Tom Gerats
Judith Strommer
Editors

Petunia

Evolutionary, Developmental and Physiological Genetics

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Tom Gerats · Judith Strommer
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Second Edition

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To Mel, Sarah and Wiert
and to Mike and Miriam

Preface

The first edition of this monograph, edited by Kenneth Sink and published in 1984, effectively summarized nearly a century of research. It has provided a guide of inestimable value to the family of Petunia researchers for more than 20 years, over which time the nature of plant research has undergone a revolution. The fairly small but dedicated group of the mid-20th century was interested in Petunia primarily as a model system for physiological, biochemical, and genetic research. 1984 was still fairly early in the molecular era, particularly for plant research, but the timing was good for a comprehensive text covering areas from which molecular biology could grow. And so it grew: as evidenced in every chapter of this book, which summarizes the progress in Petunia-based research of the past two and a half decades, the tools of molecular biology are now standard in the biologist's tool box.

In the mid-1980s the search for "the *E. coli* of plants," the organism to serve as the fundamental plant model system, had begun. In 1985 a meeting was organized at the University of Georgia in Athens, followed in 1987 by an EMBO-sponsored course at the Vrije Universiteit in Amsterdam on Petunia as a Model System. The general feeling, for reasons that emerge over and over in the present book, was that Petunia would be a superb choice as a plant model system. Early researchers like Bianchi, Cornu, Wiering, Maizonnier, de Vlaming and Farcy, and later ones like Benninck and Schram, had done their parts to prepare the way for using Petunia in molecular studies. Their work in genetics, cytogenetics, biochemistry, and physiology provided a strong foundation for molecular studies, especially on flavonoid synthesis and genome structure.

The Arabidopsis tide, however, had been building since the early 1980s, and by 1990 it was clear that the small, fast-cycling weed with its small genome was the model system of choice. A great number of young researchers became experts in Arabidopsis, and both the body of information and the available tools for Arabidopsis-based studies grew exponentially. The humble plant has served its role admirably and allowed for an incredible rate of progress in our understanding of many aspects of plant biology. Now there is a general surge of interest in comparative biology, and thus new model systems are again being sought. Petunia, as this work demonstrates, continues to offer many advantages.

Not the least of these is the spirit of the Petunia research community. The "First World Petunia Day," a small one-day meeting pretty much restricted to researchers

from Amsterdam, Wageningen and Ghent, initiated a worthwhile tradition of bringing together Petunia research and researchers. Though initially a small group, the spirit was good, the thinking was large, and over the years, the meetings attracted more and more scientists. By the "Ninth WPD," held in October 2007, more than 60 delegates joined in the informal "formal presentations" and the relaxed eating, drinking, and talking that still typify WPD meetings. It was at the "Eighth WPD," in October 2006, that the idea for a new edition of the monograph was proposed and accepted. (A check of the Petunia Platform website [www.petuniaplatform.net] will provide the reader a quick introduction to the Petunia groups and their ongoing work.)

The fresh ideas and approaches to research, together with a continuing readiness to share, have been key to the impressive progress documented in this second edition of the monograph. A cursory look at authors and references in this work will give a hint of the extent of the collaborative spirit in the Petunia community. As members continue to work together to exploit the strengths of this model system, they will continue to contribute much to the development and evolution of science, particularly in the discipline of comparative biology.

We hope that this edition of the Petunia monograph will serve the current members of the Petunia research community well and help to attract yet more excellent and collaborative workers to this elegant plant system and to the community that has made it so.

The first edition of *Petunia* will be available via Springer.com!

Guelph Judith Strommer
Nijmegen Tom Gerats

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Abbreviations

ABA	abscisic acid
ABC	model for genes specifying floral organ identity
AFBA	after flower bud appearance
AFLP	amplified fragment length polymorphism
AM(S)	arbuscular mycorrhiza (symbiosis)
AN	anthocyanin
ATP	adenosine triphosphate
AVG	2-amino-ethoxyvinyl-glycine
BA	benzoic acid
BAC	bacterial artificial chromosome
BAP	6-benzaminopurine
bc	backcross
BHLH	basic helix-loop-helix
BIBAC	binary bacterial artificial chromosome
BIL	backcross inbred line
bp	base pairs (DNA)
BSTFA	N,O bis(trimethylsilyl) trifluoroacetamide
C6	6-carbon metabolite(s)
CA	cinnamic acid
CaMV	cauliflower mosaic virus
cDNA	complementary DNA
CFP	cyan fluorescent protein
Ch, ch	chromosome
CHX	cycloheximide
cm	centimeter(s)
cM	centiMorgan(s)
CMS	cytoplasmic male sterility
CP	cysteine Protease
CYT	cytochrome

d	day(s)
D	dimensional (1D, 2D, etc.)
DAG	diacylglycerol
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
ds	double-stranded
ePVCV	endogenous Petunia Vein-Clearing Virus
EM	ectomycorrhiza
EMS	ethane methyl sulfonate
EPRV	endogenous pararetrovirus
ER	endoplasmic reticulum
EST	expressed sequence tag
ETR	ethylene resistant
EXL	expansin-like
EXP	expansin protein
FISH	fluorescence in situ hybridization
FLIM	fluorescence lifetime imaging microscopy
FM	floral meristem
FRET	fluorescence resonance energy transfer
FTIR	Fourier transform infrared (spectroscopy)
g	gram
GA	gibberellic acid
GC	gas chromatography
GFP	green fluorescent protein
GMS	genic male sterility
GSI	gametophytic self-incompatibility
GUS	β-glucuronidase
h	hour(s)
hap	hours after pollination
HDGS	homology-dependent gene silencing
HFD	his-phe-asp amino-acid signature
HV	hypervariable
IAA	indole-3-acetic acid
Ig	immunoglobulin
IM	inflorescence meristem
IPT	Isopentenyltransferase
K	potassium
kb	kilobase(s)
kDa	kiloDalton(s)

LB	Luria broth
LIS	linalool synthase
LTR	long terminal repeat
m	meter(s)
MADS	family of transcription factors with specific motifs
MCS	multiple cloning site
MeBA	methyl benzoate
MD	Mitchell Diploid (Petunia Mitchell)
MFS	major facilitator superfamily
Mg	magnesium
mm	millimeter(s)
mM	millimolar
MP	movement protein
MS	mass spectrometry
mt	mitochondrial
MYA	million years ago
MYB	million years before; transcription factor with specific motif
myc	mycorhhiza
MYC	transcription factor with specific motif
MW, mw	molecular weight
N	nitrogen
NAD	nicotinamide adenine dinucleotide
NLS	nuclear localization signal
nod	nodulation
P	phosphorus
P_i	orthophosphate
PA	phosphatidic acid
PAGE	polyacrylamide gel electrophoresis
PAM	periarbuscular membrane
PCD	programmed cell death
PCF	petunia CMS-associated fused gene
PCR	polymerase chain reaction
PDMS	polydimethylsiloxane
pI	isoelectric point
PIP_2	phosphatidyl inositol 4,5-bisphosphate
pm	picomole(s)
PPA	prepenetration apparatus
PPR	pentatricopeptide repeat
PR	pathogenesis related
PRV	pararetrovirus
PSC	pseudo-self-compatibility

PTGS	post-transcriptional gene silencing
PT	phosphate transporter
PVX	Potato virus X
QTL(s)	quantitative trait locus (loci)
RAM	root apical meristem
RAPD	random amplification of polymorphic DNA
rDNA	ribosomal DNA
RFLP	random fragment length polymorphism
RH	RNase H
RIHC	RNA-induced silencing complex
RIL	recombinant inbred line(s)
RNA	ribonucleic acid
RNAi	RNA interference
RNase	ribonuclease
RNS	root nodule symbiosis
ROS	reactive oxygen species
RT	reverse transcriptase
SAM	shoot apical meristem; S-adenosyl-L-methionine
SC	self-compatibility
SCF	ubiquitin E3-ligase complex
SGN	Solanaceae Genomics Network
SI	self-incompatibility
siRNA	small interfering RNA
SLF	S-locus encoded F box
sp(p)	species (plural)
SPME	solid phase micro-extraction
ss	single-stranded
ssp; subsp(p)	subspecies (plural)
SYM	symbiosis
TD	Transposon Display
tDNA	transgene DNA

TGMV	tobacco golden mosaic virus
TGS	transcriptional gene silencing
TIGR	The Institute for Genomic Research
TMCS	trimethylchlorosilane
TMV	tobacco mosaic virus
TRV	tobacco rattle virus
TSD	target site duplication
TUNEL	terminal transferase-mediated dUTP nick end-labeling
UI	unilateral incompatibility
UV	ultraviolet
VIGS	virus-induced gene silencing
VLP	virus-like particle
WT	wild type
YFP	yellow fluorescent protein
YEP	yeast extract bacto-peptone
μm	micron(s)

Chapter 1
The Genus *Petunia*

João Renato Stehmann, Aline P. Lorenz-Lemke, Loreta B. Freitas, and João Semir

Abstract The common garden petunia, *Petunia hybrida*, is derived from *P. integrifolia* and *P. axillaris*, two of many *Petunia* species endemic to South America. The geographic distribution includes temperate and subtropical regions of Argentina, Uruguay, Paraguay, Bolivia, and Brazil, with a center of diversity in southern Brazil. The presence of seven chromosomes and a number of morphological, anatomical, and biochemical characteristics differentiate the genus from its sister taxon, *Calibrachoa.* Included in this chapter is a taxononomic guide for the 14 currently recognized species, some of them restricted to very small geographic areas. Species diversity is in danger of diminishing significantly due to human intervention, particularly in the form of grassland destruction.

1.1 Historical Review

Petunia Jussieu (Solanaceae) is best known for the garden petunia, an ornamental hybrid widely cultivated around the world. There are many cultivars with a broad range of flower color and size, and the market of their seeds represents an important economic resource for many countries. Its generic epithet comes from "petum" or "betum," an indigenous name given to the tobacco, *Nicotiana tabacum* L., that roughly resembles one of the first two species described in *Petunia, P. nyctaginiflora* Juss. (=*P. axillaris* [Lam.] Britt. et al.) (Fries 1991). Due to some morphological similarities, such as annual growth habit, five stamens, capsular fruits, and small seeds, *Petunia* and *Nicotiana* G. Don were historically included in the same infrafamiliar taxonomic groups (Wettstein 1895; D′Arcy 1991; Hunziker 1979, 2001). However, recent phylogenetic studies based on molecular data suggest that *Nicotiana* and *Petunia* are not so closely related and should be placed in different subfamilies (Olmstead and Palmer 1992; Olmstead, Sweere, Spangler, Bohs, and Palmer 1999; Olmstead and Bohs 2007).

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Petunia was originally described by Jussieu (1803) based on material collected in Montevideo, Uruguay, by Commerson. Two very distinct species, *P. parviflora* and *P. nyctaginiflora*, were described in the same paper, the first with short funnelform corolla and the second salverform with a long corolla tube. Earlier, in 1793, Lamarck described the latter species as *Nicotiana axillaris* Lam. During the next four decades, species of *Petunia* were variably described in different genera of the Solanaceae such as *Nicotiana* (Lehmann 1818), *Fabiana* Ruiz & Pav. (Saint-Hilaire 1824), *Calibrachoa* La Llave & Lex. (La Llave and Lexarza 1825), *Salpiglossis* Ruiz & Pav. (Hooker 1831), and *Nierembergia* Ruiz & Pav. (Graham 1833).

In 1846, Miers published a revision of the South American Solanaceae in which he recognized 10 species of *Petunia,* half of them described for the first time. His illustrations of the species were presented later, in 1850. In the same year, but two months later, Sendtner published his revision of Solanaceae in the *Flora Brasiliensis*, with 13 species of *Petunia*, nine of which were new. In de Candolle's *Prodromus*, published in 1852, Dunal presented 16 species of *Petunia,* transferred another three from *Petunia* to *Fabiana,* and described a new genus, *Leptophragma*, now considered a synonym of *Calibrachoa*.

Fries (1911) published the first monograph of *Petunia*, accepting 27 species, nine of which were new. The taxonomic treatment provided a detailed discussion of the morphology, circumscription, geographic distribution, and relationships of *Petunia* with other genera. Fries' monograph remains the latest complete revision of the genus available. During the following five decades, few new species were described (Sandwith 1926; Steere 1931; Morton 1944). The number of species in *Petunia* increased significantly only when Smith and Downs (1964, 1966) described nine new species among the flora of Santa Catarina, Brazil.

The study of the garden petunia, termed *Petunia x hybrida* (Hook.) Vilm. and now commonly known as *Petunia hybrida*, was decisive for the future of the taxonomy of the genus. The garden petunia was first obtained by hybridization in 1834 by Atkins of Northampton, a British nurseryman, and it soon spread to European gardens (Sink 1984). Today it is cultivated all over the world and is one of the most important Solanaceae utilized for ornamental purpose.

Many authors have investigated the origin of this garden hybrid and the mechanisms of genetic incompatibility found in *Petunia* (Ferguson and Ottley 1932; Mather 1943; Mather and Edwardes 1943; Stout 1952; van der Donk 1974; Linskens 1975; Sink 1984; see Chapter 5), and different species have been suggested as parents of the garden petunia.

In 1982, Wijsman considered the origin of the hybrid and concluded that it was obtained from breeding of only two biological species, one with white flowers [*P. axillaris* (Lam.) Britton et al.], and the other with purple flowers [*P. integrifolia* (Hook.) Schinz & Thell.], each one with geographic subspecies. Later, Wijsman (1983) tried to breed other species (*P. calycina* and *P. linearis*, $2n = 18$) with the parents of the garden petunia ($2n = 14$). While hybrids were obtained by crossing species with equivalent chromosome number, all crossings between species with different chromosome numbers failed. Wijsman and de Jong (1985) concluded that these groups distinguished by chromosome number (and some morphological

characters) must be treated as different genera and proposed to keep in *Petunia* only the species related to *Petunia parviflora* Juss. ($2n = 18$), the type species of the genus as established by Britton and Brown (1913) using the mechanical method of typification (first species cited in the protologue). Species of *Petunia* with $2n = 14$ were transferred to *Stimoryne* Rafin., the next generic available synonym.

As the taxonomic proposal of Wijsman and de Jong (1985) would change the name of the garden petunia, Wijnands and Bos (1986) proposed to conserve *Petunia nyctaginiflora* Juss. ($2n = 14$) as the type of *Petunia.* This proposal was accepted (Greuter et al. 1994), and those species related to *Petunia parviflora* ($2n = 18$) were transferred to *Calibrachoa* (Wijsman 1990; Stehmann and Semir 1997; Stehmann and Bohs 2007).

Further cytotaxonomic, reproductive, anatomical, and chemical studies related to species of *Petunia* and *Calibrachoa* have corroborated Wijsman's decision to split *Petunia* into two genera. All species investigated in *Petunia* have a chromosome number of $n = 7$ (Watanabe et al. 1996a), while in all *Calibrachoa* species examined to date, $n = 9$ or 18 (Stehmann, Semir, Dutilh, and Forni-Martins 1996; Watanabe et al. 1996b). Chromosome counts assign the basic numbers of $x = 7$ and $x = 9$ to *Petunia* and *Calibrachoa,* respectively. Interspecific cross-incompatibility between *Petunia* species with different chromosome numbers, as reported by Wijsman (1983) and Watanabe et al. (1996a), demonstrates that the groups are genetically isolated and they could not hybridize in natural conditions, even if they share the same pollinators. Anatomical features are useful to distinguish the genera. Reis, Sajo and Stehmann (2002) studied leaf anatomy in 16 species of *Calibrachoa* and seven species of *Petunia.* Fourteen *Calibrachoa* species have endodermis surrounding the vascular bundles, formed by well-developed parenchymatic cells very distinct from surrounding mesophyll. Only two species, *C. parviflora* (Juss.) D′Arcy and *C. pygmaea* (R.E. Fries) Wijsman, do not show differentiated endodermis. In none of the analyzed species of *Petunia* is the endodermis morphologically differentiated. Chemical evidence also supports Wijsman and de Jong (1985). Ellinger, Wong, Benson, Gaffield, and Waiss (1992) reported that *C. parviflora* yielded none of the ergostanoids that are associated with *Petunia* species. However, only a few species were studied and further work is needed.

