopened anthers (Fig. 15.3) is a good procedure. The remaining portion of the corolla serves two purposes:

1. as a marker for flowers that have not been pollinated, to facilitate finding the flowers later and to reduce mistakes.
2. as the remaining portion of the corolla subsequently unfolds or matures, the flower is usually prime for pollination in order to get maximum seed set.

Pollen from the selected male parent can be transferred by picking out individual dehisced anthers with tweezers and then touching the anthers to the stigma (Fig. 15.4 A). This procedure conserves pollen if several crosses are to be made with a single pollen parent when only a few open flowers are available. Otherwise, a small brush can be used (Fig. 15.4 B). After a cross is made, the flower must be marked. Usually a small white tag labeled with the seed parent followed by the pollen parent is used (Fig. 15.2 B).

During warm temperature periods, seed maturation takes about 4 weeks from time of pollination. During this 4-week period, necessary steps should be taken to make sure the plants are free from disease especially *Botrytis* which will attack ripening seed pods if preventative measures are not taken. Once the top one-third of formed seed pods turns brown they can be harvested (Fig. 15.5). The pods are still firm enough to make removal easy without the pods splitting open and consequent possible loss of seed. Small coin envelopes make excellent containers for harvested seed. They must be marked properly according to pedigree.

At the time the seed pods are harvested, the degree of success should be noted. This can be done on the charted pollination worksheets. Even though the degree

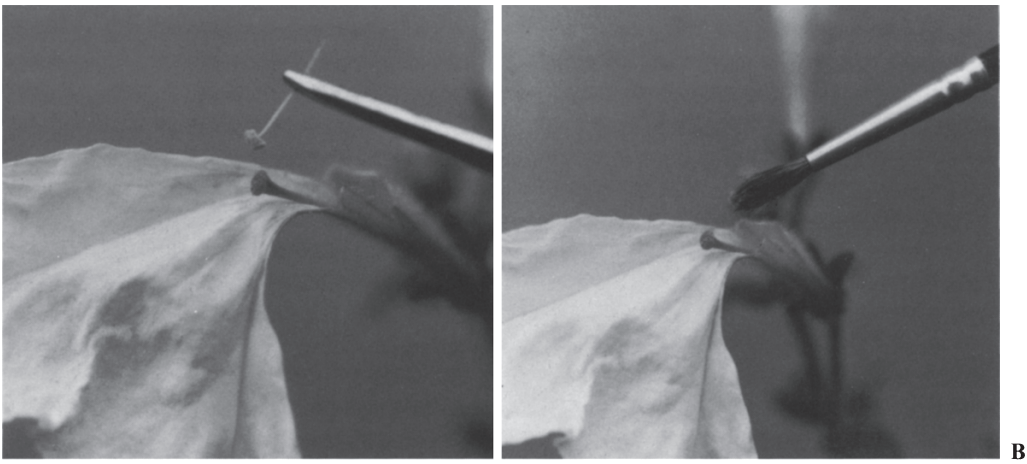


Fig. 15.4 A, B. Tools used for pollen application to *Petunia* stigmas. **A** Using a pair of tweezers. **B** Using a small artist's brush

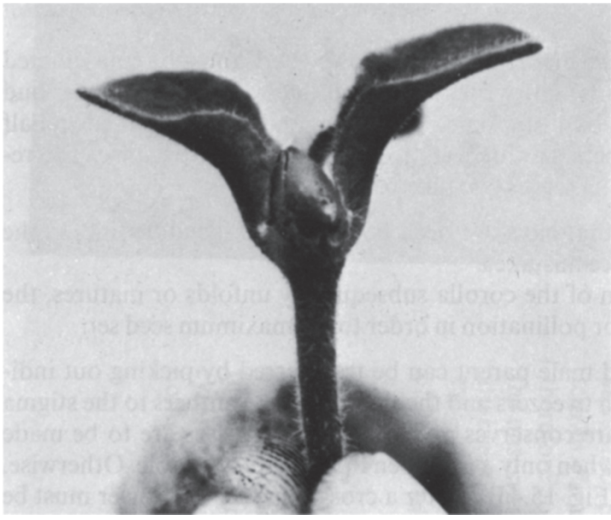


Fig. 15.5. Ripe seed capsule of *Petunia* with one sepal removed showing capsule starting to split open at tip end

of success is usually subjective, it can have a strong bearing on future plans for the parents in question.

The collected seed can be air-dried in the collection packages. The writer has found the ideal conditions for seed drying are a seed storage room with 18 °C temperature and 20% relative humidity; however, a dry area with a 21° to 26 °C temperature is also suitable for short term drying. The seed can then be cleaned as necessary using small screens or sieves (Fig. 15.6). With small batch cleaning, to clean out unwanted chaff after screening, the seed can be rolled down a slightly folded 8 in. × 11 in. blotter.



Fig. 15.6. Equipment that can be used to separate *Petunia* seed from unwanted debris especially small trial crossing lots

15.4 Inheritance and Breeding for Specific Traits

There is little published information on specific procedures used for the development of *Petunia* phenotypes. Thus, the information presented here is derived from the writer's 26 years of experience with *Petunia* and personal communications with other breeders. A student of the subject would do well to read the published works of Weddle (1947, 1976) for background information.

Successful plant breeding is dependent upon the selection pressure applied for the various characteristics desired. In the production of a specific phenotype, the use of the genotype × external factor interaction must be the basis of plant improvement (Craig 1968). The writer has found that success is dependent upon the selection pressure being applied at all stages of the development program – prior to propagation, after propagation, and mature plant performance. As the time required for selection increases, the efficiency of selection decreases which makes programs based only on the performance of mature plants questionable.

A good *Petunia* breeder must know his subject completely – genetics, plant physiology, especially plant growing procedures, and the intended use of the plants at every stage including the consumer. Once this is accomplished the breeder will develop a trained eye for making the necessary selections.

Successful *Petunia* breeders master the selection of inbreds of superior combining ability by experience, hard work, paying attention to details, and having a highly organized system. Furthermore, most programs operate on the basis of obtaining one generation a year.

Selection prior to propagation revolves around post-performance of breeding lines – combining ability. Breeders usually make more selections than can be used as a precaution against not losing a desired characteristic. Then, everytime a new generation is planned, decisions must be made as to what to plant in order not to get a population that is too large and costly to maintain.

The next selection period is after propagation and examining the seedling response. Some of the most popular *Petunia* phenotypes produce inbreds that are notably weak or show poor seed germination. This weak seed germination and growth can cause seed production problems or even be carried over into hybrid combinations.

Selection of *Petunia* at the mature plant level should take place at two different stages:

1. In the transplant pack.
2. The final planting location.

In the transplanted pack, quality characteristics such as earliness of bloom, habit as it relates to branching and height in the pack, flower production – color, substance, and size, foliage color and leaf size, disease tolerance, and pollen production can be selected or screened.

The final planting location may deal with inbreds selected from greenhouse pollination work or field planting of inbreds or hybrids for evaluation of outdoor performance. Mature greenhouse plants generally should be counted on to provide information only on pollen production and seed set because of artificial growing conditions. Field plantings, however, can be very valuable especially if numerous evaluations are made – not just one or two during the growing season. Records taken on bloom production, plant habit and vigor, flower size, substance, color intensity, disease tolerance, and "weatherability" are of prime importance. The rating for these characteristics on hybrids furnishes considerable information toward probable combining ability of the involved inbred lines and is heavily considered during the process of inbred elimination that must constantly be done.

15.4.1 Inheritance of Flower Morphology

15.4.1.1 Introduction

The inheritance of flower morphology becomes a part of any *Petunia* breeding program. The knowledge of inheritance systems involved, therefore, is very important in the selection process and the way any program is handled.

The two most important morphological characters are flower size and doubleness, and genetic study of these two traits has been extensive. The terminology for these characters is presently:

1. Grandiflora – large-flowered (gene designation *G*)	Plickert 1936
2. Multiflora – small-flowered (gene designation *g*)	
3. Double corolla – double-flowered (gene designation *D*)	Frost 1915
4. Single corolla – single-flowered (gene designation *d*)	



Fig. 15.7. Variation in *Petunia* sepal sizes. *Top row* shows multiflora calyces and *bottom row* grandiflora calyces that were selected respectively from within four different lines segregating multiflora and grandiflora plants

There is general agreement that flower size is determined by a single pair of alleles *G* and *g* (Dermen 1931; Plickert 1936; Weddle 1947; Seidel 1962; Reimann-Philipp 1962; Ewart and Walker 1964; Chlebowski 1964; Goldsmith 1968; Sink 1975). The major influence of the *G* allele is to increase flower size, but it has a pleiotropic effect of producing a coarser plant habit, larger, thicker and broader leaves, thick peduncles, and larger, thicker and broader sepals (Fig. 15.7) (Seidel 1962; Chlebowski 1964; Sink 1975; Weddle 1976). There is, however, a divergence of ideas concerning the system or systems involved in the inheritance of the grandiflora character (*G*). Although it is now possible to identify homozygous grandiflora plants and produce larger-flowered hybrids, this was not always the case; there have been wide variations from expected ratios for monogenic inheritance in segregating generations. The problem is characterized by the occurrence of fewer than expected grandiflora plants in some instances and fewer than expected multiflora plants in others. Also, the homozygous *GG* plants, when they do appear, are very weak and produce variable amounts of pollen and seed which generally give low germination percentages and weak seedlings. To explain the shortage of grandiflora plants in segregating populations, Weddle (1947) suggested that the large flower gene is closely linked to a lethal or semilethal. Differing degrees of sublethality with a pollen certation effect (Reimann-Philipp 1962) and vigor alleles interacting with sublethal genes (Ewart and Walker 1964) have also been suggested.

These explanations, however, did not resolve the instances where there were fewer multiflora plants than expected in segregating populations. This type of segregation was prevalent in the nearly true-breeding grandiflora cultivars developed by Ernst Benary between 1930 and 1945 (Weddle 1976). His Erfurt strain would breed true up to 80%–95% grandiflora. These line cultivars included Setting Sun, Colorado Sunset, Lace Veil, and White Cloud. It is of interest that some of these cultivars, according to Weddle (1976), became the foundation of many of the

presently available F_1 hybrid grandifloras developed by Pan American Seed Co. Reimann-Philipp (1962) postulates that this type of segregation is due to a system of balanced lethals (*L* normal, *l* lethal) with the lethal linked to the multiflora *g* allele; and that when the multiflora plants do appear, it is due to crossing over which he calculated to be a least 2%.

The writer has found that homozygous *GG* plants appear rarely in this material, and when they do, they are extremely weak and often pollen-sterile. Either there is considerable certation taking place, or because these cultivars are line-bred, the homozygous *GG* plants are weakened by inbreeding.

Because of the weak condition of homozygous grandiflora plants, commercial grandifloras are completely dependent upon hybrid production (Goldsmith 1968).

15.4.1.2 Inheritance of Flower Doubleness

The inheritance of flower doubleness remained elusive for a long time. From 1855 when the mutation took place, until the early 1930's, double-flowered plants were maintained by cuttings (Weddle 1976) or produced by crossing doubles to singles which resulted in both double and single plants.

In the first real attempt to understand the genetics of doubleness in *Petunia,* Saunders (1910), using two species and a *P. hybrida* grandiflora line containing doubles, was no more successful than earlier plant breeders in obtaining seed set from double flowers due to malformed or lack of pistil development. Singles, when selfed, produced all singles, and single × double crosses produced the expected singles and doubles with singles being in excess. It was assumed due to the excess of singles, that singleness was dominant a theory which was later disproved. The difficulty probably originated in the grandiflora material with the semilethal and possible certation problem, as well as the apparent weakness in doubles at the time.

Frost (1915), of *Matthiola* genetics fame, in reviewing Saunders work, put forth the correct assumption and also made the first designation for the character:

1. Because single × double gives single and double plants, and singles selfed give only single plants, the double factor (*D*) is dominant and single (*d*) is recessive.
2. Cross-pollination between the two types gives the ratio of 1*Dd*: 1*dd*.
3. The excess of singles was due to improper seedling selection.

He took the clue about the less vigorous doubles from a quote in 1914 by T. Payne, a Los Angeles seedman, who recommended that the weaker seedlings should be saved because they were more apt to produce double flowering plants.