Phytogenetic relationships have been clarified by the results of recent molecular studies. The separation into two genera was supported by RFLP chloroplast DNA (Ando et al. 2005b) and ITS, cpDNA and mtDNA analyses (Kulcheski et al. 2006). In both analyses, *Petunia* and *Calibrachoa* showed a close phylogenetic relationship and, for this reason, are considered sister groups.

1.2 Morphological Circumscription of *Petunia* and *Calibrachoa*

Some species of *Petunia* and *Calibrachoa* have sympatric distributions and share similar vegetative and floral attributes, making it difficult to recognize the genus to which they belong. To help with the recognition of each genus, we compare the principal diagnostic morphological traits below (Table 1.1).

Table 1.1 Comparative morphological traits between *Petunia* Juss. and *Calibrachoa* La Llave & Lex. (Solanaceae)

Trait	*Petunia*	*Calibrachoa*
Habit	Herbs with non-woody stems	Small shrubs or herbs, woody stems
Duration	Usually annual	Annual or perennial
Brachyblasts	Absent	Present or absent
Leaves	Ovate, elliptic, oblong, or obovate, rarely linear; flat margin	Ovate, elliptic, obovate, oblong, or linear; flat or revolute margin
Inflorescence	Monochasial with opposite leaf-like bracts	Monochasial with opposite leaf-like bracts
Aestivation	Imbricate	Reciprocative (except in *C. pygmaea*)
Symmetry	Actinomorphic or zygomorphic	Zygomorphic (except in *C. pygmaea*)
Calyx	Ribs usually not conspicuous; deeply lobed (except in *P. altiplana* and some coastal populations of *P. integrifolia*); lobes linear or enlarged toward the apex	Five or ten ribs; lobed usually to the middle, lobes usually narrowed toward the apex
Corolla	Funnelform, campanulate, or salverform; purple, red (bright or orange), pink, or white	Funnelform (salverform, ventricose, and apically constricted in *C. pygmaea*); purple, red, pink, or whitish
Anthers	Yellow, bluish, or violet	Yellow
Seed coat	Cells with wavy anticlinal walls	Cells with straight anticlinal walls

In nature, *Petunia* species are mostly annual with herbaceous stems and brachyblasts absent or poorly developed. It is difficult to get information about life history from herbarium material; additional information can be obtained by growing plants in greenhouses, but there they do not behave as they do in nature, especially in the subtropical regions where most species of *Petunia* grow. In southern Brazil the winter is very cold, with minimum temperatures below 0°C; thus, frost and snow must affect the survival of individuals. Since *Petunia* species are usually not lignified and they lack any special underground system (except for coastal populations of *P. integrifolia*), we can assume that most species, such as *P. axillaris*, *P. integrifolia*, *P. reitzii* L.B. Sm. & Downs, and *P. bonjardinensis* T. Ando & Hashim., are annuals. *Calibrachoa* species are annual or perennial. The perennials have a shrubby habit with basal woody stems, often bearing brachyblasts.

Leaf morphology in *Petunia* is more uniform than in *Calibrachoa*. In *Petunia* the leaves are usually sessile, more rarely petiolate, ovate, obovate, or elliptic, rarely linear, and the surface and margin are flat. In *Calibrachoa*, leaves are also commonly sessile, but their form is extremely variable. There are elliptic, ovate, obovate, oblong, and linear leaves, with flat or revolute margins. The revolute margin is not found in any *Petunia* species. Reis et al. (2002) showed that leaves of

Calibrachoa are quite diversified, both externally and internally, and its species can be separated according to the type of margin, distribution of stomata on leaf surfaces, organization of the mesophyll, and morphology of trichomes.

The inflorescences in both genera are sympodial, with monochasial growth, whereby each flower is always associated with two opposite, leaf-like bracts (one sympodial unit). The sympodial pattern is typical for almost all species of Solanaceae (Bell and Dines 1995). Secondary branches arise subsequently in the axils of basal bracts, whereupon the appearance of the inflorescence may become dichasial. Development of the sympodial unit is normally continuous, but sometimes it can be interrupted such that the flower is not produced at a specific node. Fries (1911) and Danert (1958) described and illustrated the characteristic inflorescence branching of *Petunia* s.l. To distinguish *Petunia* and *Nicotiana,* Smith and Downs (1966) as well as Hunziker (1979) described *Petunia* with solitary flowers. However, the flowers of *Petunia* and *Calibrachoa* cannot be considered solitary, because they are arranged in a sequence of sympodial units that constitute an inflorescence. These sympodial units with two opposite leaf-like bracts are unique to *Petunia* and *Calibrachoa* in Petunioideae, and clearly constitute an apomorphic character of this group.

The calyx of most *Petunia* species is deeply lobed with linear or spatulate lobes, usually enlarged toward the apex. Calyx lobed to the middle appears only in flowers of *P. altiplana* T. Ando & Hashim. and coastal populations of *P. integrifolia.* In *Calibrachoa* the calyx is usually lobed to the middle, and the lobes are often acute and narrowed toward the apex. Exceptions can be found in *C. micrantha* (R.E. Fries) Stehmann & Semir and *C. pygmaea*, in which the calyx can be cleft up to 2/3 of its length. However, the lobes of the calyx of *C. micrantha* are ovate-lanceolate and narrowed at the apex, while in *C. pygmaea* they are linear and obtuse at the apex. Another useful characteristic of the calyx is the presence of marked ribs. In *Calibrachoa*, calyx ribs are prominent in most species, except in *C. pygmaea* and *C. parviflora*, which have a more fleshy calyx with less-evident ribs. Conversely, most *Petunia* species lack calyx ribs.

Fries (1911) and Smith and Downs (1966) used the degree of calyx partition as a diagnostic characteristic in *Petunia* s.l. Wijsman and de Jong (1985) observed that this distinguished most species belonging to the groups with different chromosome numbers. The deeply lobed calyx described in *P. parviflora* [= *C. parviflora*] was considered by them to be an artifact and treated as a pentapartite, not pentafid, calyx with lobes connected to a thin membrane. The halfway-lobed calyx of most *Calibrachoa* may reflect the higher level of fusion of the lobes and the lateral veins, forming ten thick ribs. These ribs are quite evident in the calyx of the other related genera, such as *Fabiana* (Barboza and Hunziker 1993) and *Nierembergia* (Millan 1946), in which the level of fusion is higher.

Studies on vasculature and structure of the calyx are important to the taxonomy of Solanaceae. D'Arcy (1986) described the solanaceous general calyx as a whorl of five lobes nerved by five primary traces that branch into the lobes, forming a pair of lateral veins with minor leaf-like venation. He pointed out a tendency of the calyx lobe and veins to fuse in different levels, giving rise to main traces or ribs in the

fused area. In *Lycianthes* the calyx nervation consists of primary traces with fused laterals leading to ten teeth in two series, and this pattern serves to distinguish it from *Solanum*, with which it shares poricidal anthers.

Calibrachoa and *Petunia* have different aestivation patterns, and this trait distinguishes the genera. *Petunia* species show imbricate aestivation (Fig. 1.1A), whereas most *Calibrachoa* species have reciprocative aestivation (Fig. 1.1B). This term was coined by Miers for instances in which the anterior induplicative lobe covers the four others, which are conduplicate (Hunziker 2001). Within *Calibrachoa*, only the *C. pygmaea* corolla seems to have an imbricate-reciprocative aestivation.

Aestivation was confirmed as an unambiguous characteristic that differentiates *Calibrachoa* and *Petunia* species, as Wijsman and Jong (1985) predicted. The *Calibrachoa* aestivation pattern is similar to the conduplicate pattern described for *Nicotiana* by Goodspeed (1954). However, in *Nicotiana* the conduplicate type is extremely variable in its degree of spirally. Within one species, *N. tomentosa* Ruiz & Pav., conduplication shifts via intermediate races into imbrication. In the Solanaceae, aestivation has long been used to distinguish subtribes (Baehni 1946) or tribes (Hunziker 1979). The distinct ontogenetic patterns of corolla development observed in buds of *Petunia* and *Calibrachoa* provide evidence that they are not

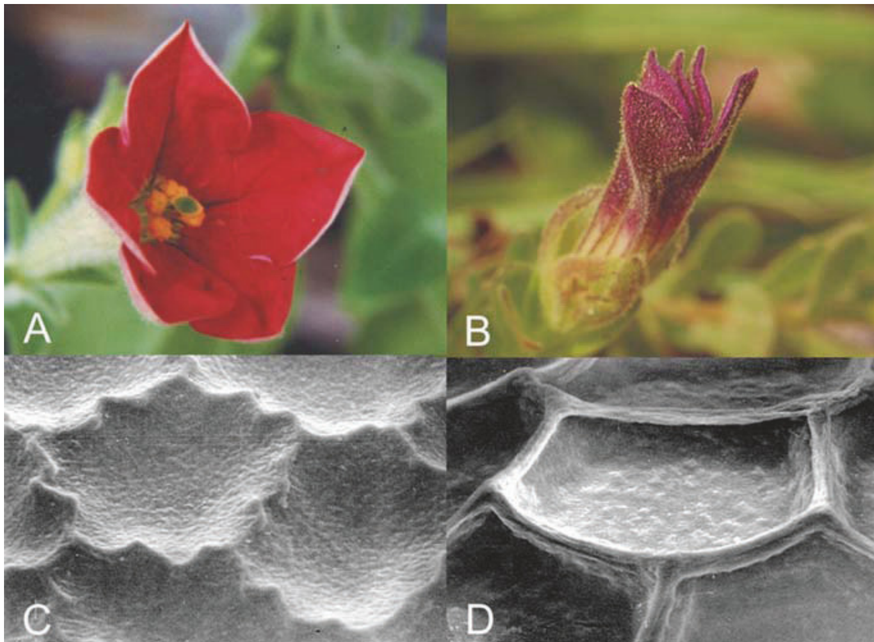

Fig. 1.1 Distinct patterns of corolla aestivation in *Petunia* and *Calibrachoa*. (**A**) *Petunia exserta* with imbricate aestivation. (**B**) *Calibrachoa sellowiana* with reciprocative aestivation. Seed coat of *Petunia* and *Calibrachoa* observed by SEM: (**C**) anticlinal walls are wavy in *Petunia* (*P. integrifolia*) and (**D**) straight in *Calibrachoa* (*C. dusenii*)

closely enough related to represent infrageneric taxa, for example, subgenera, but rather support their distinction at the generic level.

The general ontogenetic pattern of corolla aestivation is completely different in the two genera. After initiation of the corolla primordia, the lobes start the differentiation and growth process. The corolla lobes in *Petunia* become imbricate at the median stage of development, before elongation of the tube. At this same stage of bud development, corolla lobes in *Calibrachoa* become folded and aestivation shows an induplicate pattern. Later the one basal lobe turns upward, enfolding the four others. Anatomical studies are necessary to better describe these patterns.

The corolla color in *Calibrachoa* and *Petunia* species is usually purple, but both genera may also show whitish, reddish, or pinkish flowers. Species with a funnelform corolla may have a yellow corolla throat in *Calibrachoa*, but in *Petunia* yellow is never associated with this type of flower. It is also worthwhile to note the yellow color of the anthers in all *Calibrachoa*, but ranging from yellow to bluish or violet in *Petunia*. Ando et al. (1999) studied in detail the occurrence of floral anthocyanins in 20 taxa of *Petunia* and recognized four distinctive colors: white, purple (and red-purple), orange-red, and bright red.

The corolla shape typically found in *Calibrachoa* is funnelform. Only *C. pygmaea* shows a salverform, ventricose, and apically constricted corolla. *Petunia* shows various types of corolla, such as funnelform, campanulate, and salverform (Fig. 1.2). The limb is also quite diverse in *Petunia*, with lobes rounded, obtuse, retuse, or acute. In *P. exserta* Stehmann, a hummingbird-pollinated species, the corolla lobes become more cleft and reflexed with age.

Petunia and *Calibrachoa* have small foveolate-reticulated seeds (less than 1.4 mm), and seed coats observed under SEM show different patterning. Seed-coat anticlinal walls are wavy in *Petunia* (Fig. 1.1C) and straight in *Calibrachoa* (Fig. 1.1D). Bahadur, Venkateshwarlu and Swamy (1989) had previously described the different patterns of seed-coat morphology in *Petunia* s.l., but did not make any comments about the taxonomic implications of their discovery. Stehmann and Semir (1997) reported seed-coat epidermal cells as a fundamental diagnostic characteristic to distinguish the two genera. In order to check the assumption of those authors, Watanabe et al. (1999) studied seed coats in 45 taxa of the genus *Petunia* s.l. Three different seed-coat epidermal patterns were described: (1) wavy middle lamellae and anticlinal walls, (2) wavy middle lamellae embedded in straight anticlinal walls, and (3) straight middle lamellae and anticlinal walls. In fact, these three groups correspond to the two groups, one with wavy anticlinal walls (*Petunia* species) and the other with straight anticlinal walls (*Calibrachoa* species). The middle lamellae are not well characterized by SEM, and the results of this analysis must be treated with caution. The seed-coat epidermis, associated with aestivation pattern, clearly distinguishes *Petunia* from *Calibrachoa.*

There are few SEM descriptions of seed coats for related genera in Solanaceae. In *Nicotiana* seed-coat epidermal cells, there are variable types of anticlinal walls. Most *Nicotiana* species have wavy anticlinal walls, but straight anticlinal walls also occur in this genus (Goodspeed 1954). Features of the seed-coat epidermal wall

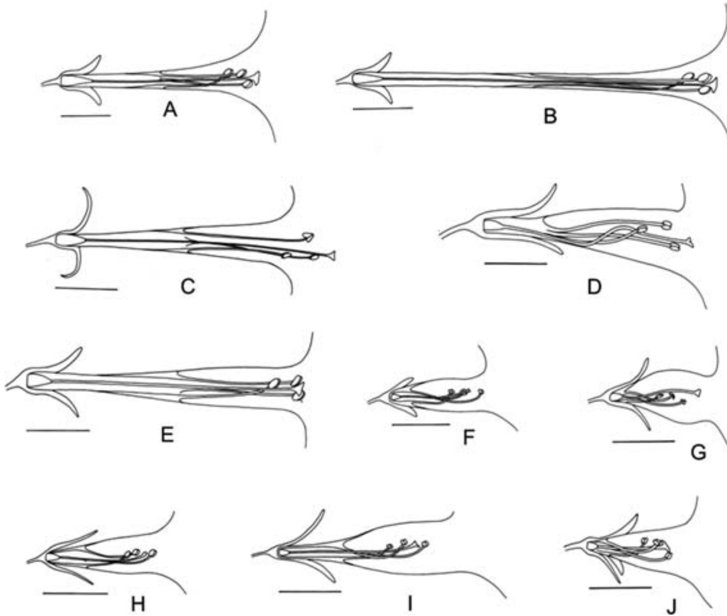

Fig. 1.2 Major patterns of Petunia flower arrangements. Salverform corolla: (**A**) *P. axillaris* subsp. *axillaris;* (**B**) *P. axillaris* subsp. *parodii*; (**C**) *P. exserta;* (**D**) *P. mantiqueirensis*; (**E**) *P. secreta.* Funnelform or campanulate corolla: (**F**) *P. altiplana*; (**G**) *P. bonjardinensis;* (**H**) *P. integrifolia;* (**I**) *P. reitzii;* (**J**) *P. scheideana.* Scale bar = 1 cm

are characteristics known to have taxonomic importance in *Physalis* (Axelius 1992) and *Schwenckia* (Carvalho, Machado and Bovini 1999), as well as in other families like Cactaceae (Barthlott and Voit 1979) and Campanulaceae (Shetler and Morin 1986). Seed-coat attributes have been considered valuable characteristics to recognize species or genera, or even tribes and subtribes (Barthlott 1981).