T. Sakata, Japan is given credit for introducing the first all double *Petunia* hybrids (Weddle 1976; Howland 1981). He discovered the production secret in the early 1920's (Howland 1981) and had an international monopoly on double *Petunia* seed from 1928 until the mid 1940's. The next breeder to unlock the secret was R. Simonet, Edmonton, Canada who developed the multiflora cultivar America, a 1943 All-America Winner. Weddle (1947) published how this had been accomplished through the work he had done on doubles while employed by the Burpee Seed Company. Under proper growing conditions, at the proper time of year, he found that many heterozygous double plants have functional pistils

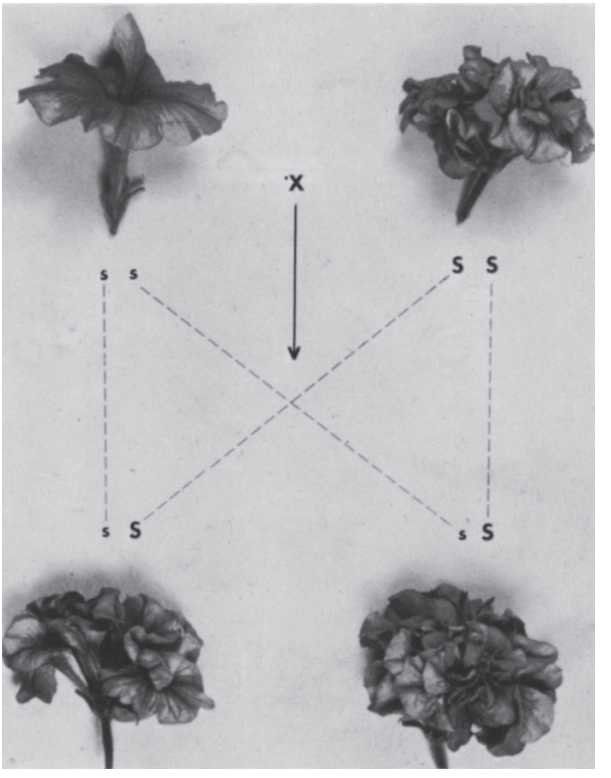


Fig. 15.8. Crossing system to produce F_1 hybrid multiflora, double *Petunia* plants

and set seed. He was then able to select homozygous double plants from the resulting plants which could be used in producing all double hybrids. The homozygous *DD* plants were female-sterile and could only be propagated by cuttings. The all double *Petunia* is, therefore, completely dependent upon the hybrid method of production, the single being the seed parent and the homozygous double the pollen parent (Weddle 1947; Goldsmith 1968; Fig. 15.8).

15.4.2 Breeding for the Multiflora Single Phenotype

The present day single, multiflora *Petunia* presently commands only a 4.7 percent average (Voight 1981) of the bedding plant growers mix of different plants grown and sold, but this phenotype has had a very significant impact on the bedding plant industry, the double flower phenotype, and the now popular grandiflora phenotype.

In the 1920's and 1930's, breeders using the efficacy of inbreeding and individual plant selection, began to develop and introduce color-type, line-bred cultivars, and the first ones were the multifloras (Weddle 1976). The line-bred cultivars Pink Gem 1934, Rose Gem 1936, Flaming Velvet 1936, Dainty Lady 1936, Burgundy 1937, and Blue Gem 1938 were All-America Winners (Anonymous 1979). The

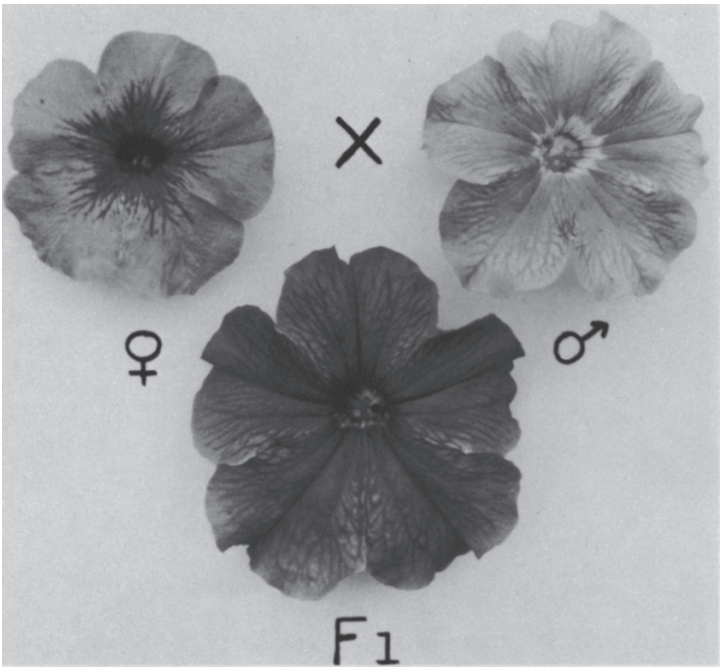


Fig. 15.9. Crossing direction to produce F_1 hybrid grandiflora *Petunia* using the multiflora parent as the seed parent

first red *Petunia* was the line bred cultivar Fire Chief, a 1950 All-America Winner. Fire Chief was developed by S. Sinclair, a Bodger Seeds plant breeder, over a ten year period with the line-bred cultivars Salmon Supreme and Cheerful as ancestors (Bodger H. 1980 pers. commun.).

Also, in the late 1940's C. Hope, was developing inbred, red multifloras but had not introduced any due to low seed productivity (Hope C. 1980 pers. commun.). His reds were developed from crosses between Dazzler, a deep ruby multiflora with a yellow throat, and some Deep Salmon grandiflora lines. After Fire Chief was introduced, Hope crossed it with his reds and selected a hybrid combination which became the 1953 All-America Winner, Comanche. This cultivar became very successful and is still available. It proved that expensive hybrid seed could be marketed with success, and from that time on all major *Petunia* introductions have been hybrids.

Inbred, single multiflora *Petunia* is also used as one parent for all double and grandiflora hybrids (Figs. 15.8 and 15.9). The development of multiflora inbred lines, therefore, is of great importance to any *Petunia* breeding program.

In developing multiflora inbreds the selection of material from the best new hybrids on the market and experimental crosses within an existing program is something that should be done every year. Open-pollinated F_2 seed can be collected from the field and planted the following year. With experience, the selections from F_2 and F_3 generations can be done from flats in late spring, growing

the plants under regular bedding plant production procedures. The writer has found it generally sufficient to select two dozen plants. The selections are enclosed in greenhouses for selfing. Most *Petunia* programs use a pedigree system for keeping track of the numerous inbred lines.

Minor genes are known to amplify the expression of doubleness and flower size and selection for these are important in the multiflora phenotype. Plants with small petaloid formations or corolla fringing should be considered as possible material for future double crosses. A higher percentage of these types will segregate from material derived from a double background.

Plants with large flowers should be considered for future crosses stressing a larger flower size. Selections here must be done carefully because larger flower types seem to be linked with poor branching and coarse, open habits not presently considered desirable for garden performance. These characteristics have also been associated with earliness (Weddle 1976).

Selections from the F_4 generation can be handled the same way with regard to selecting a few desirable plants for the greenhouse; however, the remaining plants are planted in the field to record performance under the stress of outdoor environmental conditions. Any new *Petunia* breeder will be shocked the first time the field performance is witnessed against what has previously been observed under greenhouse conditions. Many selections will be found to be very poor for outdoor performance and such information is very valuable in eliminating many inbred lines from further consideration.

A system of handling the plants in the greenhouse must be well thought out and carefully controlled otherwise efficient use of labor and greenhouse space will not take place, and this can be very costly. The selected plants can be potted up using movable containers to facilitate handling and must be supported in some fashion such as with two bamboo stakes to each pot and the use of Twist-Ems to contain the plants in an upright fashion for ease of pollination (Fig. 15.10). Very narrow benches with some type of support netting can also be used; two rows of plants with an aisle on each side facilitates maintenance and pollination.

As information gathered from the seed flat, pack flat, and field performance stages is reviewed, experimental crosses are set up and carried out. Crosses exhibiting successful seed set are planted the following year and compared to commercial cultivars in pack and field evaluations. Should any cross show superior results a trial seed production block is set up, usually the next year accompanied by critical performance tests. Expanded pack trials are conducted together with numerous field trials at the breeder's location and on public trial grounds. If the experimental cross passes these tests, a full production cycle is planned the next year and the new cultivar is released the following year. This whole process from the first selection for inbred development until a new inbred is used as a parent for hybrid production can require 8–10 years.

Over several years a series of compatible parental lines will develop. A system for maintenance and stock seed increase must be established. These advanced generation lines, F_6 and older, can be jeopardized if self-pollination is rigorously followed. Deleterious characters can appear very rapidly and prove difficult to eliminate. This problem can be solved by growing out four dozen plants rather then the normal two dozen and selecting the best 6–8 plants. These plants can then



Fig. 15.10. Typical multiflora, inbred *Petunia* plant maintained for crossing under controlled greenhouse conditions

either be sib-mated or mass-pollinated or sib-mated one year and massed the next. This system will help maintain enough heterogeneity to keep most of the deleterious characters from appearing.

15.4.3 Breeding for the Grandiflora Single Flower Phenotype

Single, grandiflora *Petunia* plants presently command a bedding plant mix average of 16.3% which is the largest percentage of all flowering bedding plants grown (Voigt 1981). Commercial cultivars from seed were being introduced by E. Benary, Erfurt, Germany, and T. Sakata, Yokohama, Japan, by 1930 (Weddle 1976). These were line-bred cultivars and did not breed true for the grandiflora character.

Weddle was first to develop true breeding, grandiflora *Petunia* (Weddle 1947). His first hybrid single cultivar Ballerina, introduced by Pan-American Seed Company, was a 1952 All-America Winner (Anonymous 1979). It was followed by several other cultivars from Pan-American, Harris, and Sakata seed companies and later Geo. J. Ball and Goldsmith Seeds.

A multiflora inbred is one parent in any grandiflora hybrid cultivar, usually the seed parent; therefore, multiflora inbred development must be a part of any grandiflora program. Since homozygous grandiflora lines can only be used successfully in a program if they are grown under controlled greenhouse conditions, the field performance of multiflora lines is very important.



Fig. 15.11. Representative plants selected from an F_2 segregating line for multiflora-grandiflora plants. *Left to right* one multiflora plant, three heterozygous grandiflora plants, followed by two weak, homozygous grandiflora plants

Due to their genetic behavior, development of grandiflora inbreds requires special procedures. The first time such plants are selected from a heterozygous population, a higher percentage of usable, homozygous *GG* plants will come from a population derived from crossing two heterozygous grandiflora lines. The next best combination is selecting in the F_2 generation of F_1 hybrids. The resulting seed should be sown thinly and germinated under the best conditions possible. As the seedlings are being transplanted, only the weak ones should be saved. The transplanted material should be watched very closely in culture and grown at a night temperature of no lower then 18 °C. By the fourth week after transplantation, the difference in vigor between possible homozygous plants and the other segregates is quite noticeable. A plant within a line that is weaker in growth and which has lighter terminal foliage and soft stems and leaves should be considered as a possible homozygous large-flowered plant (Fig. 15.11). Later, as the selected plants bloom, any that still show a weak habit and very large, soft sepals when compared within each line should be kept and selfed (Fig. 15.12). If selfed seed can be obtained, usually vigor can be selected for in succeeding generations. The use of sib-matings can sometimes help immeasurably in obtaining increased vigor and fertility.

The procedure for handling the plants from that stage in the greenhouse is the same as described for multifloras. The homozygous, large-flowered inbreds, however, must be carefully cultured as growth problems appear quickly if the soil pH or minor elements, especially iron, are imbalanced.

15.4.4 Breeding for the Double Flower Phenotype

The percentage double *Petunia* in the crop mix of bedding plant growers is 3.1% (Voight 1981). This includes both the multiflora and grandiflora doubles.

Grandiflora doubles were the first F_1 hybrid flower seed to achieve any economic importance (Weddle 1976). The first cultivars of all double *Petunia* plants



Fig. 15.12. A range in grandiflora flower sizes as selected from five separate homozygous, grandiflora *Petunia* lines

introduced by T. Sakata were heterozygous for flower size (Weddle 1976), but obtaining all double plants alone was considered such a major accomplishment that his Victorious Mixed was a 1934 All-America Winner, followed by Victorious Gaiety and Orchid in 1938, Victorious Apple Blossom in 1939, and Blue Brocade in 1941 (Anonymous 1979).

The first all double multiflora cultivar America thought to be developed by R. Simonet was introduced in the U.S. by the Burpee Seed Company in 1943 (Weddle 1976). The Burpee Seed Company then introduced Mrs. Dwight D. Eisenhower and Rose Marie in 1947; both were All-America Winners. These were bred by C. Weddle. Pan-American Seed introduced the all double Glorius mixture in 1946 (Weddle 1947). This mixture was at first heterozygous with regard to flower size (Hope C. 1980 pers. commun.), and later improved in the early 1950's. Subsequently, Sakata introduced an improved Victorious strain which was all double and all large-flowered. This was followed by the high pack performance Bouquet series from Goldsmith Seeds and Superstar series from Harris Seeds.

Later Pan-American Seed Co. introduced the all double multiflora Cherry-Tart, a rose and white bicolor. Cherry-Tart had superior garden performance and is still considered to be one of the best cultivars ever introduced. The popularity of Cherry-Tart has led to a whole Tart series followed by Sakata's Empress series, Goldsmith's Delight series, and the Cosmic series from Harris Seeds.

The popularity of the double *Petunia* and the number of cultivars available has declined. The decrease in popularity is possible due to the fact that doubles have not been improved as much in garden performance when compared with many new single cultivars. The seed is also expensive, probably reflecting the costly procedures of maintaining and propagating the homozygous, double pollen parents by cuttings. Any present day double *Petunia* program must include inbred lines of single grandiflora and double multifloras, and each make their own unique contribution to the final product.