1.3 Taxonomy

Petunia comprises annual or perennial herbs, up to 1 m tall, with erect, ascendant, decumbent, or procumbent stems, rarely rooting at the nodes. The leaves are sessile or petiolate, with blades elliptic, ovate or obovate, more rarely rounded or linear, membranaceous, somewhat juicy, flat, and usually without marked venation. Inflorescences are sympodial, with monochasial growth, whereby each flower is always associated with two opposite, leaf-like bracts. The calyx is green, deeply lobed, with a very short tube and long linear or spatulate lobes with usually inconspicuous ribs. The corolla is funnelform, campanulate, or salverform, with imbricate aestivation, tube 1.5–7 cm long, and purple, white, red (bright or orange), or pink limb. The five stamens are variously adnated to the corolla tube, usually included and arranged in

three levels: one short, two middle, and two longer (more rarely only in two levels). Anthers are ventrifixed, yellow or violaceous in color. The ovary is glabrous, surrounded by a lobed nectary, with filiform style, and disciform or lobed stigma. The stigma can be placed among the anthers of the didynamous pair of stamens, at the same level or above the longest pair, sometimes exserted to the corolla tube. The fruits are capsular, many seeded, with peduncle inflexed or deflexed at mature stage. Seeds range in size from 0.4 to 1.4 mm long and present seed coats with wavy anticlinal walls.

We recognize 14 species distributed in subtropical and temperate South America. *Petunia axillaris* and *P. integrifolia*, parents to the familiar garden hybrid, have the largest distribution. Several species are narrow endemics, for example, *P. bajeensis* T. Ando & Hashim., *P. bonjardinensis, P. exserta, P. mantiqueirensis* T. Ando & Hashim., *P. reitzii, P. saxicola* L.B. Sm. & Downs, and *P. secreta* Stehmann & Semir. Species of *Petunia* can be found in sunny, partially shaded, or completely shaded sites. *Petunia altiplana, P. axillaris, P. inflata,* and *P. integrifolia* are easily found in disturbed places such as roadside slopes, especially in rocky ground; *P. scheideana* and *P. mantiqueirensis* occur in partial shade at edges of *Araucaria* forests; and *P. exserta* grows in the shaded relief from shallow caves sculpted by the wind in sandstone.

The morphological circumscription of certain species is not easy, especially those related to *P. integrifolia,* characterized by purple and funnelform corolla, violaceous pollen, and stigma placed among the anthers of the didynamous pair of stamens. In a wide sense, this group corresponds to Wijsman's *P. integrifolia* complex (*P. integrifolia, P. inflata,* and *P. occidentalis* R.E. Fr.) and others to specific (*P. interior* T. Ando & Hashim., *P. riograndensis* T. Ando & Hashim., *P. littoralis* L.B. Sm. & Downs and *P. bajeensis*) and infraspecific (*P. integrifolia* subsp. *depauperata* (R.E.Fr.) Stehmann) taxa. This complex group of taxa called "integrifolia" comprises at least four distinct genetic lineages (Lorenz-Lemke, unpublished data).

A key to recognizing the species of *Petunia* is presented below, together with comments about morphology, nomenclature, geographic distribution, and habitat.

1.3.1 Key to the Native Species of Petunia

1a. Corolla salverform (tube cylindrical or slightly enlarged toward the apex).
2a. Corolla white. **2. *P. axillaris***
2b. Corolla purple or reddish.
3a. Corolla purple; anthers and stigma included in the corolla tube; heliophilous plants.
4a. Plant erect; corolla tube and throat purple; filaments adnate nearly to middle of corolla tube; pollen yellow. **14. *P. secreta***
4b. Plant procumbent, ascendant or climbing; corolla tube and throat whitish and purple reticulate-veined; filaments adnate below the middle of tube; pollen bluish or violet. **9. *P. mantiqueirensis***

3b. Corolla reddish; anthers and stigma exserted from corolla tube; sciophilous plants. **5. *P. exserta***
1b. Corolla funnelform or campanulate (tube clearly enlarged toward the apex).
5a. Corolla pink to bright red.
6a. Filaments adnated >9 mm to corolla tube base; stigma slightly exserted above the anthers of longest pair of stamens. **12. *P. saxicola***
6b. Filaments adnated up to 8 mm to corolla tube base; stigma located below the anthers of the longest pair of stamens. **11. *P. reitzii***
5b. Corolla purple.
7a. Stigma exserted above anthers of the longest stamens. **4. *P. bonjardinensis***
7b. Stigma located at the same level or below the anthers of the longest pair of stamens.
8a. Stigma located at the same level to the anthers of the longest pair of stamens; corolla throat pale purple to whitish with contrasting deep-purple reticulation; stigma >1.5 mm long, vertically two lobed. **13. *P. scheideana***
8b. Stigma located below the anthers of the longest pair of stamens; corolla throat purple with dark purple stripes or reticulation; stigma <1.2 mm long, not two lobed (except *P. occidentalis*).
9a. Plant repent, rooting at the nodes; leaves widely obovate or orbicular, usually rounded to the base, more rarely attenuate or short attenuate; calyx halfway lobed. **1. *P. altiplana***
9b. Plant erect, ascendant or decumbent, not rooting at the nodes; leaves ovate, elliptic, oblanceolate, or obovate, rarely orbicular, with attenuate or long-attenuate base; calyx deeply lobed (less so in coastal populations) (*Petunia integrifolia* complex).
10a. Anthers with channeled lobes at dehiscence. **8. *P. interior***
10b. Anthers with lobes flat at dehiscence.
11a. Plant viscid; leaves with prominent venation; corolla mouth reniform in frontal view, with intruded throat. **3. *P. bajeensis***
11b. Plant not obviously viscid; leaves with obscure venation; corolla mouth elliptic in frontal view, with flat throat.
12a. Stems decumbent; capsule subglobose with peduncle deflexed. **7. *P. integrifolia***
12b. Stems usually erect or ascendant; capsule ovoid with peduncle inflexed or weakly deflexed.
13a. Corolla limb 25–40 mm in diameter, filaments adnated <5 mm to the corolla tube base. **6. *P. inflata***
13b. Corolla limb 20–25 mm in diameter, filaments adnated >7 mm to the corolla tube base. **10. *P. occidentalis***

1. ***Petunia altiplana*** T. Ando & Hashim. (Figs. 1.2F and 1.3A–B) – This species is easily recognized by its rooting stems, usually broadly spatulate leaves, purple corolla, and stigma located below the anthers of the longest pair of stamens. Its repent habit of usually forming a round mat is unique to the genus. The radial growth pattern plus the massive blooming permit the use of the species for ornamental

Fig. 1.3 Petunia species. (**A**) and (**B**) *P. altiplana*; (**C**) *P. axillaris*; (**D**) *P. bajeensis*; (**E**) *P. bonjardinensis*; (**F**) *P. exserta*

purposes. This species is distributed in the highlands of Santa Catarina and Rio Grande do Sul, Brazil, in altitudes from 800 to 1200 m, and grows in outcrops or exposed roadside slopes (Ando and Hashimoto 1993).

2. ***Petunia axillaris*** (Lam.) Britton et al. (Figs. 1.2A–B and 1.3C) – It can be readily identified by its erect habit, salverform white corolla, yellow pollen, and inflexed pedicel in fruit stage. The flowers emit a scent at dusk and are visited by sphingid hawkmoths (Galeto and Bernardello 1993; Ando et al. 2001;

see Chapter 2). It exhibits the largest geographic distribution in the genus and is known to occur in Brazil (Rio Grande do Sul), Argentina, Uruguay, Paraguay, and Bolivia. Three allopatric subspecies have been accepted based on corolla tube length and stamen arrangement (Ando 1996; Kokubun et al. 2006). Individuals of *P. axillaris* are heliophilous and inhabit rocky sites, but can also be found along roadsides.

3. ***Petunia bajeensis*** T. Ando & Hashim. (Fig. 1.3D) – The species is characterized by its viscid vestiture, foliose stems forming a cushion-like structure, ovate, elliptic, or oblong leaves with prominent primary and secondary veins (showing the marked brochidrodomous venation), purple funnelform corolla, stamens adnated more than 7 mm to the base of the corolla tube, stigma located below the anthers of the longest pair of stamens, and deflexed pedicel in fruit stage. Vegetatively, the individuals of this species roughly resemble more robust plants of *P. bonjardinensis,* but the morphology of the flowers does not differ from that of *P. integrifolia* except for the larger size of the floral parts. To date found only in the extreme southern region of Rio Grande do Sul, Brazil, in the municipalities of Bajé, Canguçu, and Lavras do Sul, it can be found growing along roadside slopes (Ando and Hashimoto 1998).

4. ***Petunia bonjardinensis*** T. Ando & Hashim. (Figs. 1.2G and 1.3E) – The decumbent habit, with very fragile stems, villose vestiture, campanulate and purple corolla, stigma positioned above the anthers of the longest pair of stamens, and deflexed peduncles in fruit stage are characters permitting clear identification of the species. *Petunia bonjardinensis* is endemic to a small area near to the border of the southern Brazilian plateau, in the municipality of Bom Jardim da Serra, Santa Catarina (Ando and Hashimoto 1993), where it is not difficult to find individuals growing on roadside slopes.

5. ***Petunia exserta*** Stehmann (Figs. 1.2C and 1.3F) – *Petunia exserta* is unique in the genus, showing red (red-orange) corolla and distinct exserted stamens and stigma, attributes associated with hummingbird pollination (Stehmann 1987; Lorenz-Lemke et al. 2006; see Chapter 2). It shares the erect habit, salverform corolla, yellow pollen, and inflexed stalk with *P. axillaris* and *P. secreta.* This strictly endemic species is known only from the "guaritas" and adjacent areas, at the municipality of Caçapava do Sul, Rio Grande do Sul, Brazil, growing in shallow caves sculpted by the wind in sandstone towers.

6. ***Petunia inflata*** R.E.Fr. **–** *Petunia inflata* can be recognized by its ascendant habit, purple corolla with slightly constricted tube, and capsule with usually inflexed fruit-stalk. This species was originally described as differing from *P. violacea* [=*P. integrifolia*] because of its inflexed pedicel in the fruiting state and disjunct geographic distribution (Fries 1911). Smith and Downs (1966) considered *P. inflata* a synonym of *P. integrifolia,* but Wijsman (1982) resurrected the taxon at the subspecific level under *P. integrifolia,* a widespread species in southern South America with three geographic subspecies: *P. integrifolia* susbp. *integrifolia*, *P. integrifolia* subsp. *inflata* (R.E.Fr.) Wijsman, and *P. integrifolia* subsp. *occidentalis* (R.E.Fr.) Wijsman. In a recent study based on morphometric analysis of cultivated material, Ando et al. (2005a) accepted *P. inflata* as distinct from *P. integrifolia* and pointed

out the unfolded and straight calyx lobes as useful diagnostic characters. They also reported the existence of a hybrid zone in northwestern Rio Grande do Sul, Brazil.

7. ***Petunia integrifolia*** (Hook.) Schinz & Thell. (Figs. 1.2H and 1.4A) – This species is recognized by the following suite of morphological characters: decumbent stems, elliptic or obovate leaves (linear in some coastal populations), funnelform purple corolla, anthers completely opened (flat) after dehiscence, showing the bluish pollen, stigma placed below the anthers of the longest pair of stamens, and deflexed pedicels in the fruiting state. *Petunia integrifolia* inhabits the Pampas province and occurs in Argentina, Uruguay, and southern Brazil (from Rio Grande do Sul to the coast of Santa Catarina), growing on different kinds of substrata (latossols, sand-soils, and litosoils). It can also be found on disturbed areas such as roadsides or cultivated lands. This species was described and illustrated for the first time by Hooker in 1831 as *Salpiglossis integrifolia,* based on a cultivated plant at Glasgow Botanic

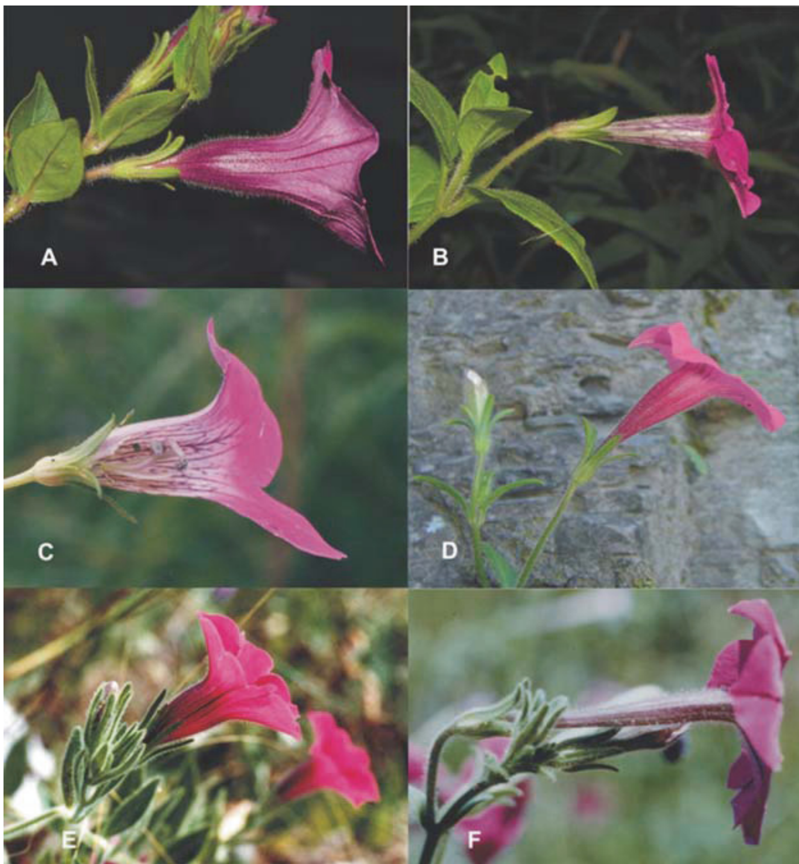

Fig. 1.4 Petunia species. (**A**) *P. integrifolia*; (**B**) *P. mantiqueirensis*; (**C**) *P. scheideana*; (**D**) *P. saxicola*; (**E**) *P. reitzii*; (**F**) *P. secreta*

Garden. These cultivated materials were obtained from seeds brought from Buenos Aires by John Tweedie in the autumn of 1830. Two years later, Lindley described and illustrated *Petunia violacea,* also based on plants obtained from Buenos Aires. For a long time, this synonym was employed as the valid name of the species in horticultural and genetic literature.

Populations growing in the sandy soils of beaches within Santa Catarina island, in Santa Catarina State, with elongated stems bearing narrow and glabrous leaves, were described by Smith and Downs (1966) as a different species, named *P. littoralis*. Ando et al. (1995), analyzing the morphological characters of infraspecific taxa of *P. integrifolia*, concluded that *P. littoralis* does not differ enough to be considered a distinct species and that the floral morphology resembles that of *P. integrifolia* var. *depauperata* (R.E.Fr.) L.B.Sm. & Downs [= *P. integrifolia* subs. *depauperata* (R.E.Fr.) Stehmann].

Based on analysis of morphological and molecular data (Lorenz-Lemke unpublished data), Stehmann and Bohs (2007) accept two subspecies: *P. integrifolia* subsp. *integrifolia* and *P. integrifolia* subsp. *depauperata* (Fries) Stehmann & Semir. The former is widespread in the Pampean region of Argentina and Uruguay, as well as in the continental southern part of Rio Grande do Sul, Brazil, while the latter occupies the quaternary deposits along the coast, from the extreme southern Rio Grande do Sul to Florianópolis, in Santa Catarina.

Ando and Hashimoto (1998) described *P. riograndensis* growing in the Serra do Sudeste, a low-altitude mountain range (600 m) that crosses southern Rio Grande do Sul, Brazil, in an east–west direction. The main morphological character distinguishing *P. integrifolia* from *P. riograndensis* is the presence of five grooves on the outer surface of the cylindrical portion of the corolla, observed when the calyx is removed. In all other vegetative and floral traits, individuals of *P. riograndensis* resemble those of *P. integrifolia,* and they could be included in the range of variation within the latter species. For this reason, *P. riograndensis* is here considered a synonym of *P. integrifolia.*

Chen et al. (2007) analyzed the genus based on *Hf1* gene sequences, and their results corroborate that the four taxa, *P. integrifolia* (including the two infraspecific taxa), *P. riograndensis* and *P. littoralis,* could be treated as conspecific. In our opinion, the acceptance of a wide circumscription of *P. integrifolia* is the best decision while taxonomic and evolutionary relationships are not totally clear.