In the early history of the double, the *D* allele was maintained with the grandiflora *G* allele an unwise procedure which plant breeders later found involved all the problems of developing homozygous, large-flowered inbreds. Eventually the *D* allele was maintained with the multiflora *d* allele which gave the inbreds more vigor and could be used for both multiflora and grandiflora double production. The development of all double multiflora inbreds, however, is still time-consuming and expensive because the homozygous plants are female-sterile, producing only anthers (Natarella and Sink 1971). Even the heterozygous doubles do not form many functional pistils; but if the plants are not overgrown and are grown a little on the hard side, they will form a greater number of pistils during the months of September and October and again in April. This is the procedure Weddle (1947) had discovered and which the writer also found while developing double grandiflora cultivars.

The first step towards developing multiflora, homozygous double inbreds is either to cross two similar multiflora heterozygous double plants together or self a particularly good plant from a hybrid cultivar and select several heterozygous plants for selfing for two generations. The heterozygous plants will generally exhibit more vigor and will not be quite as full doubles as the homozygous plants. Two selfed generations is about the limit due to vigor and sterility problems. In the generation resulting from the second self, possible homozygous double plants can be selected for testcrossing, but usually better results are realized if very similar heterozygous sister plants or very similar heterozygous plants from sister lines are crossed. Homozygous double plants can then be selected from these crosses for testcrossing. This procedure will usually provide better pollen production and more vigor in the plants which is very important for cutting production for the maintenance and seed production phases. Even after all this work only a very few will be successful as parental material, and they are always subject to disease and virus infection which has caused the loss of many excellent double parent lines over the years. The problem of vegetative reproduction for maintenance and seed production with the constant possibility of disease infection is probably the greatest single deterrent for double development. Tissue culture could possibly be of great benefit in maintaining the lines, and provides a ready source of plants for seed production, and helps keep the material disease-free.

The development of multiflora single inbreds which are used as seed parents for multiflora double hybrids are bred simultaneously within a regular multiflora development program.

Usually the single-flowered multiflora and grandiflora inbreds which exhibit the best combining ability for doubleness come from a double background and

heterozygous grandiflora, fringed, flowered material. The largest number of additive polygenes for doubleness come from these types.

Multiflora double hybrids usually have a better outdoor performance rating than most grandiflora, double hybrids; this is probably due to the fact that at least the single, multiflora parent inbred lines can be performance-tested outdoors. This is not the case for grandiflora, double hybrids when both parents are kept under cover with only the field performance of testcross hybrids providing the success or failure information of selected material.

15.4.5 Breeding for Flower Colors

It was not until around 1920 that any *Petunia* would come true to color (Weddle 1976). White and shades of rose, pink, salmon, purple, crimson, and varigated *Petunia* plants were quite common. Since then, only three new colors have been introduced – red, plum, and yellow.

In their genetic study of *Petunia* flower color, Paris and Haney (1958) found nine genes for color with one having three alleles, giving a total of 768 possible phenotypes. Later, two more additional color genes were identified by Paris and Goldsmith (1959).

Any major *Petunia* program will carry a series of inbreds for all the major color classes for each of the flower types, thus drastically increasing the number of lines to maintain.

The longest project to develop a new color in modern times, and probably until the present, has been conducted by C. Hope, Costa Rica. He sought a yellow *Petunia* for 25 years and developed the multiflora cultivar Summer Sun. The background material for this development started with reds which had a bright yellow throat; years of recombination crosses, backcrosses, and rigorous reselection by the breeder, expanded the yellow color to the whole corolla.

15.5 Disease Considerations

Petunia is subject to disease attack in all the stages of plant production. Bedding plant producers are faced with the "damp-off complex" associated with *Pythium, Rhizoctinia, Phytophthora, Alternaria,* and *Botrytis* in seed and transplant flats. These diseases are usually kept in check with proper sanitation, sterilization, and preventative procedures, but become much more costly and troublesome during the expanded transplant and production stage.

Petunia outdoor performance is outstanding under sunny, warm, dry weather conditions and is at a disadvantage only under very humid, wet weather and poorly drained soil conditions which set the stage for various diseases. Again, *Botrytis, Phytophthora, Alternaria* along with *Sclerotinia* can attack the plants.

The garden disease problem appears to have become so severe that some home gardeners are reluctant to plant *Petunia* if more disease-resistant plants can be used.

15.5.1 Breeding Disease Resistance

Most modern *Petunia* breeding programs have neglected breeding for disease resistance. This disinclination may stem from the fact that such research seems difficult, unrewarding, and expensive to conduct, especially in the light that what resistance, if any, which has been found has not been identified or studied genetically.

A little progress has been achieved with resistance to *Botrytis* where differences between cultivars have been observed. This success has been achieved more by chance than by design; what is associated with *Botrytis* resistance and whether a gene or genes exist for resistance is no known.

All the major *Petunia* diseases mentioned will probably be studied in the near future through economic necessity if *Petunia* is to maintain it's position as the number one bedding plant crop.

15.6 Seed Production

Until 1950 when the impact of F_1 hybrids was beginning, most *Petunia* seed production was handled as field production with line-bred material. In 1949 the value of *Petunia* seed production in the United States amounted to a little more than U.S.$ 231,000 mostly produced in California (Hawthorn and Pollard 1954). By 1970 the value had dropped to slightly less then U.S.$ 86,000 (Craig 1976). The drop in value reflects the popular use of F_1 hybrids today. Though much more expensive, they are preferred for their quality.

15.6.1 Field Production

Field production has graduated to a large extent from line-bred material to the production of F_2 seed from selected hybrids. This has greatly increased productivity and cut costs because it is not necessary to plant as much acreage as was needed for line-bred material. The seed produced is used largely for the packet seed segment of the seed industry.

The seed in the past was sown into the production fields, but now much of the acreage is transplanted. After a season of growing, the plants are cut and placed on canvas for drying (Hawthorn and Pollard 1954; Craig 1976). After threshing, the seed is processed and germination-tested before packaging.

15.6.2 Hybrid Seed Production

15.6.2.1 Hybrid Production Centers

Hybrid *Petunia* seed is almost entirely produced by hand emasculation and pollination in Central America and Japan. Production has also been done in Italy,

India, and Africa but has never reached the level of operations in Central America. C. Hope set up a seed production station near Turrialba, Costa Rica and agreed to produce seed for the Pan-American Seed Company he helped form in 1946 (Howland 1980). The operation was soon moved to a better climate area near Cartago and was named Linda Vista. Today, Linda Vista is two farms totaling 400 acres employing 800 people.

The first crop of seed for Pan-American Seed was produced in 1947. Subsequently, Linda Vista started producing seed on a contract basis for other seed companies. It is estimated that two-thirds of the hybrid *Petunia* seed produced in the world originates from this operation (Ford 1981).

Goldsmith Seeds now has an operation in Guatemala and Pan-American Seed has set up an operation in Honduras.

15.6.2.2 Handling of Parental Plants

Petunia seed production is a very labor-intensified endeavor and labor accounts for 60% to 70% of the seed cost (Ford 1981). The breakdown of time expenditures for efficient production methods with fertile seed parents is 40% for emasculation, 15% for pollen collection and storage, 30% for hand pollentation, and 15% for selective harvesting of seed pods (Frankel and Galun 1977).

Poor seed production capabilities of a hybrid combination only compound the seed costs. The average seed productivity of multiflora seed parents is about 1.5 g per plant and for grandiflora seed parents 0.5 g per plant (Hope C. pers. commun.). Plants of seed parents are usually grown on narrow benches with two rows of plants supported by a netting system, with an aisle along each side of the benches to facilitate work on the plants (Fig. 15.13). Emasculations, pollinations, and seed collection take place every day.

Several methods have been tested to reduce the cost of emasculation, but none have been really successful. Cytoplasmic male sterility became available and was attempted in the 1950's, but was not a success. Cytoplasmic male-sterile-produced F_1 hybrids have been found to be almost always inferior to their fertile counterparts. Reduction in flower size, inferior flower color, and especially, blindness and bud blasting have been the problems encountered (Ewart and Walker 1958; Goldsmith 1968; Frankel and Galun 1977). A few hybrids have now appeared which are utilizing the cytoplasmic male-sterile system with a degree of success. The grandiflora cultivar Pink Magic appears to be the best product of this experimentation. Around 1970, C. Hope found a mutant floral type in a single-flowered plant exhibiting a second set of sepals which replaced the corolla tube (Fig. 15.14). Sink (1973, 1975) designated this mutant type as *apt* (apetalous condition) and found the character to be a monogenic recessive trait. This apetalous condition was seriously screened by the writer after observing the mutant in 1974 for possible use in seed production, because it meant that the emasculation step of removing a portion of the corolla on normal-flowered inbreds to remove the anthers before anthesis could be eliminated. Two problems, however, surfaced which generally eliminated the character for seed production. Since the corolla tube color is lacking in the apetalous phenotype, extensive purity testing would be necessary during backcross incorporation into inbred lines and seed produced



Fig. 15.13. A *Petunia* F_1 hybrid seed production house at Linda Vista SA, Cartago, Costa Rica



Fig. 15.14. Branch of apetalous *Petunia* plant showing apetalous flowers

from apetaleous seed parents. The risk of contamination, especially during the seed production phase, was considered too high. Also, it was observed that the second set of sepals did not behave like normal sepals. They had a tendency to die back, leaving dead plant material surrounding developing seed pods where a *Botrytis* infection would develop.

Presently, C. Hope (1981 pers. commun.) is investigating an approach using extended styles which would place the stigmas beyond the anther ring. The ultimate procedure may be the use of a genic male-sterile system. Goldsmith (1968) mentioned that such a system had been used but he felt that problems of maintaining such lines vegetatively were greater than producing seed by emasculation and pollination. The type of system employed or how the character was inherited was not mentioned.

Frankel (1971) found a genic male-sterile in his graft-transmitted cytoplasmic male-sterile material which was inherited as a monogenic recessive. Such a system, once incorporated into parental lines, would be maintained by backcrossing giving a one to one ratio of sterile to fertile plants. To obtain sterile plants for seed production, a backcross generation would need to be flowered in transplant flats with the expected 50% sterile plants selected out. Even the 50% fertile plants could be used, if necessary, following the normal emasculation procedures.

If tissue culture were available, sterile plants could be produced in mass once the desired level of homozygosity was established after the character was introduced into parental lines.

15.6.2.3 Pollen Collection

Pollen parents are generally grown under cover to protect the plants from adverse weather conditions, and a ratio of two pollen plants to every seed plant is generally used. One of two procedures is generally used for pollen collection depending upon the equipment available and how each seed production organization perceives the way the collection should be handled.

One procedure is to pick flower buds just before anthesis. The anthers can be removed by hand or the back one-third of each bud can be cut off and the remaining section placed on a vibrating screen or pan which causes the anthers to separate from the corolla. The anthers are then dried until they dehisce at which time the pollen is sieved by hand onto a smooth surface and scraped into a storage container.

A second procedure is to use a small suction apparatus and vacuum the pollen from the open flowers on the plants before or after they have been picked. The result is generally pure pollen which can be used immediately or stored.

Petunia pollen can be stored for a short period of time in sealed containers at 4 °C. The pollen, after air drying can also be stored for a year or more at temperatures between −10 °C and −35 °C (Frankel and Galun 1977). Presently C. Hope (1982 pers. commun.) stores pollen up to 2½ years and has confirmed that the pollen has been viable for as long as five years.

Petunia pollen is often diluted by inert talcs for economic use and even distribution. The material used can be a distinct color so that the pollinators can see which flowers have been pollinated in order not to double pollinate and, thus,

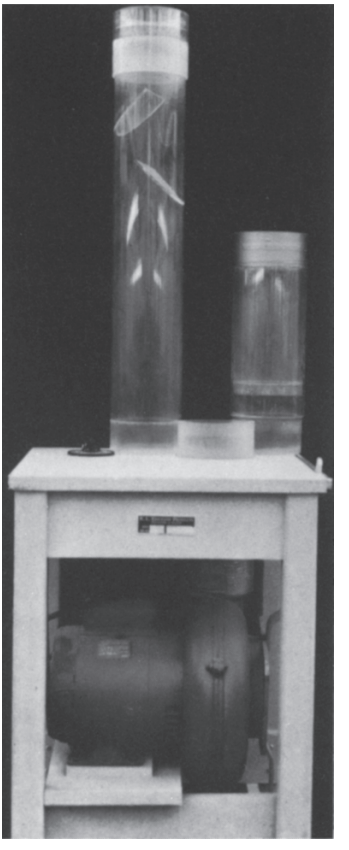


Fig. 15.15. An E.L. Erickson Products air column seed cleaner that can be used for cleaning out small-sized, lightweight, inert material from harvested *Petunia* seed. The air column has been disconnected for picture purposes

waste pollen. Diluent material should not stick to the pollen, or lump, but be similar in size and weight to the pollen grains, should not be hygroscopic and should be inert to the pollen and stigma (Frankel and Galun 1977). The dilution percentage must be determined for each case. Such a practice has often allowed the number of pollen plants to be reduced, thus, cutting production costs.

15.6.2.4 Pollination Procedures

Air humidity, temperature, pollen dilution, and amount of stigmatic secretion can be very critical for pollen germination, and stigma receptivity may be short or long in duration. This information must be determined for each hybrid in order to maximize seed set.

Many red *Petunia* plants will set little seed if pollinated immediately after emasculation, but will set abundantly a day later when the stigmatic secretion can be observed. The stigmatic secretion may also be minimal and in such cases, undiluted pollen is adviseable. Under these circumstances the writer prefers the emasculation process where a portion of the corolla is left undisturbed in order to guage more accurately when the stigmas will be most receptive.

Pollen is applied by hand using pipe cleaners, small artist brushes, and home made applicators made of small foam rubber sections on the tip of a wire.

15.6.2.5 Seed Harvesting, Cleaning, Testing, and Storage

Petunia seed pods will ripen on the plant generally 4 weeks after pollination under daytime temperatures of 20 °C to 29 °C. The ripe seed pods are harvested by hand and allowed to air dry further in a warm, dry area. Once fully dry, the pods split open and most of the seed will separate upon shaking. This can also be done on a proper seed-separator sieve. The seed can then be cleaned by an air column seed cleaner to get rid of small inert material and light-weight seed that is usually of questionable value (Fig. 15.15).

A germination sample is usually taken and the seed is put in proper storage under low humidity which helps the further drying of seed. Many *Petunia* hybrids will not give a good germination test until after 3 months of storage.

Petunia seed will maintain good viability for 3–5 years if stored at 18 °C at a relative humidity of 20%.

15.7 Future Considerations

Tissue culture could become very important in the production of parent plants that are difficult to maintain or which have special genetic characters for seed production that help lower the cost of production. There is no question that studies must be made to identify which resistances to disease can be found for natural incorporation into new cultivars. New color combinations should be possible after protoplast fusion processes are fully developed. Then, possibly, the colors of the *Salpaglossis* can be brought into the *Petunia*.

16 Seed Physiology

H. M. CATHEY[1]

16.1 Introduction

An artifical environment is needed to insure production of uniform, consistent, and disease-free *Petunia* seedlings. Considerable research experience with *Petunia* seed germination has revealed the basic requirements for germination and the number of days required to produce a seedling with a strong root system and well developed leaves. Furthermore, sowing and transplanting procedures have been developed to provide consistent results on a year-round basis. This chapter will be devoted to a review of the important role that temperature, light, gas exchange, and seed dormancy play in the physiology of *Petunia* seed germination.

16.2 Temperature

The seed of wild *Petunia* plants brought back for cultivation from tropical South America have variable optimum temperatures for both germination and subsequent seedling growth. Some will germinate over a wide range (8°–30 °C); others only when held within a narrow range (18°–22 °C). Most *Petunia* seeds in the native habitat are subjected to diurnal fluctuations of temperature – warm during the daytime and cool during the night. Thus, from a practical viewpoint, when constructing a seed environment facility regulatory equipment capable of providing fluctuating and constant temperatures should be included. Table 16.1 presents information on the optimum temperature during the germination process which may be considerably different than that at which the seedlings will be grown to maturity. In general, *Petunia* seed should be maintained at 20 °C until the seedlings fully expand. They can then be moved to another environment which permits their growth to be optimally controlled.

16.3 Light

The action spectrum of light as a controlling factor in the germination of light-sensitive seed indicates that the energy of radiation of different wave bands is

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Table 16.1. Effect of temperature on the percent germination of *Petunia* seeds held in the dark for 5 days

Cultivar	Temperature °C				
	10.0	15.6	21.1	26.7	32.2
Comet	62	82	91	88	67
Maytime	2	5	5	1	0

required for a 30% response (30 out of 100 seeds) (Toole 1961). Red light, for example, promotes germination of seeds of Grand Rapids lettuce over the range 360–700 nm with a maximum effect at 660 nm (Borthwick 1965). Far-red light inhibits germination by radiation in the region 700–780 nm with a maximum effect at 730 nm (Borthwick 1964). Potentially promoted seed by exposure to red light can be inhibited by exposing them to far-red radiation and promotion and inhibition are repeatedly reversible. This author found that this same system regulates the germination of *Petunia* seed.

The photoreceptive pigment was detected in plants in the 1960's by the use of a dual-beam spectrophotometer (Toole 1973). The pigment was named phytochrome. It occurs at very low concentrations in the plant cell cytoplasm. When partly purified, it is blue-green in coloration. The response of phytochrome to red and far-red light and to dark is depicted in the following diagram:

$$\text{Pr P-660} \xrightarrow{\text{Red}} \text{P-730 (Pfr)}$$

$$\xleftarrow[\text{Far red}]{}$$

$$\text{Inactive} \xleftarrow[\text{Dark}]{} \text{Active}$$

The $P\text{-}_{730}$ is the active form of phytochrome. Germination of *Petunia* seed occurs under the following situations:

- Active form reverts to inactive in dark controlled by temperature.
- Amount of Pfr required to promote germination varies with each cultivar of *Petunia*, age of seed, and environment.
- Amount of Pfr available to promote germination varies with each cultivar of *Petunia*, age of seed, and environment.
- Time of exposure following the seed imbibition of water and the duration of light are critical for completion of the processes involved to insure optimum germination.

Thus *Petunia* seeds can possess all degrees of response to light, from no detectable germination response, to germination only when exposed to light at a specific temperature for a specific number of min to days (Toole 1973).

Certain *Petunia* cultivars, such as Maytime, required light for germination (Table 16.2); others, such as Comet, germinated equally well in the light or dark (Fig. 16.1). The amount of light required to promote germination was very low; five foot-candles for only 10 min given 24 h after planting the seeds promoted

maximum seed germination of Maytime (Table 16.2). Fluorescent lamps are the standard artificial light source to promote seed germination of *Petunia*. Cool white lamps, high in red and blue, are most frequently used.

Fluorescent lamps made with phosfors other than cool white were also tested. None were found to be consistently better than cool white on the *Petunia* cultivars tested (Table 16.3). Cool White fluorescent lamps, placed 30 cm above the seed is a simple method to insure adequate red light for the light-requiring *Petunia* cultivars. Only certain *Petunia* cultivars exhibit a light requirement for germination (Cathey 1969a). In recent tests the majority of cultivars gave high germination percentages (greater than 90%, when exposed to light.

Table 16.2. Effect of duration of red light on the percent germination of Maytime *Petunia* held for 5 days at 21.1 °C. Seeds were held in the dark for 24 h followed by the red light exposure

Red light (footcandle)	Time of Exposure		
	1 min	2 min	10 min
0	3	2	1
1	12	24	51
5	24	48	96
10	80	93	94

Table 16.3. Effect of various light sources on percent seed germination of Comet and Maytime *Petunia* held for 5 days at 21.1 °C

Cultivar	Light condition				
	Dark	Fluorescent			
		Cool, White	Red	Blue	Far, Red
Comet	99	100	98	98	92
Maytime	2	95	92	98	64

Table 16.4. Effect of imbibition time on the R (red) – FR (far red) response on seed germination of Maytime *Petunia*. Seeds were held for 5 days at 21.1 °C

Imbibition (h)	Dark	R	FR	R+FR	FR+R
4	4	75	1	1	75
24	4	94	30	26	96
48	6	97	33	60	94
92	4	95	31	67	98
96	6	96	35	68	97

Table 16.5. Effect of imbibition time on the relative promotiveness of far red on germination % in Maytime *Petunia*. Seeds were held for 5 days at 21.1 °C

Imbibition time	Light treatments	Germination %
4 h	1 min red	59
	1′R+4′FR	2
	4′FR	2
16 h	1′R	83
	1′R+4′FR	6
	4′FR	9
24 h	1′R	80
	1′R+4′FR	37
	4′FR	39
40 h	1′R	92
	1′R+4′FR	59
	4′FR	63
48 h	1′R	88
	1′R+4′FR	42
	4′FR	51
64 h	1′R	89
	1′R+4′FR	60
	4′R	51
Dark	FR	21

Table 16.6. Effect of imbibition time on the relative promotiveness of low intensity far red on seed germination % in Maytime *Petunia*. (Far red was obtained from four 75W incandescent lamps and red and blue cellophane filter). Seeds were held for 5 days at 21.1 °C

Imbibition time	Far red irradiation	Germination %
0 h	2 h	2
	4 h	6
	8 h	5
	16 h	15
	24 h	23
24 h	2 h	21
	4 h	38
	8 h	37
	16 h	52
	24 h	55
48 h	2 h	40
	4 h	40
	8 h	52
	16 h	69
	24 h	64

The R (red) promotive effects on the germination of *Petunia* seed could be reversed by subsequent FR (far red) exposure (Table 16.4). Far red completely reversed the promotive effects if given to seeds which had imbibed for 4 h. Seeds imbibed up to 24 h exhibited extreme sensitivity to far red as only ca. 30% of the seed germinated (Table 16.5). However, the promotive effect of FR on the germination of seeds increased when the seeds were allowed to imbibe up to 48 h and were then exposed to 24 h of far red (Table 16.6). The photo-reversibility from F-FR was lost when the seeds became extremely photo-sensitive thus R-FR could be demonstrated only with seeds imbibed for 24 h or less. Longer imbibition times appeared to alter the R-FR system, thus permitting FR to become promotive.

16.4 Dormancy in Seeds

Freshly harvested *Petunia* seeds possess endogenous inhibitor(s) which block the germination process. Apparently, chemical changes which must occur to permit the mobilization of the factors involved in embryo elongation and water uptake of the emerging seedling or due to morphological changes which must occur to permit the maturation of the embryo and its subsequent development or finally,

Table 16.7. Effect of selected chemicals on the seed germination % in Comet and Maytime *Petunia* held for 5 days at 21.1 °C

Cultivar	Test solution			
	Water	KNO_3 (0.2%)	Thio-urea (0.2%)	GA (100 PPM)
Comet	92	90	90	93
Maytime	10	41	3	100

Table 16.8. Effect of gibberellic acid (ppm) on the germination % in *Petunia* seeds held in the dark for 5 days at 21.1 °C

Cultivar	PPM				
	0	0.1	1.0	10.0	100.0
Comet	91	92	94	93	94
Maytime	0	1	8	57	99

due to anatomical changes which must occur to permit gas and water exchange through impermeable membranes.

Relative to dormancy, the assumption is made that the seeds have been harvested without mechanical injury, cleaned, moisture content reduced, and the seeds have been held for several months in a cool (10 °C) and dry environment. Most *Petunia* seeds held at room temperature (18°–22 °C) for at least 3 months after ripening exhibit maximum potential to germinate. The recent development of vacuum packing of seed and storage in a cool temperature has greatly prolonged the viability of *Petunia* seed and, thus, the subsequent vigor of the seedlings. We can assume that the seed, once tested by the seed analyst and released by the seedsmen, should produce prompt germination and vigorous seedlings for at least two growing seasons if they are held dry and cool. Many new *Petunia* cultivars have been selected to minimize problems during the germination process. Significant developments have been made to insure that the new introductions will produce uniform and vigorous seedlings and thus are superior to the old breeding lines.

The dormancy of *Petunia* seed as related to the light requirement can be overcome by imbibing seeds in potassium nitrate or gibberellic acid solutions (Table 16.7). Seedlings which germinated in a 0.2% solution of KNO_3 exhibited marginal chlorosis on the cotyledons and damage to the emerging radicals. Thio-urea had no promotive effect on the germination. Seeds imbibed in gibberellic acid germinated immediately, uniformly, and in response to a dosage range (Table 16.8). Gibberellic acid at a concentration of 100 ppm promoted the germination of all seeds and also substituted for their light requirement.

17 Plant Physiology

H. M. CATHEY [1] and L. E. Campbell [2]

17.1 Introduction

Petunia has been the authors' model plant species for the past 25 years for studying the influences of environmental factors and their interactions with growth regulators and stress conditions on growth and flowering. *Petunia* plants possess many features which make them a desirable research model:

1. F_1 Hybrids – Modern breeding lines are uniform in form and type, from seed batch to batch.
2. Rapid Growth – We can accelerate the growth process(es) from seed germination to seed harvest within a 4–6 week span, thus facilitating a rapid turn over of experiments.
3. Measurable – The size of the parts of a *Petunia* plant facilitate easy measurement. Its leaves, stems, flowers, and roots are of such a size and sturdy form that they can be readily handled and measured.
4. Growth displays – Every stage of development offers extremely interesting and physiologically valid growth systems to study.
5. Model – The responses of *Petunia* plants have served as a model system for studying many kinds of physiological responses such as seed germination, seedling growth, flowering, and stress tolerances. Findings with *Petunia* plants have been transferred relatively easily to the understanding of the growth systems functioning in other bedding plants.
6. Economic Value – *Petunia* plants, regardless of the changes in fashion, design concepts, and stress problems, continue to be the most valued bedding plant.

The growth of *Petunia* plants falls into a logical set of interconnecting steps. Each stage can be studied over a relatively short period of time. The information from each stage, however, can be fitted into the overall pattern of growth and development. We will discuss each stage and environment and then present an overview.