8. ***Petunia interior*** T. Ando & G. Hashim. – This species is extremely similar to *P. integrifolia,* from which it can be distinguished only by minor characters such as the stem often divided into three branches around the node bearing the first flower and the channeled lobes of the dehisced anthers. After dehiscence, the anthers do not twist as in all other species of the genus, but orient the pollen upward relative to the ground. The ascendent stems and the corolla form and size resemble those of *P. inflata,* but the orientation of the pedicel is different. Its geographic distribution ranges from northwestern Rio Grande do Sul and western Santa Catarina (with some disjunct places) in Brazil to the province of Misiones, Argentina (Ando et al. 2005a).

9. ***Petunia mantiqueirensis*** T. Ando & Hashim. (Figs. 1.2D and 1.4B) – This species is characterized by its long stems, reaching up to 4 m, petiolate,

decurrent, ovate or elliptic leaves, purple, tubulose-funnelform corolla (30–35 mm), with reticulate-veined throat, stigma slightly exserted above the anthers at the same level of the corolla limb, and weakly deflexed pedicel in fruiting stage. The geographic distribution of *P. mantiqueirensis* is restricted to the Serra da Mantiqueira, in Minas Gerais, southeastern Brazil, where few populations are known. Individuals of *P. mantiqueirensis* are shade tolerant and grow on the border of the *Araucaria* or montane forests, as well as on more open places, at altitudes ranging from 1000 to 1700 m above sea level (Ando and Hashimoto 1994).

10. ***Petunia occidentalis*** R.E.Fr**.** – The erect or ascendant habit, the small flowers with purple funnelform corolla showing a long cylindrical base (>8 mm), the weakly didynamous stamens, stigma bilobed, positioned slightly above the anthers of the longest pair of stamens, and inflexed fruit-stalk are characters that clearly distinguish this species from other *Petunia.* Its geographic distribution is restricted to the Sub-Andean mountains (from 650 to 2000 m of altitude) in northwestern Argentina (Jujuy, Salta) and southern Bolivia (Tarija), being separated from the other *Petunia* species by the Chaco, a large, flat region covered by a dry forest, in northern Argentina, Bolivia, and Paraguay (Fries 1911; Tsukamoto et al. 1998).

11. ***Petunia reitzii*** L.B.Sm. & Downs (Figs. 1.2I and 1.4E) – This species is distinguishable by its ascendent habit, bright red, funnelform corolla, with filaments adnated less than 8 mm to the base of the corolla tube. *Petunia reitzii* is endemic to the oriental border of the southern Brazilian plateau in Santa Catarina and seems to be restricted to a small area between the municipalities of Bom Retiro and Urubici, at altitudes of about 1000 m and associated with *Araucaria* forest. It grows on the walls of small cliffs beside rivers, hanging freely in space (Ando et al. 1999), but can also be found along exposed roadside slopes.

12. ***Petunia saxicola*** L.B. Sm. & Downs (Fig. 1.4D) – This species is similar to *P. reitzii*, especially in the bright red color of the corolla, but *P. saxicola* has a longer corolla tube, reaching 40–45 mm, with filaments adnated more than 9 mm to the base of the corolla tube, stigma slightly exserted above the anthers of longest pair of stamens, and glabrous leaves. The saxicolous habit of this species is unique in the genus, and individuals are found growing on humid and rocky escarpments of a small area of the border of the southern Brazilian plateau, in the municipality of Otacilio Costa, Santa Catarina. Only one population of *P. saxicola* is known to exist.

13. ***Petunia scheideana*** L.B. Sm. & Downs (Figs. 1.2 J and 1.4C) – *Petunia scheideana* is characterized by its very variable habit, with long-branched stems, sometimes up to 3 m long (either procumbent, ascendant or climbing), usually petiolate and glabrous leaves (except margin and ribs), ovate or elliptic blades, funnelform purple corolla with short tube (13–15 mm long) showing a deep purple reticulate-veined throat, anthers of the long stamens evidently separate from each other, pollen bluish, stigma bilobed placed at the same level as the anthers of the longer pair of stamens, and pedicels usually elongated, weakly deflexed in the fruiting stage. The geographic distribution ranges from higher altitudes (800–1000 m) in Paraná and Santa Catarina, Brazil, often associated with *Araucaria* forests, westward into the lowlands of extreme northern Misiones, Argentina (about 200–300 m). In Brazil, it can be found in the ecotonal zone between grasslands

and forests, climbing in the shrubby or arboreal vegetation along the border of the *Araucaria* forest or more spreading in open areas or roadside slopes. Nevertheless, in Argentina, Ando, Soto, and Suarez (2005c) reported its occurrence at open roadsides within thick forest (subtropical semideciduous forest), and not in *Araucaria* forest.

Petunia guarapuavensis T. Ando & Hashim. is treated here as a synonym of *P. scheideana.* Ando and Hashimoto (1995) compared their new species only to *P. integrifolia,* but not to *P. scheideana,* with which it shares all vegetative and floral attributes. The geographic distribution of *P. guarapuavensis* is in the more continental region of the Serra Geral, the Guarapuava High Plateau and adjacent areas of Santa Catarina, and must represent only disjunct populations of *P. scheideana*.

14. ***Petunia secreta*** Stehmann & Semir (Figs. 1.2E and 1.4F) – *Petunia secreta* is an annual, erect or ascendant, easily recognizable by its purple and salverform corolla, yellow pollen, and erect fruit-stalk. The corolla form is shared only with *P. exserta* (red-orange) and *P. axillaris* (white). *Petunia secreta* is endemic to the place called "Pedra do Segredo" and adjacent areas around the municipality of Caçapava do Sul, in Rio Grande do Sul, southern Brazil. It is clearly heliophilous, inhabiting the top of conglomerate sandstone towers at about 300–400 m elevation and visited by bees (Stehmann and Semir 2005).

Hunziker (2001) accepted *P. patagonica* as belonging to the genus, but we are considering it a doubtful name. This species was originally described under *Nierembergia* by Spegazzini (1897) based upon material collected in San Jorge Gulf, Argentina. Millan (1946) transferred this species to *Petunia,* but its morphological characters (linear leaves, solitary flowers, and campanulate calyx with obtuse and short lobes) do not match those described for the genus (Table 1.1). Further studies are necessary to clarify the identity of this taxon

1.4 Patterns of Geographic Distribution

The genus *Petunia* is endemic to South America, with subtropical distribution ranging from 22° to 39°S (Fig. 1.5). The major species richness is found in Brazil (13), followed by Argentina (5), Uruguay, Paraguay, and Bolivia (2). All species occur in Brazil, except for *P. occidentalis,* which has a disjunct distribution restricted to the Sub-Andean mountains in northwestern Argentina and southern Bolivia.

We can distinguish two principal areas of occurrence (centers of diversity) of *Petunia* species, both located in southern Brazil, where the genus must have radiated and spread in recent times (Kulcheski et al. 2006): (a) lowlands of the Pampean region (Fig. 1.5A) and (b) highlands of the southern Brazilian plateau (Fig. 1.5B). The two areas, where nine species (64%) are known to occur, are included in the Pampean and Paranense provinces, respectively (Cabrera and Willink 1980).

The area of highest richness is located at low altitudes, in a region known as Serra do Sudeste, included in the Brazilian pampa. The Pampas occupy a vast region in Argentina (Buenos Aires, La Pampa, Santa Fe, and Córdoba), Uruguay, and

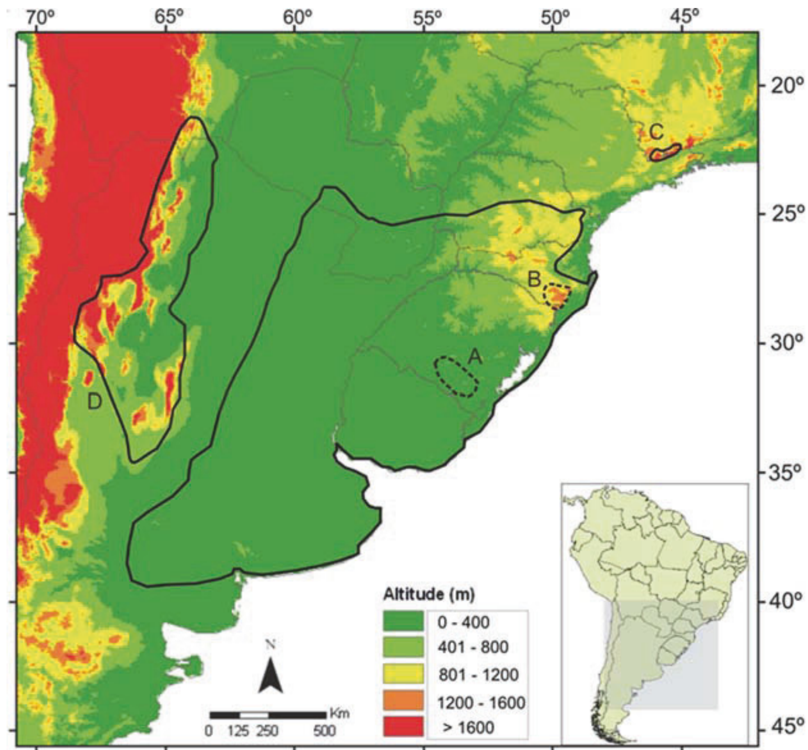

Fig. 1.5 Geographic distribution of *Petunia* (*solid lines*). The two centers of diversity (*dotted line*) (**A**) Serra do Sudeste in Rio Grande do Sul, Brazil, and (**B**) Highlands of Serra Geral in Santa Catarina, Brazil, are indicated, as well as the major disjunct regions (**C**) Serra da Mantiqueira, in Minas Gerais, Brazil, and (**D**) the Sub-Andean region in Argentina and Bolivia

southernmost Brazil and are covered by temperate grasslands. The Serra do Sudeste and neighboring places in southern Rio Grande do Sul have a low-altitude mountain range (reaching 600 m in some areas of the Escudo Sul-Rio-Grandense) and a set of diverse edaphic conditions. *Petunia integrifolia* and *P. axillaris,* parental species of garden petunia, are sympatric in these areas (Ando et al. 2001). Five species of *Petunia* grow in Serra do Sudeste, three of them strict endemics (*P. bajeensis, P. exserta,* and *P. secreta*).

The second area comprises the border of the Serra Geral (a large escarpment ranging from Minas Gerais to Rio Grande do Sul) in the state of Santa Catarina. In this area, species of *Petunia* grow associated with grasslands, along forest borders, or on outcrops associated with *Araucaria* moist forests, at altitudes ranging from 800 to 1800 m. For this area we can list the occurrence of four species, three of them strict endemics, *P. bonjardinensis, P. reitzii* and *P. saxicola,* restricted to the higher area of the plateau in Santa Catarina.

There are two major disjunct areas of occurrence of *Petunia*: the Serra da Mantiqueira in Minas Gerais, Brazil, and the Sub-Andean region in Argentina and Bolivia (Fig. 1.5C, 1.5D). In the first, only *P. mantiqueirensis* is found, an endemic species phylogenetically related to the Brazilian highland group. The geographic barrier corresponds to the Atlantic rainforest and savanna that cover almost all the São Paulo state. The Sub-Andean region is separated from the core *Petunia* distribution by the Chaco, a large and drier region, and inhabited by two taxa: *P. axillaris* subsp. *subandina,* which grows from San Luis, Argentina, toward Tarija, Bolivia (Ando 1996; Kokubun et al. 2006), and *P. occidentalis*, with distribution restricted to northern Argentina and adjacent areas in Bolivia (Fries 1911; Tsukamoto et al. 1998).

The subtropical range of *Petunia,* with centers of diversity in southern Brazil, overlaps with that of *Calibrachoa.* The two groups are widely separated geographically from the core distribution of the other herbaceous Petunieae (sensu Olmstead and Bohs 2007) (except in part for *Bouchetia* Dunal and *Nierembergia*). As *Petunia* and *Calibrachoa* are closely related (Kulcheski et al. 2006) and reproductively isolated (Wijsman 1983; Watanabe et al. 1996a), we suppose that they had a congruent biogeographic history with radiation and expansion. The morphological floral similarity of many species of *Petunia* and *Calibrachoa,* basis of the taxonomic generic confusion, must represent an example of convergence. Studies realized by Wittmann, Radtke, Cure, and Schifino-Wittmann (1990) indicated that some species of both genera are melittophilous and share similar groups of pollinators. During field work we could observe the occurrence of syntopic pairs of mellitophilous species of *Petunia* and *Calibrachoa* in almost all areas of southern Brazil. In Petunieae, pollination by bees was reported for the genera *Nierembergia* (Cocucci 1991) and *Petunia* s.l. (Wittmann et al. 1990). Nevertheless, species of *Nierembergia* are pollinated primarily by oil-collecting bees, while *Petunia* and *Calibrachoa* are visited by bees searching for pollen and nectar (Wittmann et al. 1990; Ando et al. 2001; Stehmann and Semir 2001).

1.5 Evolutionary Relationships and Endemic Species Conservation

Classification of subfamilies and tribes of Solanaceae has changed significantly in the last decade. Using the circumscription of Fries (1911), Hunziker (1979) considered *Petunia* s.l. related to *Nicotiana,* belonging to the tribe Nicotianeae, subfamily Cestroideae (Hunziker 1979). After the proposal of Wijsman and de Jong (1985) and its nomenclatural consequence (Brummitt 1989), D'Arcy (1991) accepted the genus *Calibrachoa* as distinct within Nicotianeae. Olmstead and Palmer (1992) investigated subfamilial relationships and character evolution in Solanaceae based on chloroplast DNA phylogeny, reporting that *Petunia* s.s. (*P. axillaris* with $2n = 14$) and *Fabiana* constitute a sister group and that both genera plus *Brunfelsia* L. might best be combined to form a new tribe. *Fabiana* is a Patagonian and Andean

genus with about 15 species, consisting of small ericoid shrubs or chamaephytes (Barboza and Hunziker 1993). It shares morphological characters with some *Calibrachoa* (but not *Petunia*), such as the woody habit, brachyblasts, linear leaves, a funnelform corolla, and a basic chromosome number of $n = 9$. However, they differ in their geographic distributions, with *Fabiana* in the Andes, and *Calibrachoa* (as well as *Petunia*) in the Pampas and the southern Brazilian Plateau.

Data of Olmstead et al. (1999) based on chloroplast DNA variation delimited a traditional Cestroideae as five smaller, monophyletic informal groups: Cestroideae, Petunioideae, Schizanthoideae, Nicotianoideae, and Schwenckioideae. But in a recent summary published by Olmstead & Bohs (2007), the five major clades were treated as tribes of Cestroideae, with *Petunia* plus eight genera placed in Petunieae. The phylogenetic relationships within Petunieae remain unresolved and further work is needed, especially in *Brunfelsia, Fabiana, Leptoglossis* Benth., and *Nierembergia*.

The evolutionary history of *Petunia* Juss. has been recently investigated through different genetic markers. Short genetic distances among the species and consequent poorly resolved phylogenies were the general pattern, indicating recent diversification. Ando et al. (2005b) analyzed 52 taxa of *Petunia* s.l. by plastidial RFLP markers; Kulcheski et al. (2006) investigated 11 *Petunia* s.s. taxa with eight sequence markers for the three plant genomes, and Chen et al. (2007) studied 19 Petunia s.s. for two sequence markers, one nuclear, the other plastidial. Ando et al. (2005b) and Kulcheski et al. (2006) detected two major groups: one corresponds to highland species (altitude over 800 m) and the other to lowland species (altitude below 800 m). The three papers confirmed the genus monophyly, the *Calibrachoa* position as the sister taxon, and the large genetic distance between the clade containing *Petunia* plus *Calibrachoa* and the other genera of the tribe Nicotianeae.

While species description and delimitation have traditionally been based on morphology, biologists have recognized that morphological divergence may have little relationship to the degree of genetic differentiation between specific lineages (Orr 2001). Therefore, it could be difficult to predict the genetic cohesiveness of a group based on its morphological differentiation or taxonomic status alone. For instance, Schaal, Hayworth, Olsen, Rauscher, and Smith (1998) showed many examples of infraspecific differentiation in plants, with the occurrence of isolated lineages across its range. On the other hand, genetic exchange between well-established, morphologically distinct species is a widespread phenomenon in plants (Mallet 2005). Additionally, groups that undergo adaptive radiation frequently exhibit rapid morphological differentiation and may present little genetic divergence and weak reproductive barriers between species (Seehausen 2004; Ando et al. 2005b).