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17.2 Fluorescent Lamps – Low Intensity

Since the 1940's fluorescent lamps have been the basic light source used in controlled-environment studies and indoor gardens. In their early experiments, Borthwick and Hendricks (1961) observed that some plants did not elongate, branch, or flower as they did when grown under sunlight. As with the sunshine carbon arc, desired growth characteristics were observed with incandescent-filament lamps supplemented with cool white fluorescent lamps. Although it provided only about 10% to 20% of the visible radiant energy in the chamber, the incandescent addition created an environment in which stem elongation, leaf color, branching, and flowering were typical for the species. Incandescent lamps also nearly doubled at least the heat and infrared radiation, which made it difficult to maintain uniform temperatures in the chambers (Campbell et al. 1975).

To avoid the heat and short life of incandescent lamps, lamp manufacturers tried to develop alternative fluorescent lamps with radiation for the leaf absorption spectra peaking in the blue (B) (430 nm), red (R) (660 nm), and far-red (FR) (730 nm) wavelength regions. The first lamps were intensely purple with little output in the yellow-green (500–600 nm). They provided B and R but little FR radiation. We now have a wide range of special-use fluorescent lamps, but their R-FR output in relation to that in INC lamps is low.

Fluorescent lamps are used for display and maintenance of plants. They also provide a method for growing *Petunia* seedlings for subsequent growth in greenhouses, home, or garden. Lighting is emphasized to produce dark green plants that retain a compact growth habit for many months (Cathey et al. 1978).

17.2.1 Comparative Effects of Seven Fluorescent Light Sources

Acceptable *Petunia* plants were grown under all seven kinds of fluorescent lamps in all cases (Table 17.1). Differences between lamps could be explained in terms of the environmental requirements of the particular plants. *Petunia* seedlings were

Table 17.1. Mean fr. wt. height (or leaf length), and node no. of plants grown under Cool-White (CW), Warm-White (WW), Gro-Lux (GL), Agro-Lux Wide-Spectrum (GL-WS), Agro-lite (AGRO), Experimental Lamp (EXP), and Vita-Life (VITA) fluorescent lamps for 16 h daily at 20 °C

Species and factor	CW	WW	GL	GL-WS	AGRO	EXP	VITA
Petunia × *hybrida* Hort. Vilm.-Andr. "Pink Cascade" (petunia)							
Fr. wt. (g)	10.0b[a]	10.8b	10.2b	10.4b	10.4b	8.9a	10.4b
Fr. wt. relative to CW	100	108	84	102	104	89	104
Leaf length (cm)	10.0a	10.0a	8.9a	13.5ab	10.2a	16.5b	11.0a
Leaf length relative to CW	100	100	89	135	102	165	110
Node no.	12.2	12.2	11.6	12.4	10.4	12.2	12.1 N.S.
Node no. relative to CW	100	100	95	102	85	100	99

[a] Mean separation, within rows, by Duncan's multiple range test, 5% level

grown under less than optimum light. GL and EXP lamps were generally the least effective light sources in terms of fr. wt. CW, WW, GL-WS, AGRO, and VITA were equally effective as judged from fr. wt. of plants. The EXP lamp, which was higher in FR than the other lamps, promoted greater stem elongation. GL, which was high in R but lower in FR than other lamps, provided the least stem elongation. At the end of the experiment, the *Petunia* plants were in a rosette stage of growth and did not exhibit maximum stem elongation. The number of nodes formed on the plants under the seven kinds of lamps remained the same.

17.2.2 Comparative Effects of Cool-White Fluorescent and Incandescent Lamps Applied at Different Durations

Plants survived and grew under all light treatments in all cases (Table 17.2). Reduction of daylength from 12 to 6 h significantly reduced fr. wt. With 12-h periods of intermittent lighting (2 min or 60 min light/dark periods), the 2 min periods were more effective in promoting growth than were the 60 min periods, although not always significantly. Simultaneous addition of INC to CW fluorescent for 12 h increased fr. wt. of plants. Again, plants grown under 6 h of CW + INC light weighed less than those grown under 12 h of CW + INC daily. *Petunia* plants exposed to the 2-min cycles of CW t o INC tended to weigh more than those grown with 60 min cycles.

These results support the general belief that the efficiency of the lamp in converting power to visible radiation is the most important factor in selecting artificial lights for *Petunia* plant growth. Light quality or spectral distribution is a second order of importance (Cathey and Campbell 1975). Rapid growth and prompt flowering of *Petunia* did not occur solely through use of any one of the fluorescent lamps tested. None emitted levels of R and FR equal to that from INC or the sun, which would have brought any photoperiodically-controlled growth system into maximum effectiveness. Fluorescent lamps that had the highest levels of FR (AGRO, GL-WS EXP) still had much less than that from INC lamps or

Table 17.2. Mean fr. wt. and flowering time of plants grown under cool-white (CW) fluorescent lamps, with and without incandescent (INC) lamps for 6 and 12 h daily or 1 or 30 min on and off for 12 h daily at 20 °C

Species and factor	CW 6 h	CW 12 h	CW 1 min cycle 12 h	CW 30 min cycle 12 h	CW + INC 6 h	CW + INC 12 h	CW + INC 1 min cycle 12 h	CW + INC 30 min cycle 12 h
Petunia × *hybrida*								
Fr. wt. (g)	5.7[a]	18.1	7.1	6.4	10.2	37.1	12.1	9.4
Fr. wt. relative to CW-12 h	31a	100c	39a	35a	56ab	205d	67b	52ab

[a] Mean separation, within rows, by Duncan's multiple range test, 5% level

the sun. Amounts of UV from the fluorescent lamps did not approach the amount of UV radiation from the sun.

Development of foliage color of *Petunia* plants followed a trend opposite that observed on stem elongation. Thus, a lamp that promoted stem lengthening invariably created a growth system in which the foliage color tended toward pale green. Amount, frequency, and combinations of nutrients did not overcome the pale color of the foliage. This aspect of lighting is often confused by research workers. Foliage color, created by the lighting, was not an indication of the effectiveness of a light source. In fact, there is no ideal artificial light for plant growth. When plants were grown under artificial light, we observed all three major (photosynthesis, photochrome, and high energy reactions) functioning simultaneously to control the rate of leaf formation, internode lengthening, weight accumulation, and flowering.

The use of 2 or 60 min light-dark periods over 12-h days did not appear to favor rapid growth and early flowering as much as the addition of INC lamps. The damaging effects of light-dark cycles, as reported by Allard and Garner were not evident. The 12 h daily dark period permitted reversion of the active form of phytochrome and the maintenance of green leaves and short internodes. Cutting the total lapse of time of lighting the 6 h, but giving it in light-dark periods of 2 and 60 min, did not increase the effectiveness of lighting that was given over 12 h daily. These results suggest that the same amount of energy given over 6 or 12 h promotes equal growth responses. The addition of INC, which produces nearly equal R (660 nm) and FR (730 nm) radiation, showed that total energy and the presence of FR were essential for rapid growth.

We tried to achieve photoperiodic effects while testing the possibility of reducing energy consumption. It was not expected that any fluorescent lamp alone could achieve the growth responses observed with CW + INC combination. When using artificial light sources it should be understood that most "white"-appearing light sources may be used for growing *Petunia* plants. Interconversion from one lamp to another can be achieved with nearly equal effectiveness.

17.3 Photoperiod, Kind of Light, Temperature

The effects of (a) photoperiod, (b) kind of light (incandescent or fluorescent) used to extend the photoperiod, and (c) temperature on growth and flowering of *Petunia* plants were studied during 1958 and 1959 at Beltsville, Maryland (Piringer and Cathey 1960).

17.3.1 Effects of Photoperiod

Young *Petunia* seedlings were very responsive to daylength during the first month. The seedlings on 8-h days were less than 1.5 cm tall with six to eight leaves fully expanded, while those on 16-h days had the same number of expanded leaves but were much taller.

Ballerina seedlings from the 16-h photoperiod were grown for an additional 65 days on various other photoperiods (Table 17.3). Main-stem length increased as the photoperiod was longer. More nodes and shorter internodes occurred on plants on the short photoperiods, and differences in main-stem length were due primarily to differences in internode length.

The longer the photoperiod the earlier the plants flowered, both chronologically and morphologically. After 65 days of photoperiod treatment flower buds were microscopic on 8-h, macroscopic on 9- and 10-h, and open on 12-h and longer photoperiods. The longer the photoperiod, the lower the node on the main stem at which the first flowers appeared. More flowers per main stem formed in a given time on the long photoperiods because of earlier flowering.

Plants on photoperiods of 12 h or longer were mainly single-stemmed, while plants on photoperiods of 10 h or less were highly branched at the base. Each branch had flower buds, which developed to anthesis before flower buds on the main stem. The stem lengths, node numbers, and node at which first flowers occurred on branch stems were influenced by photoperiod the same as those on the main stem. On 8-h photoperiods the basal branches were well developed within 6 weeks on Ballerina *Petunia* and within 8 weeks on the other varieties. The times of flowering of the early and late varieties were profoundly affected by photoperiod. Each variety retained it characteristic blooming time on the 8-h photoperiod. However, on the 16-h photoperiod with incandescent light, plants of both varieties flowered early and within the same week.

Fasciation of the main stem of the variety Dwarf Pink Glory was induced during 8 weeks on 8-h photoperiods. The fasciation occurred on approx. 50% of the plants in a given experiment. In plants moved to the field in mid-May, the fasciated main stems became about 1 cm thick and 16 cm broad. Basal branches arising from the fasciated stems were normal in all respects.

17.3.2 Effects of Photoperiod and Kind of Light

The effect of kind of light used to extend the photoperiod was studied for Ballerina (Table 17.3) and for the early and late varieties. Incandescent and fluorescent lamps were used for 8 h following natural day light to provide 16-h photoperiods. Stem lengths of Ballerina plants on incandescent light were longer than those of plants on fluorescent light because the internodes were significantly longer. Plants of the three varieties studied flowered early and on single stems on the 16-h photoperiods regardless of light source. However, on the incandescent light Ballerina flowered about 2 weeks earlier (Table 17.3) and the late variety 3 weeks earlier than they did on the fluorescent light. The kind of light used on the 16-h photoperiods caused no significant difference in the times of flowering to the early variety.

17.3.3 Effect of Photoperiod and Temperature

Plants on 9-h photoperiods at all night temperatures had typical short main stems due to short internodes and basal branching characteristics of plants on short

Table 17.3. Growth and flowering of main stems of *Petunia hybrida* var. Ballerina[a] during 65 days[b] in the greenhouse on various photoperiods and two kinds of supplemental light on the longest photoperiod

Photo-period	Supplemental light		Stem						
	Duration	Kind	Length	Nodes	Mean internode length	Period to first anthesis	Nodes to first flower	Total flowers per plant	Total basal branches per plant
Hours	Hours		cm	Number	cm	Days	Number	Number	Number
8	0	None	6.1A	39.7A	0.16A	>65. A	36.8A[c]	0. A	2.4A
9	1	Incandescent	9.0A	36.7B	0.25A	>65. A	34.1B[d]	0. A	4.1A
10	2	Incandescent	13.3B	32.9C	0.41B	>65. A	29.3C	0. A	4.4A
12	4	Incandescent	26.8D	27.0D	0.99D	55.7D	20.5D	3.8B	0. B
14	6	Incandescent	31.1E	26.5D	1.12E	49.9C	18.8DE	5.7C	0. B
16	8	Incandescent	44.8F	27.2D	1.63F	41.2D	18.2E	7.5 D	0. B
16	8	Fluorescent	18.8C	27.5D	0.69C	56.6B	22.4D	3.3B	0. B

[a] The plants per treatment. Different upper case letters after means indicate that they are significantly different at the 1% level, whereas the same letter indicates that they are not different

[b] December 15 to February 18

[c] Microscope flower buds

[d] Macroscope flower buds

Table 17.4. Growth of the *Petunia hybrida* var. Ballerina[a] after 50 days in the greenhouse[b] on 9 h days[c] and four different night temperatures[d]

Night temperature	Main stem				Basal branches[e]		
	Length	Nodes	Mean internode length	Number	Length	Nodes	Mean internode length
°F	cm	Number	cm		cm	Number	cm
50	6.0b	22.4B	0.27b	6.8a	11.1c	15.1c	0.73bc
60	5.4b	26.2A	0.21b	7.2a	13.8b	22.1a	0.62c
70	12.0a	29.0A	0.44a	6.8a	18.0a	19.6b	0.85ab
80	9.6a	27.4A	0.35ab	8.2a	19.4a	22.8a	0.93a

[a] Five plants per treatment. Different upper-case letter after means in any one column indicate that means are significantly different at the 1% level; and different lower case letter indicate that the means are significantly different at the 5% level. The same letter indicates no statistical difference
[b] September 10 to October 30
[c] Eight h of natural light supplemented with 1 hour of low-intensity incandescent light
[d] Night temperatures were given to the plants from 4 p.m. to 8 a.m. daily
[e] Means represent the total number of branches for the five plants in each treatment

days, but developed shorter internodes at 10 °C and 15.6 °C than at other night temperatures (Table 17.4). The number of basal branches formed was not affected by night temperature, but longer branch stems developed as the night temperatures were higher (Post and Weddle 1940). Plants on 16-h photoperiods were essentially single-stemmed at night temperatures above 15.6 °C (Table 17.5). At 15.6 °C and 21.1 °C they had some basal branches, but the number of branches was less than one-half the potential number of 9-h photoperiods (Table 17.4 and 17.5).

Flower buds did not occur on the main stems of plants during 50 days on 9-h photoperiods. Flowering on the 16-h photoperiod was significantly earlier at 26.7 °C than at 21.1 °C, and flowering at both these temperatures was significantly earlier than that at 15.6 °C and 10 °C, where flowers were apparent on plants only by microscope dissection. When Ballerina, Cotton Candy, Comanche, and Dwarf Pink Glory *Petunia* were grown at minimum daily temperatures of 15.6 °C and 21.1 °C on 8-h and natural days from mid-March to mid-May, plants of all varieties had the typical short main stems on 8-h photoperiods and longer stems on natural days (11.7–14.2 h). For each photoperiod, stems of all varieties were longer at 21.1 °C than at 16.6 °C. All varieties flowered within a week on a given photoperiod and temperature. Plants that were on 8-h photoperiods for 83 days before transfer to the field flowered in about 100 days from seeding regardless of temperature. On natural days, plants flowered in about 70 days at 21.1 °C and in 75 days at 15.6 °C.

All varieties had the same basal-branching response to photoperiod and temperature. Plants developed the fewest branches on natural days at 21.1 °C. No difference in the number of basal branches occurred between plants of a given variety on natural days at 15.6 °C and on 8-h photoperiod at 15.6 °C. The response of Ballerina in this experiment agrees in general with its responses in the

Table 17.5. Growth and flowering of main stems of *Petunia hybrida* var. Ballerina [a] during 50 days in the greenhouse [b] on 16 h days [c] and four different night temperatures [d]

Night temperature		Main stem				
		Length	Nodes	Mean internode length	Basal branches	Days to beginning anthesis
°F	°C	cm	Number	cm	Number	Number
50	10.0	12.0D	27.0C	0.44d	3.0A	–c [e]
60	15.6	21.2C	27.6C	0.79c	1.0B	–c [e]
70	21.1	28.8B	31.0B	1.04b	0.0C	48.0b
80	26.7	41.0A	34.6A	1.32a	0.8B	42.8a

[a] Five plants per treatment. Different upper-case letter after means in any one column indicate that means are significantly different at the 1% level; and different lower case letter indicate that the means are significantly different at the 5% level. The same letter indicates no statistical difference
[b] December 15 to February 3
[c] Eight hours of natural light plus 8 h of low intensity incandescent light
[d] Night temperatures were given the plants from 4 p.m. to 8 a.m. daily
[e] Plants had macroscopic flower buds

previous experiments in that the longer the daylength, the fewer the basal branches. The number of basal branches was further reduced by high temperatures on a given daylength (Roberts and Struckmeyer 1938 a, b).

17.3.4 Effects of Previous Photoperiod on Growth in Field

In one experiment plants of Ballerina, Cotton Candy, Comanche, and Dwarf Pink Glory were given 8-h and natural photoperiods and 15.6 °C and 21.1 °C daily minimum temperatures on each photoperiod. In mid-May, after 9 weeks of treatment, a total of 12 weeks from seed sowing, the plants were moved to the field for further observation. Natural daylengths in mid-May were longer than 14 h. All plants flowered by early June. By mid-June, plants of all varieties that had been on natural days at either temperature were tall and the flowering main stems had toppled.

Within the same period, plants of all varieties that had been on 8-h photoperiods had short erect flowering stems that were somewhat taller at 21.1 °C than at 15.6 °C. However, plants of all varieties from the 8-h photoperiod at 15.6 °C were diatropic. When the diatropic plants were set into the field, the growing points of the branches grew upwards almost immediately and flowered before the main stem.

17.4 Sole Light Source in Growth Chambers

The major theories of how light regulates the growth and flowering of plants have not changed in emphasis since the landmark paper by Garner and Allard (1920).

Table 17.6. Comparative plant growth in response to exposure to fluorescent (CWF), high-pressure sodium (HPS), and low-pressure sodium (LPS), lamps with and without supplemental incandescent (INC) lamps, light sources are given in Table 17.4 and 17.5

Light source	15 days[a] *Petunia* Pink cascade		
	Main stem height % cm	Nodes % no.	Fr. w. of tops % g
CWF	100a = 4.2	100a = 16.5	100a = 36.7
CWF + INC	85a	97a	111ab
LPS	100a	98a	100a
LPS + INC	112a	115a	153b
HPS	100a	101a	112ab
HPS + INC	112a	104a	120ab

[a] Columns within groups followed by identical letters are not significantly different at the 5% level. Data normalized to 100% for CWF value as controls

It has been found that through proper use of light many plants can be timed or regulated to flower or fruit at any time of the year. To use these light responses we need to understand the physiological changes that are caused in plants by natural day length and how we can supplement, override, or substitute the reaction controlling these changes. The following experiments were designed to determine the responses of *Petunia* to controlled light with respect to level, duration, and spectral content in a manner that identified the contribution of each factor.

Despite all of the research on light sources, we still have only meager information on the radiation requirements of most plants. For maximum efficiency of light utilization data are needed on: the minimum intensity and duration of light for survival (the compensation point); the minimum intensity and duration of light for control of flowering; the relative effects of dim and intense light on growth; and the optimum ratios of the various wavelength intervals in the visible and infrared regions.

The comparative growth of petunia in response to CWF, LPS, and HPS is presented in Table 17.6. As a sole light source CWF, LPS, and HPS were equally effective in promoting growth of *Petunia*. The simultaneous addition of INC light to LPS helped to increase the weight of the *Petunia* plants without altering the node number of plant height. The plants grown under LPS and INC lamps flowered as soon as those grown under CWF and HPS lamps; the stems, however, were shorter, the number of basal shoots were increased, and the leaves were greener and thicker than they were with the other two lamps. These observations were reflected in the significantly increased fresh weight of the plants.

Based on these results, we can conclude that LPS can be used as a suitable light source to start and grow *Petunia* plants in a controlled environment. Its effectiveness is b..sed on the total amount of light in the 400–850 nm region and suggests

that Action Spectrum II for intense light dominates the photoreaction that controls growth under these conditions. In an environment of LPS alone, the plants were compact with intensely green leaves.

17.5 Light Source Interaction with Iron Nutrition

Brown et al. (1979) determined that Fe^3 in ferrozine [disodium salt of 3-(2-pyridyl)-5,6-bis (4-phenylsulfonic acid-1,2,4-triazine)] solutions was reduced to Fe^{2+} under CWF + INC but not under LPS + INC lamps. The ferric-ferrozine solution was used as an assay for the active (Fe^3 to Fe^2) wavelengths in the radiation produced by various lamp combinations. Our objectives were to determine: (a) the wavelengths that cause reduction of Fe^{3+} to Fe^{2+}; (b) the amount of chlorophyll produced in leaves; (c) the mineral element concentrations; and (d) the growth yield of *Petunia* under LPS and CWF lamps with and without INC lamps. *Petunia* plants were green under both LPS and CWF lamps. The effect of temperature (22 °C and 28 °C) on the above-mentioned factors was determined in environmental control cabinets. Their hypothesis was that Fe^{3+} reduction to Fe^{2+} in leaves by CWF lamps makes Fe^{2+} more available for plant use and enhances the biochemical reactions essential to chlorophyll formation. Fe^{3+} is transported to plant tops as Fe^{3+} citrate. The Fe concentration could be the same under LPS and CWF lamps, but available Fe could differ dependent on reduction of Fe^{3+} to Fe^{2+}. Ferrous iron is more available for plant use that Fe^{3+}.

17.5.1 Reduction of Fe^{3+} to Fe^{2+} in Solution

Reduction of Fe^{3+} to Fe^{2+} was similar in ferrozine-$FeCl_3$ solutions exposed to FS 20, BLB, and Philips' BL lamps. Reduction in a beaker covered with a CS 7-54 filter was about 0.7 that in the open beakers exposed to the FS 20 lamps. In beakers covered with filter CS 5-60 the reduction was about 0.3 that in the open beakers exposed to BL lamps.

Although very little Fe^{3+} was reduced to Fe^{2+} when the solutions were exposed to the no UV blue lamps, still less was reduced when filters CS 5-60 or CS 3-71 were placed over the ferrozine-$FeCl_3$ solutions. During the 3-h exposure of the ferrozine-$FeCl_3$ solution to light, the CWF + INC reduced nearly 20 times more Fe^{3+} to Fe^{2+} than the LPS + INC light. Reduction did not occur in the covered solutions.

In the environmental control cabinets, CWF or CWF + INC light reduced significantly more Fe^{3+} to Fe^{2+} than did LPS or LPS + INC light (Table 17.7). Generally more Fe^{3+} was reduced to Fe^{2+} at 28 °C than at 22 °C and also with the addition of INC light. The latter adds some UV and blue radiation.

17.5.2 Growth of Plants

Experiment 1. In the growth rooms, *Petunia* plants were green and had about the same chlorophyll concentrations under both light sources (LPS + INC; CWF +

Table 17.7. Fe^{3+} reduced to Fe^{2+} (Fe^{3+} ferrozine) in ferrozine $FeCl_3$ solutions after 4 h and chlorophyll concentrations of *Petunia* as affected by LPS, LPS + Inc, and CWF + Inc light at 22 °C and 28 °C

Cultivar and treatments	Temperature C	Fe^{2+} ferrozine μM 4.0 h	mg/g frech wt.	Chlorophyll Chl.a/Chl.b Petunia	28C/22C
LPS	22		0.90ab[a]	2.0	1.1
LPS + Inc	22		0.84b	2.0	1.0
CWF	22		1.04ab	2.1	1.1
CWF + Inc	22		0.83b	2.0	1.1
LPS	28		1.00ab	1.8	–
LPS + Inc	28		0.84b	2.0	–
CWF	28		1.12a	2.0	–
CWF + Inc	28		0.90ab	2.3	–

[a] For each plant within each column, values followed by the same letter are not significantly different at the 1% level, according to Duncan's multiple range test

INC). *Petunia* yields (g dry wt.) were about the same under the two light sources. The *Petunia* plant had about the same nutrient element concentrations except for K (%), Ca (%), and Zn (μg/g) as follows: these were about 7.2, 1.0, and 62. As for the other nutrient elements, P was about 0.50%, Mg 0.50%, Mn 70 μg/g, Fe 110 μg/g, B 50 μg/g, and Cu 5 μg/g.

Experiment 2. *Petunia* plants were green and had about the same chlorophyll concentrations under both types of lamps. For *Petunia*, the chlorophyll a/chlorophyll b ratio and chlorophyll 28 C/22 C ratio were about the same under all light sources (Table 17.7). The Cu concentration in *Petunia* was not affected. No consistent differences in Fe concentrations were noted.

17.6 Supplemental Lighting in Greenhouses

The growth of *Petunia* (Table 17.8) as judged by fr. wt. after 5, 10, 15, and 20 days showed that the natural light in a greenhouse during winter was inferior to that in a growth chamber for growing seedlings. Extending the daylength to 16 h with low-intensity INC lamps (216 lx) did not provide a substantial increase in the growth of the plants, even though the petunias flowered much earlier than those grown on 8-h and natural short days. Supplementing the natural light with 16 h of LPS or HPS provided growing conditions for the plants that were equal to the one in the growth chamber (Cathey and Campbell 1977).

The differences in sensitivity to light sources of *Petunia* plants became apparent early in their development. The traditional LD treatment was 8.9 h–12 h natural light plus 0.216 klx of INC from 2000 to 0400. Data from these plants were designated as the 100% response or baseline. This photo-environment produces LD effects on most plants when growing time is sufficient but the experiments reported here generally did not last long enough to provide such photoperiodic effects (early flowering, delayed flowering, vegetative growth, or dormancy). The

Table 17.8. Comparative fr. wt. of *Petunia* seedlings grown 5 to 20 days under exposure to natural daylight (ND), fluorescent (CWF), low-pressure sodium (LPS), high-pressure sodium (HPS), and incandescent light (INC)

Environment	Fresh weight to tops at harvest[a]			
	5 days % g	10 days % g	15 days % g	20 days % g
Growth Chamber				
CWF + INC 16 h	100c = 3.7	100c = 13.7	100b = 40.7	100b = 64.7
Greenhouse:				
ND (8 h)	57a	43a	28a	34a
ND (9.7–11.0 h)	57a	70b	56a	63a
ND + 16 h INC (216 lx)	77b	56a	54a	57a
ND + 16 h LPS (22 klx)	91c	96c	92b	88b
ND + 16 HPS (17 klx)	91c	96c	94b	83b

[a] Columns within groups followed by identical letters are not significantly different at the 5% level. Data normalized to 100% for CWF + INC (growth chamber) as controls

growth responses (height, weight, or flowering) to supplementary lighting with high- and low-pressure sodium lamps were apparent from the beginning of the tests and occurred at rates that previously have been associated only with controlled-environment studies. The lighting supplemental to the natural days of winter provided an environment in which height or weight increases were much faster than rates previously observed for *Petunia* under conventional greenhouse conditions (Carpenter 1974; Cathey and Campbell 1974, 1979).