In recent years, a recurring claim with regard to the species problem is that most species concepts have implicit similarities and are consistent with the idea that species are evolving lineages or populations (Hey 2001). The phylogeographic approach may be the needed bridge between the species concepts and empirical studies, offering a means to understand the historical processes that have influenced the structure of genetic variation now observed in the species (Avise 2000). Plant phylogeographic studies, however, remain rare in comparison to those of animals,

owing in part to the difficulty of obtaining markers with an appropriate level of intraspecific polymorphism (Schaal et al. 1998; Shaw et al. 2005). These investigations, however, increased in recent years once nuclear (Strand, Leebens-Mack, and Milligan 1997; Olsen and Schaal 1999; Gaskin and Schaal 2002) and plastid (Maskas and Cruzan 2000; Lorenz-Lemke et al. 2006) sequences, as well as nuclear microsatellites (Collevatti, Grattapaglia, and Hay (2001), which exhibit adequate variation, began to be identified. The phylogeographic approach has allowed, in some cases, detailed understanding of populational and historical processes, especially in plants of economic and ecological interest (Schaal et al. 2003). Since phylogeography is applicable to problems both below and above species boundaries, its analysis can cover a wide range of plant evolutionary patterns (Schaal et al. 1998).

Lorenz-Lemke et al. (2006) investigated the molecular diversity of the endemic *Petunia exserta* and its closely related species *P. axillaris* subsp. *parodii* and the first case of natural interspecific hybridization between *Petunia* species. *P. exserta* is the only ornithophilous species of the genus and is characterized by showy red flowers with anthers and stigma strongly exserted. In spite of its exuberant color, *P. exserta* is not the parent of red-flower commercial hybrids, having been discovered some decades after these hybrids had been produced (Griesbach, Stehmann and Meyer 1999; Ando et al. 2000). Endemic in a very small area (about 500 km^2) of the Serra do Sudeste region, it has so far been found growing within shallow caves in the rock (shelters) on sandstone towers, which seems to be a very restricted and inhospitable environment for the other species of this genus (Stehmann 1987). *P. exserta* and *P. axillaris* are closely related species (Ando et al. 2005b; Kulcheski et al. 2006) that share diverse morphological characteristics. *P. axillaris* displays white flowers that emit a strong fragrance at nightfall and produce a considerable amount of nectar to attract nocturnally active hawkmoths (Sphingidae) (Ando et al. 2001), see Chapter 2. Its distribution range includes Bolivia, Argentina, Uruguay, and Brazil's extreme south, and it can be found in rocky outcrops and also in disturbed habitats such as the margins of highways (Ando 1996). In various locations the distribution of *P. axillaris* overlaps that of the other species of the genus, and it is possible to achieve artificial crossings between them and *P. axillaris* (Watanabe et al. 1996a). The analysis of *trnH-psbA*, *trnS-trnG,* and *psbB-psbH* chloroplast (cp) DNA markers by Lorenz-Lemke et al. (2006) revealed 13 haplotypes, and the network showed two main genetic clades, which probably represent the original gene pool of the two species in the region. In general, individuals of a given population presented the same haplotype, independent of phenotype, corroborating the hybridization hypothesis. Field observations suggest that hummingbirds are responsible for the interspecific gene flow. Analysis of molecular variance (AMOVA) revealed high interpopulational diversity among the towers. The low rate of gene flow between populations is possibly related to the autochoric seed dispersion system, wherein dispersion is entirely by free fall or explosive propulsion by a fruit that opens suddenly or by a trip lever (van der Pijl 1982).

Hybridization with a widespread congener can create serious consequences for rare plant species (Levin, Francisco-Ortega, and Jansen 1996). The low levels of interspecific genetic variability disclosed by diverse molecular markers (Kulcheski

et al. 2006; Lorenz-Lemke et al. 2006) indicate that the separation between *P. axillaris* and *P. exserta* is extremely recent. Habitat shift and modifications of floral traits are some of the probable factors involved in the isolation between these species. Therefore, hybridization with *P. axillaris* can constitute a risk for the maintenance of *P. exserta*'s typical populations, as it allows for the dilution or loss of the unique adaptations of this species. A question that remains is whether the hybridization between *P. axillaris* and *P. exserta* is recent (possibly related to anthropogenic disturbance) or a more ancient historical process. A considerable change in the floral composition of the Serra do Sudeste region took place with the start of more intensive human colonization around 1800, as the introduction of agricultural food and forage crops led to the almost complete degradation of the original vegetation. Some 30 endemic plant species with very restricted distributions are found in this region, and among these, *P. exserta* seems to require special attention due to the factors presented here, such as very strict distribution and habitat requirements (Guadagnin, Larocca, and Sobral 2000). Reduction in the number of individuals, and even local population extinctions, have been documented for *P. exserta* (Guadagnin et al. 2000), and the species has now been added to the local list of endangered species (http://www.sema.rs.gov.br/sema/html/pdf/especiesameacadas.pdf). In addition to *P. exserta*, the occurrence of other endangered species indicates the need for the establishment of conservation units in certain Serra do Sudeste areas, especially in the rocky outcrops where these taxa are predominantly found.

In the southern and southeastern Brazilian highlands, another *Petunia* group shows morphological and genetic patterns that indicate recent speciation. Lorenz-Lemke (unpublished data) have analyzed the sequences of plastidial markers of seven closely related taxa (*P. altiplana*, *P. bonjardinensis*, *P. guarapuavensis* [treated as a distinct species], *P. mantiqueirensis*, *P. reitzii, P. saxicola,* and *P. scheideana*) that occur associated with grasslands, and link their reproductive isolation to geographic discontinuity. These species are melittophilous (pollination carried out by bees), self-incompatible (Tsukamoto et al. 1998), and genetically close (a monophyletic group, according to Kulcheski et al. 2006). As the natural pollinators are shared and artificial interspecific crossings result in fertile hybrids (Watanabe et al. 1996a), it was suggested that geographic isolation is the major factor involved in the maintenance of species integrity. All data suggest that the diversification of this "highland clade" may be a product of allopatric speciation triggered by habitat range shifts through the Pleistocene period. The dating analyses by Lorenz-Lemke (unpublished data) indicated a Pleistocene radiation and a high diversification rate, comparable to plant groups that undergo continental or island radiation (Baldwin and Sanderson 1998; Klak, Reeves, and Hederson 2004; Hughes and Eastwood 2006). The lack of well-differentiated clades between the species and haplotype sharing suggest the persistence of ancestral polymorphisms through speciation events and/or past interspecific hybridization. AMOVA revealed a high level of population structuring, distinct seed dispersion abilities between the species, and the influence of the Pelotas river on the genetic structure of *P. altiplana* and *P. bonjardinensis*. Five of the species studied are narrowly endemic and critically threatened by habitat loss due to *Pinus* forestation and diverse agricultural use of grassland areas.

The remaining grassland areas of the southern and southeastern Brazilian highlands are sparse and small, due to historical and current processes. Paleoenvironmental reconstructions from pollen records confirmed that these grasslands were natural formations that covered the Brazilian highlands uninterruptedly since at least the last glacial age (Behling 2002). These data also demonstrated that their biodiversity has suffered anthropogenic disturbances since the early Holocene, with the increase of fire events probably related to the onset of occupation by Amerindians. Currently, large grassland areas have been replaced by exotic pine forests and different agricultural practices, which represent the main threats for these floristic formations (Behling and Pillar 2007).

The *Petunia* species are directly affected by grassland destruction. Besides being restricted to this biome, the greater number have an exceptionally small geographic distribution, the extreme situation occurring with *P. saxicola*, for which only one population is known. It is not known if these micro-endemisms are natural or have suffered anthropogenic influence. Moreover, the cpDNA results indicated that seed flow between the populations is naturally low at this biome. Furthermore, the present changes are happening at a fast pace and can have significant effects in the demographic dynamics of these species, especially in reducing the genetic diversity (that already is very limited) and increasing random genetic drift effects (Ellstrand and Elam 1993). As the largest portion of their genetic diversity is interpopulational, most of these *Petunia* populations should be considered as separate management units.

P. reitzii and *P. saxicola* were already included in the "List of Threatened Brazilian Flora," classified as critically endangered (*2004 IUCN Red List of Threatened Species*, available at www.iucnredlist.org). The species-range information and the genetic data obtained by Lorenz-Lemke (unpublished) suggest that *P. mantiqueirensis*, *P. bonjardinensis,* and *P. scheideana* should also be included. Beyond their great biological value as an endemism center, the Brazilian highlands deserve special attention due to their remarkable flora richness, shaped by the convergence of tropical and temperate taxa (Rambo 1951a, b). These transitional regions are extremely important for the diversification and speciation processes, and to preserve populations that occur along these areas can be a protection strategy for possible biological responses to environmental or climatic changes (Smith, Kark, Schneider, and Wayne 2001).

In the *Petunia* genus, the integrifolia group presents the most complex taxonomy and comprises species, varieties, and subspecies. These taxa, in which the garden petunia parentals are included (*Petunia* x *hybrida* pink flowers), show one of the widest distributions in South America, since they can be found in Argentina, southern Brazil, and Uruguay. This set of organisms (*P. integrifolia* subsp. *integrifolia*, *P. integrifolia* subsp. *depauperata*, *P. littoralis*, *P. interior*, *P. inflata*, *P. riograndensis,* and *P. bajeensis*) presents very similar morphological characteristics, in which it is difficult to outline stable entities. Some floral characteristics are shared by all these taxa, such as a magenta or purple infundibuliform corolla, violaceous pollen, and stigma located between the anthers of the larger or median-sized stamens. On the other hand, the considerable variation seen in vegetative character and habitat

among taxa in the integrifolia group, observed across its wide distribution area, led to various changes in its taxonomic status through the years (Fries 1911; Smith and Downs 1966; Wijsman 1982; Ando et al. 1995, 2005a). Species distinction is usually made by geographic distribution, as well as by overlapping vegetative and floral characteristics.

Using a phylogeographic approach, Lorenz-Lemke (unpublished data) analyzed the taxa that comprise the integrifolia group with the intent to better understand the diversification processes and to contribute to a more adequate delimitation of its units. Plastidial markers showed that this group comprises four distinct lineages clearly delimited geographically and that the actual integrifolia taxonomy should be revised. *P. bajeensis* and *P. inflata* seem to form two taxonomic units independent of the other forms of the group. Genetic differences were not found between *P. integrifolia* subsp. *integrifolia* and *P. riograndensis*, between *P. integrifolia* subsp. *depauperata* and *P. littoralis*, or between *P. inflata* and *P. interior*. These features suggest that the few morphological characters that distinguish them are not appropriate for taxonomic delimitation. Environmental heterogeneity (especially luminosity and edaphic factors) and phenotypic plasticity are probably related to the morphological variability found among its natural populations. The existence of well-established clades indicates that these taxa are historically isolated. While the other three clades occur in a geologically ancient area, the *P. integrifolia* subsp. *depauperata*/*P. littoralis* clade occurs in areas that were under strong influence of the Pleistocene marine transgression/regressions. This lineage has its occurrence limited to quaternary sediments of the coastal plain of southern Brazil. Using the accurate geological age of this area as a calibration point for the continental/coastal lineages divergence (400,000 ybp; Villwock and Tomazelli 1995), a substitution rate was calculated. This result will make possible better inferences about the evolutionary history of the *Petunia* and its sister group *Calibrachoa*, situating the diversification events in the climatic, floristic, and geologic contexts in which they occurred.

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Chapter 2
Petunia as a Model System for the Genetics and Evolution of Pollination Syndromes

Thomas Gübitz, Maria Elena Hoballah, Alexandre Dell'Olivo, and Cris Kuhlemeier

Abstract In recent years Petunia has become a promising model system for studying the genetics and evolution of pollination syndromes. Here we provide a brief introduction to the issue of pollination syndromes, explain why Petunia is a suitable model for its study, present useful background information about pollinators and plants, review recent studies, and discuss questions related to the genetics and evolution of Petunia pollination syndromes.

2.1 Petunia as a Model for the Evolution of Pollination Syndromes

Flowering plants often feature pollination syndromes, sets of traits such as flower color, morphology, scent and nectar production, which appear to fit the morphology and behavior of specific pollinator types (Faegri and van der Pijl 1979). The general correspondence of floral traits and pollinators makes it possible to predict which pollinators are attracted to a given flower phenotype. The genus *Petunia* features three pollination syndromes: bee, hawkmoth, and hummingbird pollination. The bee-pollinated Petunia species, such as *Petunia integrifolia*, feature purple flowers with a wide floral tube into which bees crawl in order to reach pollen and nectar. These bee-pollinated flowers produce little nectar and hardly any scent. The hawkmoth-pollinated *Petunia axillaris* flowers feature white corollas with narrow tubes, allowing only nectar feeders with long probosces to reach the abundant nectar. Characteristic for hawkmoth-pollinated flowers, *P. axillaris* flowers produce large amounts of odor after dusk. *Petunia exserta* flowers bear the hallmarks of hummingbird pollination: the flower corolla is red, the petal limbs reflexed, anthers and style exserted, and the flowers emit no detectable scent. Similar to hawkmoth-pollinated flowers, *P. exserta* flowers have a long tube carrying copious amounts of nectar.

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Published phylogenies (Ando et al. 2005; Kulcheski et al. 2006) suggest that bee pollination in Petunia species such as *P. integrifolia* represents the ancestral state in the genus and that hawkmoth pollination in *P. axillaris* evolved from bee pollination in the *P. axillaris* ancestor. Nuclear DNA sequences suggest that hummingbird pollination in *P. exserta* has evolved from hawkmoth pollination (unpublished data).

2.1.1 The Complexity of Pollination Syndromes

It is generally thought that selection by pollinators drives floral evolution and also explains evolutionary shifts in pollination syndromes. The underlying idea is that plants increase their fitness by adapting to the most efficient (and abundant) pollinator, that is, the pollinator that transfers the most pollen to conspecific flowers. Despite the frequently observed match of pollinators and flower types, there are some controversies surrounding pollination syndromes. First, the association of a single plant species and a single pollinator is rarely as exclusive as is found in some orchid species. In fact, flowers corresponding to a particular pollination syndrome are sometimes found to be visited by other (non-specialist) pollinators. In some cases the expected specialist pollinator, deduced from a pollination syndrome, may be rare or even absent and other (non-specialist) pollinators may be more important for pollination (Herrera 1996). Besides such empirical criticism, evolutionary theory predicts that extreme dependence of a plant species on one or a few pollinators always carries a risk of extinction, because if pollinators decline, so will the adapted plant species (Waser, Chittka, Price, Williams, and Ollerton 1996; Johnson and Steiner 2000; Fenster, Armbruster, Wilson, Dudash, and Thompson 2004). Furthermore, in addition to pollinators, other agents such as nectar robbers, herbivores, or pathogens may interact with floral traits and affect the evolution of these traits (Fenster et al. 2004). Despite these caveats, the pollination syndrome concept is useful for generating hypotheses about likely pollinators and the adaptive value of floral traits in relation to these pollinators.

2.1.2 Phenotypic and Genetic Approaches to Pollination Syndromes

Major aims for the study of pollination syndromes are to demonstrate the adaptive value of floral traits and to elucidate how these traits have come about. The adaptive value of pollination traits can be tested in two ways: by phenotypic manipulation or by genetic approaches. For decades ecologists have manipulated natural or artificial flowers in order to test the attractiveness of particular floral features to pollinators, that is, altering the size of attractive petals or bracts (Fenster et al. 2004). An alternative approach to produce phenotypes for testing is to cross taxa displaying divergent pollination traits. This approach allows for the establishment of lines carrying chromosomal fragments determining the phenotype of one taxon in the

genetic background of another. Usually near-homogeneous lines are obtained from F2 or backcross (BC) populations by selfing individual lines for several generations (usually $n > 4$) generating recombinant inbred lines (RIL) or backcross inbred lines (BIL).