17.6.1 HPS and LPS with Equal Energies

Petunia plants exhibited equal growth responses to supplemental lighting (HPS, LPS) of 42 W/m^2 (400–700 nm) from 0800 h to 2400 h (Tables 17.9 and 17.10, treatments 4 and 5). The responses to HPS and LPS compared in various combinations are presented in the following sections (Cathey and Campbell 1979). LPS was more effective than HPS on the promotion of early flowering *Petunia*.

17.6.2 LPS with Energy Halved

Petunia plants exhibited a significantly greater growth response to 42 (400–700 nm) than to 21 W/m^2 LPS from 0800 h to 2400 h.

17.6.3 LPS with Equal Energies During Day or Night

Growth of the *Petunia* plants was the same with supplemental LPS during the day (0800 h–1600 h) or during the night (2000 h–0400 h) at an intensity of 42 W/m^2 (400–700 nm).

Table 17.9. Supplementary lighting treatments used

Treatment	Daylength + type of radiation[a]	Duration		Illuminance (klux)	Irradiance (W m^{-2})	
		H	Time		400–700 nm	700–850 n
1.	SD	8	0800–1600	–	–	–
2.	ND	–	–	–	–	–
3.	ND+INC	8	2000–0400	0.216	0.9	1.0
4.	ND+HPS	16	0800–2400	17	42	15.5
5.	ND+LPS	16	0800–2400	22	42	6.0
6.	ND+LPS	8	0800–1600	22	42	6.0
7.	ND+LPS	8	2000–0400	22	42	6.0
8.	ND+LPS	16	0800–2400	11	21	3.0

[a] Measured 0.25 m above bench SD = Short Day (8 h); ND = Natural Day; INC = Incandescent; HPS = High-Pressure Sodium; LPS = Low-Pressure Sodium

Table 17.10. Effects of supplementary lighting treatments on growth responses of *Petunia* plants

Species Variable	Treatment							
	1	2	3	4	5	6	7	8
Petunia × *hybrida* Vilm. – **LD-F**								
Petunia "Pink Cascade"								
Weight (g)	24.0	40.6	44.7	62.3	77.8	65.7	70.0	68.5
(%)	54a	90b	100b	139c	174d	147c	136c	153c
Height (cm)	4.3	15.0	20.6	8.5	7.3	15.8	18.0	7.3
(%)	21a	73b	100c	41a	35a	77b	87b	35a
Node (no.)	21.8	24.5	24.3	23.3	25.0	24.5	24.5	25.3
(%)	90	101	100	96	103	101	101	104 N.S.

17.6.4 LPS with Equal Energies for 16 h (Day and Night) and 8 h (Night)

Petunia plants exhibited equal growth responses (height or weight increase, early flowering) to 16 h of 21 W/m^2 LPS (0800 h–2400 h) and 8 h of 42 W/m^2 LPS (2000 h–0400 h).

With this background, we concluded that HPS and LPS, when given as a supplemental light treatment in the greenhouse from morning (0800 h) to midnight (2400 h), were equally effective in initiating photosynthetic activity. Next, we compared the lamps at 42 W/m^2 in the 400–700 nm region. HPS emitted about three times more energy in far-red (700–850 nm) than LPS (Table 17.9). HPS and LPS equally accelerated increased fresh weight, height, and early flowering which were not correlated with radiation in the far-red region. The number of nodes initiated on the plants grown with the supplemental lighting, however, was not as large as the number of nodes reported (Krizek et al. 1966, 1972) in seedlings started in controlled environments. This showed that the HPS and LPS lighting

were increasing the net photosynthesis (growth) of each leaf and stem on the plants rather than reducing the time required for leaf initiation. Thus, the natural light conditions, even though night interruptions with INC were given to create a LD photoperiodic effect, were inadequate for the plants to achieve their optimum rates of photosynthesis. The supplemental lighting treatments with HPS and LPS on some annual and tropical foliage plants increased the photosynthetic activity of the leaves and significantly increased fresh weights and heights, and accelerated flowering (Cathey and Campbell 1980).

This dependency on supplement lighting for achievement of rapid growth was further supported by the LPS treatments involving 21 W/m^2 for 16 h and 42 W/m^2 for 8 h during the day (0800 h–1600 h) and the night (2000 h–0400 h). *Petunia* plants grew significantly better (fr. wt., height, early flowering) than the long day INC controls. *Petunia* did not, however, exhibit a greater response to day than to night supplemental lighting. The plants grew equally well when lighted 16 h daily (0800 h–2400 h) with 21 W/m^2 or 8 h daily (2000 h–0400 h) with 42 W/m^2.

Effectiveness, as measured by vegetative growth or flowering time, of the supplemental lighting in the greenhouse was generally unrelated to the observed photoperiodic responses of the plants. We observed that supplemental light with HPS and LPS (day or night) equally accelerated growth of LD plants (Cathey and Campbell 1981).

17.7 Chemicals

The kinds of chemicals tested, their analogs, formulations, and carriers, methods of application, and interactions with the environment involve an infinite number of experimental combinations (Cathey 1970). From these trials have come major commercial uses for growth control of *Petunia* plants (Cathey 1969 a).

Chlorphonium chloride.
The interaction between tributyl (2,4-dichloro benzyl) phosphonium chloride (called Phosfon-D) and the environment on growth of *Petunia* was reported by Cathey and Piringer (1961). Since chlorphonium chloride destroyed chlorophyll in the leaves, its application was limited to a preplanting mix or a soil drench. Further, the rates were a quarter to a half ton of the chemical per acre, and it persisted in the soil to affect subsequent crops. Thus, the potential use of chlorphonium chloride was limited to use on potted plants.

Chlormequat.
Cycocel (2-chloroethyl) trimethylammonium chloride (called Cycocel or CCC) was effective on 44 of the 55 species tested by Cathey and Stuart (1961), but even massive dosages had no effect on *Petunia*.

Ancymidol.
2-cyclopropyl-2 (4-methoxyphenyl)-5-pyridinemethanol (called AREST), applied as a foliar spray or a soil drench was extremely broad in activity on plants (Cathey 1975). It was inactive, however, on *Petunia*.

Daminozide.
Butanediotic acid mono (2,2 dimethyl hydrazide) (called B-Nine) was reported by Riddell et al. (1962), as an effective growth retardant for application to *Petunia* foliage (Cathey 1969 b). Effective use of B-Nine depended on time of application, dosage, frequency of application, relation to night temperature, and relation to photoperiod (Cathey 1972). These points will be discussed in order.

17.7.1 Effective Time

The most effective time to treat a Comanche Improved *Petunia* plant was at the time the stem was beginning to elongate rapidly (Table 17.11). Comanche Improved plants reached this stage 42 days after planting the seeds at 15.7°–18.1 °C night temperature. The effects of daminozide applied earlier than this stage of growth did not persist until the plants were in flower. Treatments after the flower buds were visible were much less effective than treatments when growth was rapid. Treatments just after transplanting had little effect on the plants. As a result of retarded elongation of the main axis of the plant, the basal branches developed and soon formed flower buds.

The dosage to obtain nearly maximum response of Comanche Improved *Petunia* to daminozide was 0.5% for one foliar application 42 days after planting the seeds. Overtreatment with daminozide produced plants that were only slightly shorter than ones sprayed with 0.5%, and the growth retardation on all plants treated persisted 4–6 weeks. No carry-over to other shoots produced on the plants was observed, and often treated plants eventually grew taller and weighed more than untreated ones of the same age and growing conditions.

Applications of daminozide every 4–6 weeks were necessary to keep the plants under control of the growth retardant. Dividing the dosage of daminozide into two equal parts and applying the chemical several days apart were no more effec-

Table 17.11. Effect on stem length of Comanche Improved and Pink Cascade *Petunia* of a 0.5% daminozide foliar spray of applied various days after the planting of seeds. Plants grown on natural days with incandescent light at 20 foot-candles from 10 p.m. to 2 a.m. Night temperature, 18.7 °C

Time of application of 0.5% daminozide spray (days after planting)	Stage of growth of Comanche Improved	Height	
		Comanche Improved (in.)	Pink Cascade (in.)
27	Recently transplanted	20.7	17.4
32	Leaves touch rim of pot	19.6	15.7
37	Stem starts to elongate	15.9	14.3
42	Elongate stem visible	13.4	12.8
47	Stem elongating rapidly	12.2	12.6
52	Flower buds just visible	17.6	15.8
57	Flower buds large in size	20.9	16.4
Untreated plant		22.0	16.6

tive than 1-spray treatment at the time the plants were most responsive to the growth retardant. Applications of daminozide and additional artificial lighting of plants being held at temperatures lower than 15.7 °C had little or no effect on subsequent growth. Several weekly applications were required for an effect at temperatures less than 15.7 °C. For daminozide to be effective, the plants must be actively elongating before treatment. As a safeguard, the foliage of treated plants was protected from splashing water. Daminozide penetrated the leaves of petunia slowly and could be easily washed from the leaves during the first 24 h.

17.7.2 Side Benefits

Plants treated with daminozide exhibited several side benefits that greatly enchanced their usefulness. The treated plants were much less susceptible to water stress. After a period of cloudy weather, followed by a bright day, the treated plants did not wilt but continued to grow. Untreated plants wilted and were easily overwatered. The amount of water lost from a properly treated plant was less than from an untreated one. This suggested that the plant would wilt later and require less frequent watering than untreated plants. The treated plants were much less susceptible to air pollutants. Foliage was often damaged on plants grown in air-washed pan- and fan-cooled houses. The leaves on untreated plants collapsed when exposed to smog and never recoverded. The treated plants grew without apparent injury. Some cultivars, particularly the white-flowered ones, were extremely susceptible to smog.

17.7.3 Daminozide and Photoperiod

Seeds of Comanche Improved *Petunia* were planted February 25 to determine how photoperiod and daminozide should be used in the production of *Petunia* plants for sale from May 1 to June 15. The plants were grown as described in the preceding section at a minimum night temperature of 15.6°–18.3 °C. March 20, seedlings in the 5th to 6th leaf stage were transplanted to 5- and 7-cm clay pots and to 13 × 20-cm fiber trays 3-cm deep. Plants were placed on 8-h days, natural days and natural days with incandescent light from 2 300 h to 0 100 h or 1 600 h to 0 800 h and were given sufficient space to allow for full development.

At flowering, starting in early May, the plants given additional incandescent light were too tall for commercial use. By the addition of foliar sprays of daminozide applied 42, 47, or 52 days after planting the seeds, elongation of the main stem was retarded and the plants were useful as compact, flowering, potted plants. Applications of daminozide to plants grown with 16 h of additional light caused more retardation than on those lighted for 2 h. Spray applications of daminozide to plants elongating slowly, as did those on natural days and those lighted for 2 h, were much less effective than those given plants elongating rapidly. Spray applications of daminozide to plants grown on 8-h days delayed flowering and retarded elongation of the basal branches; the number of basal branches produced was unaffected.

May 15, plants from 8-h days and natural days were placed outdoors. Half of the plants from each day length had been sprayed with 0.5% daminozide April 12. The plants from natural days were in bloom at the time they were planted outdoors. Those grown on 8-h day without a daminozide treatment were producing a few flowers on side branches; the ones treated with 0.5% daminozide were compact and nonflowering. May 25, part of the plants in each group had the tops of the main shoots removed. The performance of the various groups of plants was observed throughout the summer. The *Petunia* plants grown on 8-h days in the seedling stage and treated with daminozide produced a carpet of flowers throughout the summer. They came into bloom 3–3½ weeks after planting, and every basal branch became a flower-producing shoot. Each plant covered an area of the greatest diameter observed in the trial. The plants grown on 8-h days in the seedling stage without daminozide produced bedding plants that were taller but with less diameter than those treated with daminozide. The plants grown on natural days in the seedling stage soon toppled in the border, and few basal branches developed. A similar lot of plants from natural days which had been sprayed with daminozide developed more flowering branches than those without daminozide, but the display effects of plants grown on 8-h days were much superior to those of plants grown on natural days. The plants grown with additional artificial light were undesirable for bedding plant purposes. The plants that had the main stem removed May 25 eventually produced useful bedding plants, but 6–8 weeks ensued before flowers were present.

17.7.4 Number of Sprays

Other lots of plants in the border were sprayed with daminozide. The treatment was timed with the first appearance of elongation on the *Petunia* plants. In this case, the sprays were started June 7. Some plants were sprayed only once; others were sprayed two or three times at 3-week intervals. Spray applications of daminozide to *Petunia* plants in the border retarded growth, and, when repeated at 3-week intervals, they continued to control growth through the summer. When treatment with daminozide was discontinued, the plants resumed normal growth 4–6 weeks later.