Genetic compatibility of plant taxa also allows for the genetic dissection of intra- or interspecific quantitative differences by quantitative trait locus (QTL) mapping. For this, a segregating population derived from an initial cross of the two parental lines of interest is phenotypically and genetically characterized. First, a genetic linkage map is established from the genotypic marker data, followed by a test of association between genetic markers and phenotypic values. The QTL approach takes the linkage of markers into account in order to estimate the contribution of chromosomal regions to the phenotypic variation in the segregating population. QTL analysis can also provide estimates of the size effect and the number of loci contributing to the variation in a trait. This is of particular interest for the genetic dissection of pollination syndromes. Determining the number of genes involved and their quantitative effects is critical for understanding the evolutionary transition from one pollination syndrome to another. Initial QTL analysis can be followed by positional cloning of genes underlying trait differences. Cloning of these genes will allow for unraveling of the molecular basis and the evolution of phenotypic variation. Finally, transgenic experiments may allow for the manipulation of single genes to test their phenotypic effects.

2.1.3 Petunia as a Model System for Studying Natural Variation

Among other emerging model systems for the study of pollination syndromes, such as *Ipomoea, Aquilegia, Penstemon* and *Mimulus*, *Petunia* occupies a special role, as it is, at present, the only genus that is genetically accessible, with several available genetic tools (Galliot, Stuurman, and Kuhlemeier 2006a). Among other traits, a relatively short generation time, ease of culturing, and particularly, the availability of transposon tagging (see Chapter 17) and genetic transformation (see Chapter 19), make Petunia an excellent system for studying the genetics of phenotypic traits. Transposon tagging is usually performed in a *Petunia hybrida* background (W138) that has about 200 active copies of the transposon *dTph1* (Gerats et al. 1990). QTL from wild species can be tagged with this system by introgression into this *P. hybrida* background. Hence, BIL produced from introgressing *P. axillaris parodii* S7 or *P. integrifolia inflata* S6 chromosomal fragments into a W138 *P. hybrida* background provide a resource for gene isolation (Stuurman et al. 2004). Further, *dTph1* activity can be almost entirely shut down by introgression of a *P. integrifolia inflata* chromosomal fragment on chromosome I, allowing for the stabilization of tagged mutants (Stuurman and Kuhlemeier 2005).

The possibility of testing the preference of pollinators for specific flower traits is essential for establishing the adaptiveness of these traits. In Petunia at least one natural pollinator, the hawkmoth *Manduca sext*a, can be reared in the laboratory and is commercially available.

2.2 Evolution of Hawkmoth from Bee Pollination Syndrome

Flowers representing bee and hawkmoth pollination syndromes differ markedly in flower morphology, color, scent, and nectar production. Bee-pollinated flowers tend to have shorter and wider corolla openings than hawkmoth-pollinated flowers, which often feature long corolla tubes. Many bee-pollinated flowers appear morphologically less specialized than most hawkmoth-pollinated flowers; that is, their morphology allows a broader spectrum of pollinators to access pollen and nectar.

P. integrifolia and *P. axillaris* correspond to this pattern of trait differences in bee and hawkmoth pollination syndromes. Here, we focus on *P. integrifolia* (subspp. *integrifolia* and *inflata*) representing bee pollination and *P. axillaris* (subspp. *axillaris and parodii*) representing hawkmoth pollination. Although *P. integrifolia* and *P. axillaris* may occur in sympatry, and cross-pollination by hand produces viable seed, the failure to observe natural hybrids suggests that one or more prezygotic isolation barriers exist (Ando et al. 2001). Ethological isolation (due to visitation by different pollinators) is one likely factor contributing to this isolation barrier.

P. integrifolia is primarily visited by solitary bee species, in particular by species of *Callonychium*, *Calliopsis,* and *Leioproctus* (Figs. 2.1 and 2.2). The *Calliopsis*

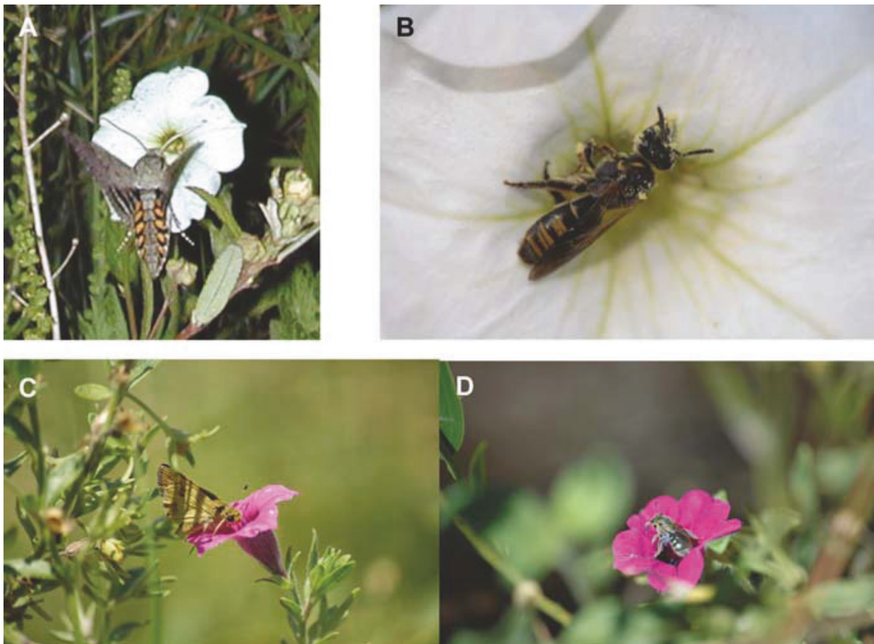

Fig. 2.1 Pollinators of *P. axillaris* and *P. integrifolia* in the wild in Uruguay. (**A**) A nocturnal sphinx moth (*Manduca* spp.) nectar feeding on *P. axillaris*: (**B**) An unidentified solitary bee collecting pollen on *P. axillaris*: (**C**) A diurnal butterfly (*Hylephila phyleus*) probing for nectar on *P. integrifolia*, (**D**) A solitary bee collecting pollen on *P. integrifolia*

Fig. 2.2 *P. integrifolia* flowers are used by some solitary bees as mating sites. Here a male and female *Calliopsis* sp. are seen *in copula*. Note the color match between eyes of the bees and anthers of the flower

species observed on *P. integrifolia* in Uruguay is a small solitary bee that uses Petunia flowers not only to feed on nectar and pollen but also as mating places (Fig. 2.2). We often observed males waiting inside the floral tube and then attempting to mate with a female as soon as she landed, suggesting that the Petunia flowers function not only as a feeding resource but also as a rendézvous site for the bees. Similar behavior was observed in *Callonychium* species on purple-flowering *Petunia* species in Brazil (Wittmann, Radtke, Cure, and Schifino-Wittmann 1990).

These observations indicate a strong mutualism between these solitary bee species and some purple Petunia species. *P. axillaris* is nocturnally visited by sphingid moths, in Uruguay, mainly *M. sexta* and *Eumorpha vitis* (Table 2.1), and diurnally by solitary bees (mainly *Halictus* spp.). Day and night pollinator exclusion experiments have shown that *P. axillaris* is effectively pollinated by both diurnal and nocturnal pollinators (Hoballah et al. 2007), suggesting that at least in some habitats bee pollinators can be also effective pollinators of *P. axillaris*. However, the sets of bee species observed on *P. axillaris* and *P. integrifolia* differed markedly (Hoballah et al. 2007), suggesting the species are visited by different bee species. In this context it is worth noting that the abundance of pollinators may vary spatially and temporally. Observation of pollinators in an artificial population of both Petunia species in Uruguay in 2007 showed that some bee species visit both flower types (unpublished results). More observational data, in particular from natural sympatric populations, will be required to determine the extent to which both Petunia species are visited by the same sets of bee species.

Whether bees and hawkmoths have an innate preference for either flower type was tested under controlled conditions in the greenhouse. Naïve *Bombus terrestris* (although not a natural pollinator of Petunia) and naïve *M. sexta* were given the choice of *P. axillaris* or *P. integrifolia* in a greenhouse. The bumblebees clearly preferred to feed on flowers of *P. integrifolia* species and the hawkmoth *M. sexta* clearly preferred *P. axillaris* flowers (Hoballah et al. 2007).

Table 2.1 Species and locations of flower visitors collected from *P. axillaris* and *P. integrifolia* flowers in the natural habitat in Uruguay. Abbreviations for the collection locations: R: Rivera; LC: Las Cañas; PV: Puerto Viejo; C: Carmelo; PF: Playa Fomento; M: Minas; PA: Playa Agraciada;?: precise location unknown

Petunia species	Visitor species (family, subfamily)	Location, month, and year of collection
P. integrifolia	*Calliopsis* sp. (Apidae, Colletinae)	R 01.05, LC 02.07
P. integrifolia	*Calliopsis* sp. (Apidae, Colletinae)	PV 01.05
P. integrifolia	*Halictus* sp. (Apidae, Halictinae)	R 01.05
P. integrifolia	*Lasioglossum* sp. (Apidae, Halictinae)	R 01.05
P. integrifolia	*Lasioglossum* sp. (Apidae, Halictinae)	PV 01.05
P. integrifolia	*Leioproctus* sp. subgen. Hexantheda (Apidae, Colletinae)	? 11.02
P. integrifolia	*Leioproctus enneomera* (Apidae, Colletinae)	R 01.05
P. integrifolia	*Leioproctus enneomera* (Apidae, Colletinae)	PV 01.05
P. integrifolia	*Hylephila phyleus* (Hesperiidae, Hesperiinae)	JI 02.05
P. axillaris	*Halictus* sp. (Apidae, Halictinae)	? 11.02
P. axillaris	indet. panurgine genus (Apidae)	JI 01.04
P. axillaris	*Halictus* sp. (Apidae, Halictinae)	C 02.04
P. axillaris	*Lasioglossum* sp. (Apidae, Halictinae)	C 02.04
P. axillaris	*Manduca diffissa* (Sphingidae, Sphinginae)	PF 11.02, C 02.07
P. axillaris	*M. sexta* (Sphingidae, Sphinginae)	R 02.05, JI 02.07, C 02.07
P. axillaris	*Eumorpha vitis* (Sphingidae, Macroglossinae)	C 04, JI 02.06, 02.07
P. axillaris	*Eumorpha labruscae* (Sphingidae, Macroglossinae)	C 2007,
P. axillaris	*Agrius cingulata* (Sphingidae, Sphinginae)	M 02.05
P. axillaris	*Erinnyis ello* (Sphingidae, Macroglossinae)	C 02.06
P. axillaris	*Hyles lineata* (Sphingidae, Macroglossinae)	C 02.07
P. axilaris	*Diabrotica emorsitans* (Chrysomelidae, Galerucinae)	C 02.04
P. axillaris	*Chrysodina cupricollis* (Chrysomelidae, Eumolpinae)	C 02.04
P. axillaris	*Dahlibruchus* sp. (Chrysomelidae, Bruchinae)	JI 01.04
P. axillaris	harvester ants (unidentified genus)	PA 02.06
P. axillaris	Crabspider (unidentified genus)	

In addition to more or less efficient pollinators, the evolution of floral traits may be affected by nectar robbers, herbivores, pathogens, and pollinator predators. Apart from bees and hawkmoths, we observed occasional visits by hummingbirds, Lepidoptera, and Coleoptera to *P. integrifolia* or *P. axillaris* flowers in Uruguay (Table 2.1). So far no pathogens affecting *P. integrifolia* or *P. axillaris* flowers in the wild have been described. *Xylocopa* bees are occasional nectar robbers of *P. axillaris* but have not been observed on *P. integrifolia*. The most commonly observed herbivore of floral tissue in Uruguay was the chrysomelid beetle *Diabrotica*, which can be found on both Petunia species but more often on *P. axillaris* (Fig. 2.3). *Diabrotica* specimens were also observed to carry Petunia pollen and hence may play a minor role as pollinators. On one occasion *P. axillaris* flower petals were observed being harvested by ants. The only predators of pollinators occasionally observed

Fig. 2.3 Herbivores and pollinator predators on Petunia flowers. (**A**) unidentified cricket and (**B**) *Diabrotica* sp. beetle feeding on *P. axillaris* flowers. (**C**) crabspider waiting for prey on a *P. integrifolia* flower and (**D**) crabspider waiting for prey on a *P. axillaris* flower

mainly on *P. axillaris* flowers were crabspiders preying on bees and diurnal butterflies (Fig. 2.3). The observed crabspider species are most likely too small to overpower hawkmoths; however, a potentially deterring effect on hawkmoths has not been tested. An overview of flower visitors observed in field sites in Uruguay is given in Table 2.1. One may conclude that bees and hawkmoths are the main visitors of Petunia flowers and that other flower visitors are likely to play only a minor role in the pollination ecology of Petunia species in Uruguay. In the following paragraphs we will treat the four floral traits in which bee- and hawkmoth-pollinated Petunia species differ.

2.2.1 Color

Color is perhaps the most obvious difference between flowers of *P. integrifolia* and *P. axillaris*, and it is also genetically and biochemically the best characterized trait in Petunia. According to Wijsman's classical studies (Wijsman 1983) and more recent work, the floral color differences between *P. integrifolia* and *P. axillaris* can be largely accounted for by differences at six loci. In the flower limb the regulatory locus *AN2* (Quattrocchio et al. 1999) and the *hydroxylation-at-five*

(*HF1* and *HF2*) loci, encoding flavonol 3′5′-hydroxylases (Holton et al. 1993b), regulate anthocyanin amount and quality (see Chapter 13). Important differences can also be observed at shorter wavelengths invisible to the human eye. In contrast to flowers of *P. integrifolia*, those of *P. axillaris* (with the exception of *P. axillaris parodii* S7) express ultraviolet (UV)-absorbing flavonols at high levels in the adaxial epidermis of the flower limb. This difference can be explained by variation at the *FL* locus, encoding flavonol synthase (FLS) and controlling the production of flavonols (Holton, Brugliera, and Tanaka 1993a). The floral color phenotypes in Petunia species are in accordance with the bee and hawkmoth pollination syndromes. Hawkmoths have a preference for white flowers, which do not reflect UV light (White, Stevenson, Bennett, Cutler, and Haber 1994), while bees prefer UV-reflecting flowers. In the flower tube and anthers, the *AN2* homolog *AN4* (Kroon 2004) and *HF1* account for anthocyanin production. Variations in the promoter of *chalcone isomerase A* (*CHI-A* also called *Po*) (van Tunen, Mur, Recourt, Gerats, and Mol 1991) determine the accumulation of chalcone in the pollen (see below).

Choice experiments using naïve insects under controlled greenhouse conditions show that bees have an innate preference for purple-flowered accessions and hawkmoths for white-flowered ones (Hoballah et al. 2007). The MYB transcription factor AN2 controls most of the anthocyanin variation between *P. integrifolia* and *P. axillaris* (Quattrocchio et al. 1999). It had been shown that lack of anthocyanins in *P. axillaris* flower limbs involves loss-of-function mutations in *AN2* (Quattrocchio et al. 1999). Interestingly, loss of function has occurred at least five times in *P. axillaris* (Hoballah et al. 2007). Choice experiments using genetic introgression lines differing in flower color showed that the color change can significantly shift the innate preferences of hawkmoths and bees (Fig. 2.4; Hoballah et al. 2007). Choice experiments with wild-type and transgenic *P. axillaris* plants expressing *AN2* showed that *AN2* alone could confer a major shift in pollinator attractions (Fig. 2.5). Changes in flower color have been implicated in early speciation in plant taxa with different pollination syndromes (Bradshaw, Wilbert, Otto, and Schemske 1995). However, sequence analysis of *AN2* suggests that loss-of-function mutations occurred relatively recently and might have been preceded by changes in other traits (Quattrocchio et al. 1999; Hoballah et al. 2007). It can be speculated that *P. axillaris* was preceded by an ancestor with pink flowers that may have been attractive to butterflies and diurnal hawkmoths.

Anther coloration also differs strikingly between *P. axillaris* and *P. integrifolia*, with *P. axillaris* having yellow pollen and *P. integrifolia* blue pollen. *Callonychium petuniae* bees appear to mate in purple Petunia flowers (mainly *P. integrifolia* but also in *Calibrachoa* species with either blue or yellow pollen) in southern Brazil (Wittmann et al. 1990). The males wait for foraging females in or near the flowers, mating takes place on or in the flowers, and the couple go on to visit other flowers *in copula* (Wittmann et al. 1990). We observed the same behavior in the bees belonging to the genus *Calliopsis* on *P. integrifolia* in Uruguay. Eyes of the bees match *P. integrifolia* anthers in color and shape (see Fig. 2.2), leading to the intriguing speculation that male bee behavior may have been involved in the evolution of pollen color phenotype. Moreover, *C. petuniae* is thought to have tetrachromatic

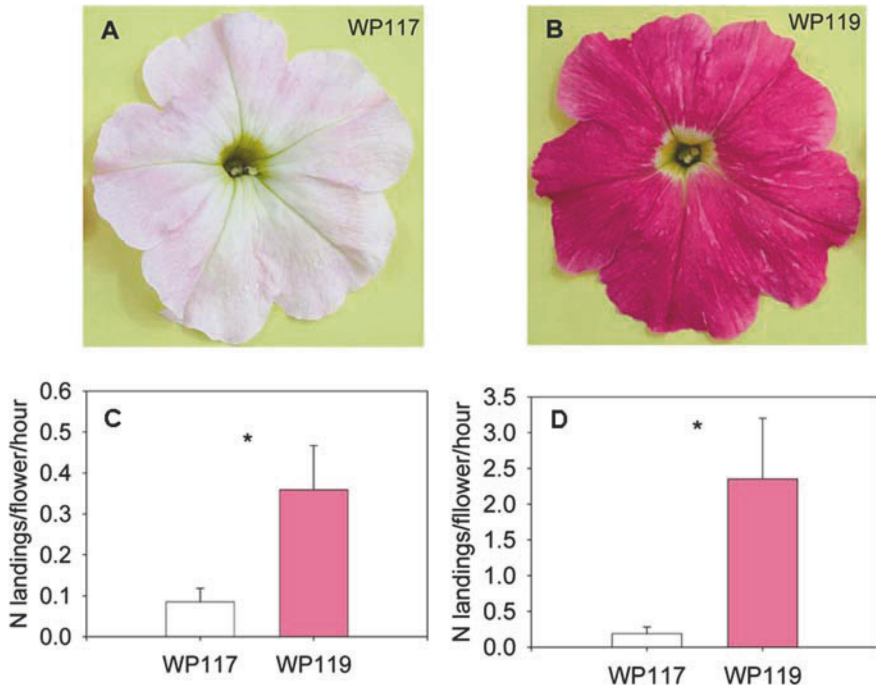

Fig. 2.4 Petunia introgression lines WP117 and WP119 are polymorphic for the *AN2* locus and affect pollinator behavior. Floral phenotypes of (**A**) line WP117, which carries a nonfunctional *AN2* allele, and (**B**) WP119, which carries an active *AN2* allele (Stuurman et al. 2004). (**C**) Mean (±SE) number of visits of diurnal butterflies in the wild habitat in José Ignacio (Uruguay) is higher for the line WP119 than for the white-flowered WP117. (**D**) Mean (±SE) number of visits of hymenopterans (Minusio, Switzerland) is also higher for line WP119 than for WP117. *Asterisks over bars* indicate the significance level of the statistical test $P = 0.01^*$. (Figures modified from Hoballah et al. 2007, ©The Plant Cell, ASBP)

vision, with receptors sensitive to red light and a strong preference for dark purple flowers, while other *Callonychium* species are much less specific in their color choice, suggesting that *C. petuniae* may have co-evolved with purple Petunia species (Wittmann et al. 1990).

2.2.2 Scent

The hawkmoth-pollinated *P. axillaris* flowers differ remarkably from bee-pollinated *P. integrifolia* flowers in the amount, quality, and timing of scent production. *P. axilllaris* flowers emit a blend of several compounds, dominated by methylbenzoate, benzaldehyde, and benzyl alcohol, during the night (Hoballah et al. 2005; see Chapter 3). The emission peak coincides with the nocturnal activity of the hawkmoth pollinators. Electroantennogram (EAG) recordings of female *M. sexta* antennae show that the three major volatiles methylbenzoate, benzaldehyde, and benzyl

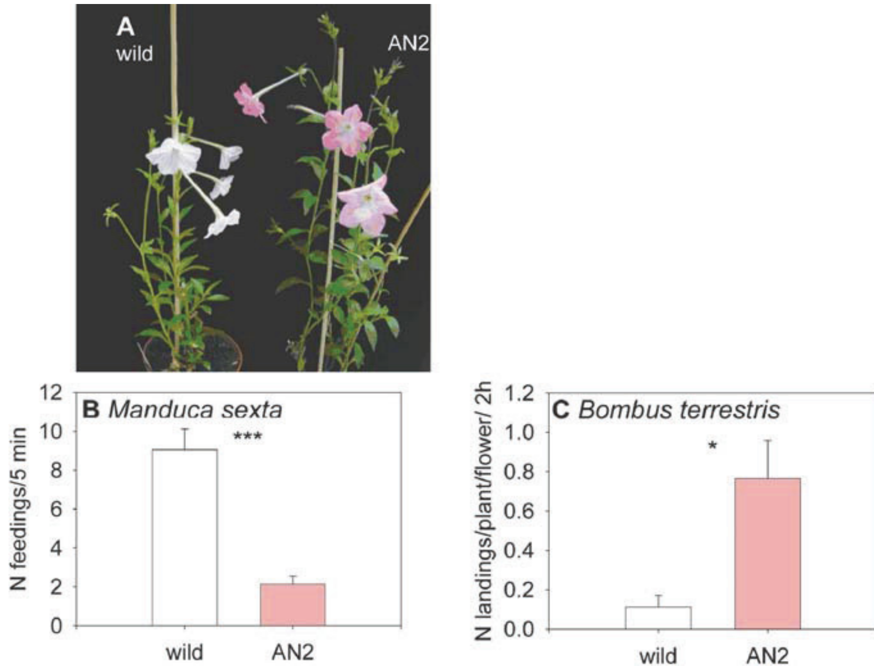

Fig. 2.5 Effect of the *AN2* gene on pollinator preference. Comparison of (**A**) wild-type and *AN2*-transformed *P. axillaris* flowers. (**B**) Mean (±SE) number of visits for feeding by *M. Sexta* is significantly higher for wild *P. axillaris* than for *AN2*-transformed flowers. (**C**) Mean (±SE) number of landings over 2 h per flower per plant of *B. terrestris* is higher on *AN2*-transformed plants than on wild-type ones. Asterisks over bars indicate significance of the statistical tests ($P = 0.0001$ ***, $P = 0.01$ ∗). (**B** and **C** modified from Hoballah et al. 2007, ©The Plant Cell, ASBP)

alcohol elicit the highest response (Hoballah et al. 2005), suggesting that these three compounds may be the most important for *Manduca* attraction. Floral scent is an essential cue for eliciting feeding behavior in *M. sexta* (Raguso and Willis 2002, 2005). This would suggest that the gain of scent attractive to hawkmoths must be an early step in the process of recruiting hawkmoth as pollinators. Whether single-scent components can elicit feeding behavior in hawkmoths or whether they interact synergistically has not been tested. Bees also use scent as a cue in their choice of flowers (Dobson 1994). However, bee-pollinated *P. integrifolia* flowers produce comparatively low amounts of benzaldehyde and only traces of a few other compounds (Hoballah et al. 2005).

Notably there is intraspecific variation in quality and quantity of floral odors in wild accessions of *P. axillaris* (Hoballah et al. 2005; Kondo et al. 2006). However, there was no clear association of flower volatile composition with the subspecific classification of these accessions (Kondo et al. 2006). Similarly, multiple *P. axillaris axillaris* accessions from different locations in Uruguay did not differ significantly in the total amount or composition of floral odor components (Fig. 2.6). Kondo

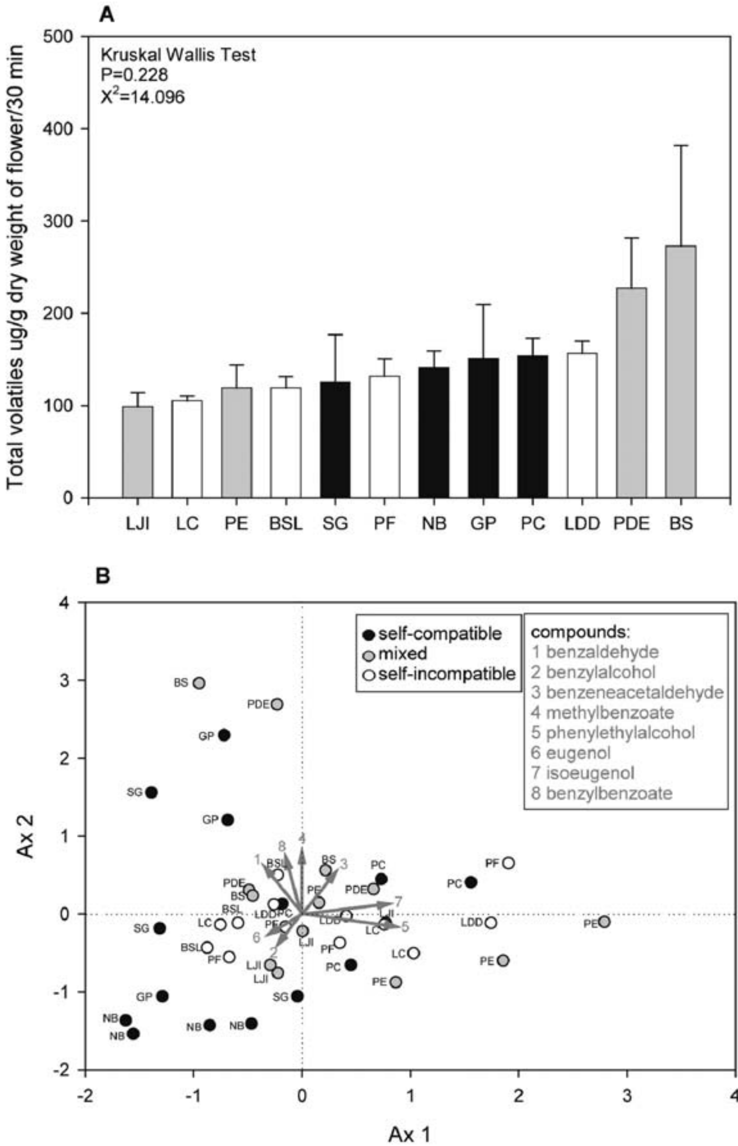

Fig. 2.6 *P. axillaris* wild accessions from Uruguay emit odor compounds that are similar quantitatively and quantitatively. (**A**) Total amounts of odor volatiles. The breeding system in each locality does not correlate with total odor production (bar fillings: black = self-compatible, gray = mixes, white = self-incompatible). (**B**) Plot of the first two principal components derived from multivariate analysis of the eight most abundant odor components. Locality labels are **LJI** = Laguna José Ignacio, **LC** = Las Canas, **PE** = Punta Espinillo, **BSL** = Barra Santa Lucia, **SG** = San Gregorio, **PF** = Playa Fomento, **NB** = Nuevo Berlin, **GP** = Gruta del Palacio, **PC** = Punta Colorada, **LDD** = Laguna del Diario, **PDE** = Punta dell'Este, **BS** = Balnearis Solis; precise coordinates of each locality are given in Hoballa et al. 2007, Table 2.1

et al. (2006) remarked that the two self-compatible accessions in their experiments produced less odor than self-incompatible lines. We did not find this trend analyzing a collection of *P. axillaris axillaris* accession samples ($n = 12$) that comprised both types of breeding systems (Fig. 2.6A).

Floral scents of plants measured in the field in Uruguay were not significantly different from those produced by seed offspring grown in the greenhouse (unpublished data). Growing plants in soils of varying nutrient content also did not show significant effects on scent production (ANOVA $P > 0.2$), while growth habit was clearly affected. Although this would suggest considerable buffering of scent production against environmental variation, there may also be considerable intraindividual variation, which could account for difficulties in demonstrating effects of environmental or genetic variation on scent production.

Fragrance intensity was quantified "by nose" in BIL of *P. hybrida* W138 x *P. integrifolia inflata* S6 (WI-BIL) and *P. hybrida* W138 x *P. axillaris parodii* S7 (WP-BIL) with *P. hybrida* W138 as recurrent parent (Stuurman et al. 2004). A QTL on chromosome VII was detected in WI- and WP-BILs (Stuurman et al. 2004). The gene *ODORANT1* (*ODO1*) (Verdonk, Haring, van Tunen, and Schuurink 2005) is a candidate for the regulation of scent production and has been mapped to chromosome VII (our unpublished results). However, in both BIL populations, the wild species allele at the QTL increased fragrance production, suggesting that the QTL represents a loss-of-function allele in the *P. hybrida* W138 line. More work will be required to evaluate *ODO1* as a candidate gene for controlling natural variations in scent production.

At present it is not established which genes required for *P. axillaris*–type scent are present in *P. integrifolia* or how many and which mutational changes would be required for gaining scent that would attract hawkmoths. Fortunately, the biochemical pathways leading to the floral volatiles emitted by Petunia flowers are under intensive study (see Chapter 3), so considerable progress in understanding natural variation in Petunia scent can be expected in the near future.

Not all volatiles produced by flowers are necessarily attractants for pollinators. Some compounds, such as isoeugenol, may rather have a function in protecting the flower from pathogens or herbivores (Hoballah et al. 2005). Moreover, volatiles that act as attractants to one pollinator may be repellents for others (Kessler and Baldwin 2007).

2.2.3 Nectar

Hawkmoth-visited flowers usually produce larger amounts of nectar that is more dilute and more sucrose rich than that of bee-pollinated flowers (Baker and Baker 1983). This difference has been observed between *P. axillaris* and *P. integrifolia* (Stuurman et al. 2004). Under greenhouse conditions, nectar concentration appeared largely independent of evaporation, and grafting experiments demonstrated that nectar production is dependent only on the scions and not on the grafting stock (Stuurman et al. 2004). However, the rate of nectar production is strongly affected

by environmental factors such as light and temperature. The total concentration of sugars (glucose, fructose, and sucrose) is about five times lower in *P. hybrida* W138 and about 10 times lower in *P. axillaris parodii* than in *P. integrifolia inflata*. The ratio of sucrose to hexose was almost 10 times higher in *P. axillaris parodii* compared to that in *P. integrifolia inflata*. In turn, the concentration of nectar is about five times higher in *P. integrifolia inflata* than in *P. axillaris parodii*.

QTL analysis of nectar volume in WI-BILs revealed two QTLs, on chromosomes II and VI; together the two QTLs account for about half the observed variation (Stuurman et al. 2004). Partial regression analysis showed that the QTL on chromosome II may be correlated with flower morphology. No QTLs were found in WP-BILs.

In a BC of *P. axillaris axillaris* N and *P. integrifolia inflata* S6, with the latter as recurrent parent, four QTLs for nectar volume, on chromosomes III, VI, V, and IV, have been detected (Galliot, Hoballah, Kuhlemeier, and Stuurman 2006b). Interestingly, all four nectar QTLs co-localized with QTLs affecting petal size. Similar correlations of nectar volume with morphology have been observed in *Nicotiana* species (Kaczorowski, Gardener, and Holtsford 2005). A sucrose–hexose ratio QTL was found in WI-BIL on chromosome IV explaining about 40% of the variation, with the *P. integrifolia inflata* allele decreasing the ratio, suggesting that it could control invertase activity (Stuurman et al. 2004).

A candidate gene for natural variation in nectar production could be *NEC1*, which affects nectar production (Ge et al. 2000, 2001). However, the RNAi knockout of *NEC1* in *P. hybrida* W115 (Petunia Mitchell) has large pleiotropic effects. Furthermore, *NEC1* and two homologs (*NEC2* and *NEC3*) have been mapped both in WI-BIL and in *P. axillaris axillaris* N x *P. integrifolia inflata* BC1 populations (Stuurman et al. 2004, unpublished data). *NEC1* is located on chromosome VII, *NEC2* on chromosome IV, and *NEC3* on chromosome VI (unpublished data), rejecting *NEC1* as a candidate, but suggesting that *NEC3* could correspond to a QTL.