17.8 Air Pollution – Sensitivity and Protection

Petunia plants have been recognized by various research workers as one of the species most sensitive to damage by oxidants including ozone. Consequently, sensitive cv. of *Petunia* as well as other plant species are used as indicators of air quality in urban centers (Craker 1972; DeVos et al. 1980). Studies involving ozone were initiated because it is the principal toxicant in photochemical smog and the most damaging air pollutant affecting vegetation in the United States (Feder 1970 a, b). Feder et al. (1969) evaluated the responses of 14 cv to ozone and other air pollutants, but most reports on *Petunia* plants include only 1 or 2 cv and do

not indicate the stage of growth at which injuries occurred, or evaluate the growth of plants after first development of air pollution injury. The literature concerned with air pollutants fails to recognize the great range of genetic material available in *Petunia* inbreds and F_1 hybrids. Their uniformity of plant habit and flowering time resulted from many years of inbreeding and selection which were needed to develop F_1 hybrids of varied growth habits and flower colors.

The literature on control of air pollution plant damage by chemical means identifies many compounds including fungicides, vitamins, the stomatal regulators phenylmercuric acetate and monomethyl ester of decenylsuccinic acid and antitranspirants (Heck 1968; Heggestad and Heck 1971). None of these chemicals have gained extensive use in agriculture largely because of the cost of chemicals and the need for frequent and thorough applications (Taylor et al. 1960). Most need to be applied just prior to an air pollution episode because their effects persist for only a few days or less if washed off by rain.

17.8.1 Comparison of Activity of Various Chemicals

Pink Cascade *Petunia* plants were treated with various chemicals which induce growth regulating responses on ornamental plants (Table 17.12). The active daminozide analogs – F724, C011, and F529 – when applied as a foliar spray were as effective as daminozide in reducing the interveninal clearing of chlorophyll and other injury induced by ozone (Cathey and Heggestad 1972). The analog C890, which is inactive as a growth-retarding chemical, was also inactive in protecting leaves against ozone.

Chemicals applied to the soil were much less effective in reducing the damage to plants than foliar sprays of daminozide and its active analogs (Table 17.12).

Table 17.12. Effect of various chemicals on ozone injury and ht of Pink Cascade *Petunia*. Spray and soil drench applied 39 days after sowing the seed. Plants were grown on long days and exposed to 45 PPHM ozone for 3 h at anthesis

Chemical Code	Dosage PPM	Method of application[a]	Ozone injury rating after 48 h	Ht at time of anthesis cm
			0–10	
H_2O control	0	Spray	8.0	20.5
Daminozide	5 000	Spray	2.0	5.6
F724	5 000	Spray	2.5	5.9
C890	5 000	Spray	7.5	16.9
COII	5 000	Spray	2.5	6.1
F529	5 000	Spray	2.3	5.4
ancymidol	10	Drench	5.0	18.3
Phosphorium chloride	100	Drench	3.5	9.4
benomyl	100	Drench	8.5	21.3
chlormequat	2 000	Drench	6.5	18.9

[a] Application: Sprayed until run off; soil drench; 100/ml of preparation

Ancymidol which possesses only slight growth retarding activity on petunias was applied to the soil at 10 ppm. The plants developed leaves with pale green margins and wilted almost daily. Chlormequat (CCC), inactive as a growth retardant on petunias, did not increase the tolerance of the plants to ozone. Phosphonium chloride (CBBP), the first chemical growth retardant known to be active on *Petunia*, induced some tolerance of the plants to ozone. Benomyl, a systematic fungicide, was also tested because it has wide biological activity on plants. Some protection from ozone injury has been reported. At the dosage (100 ppm) tested and a delay of almost 4 weeks between treatment and ozone fumigation, benomyl provided no protection from ozone injury. The plants grown in soil drenched with benomyl and fumigated with ozone developed leaves with interveinal and marginal clearing of chlorophyll. Several of the recently matured leaves were entirely white and persisted chlorophyll-free for several weeks following fumigation.

Based on these preliminary experiments, daminozide was selected as the most effective chemical growth regulator to apply to *Petunia* plants to alter their responses to ozone.

17.8.2 Responses of White Cascade *Petunia*

White Cascade *Petunia* is very sensitive to air pollution. The ambient pollution in the greenhouse with a pad and fan cooling system caused an average rating of 5.5 ozone injury on untreated plants at the start of flowering (Table 17.13). For a month prior to ozone fumigation (May, 1971) the maximum hourly average of oxidants at Beltsville was 8.0 pphm. Plants of a similar age and culture which had been treated with foliar sprays of the chemical exhibited reduced injury in direct relation to the dosage of daminozide. A single or a triple application of 0.25%

Table 17.13. Effect of dosage of foliar sprays of daminozide on ozone injury, ht, flowering time of White Cascade *Petunia*. Sprays applied 32, 39, and 46 days after sowing the seed. Plants were grown on long days and exposed only to ambient air pollution in a pad and fan cooled greenhouse

Daminozide dosage (%) H_2O control	Frequency of spray application	Ozone injury rating[a] After 48 h	Ht at time of anthesis cm	Anthesis (days)
		0–10		
	3×	5.5a	35.1a	55.2
0.125	3×	2.2b	28.1b	57.4
0.25	3×	2.2b	22.8c	57.5
0.5	3×	1.1c	15.8d	58.1
1.0	3×	0.5c	10.5e	59.0
0.25	×	2.5b	22.3c	56.4
0.5	×	0.9c	16.1d	58.3
1.0	×	1.1c	12.3e	58.9

[a] Means (20 plants per treatment) not followed by the same lower case letter differ significantly at the 5% level

daminozide reduced, but did not prevent visual damage to the chemically treated plants. Dosages of 0.5% or greater, applied once of three times, may be needed for satisfactory protection of plants from the pollution in ambient air. On several of the most recently matured leaves a few pale yellow spots appeared between the veins. Untreated plants exhibited rapid decline in the growth rates, since their older leaves turned pale green and died. Flower buds aborted and the stems lost turgor and collapsed.

17.8.3 Survey of *Petunia* cv

Plants of 65 *Petunia* cv were separated into 2 groups and exposed to various levels of ozone in growth chambers for 3 h and returned to the greenhouse. The average ozone injury for all cv combined was reduced by daminozide at all ozone levels including ambient air.

When exposed to 60 pphm ozone, injury to the *Petunia* cv varied from a few tan spots (rating 2) on Capri, Purple Plum, and Popeye to at least 70% of the leaf area dead (rating 7) on several cv. The cv are placed in six classes based on an average of our ratings after exposure of one plant of each cv to ozone at 15, 30, 45, and 60 pphm for 3 h (Table 17.14). The cv in the most tolerant class had low scores and were injured only at the highest ozone level, 60 pphm. Purple Joy in the second class of tolerants also was injured only at the highest conc. of ozone. Its injury rating, however, was higher than that of the 5 cv listed as most tolerant. The most sensitive cv, in class 6, had injury following exposure to all levels of ozone. They actually had ratings of 3 and 4 when exposed to only ambient levels of pollutants. Fandango and White Cascade in class 5 also had a rating of 3 on the control plants which were exposed only to pollutants in ambient air.

17.8.4 Morphological Changes of Daminozide-Treated Plants

The growth characteristics induced in response to applications of daminozide are well documented (Hindawi et al. 1965). The foliage was darker green, and slightly smaller in size than the untreated plants. The internode distances were reduced in relation to the concentration and frequency of applications of daminozide. The basal shoots on the treated plants developed in greater numbers than on the plants without daminozide application. Plants sprayed with daminozide did not dry out as rapidly as the untreated plants (Elkiey and Ormrod 1979 a–c). Silicone peels were made of the upper and lower surfaces of recently mature leaves. In every case, the stomates of the treated plants did not open as wide as those on untreated plants (Elkiey et al. 1979). Some cv such as Sugar Plum, maintained their stomates closed for many days of growing without apparent injury. Leaves from daminozide-treated plants were thinner in cross section than leaves from untreated plants and contained smaller cells and smaller intercellular spaces (Uhring 1978).

Table 17.14. Average ratings of 65 *Petunia* cv after fumigation with 15, 30, 45, and 60 PPHM ozone for 3 h (in 6 classes)

0–1 Most Tolerant	2–3 Intermediate I	4–5 Sensitive
Capri	Blue Magic	Albatross
Fire Gleam	Comanche	Ambassador
Pink Joy	Coral Magic	Apollo
Popeye	Glitters	Calypso
Purple Plum	Honey Bunch	Chiffon Cascade
1–2 Tolerant	Mariner	Fandango
Ace of Hearts	Maytime	Midnight Star
Apple Blossom	Mercury	Minstrel
Blue Lace	Red Joy	Red Satin
Candy Apple	Rose Cloud	Rose Joy
Cherry Blossom	Sky Magic	Rose Star
Crusader	Star Joy	Starfire
El Toro	Zig Zag	Velvet Queen
Pink Magic	3–4 Intermediate II	White Cascade
Purple Joy	Coral Satin	White Sails
Red Cap	Comino	5–6 Most Sensitive
Sugar Plum	Flirt	Fiesta
White Magic	Happiness	Harmony
	Lollipop	Pink Paradies
	Orange Bells	Snow Magic
	Pink Cascade	White Joy
	Pink Snow	
	Red Cascade	
	Sabre Dance	
	Snow Lady	
	Sugar Daddy	
	Sunburst	
	Touche	
	Victory	

17.8.5 Comparison of Activity of Daminozide and EDU

Carnahan et al. (1978) described the value of N-(2-oxo-1-imidazolidinyl ethyl)-N^1-phenylurea (abbreviated EDU for ethylenediurea) in preventing ozone injury. Cathey and Heggestad (1981) reported that EDU was more effective than daminozide in providing protection against fumigation with 0_3. For the most effective use of daminozide, greater care had to be taken than with EDU. First, it was effective only as a foliar spray. It was inoperative as a soil drench. The physiological state of the plant greatly complicated the development of ozone-tolerant plants. Wilted plants, even ones with a slight loss in turgor, did not readily take up the chemical, even though the wilting process temporarily closed the stomates and slowed growth. The plants in the flaccid state could be sprayed with daminozide without resulting in a physical change in the growth of the plants. The photoperiod on which the plants were grown further complicated the protection which could be afforded. Short days, 8 h, and extremely long days, up to 24 h, slowed or accelerated growth so much that daminozide was relatively ineffective

as a chemical protector. It was most effective when the photoperiod was longer than 12, but no more than 16 h. The recommended dosage of daminozide was twice, from 0.25%–0.5%, that suggested solely for height reduction and it had to be applied twice one week apart, starting when the plants were 29 days old. The total chemical applied was four times what is required solely for height control. The additional chemical did not bring about any significant decrease in the height of the plant, but apparently constricted leaf expansion. These slight changes in the visual appearance of the leaves were essential to close the stomates, reduce intercellular spaces, and increase the density and number of layers of palisade cells. Under no circumstances, however, was the protection afforded complete. We always observed some visible damage.

EDU, in contrast with daminozide, caused no visible changes in the form and function of the treated plants. The *Petunia* stems continued to elongate, the leaf blades expanded normally, the leaf color remained the same, and the stomates functioned normally in the presence of EDU. The chemically-treated plants were afforded complete protection when the threshold concentration of EDU, from a foliar spray and soil drench, was reached. The protection was afforded under all environmental conditions of daylength, temperature, stage of growth, cultivar, and physiological state. EDU also afforded almost immediate protective action against fumigations with 0_3 when applied as a foliar spray. A slight increase in effectiveness on petunia was observed when 1 to 3 days were allowed to lapse between application of EDU and fumigation with 0_3. The point, however, is that no lapse in time between application and fumigation was required for growth alterations and protection as with the daminozide.

EDU protected the *Petunia* leaves from 0_3 injury whether applied as a spray or as a drench on the soil. Recent studies (Bennett et al. 1981) indicate that EDU increases the quantity of certain enzymes such as superoxide dismutase and catalase, which scavenge free radicals generated in cells by the action of ozone. These catalytic oxidoreductase enzymes are involved also in the steady state 0_3 uptake process. EDU arrests senescence caused by low level ozone exposure by sustaining RNA and protein synthesis (Lee et al. 1980). The effectiveness of EDU in preventing 0_3 injury without visually altering the growth of the *Petunia* plant is a unique phenomenon for chemical growth regulators.

17.8.6 Ozone Sensitivity and Goals for Breeding

The sensitive response of various cultivars of *Petunia* to air pollution is documented by many workers (Hanson et al. 1975, 1976). Feder et al. (1969) reported White Cascade to be the most sensitive of several cv tested for sensitivity to pollutants but they did not report the tested sensitivity of this cv to ozone. We provide herein conclusive evidence that *Petunia* has a wide range in genetic sensitivity to ozone. We believe that through breeding and selection it should be possible to develop resistant cv of most ornamental plants (Elkiey et al. 1979; Hanson et al. 1976). *Petunia* cv can be further modified through the use of the chemical growth regulator daminozide, EDU, and perhaps other chemicals yet to be developed.

17.9 Summary

Growing *Petunia* is quite challenging because its whole developmental course is so rapid, the developments so varied, and the growth stages so intermixed. It is very difficult to separate the stages, work solely on one stage, and complete the experiments before flowering. Even more important are the concepts which have been established using *Petunia* as a model.

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