Nectar may contain many components in addition to various sugars, such as amino acids, scent components, repellents, and antimicrobial substances. Some of these components may act as food or attractants; others might repel pollen robbers or inefficient pollinators. However, some substances may play a more complex role. Recently, it has been suggested that toxic repellents such as nicotine may also reduce specialist pollinator feeding time per visit and hence optimize the number of possible visits per volume of nectar produced (Kessler and Baldwin 2007).

2.2.4 Morphology

P. axillaris differs from *P. integrifolia* in numerous aspects of floral morphology (Fig. 2.7). The corolla of *P. axillaris* features a longer and narrower floral tube and a much larger floral limb, and appears less zygomorphic than that of *P. integrifolia*. In addition, the positioning of style and stamen differs between the species. The two ventral anthers in *P. integrifolia* exsert beyond the style so that the style is positioned between these and the three dorsal anthers, an arrangement often found in

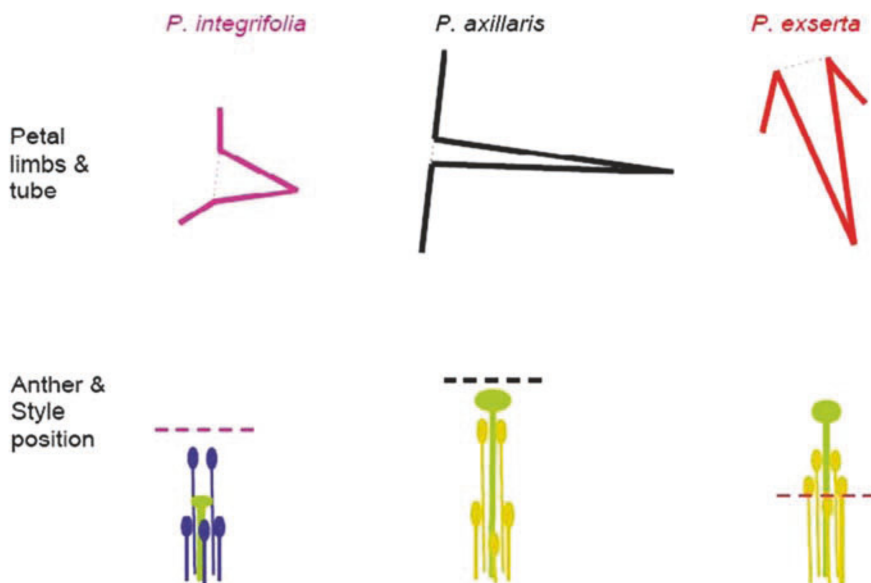

Fig. 2.7 Schematic overview of differences in corolla morphology and anther and style position in Petunia species representing different pollination syndromes

zygomorphic bee-pollinated flowers (Proctor, Yeao, and Lack 1996). In *P. axillaris* the stigma exserts beyond all anthers, putatively reducing the amount of self-pollen depositing on the style. The stigma of *P. axillaris* is also much broader than that of *P. integrifolia*, which in combination with the narrow tube could maximize the chance of non-self-pollen deposition by pollinators. In addition to floral morphology, the growth habit of the two species is different, with most *P. axillaris* accessions showing apical dominance and longer internodes while *P. integrifolia* plants are shorter, sometimes with a creeping habit. Although growth habit is certainly affected by other selective pressures such as shading and competition by other plants, hawkmoths may prefer taller plants with flowers unobstructed by vegetation, perhaps in order to avoid predators. Finally, *P. integrifolia* flowers also appear to close at night (Ando et al. 2001, MEH personal observation in Uruguay), which may be a thermo-protective strategy but may also exclude nocturnal pollinators. Empirical data confirming these notions of morphological functionality are lacking.

The genes underlying differences in the corolla phenotype in Petunia species have also been studied by QTL analysis (Stuurman et al. 2004; Galliot et al. 2006b). Three morphological domains can be defined (Fig. 2.8): D1, the length of the petal tube segment from the base of the corolla to the point where the stamina that are basally fused to the petals detach; D2, the distal tube domain between D1 and the boundary of the flower limb, defined as the maximal point of inflexion; and D3, the limb extending from D2. Among these measures, D1 shows the largest differences between *P. axillaris* and *P. integrifolia* (Stuurman et al. 2004). Stuurman et al. (2004) found five different QTLs for D1 in BIL populations in a *P. hybrida* W138 background derived from

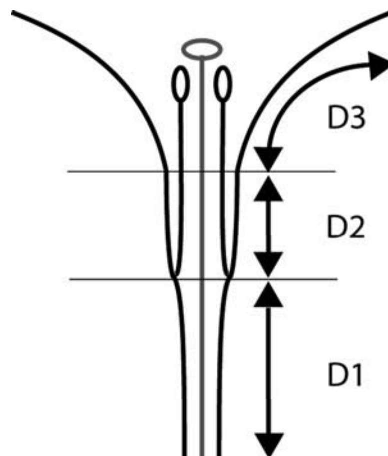

Fig. 2.8 Schematic diagram showing the three domains (**D1**, **D2,** and **D3**) in the corolla limb of Petunia flowers

crosses with *P. axillaris parodii* S7 or *P. integrifolia inflata* S6 (called WP or WI, respectively). In WI-BIL two QTLs, on chromosomes III and VII, were found. In WP-BIL three QTLs, one each on chromosomes II, IV, and VI, were detected, with the QTL on chromosome II explaining about 21% of the variation.

In a BC of *P. axillaris axillaris* N and *P. integrifolia inflata* S6 as recurrent parent, six QTLs for D1 on different chromosomes were found (Galliot et al. 2006b). The two largest QTLs (on chromosomes III and IV) contribute more than half the variation. In addition, four QTLs for D2 and four for D3 (L) were detected. Three QTLs for D2 and D3 (on chromosomes II, III, and IV) co-localize with each other and with QTLs for D1. This suggests that at least three QTLs contribute to the overall larger size of *P. axillaris axillaris* N petals compared to those of *P. integrifolia inflata*. In WI-BIL a single QTL with a large effect on the positioning of the two ventral stamens was detected on chromosome VI (Stuurman et al. 2004). This trait has been suggested to be subordinate to mechanisms controlling dorso-ventral asymmetry. To date none of the genes accounting for morphological differences in *P. axillaris* and *P. integrifolia* has been cloned.

Recently reported Petunia species such as *P. secreta* (Stehmann and Semir 2005) and *P. occidentalis* (Tsukamoto et al. 1998), with morphological phenotypes somewhat intermediate between those of *P. integrifolia* and *P. axillaris*, may represent either ancestral intermediates or hybrids of these taxa. In either case, knowledge about their pollination biology may shed some light on the pollination biology of intermediate morphs in the evolution from bee to hawkmoth pollination syndromes.

2.3 Evolution of Hummingbird from Hawkmoth Syndrome

As both hummingbirds and hawkmoths drink sucrose-rich, dilute nectar from flowers with long tubes, no large changes in these traits are required for shifting the pollination syndrome. Hummingbirds are mainly visually oriented and many

hummingbird-pollinated flowers have red pigmented corollas (Rodríguez-Gironés and Santamaría 2004). Most hummingbird-pollinated flowers have little or no scent (Proctor et al. 1996), but hummingbirds can also respond to scent components (Kessler and Baldwin 2007).

P. exserta is a recently discovered rare species endemic to southern Brazil, growing under sandstone cliff overhangs in only four locations (Stehmann 1987; see Chapter 1). *P. exserta* features typical hallmarks of hummingbird pollination: petal limbs have brilliant red pigmentation and are backward folded, style and anthers are exserted (Fig. 2.7), and the nectar is dilute, with a high sucrose–hexose ratio. We analyzed the scent of *P. exserta* and could not detect any odor compounds (unpublished data). There is anecdotal evidence suggesting that *P. exserta* is indeed pollinated by hummingbirds (Lorenz-Lemke et al. 2006). Considerable hybridization of *P. exserta* and *P. axillaris* has been observed, presumably the result of hummingbird visits to both species (Lorenz-Lemke et al. 2006). This notion is supported by our observation of hummingbirds visiting *P. axillaris axillaris* in the wild in Uruguay at sunset.

Nuclear markers suggest that *P. exserta* is closely related to *P. axillaris* and may even be nested among *P. axillaris* accessions (unpublished data). Hence, hummingbird pollination has most likely been derived from hawkmoth pollination very recently. This would be in contrast to the directional pollinator shifts suggested for the genus *Aquilegia*, in which pollination syndromes shifted from bee to hummingbird pollination and from hummingbird to hawkmoth pollination (Whittall and Hodges 2007). The putatively very recent shift to hummingbird pollination in *P. exserta* makes this species particularly appealing for testing the role of natural selection in the evolution of pollination syndrome traits. In particular, the apparent loss of scent and the accumulation of the red anthocyanin delphinidinin 3-rutinoside in *P. exserta* (Ando et al. 1999; Ando et al. 2000) are worthwhile traits for study.

Visitation by hummingbirds has been suggested for *P. reitzii* and *P. saxicola* (Ando et al. 1999). Compatible with hummingbird pollination, both species feature a different, more reddish pigment type (delphinidin 3-rutinoside-5-glucoside) than their closest known relatives. However, the morphological traits of these plants point more toward bee pollination. It would be interesting to establish the pollinator spectrum for these species and to test the significance of reddish petal pigmentation for attracting specific pollinators.

2.4 Conclusion and Outlook

Here we have tried to give an overview of our present understanding of the pollination syndromes in Petunia and to show its potential as a model system. The trait best understood at present is flower color variation. A handful of genes are known to account for much of the variation between *P. integrifolia* and *P. axillaris*. However, whether the divergence in these genes and traits was caused by natural selection or perhaps by drift is still not established. One shortcoming of this particular species

comparison is that both species are relatively divergent, which complicates the identification of the causal mutations and testing for natural selection.

The isolation of a growing number of genes involved in odor production (Schuurink et al. 2006; van Schie, Haring, and Schuurink 2006) will help to identify genes underlying species differences in this trait. In contrast to flower color and scent, we know of hardly any genes involved in establishing interspecific variation in nectar production and morphology. The prospect of isolating genes that underlie these traits would be greatly enhanced if more genomic tools for the Petunia system were available. Sequencing of genomic libraries, for example, bacterial artificial chromosomes (BACs) containing genes of interest, would be helpful and, furthermore, allow for the application of sophisticated approaches such as the testing of haplotype block structure to generate inferences about natural selection. Dissection of the genetic architecture of trait differences and isolation of major genes involved in establishing them will eventually allow us to reconstruct the major evolutionary steps in the transition from one pollination syndrome to another. One question concerning such a transition is whether particular traits such as scent or nectar have to change first and then other changes, for example, morphology or flower color, follow. Genetically reconstructing transitional phenotypes will also allow testing whether major shifts in attraction of specialized pollinators follow changes in just a few genes and whether intermediate phenotypes attract more generalized pollinators.

2.4.1 Trait Interactions

Often traits involved in different pollination syndromes co-vary, raising questions about the cause of co-variation. Do combinations of traits evolve strictly in parallel because they are selected for by pollinators, or are there functional or genetic interactions between traits? For instance, there is a correlation between tube length and nectar production. Is this the case because a longer tube simply allows the floral tube to hold more nectar, does an increase in tube length unavoidably lead to more nectar production, or is long tube length a secondary adaptation to exclude inefficient pollinators from a rich resource? Accumulation of flavonols appears inversely related to anthocyanin pigmentation. Does the competition of pigmentation pathways for the same substrate explain the correlation? Similarly, is there a functional link between scent production and reduced anthocyanin pigmentation? Do such functional correlates facilitate the evolution of fitting trait combinations?

2.4.2 Behavioral Choice Tests

Besides understanding the molecular evolution of pollination syndrome genes and genetically or transgenically manipulating pollination traits, it is crucial to assay the preferences of pollinators for floral phenotypes of interest in order to test hypotheses about the ecology and evolution of pollination syndromes. Behavioral tests can

be conducted either in the field or under controlled conditions with naïve pollinators. Both approaches have their merits and difficulties. Field experiments sample the broad diversity in pollinators and other interactors (e.g., nectar robbers and herbivores) occurring in nature and can give an understanding of how a newly evolved variant would fare under natural conditions. However, even field studies can be representative for only a given habitat and a given time frame. Testing in the wild is often very difficult (Hoballah et al. 2007), requiring favorable conditions for pollinator activity and sufficiently abundant pollinators. In contrast, testing in controlled conditions, such as a greenhouse or a flight tunnel with model pollinators, is limited to pollinators that can be reared in the laboratory. On the other hand, experiments can be replicated easily, plant material can be grown under highly standardized conditions, and transgenic plants can be used without encountering issues connected to the testing of genetically modified organisms in the field. Furthermore, testing innate pollinator preference arguably gives an indication of the genetically fixed and presumably adaptive preference in a given pollinator. Both the innate preference and the preferences modified by learning provide important information for assessing the adaptive value of floral traits.

2.4.3 Need for More Ecological and Phylogenetic Data

Obviously there is still much descriptive and experimental data needed for elucidating the ecology and evolution of the Petunia pollination syndromes. Pollinators were observed only on *P. axillaris* and *P. integrifolia* in Brazil (Ando et al. 2001) and in Uruguay (Hoballah et al. 2007). Furthermore, the importance of different pollinators for plant fitness was only partially studied in Uruguay with night and day exclusion experiments (Hoballah et al. 2007). Pollen movement between plants of the same species and between plants of different species can be studied with the use of a pollen dye. The quantity of pollen transferred by different pollinators can be studied in nature and in controlled experiments. Such experiments can reveal the degree of flower constancy of specific pollinators and the efficiency of pollinators in relation to flower types. Finally, improved taxonomic resolution of pollinators (bee species in particular) would be desirable.

2.4.4 Petunia as an Evolutionary Model System

There are a number of interesting questions that are related to, but go beyond, the issue of pollination syndromes. In *P. axillaris*, there is intraspecific variation in flower tube length. The subspecies *P. axillaris parodii* and *P. axillaris subandina* feature significantly longer floral tubes and narrower floral limbs than *P. axillaris axillaris*. It would be interesting to see whether long floral tubes are associated with a pollinator spectrum characterized by longer probosces, as would be predicted by co-evolutionary scenarios. Furthermore, does the inverse relationship between limb

size and tube length represent a genetic constraint on floral development, or is it constrained by mechanical properties of the flower, e.g., is a large limb on a long tube more easily damaged by wind?

Other aspects deserving more attention are the costs of particular traits and the tradeoffs between traits. Generating and maintaining floral tissue and nectar and scent production bear a metabolic cost (Pyke 1991), which needs to be compensated by an increase in fitness. Otherwise, variants reducing these costs should arise and spread throughout the population. Evolutionary theory would predict that where plants invest in costly traits such as nectar while pollinators cannot differentiate between cheating and non-cheating individuals, cheaters should evolve and reach a certain frequency in the population (Thakar, Kunte, Chauhan, Watve, and Watve 2003).

Besides its pollination syndromes and the importance of ethological isolation for speciation, the genus *Petunia* may also serve as an evolutionary model system to study the relevance of other prezygotic reproductive barriers, including ecophysiological and ethological barriers, and interactions of pollen with the stigma, the style and the embryo sac. As a few Petunia taxa cannot be crossed (e.g., *P. integrifolia inflata* S6 and *P. axillaris parodii* S7), but can be bridged via crosses to *P. hybrida* (Stuurman et al. 2004), introgression lines of each of these two wild species with *P. hybrida* as the recurrent parent may provide a useful means of identifying loci that are involved in establishing post-pollination barriers, and hence speciation genes *senso stricto* (Coyne and Orr 2004).

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Chapter 3
Benzenoids Dominate the Fragrance of Petunia Flowers

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Abstract In the last decade *Petunia hybrida* has emerged as the model of choice to study volatile benzenoid and phenylpropanoid synthesis, emission and regulation. These volatiles are synthesized predominantly in the corolla limb and emission is highly regulated, with a circadian rhythm, during corolla development, pollination and senescence. With all the biochemical and molecular tools available, much of our understanding of volatile benzenoids/phenylpropanoids has been obtained with Petunia, as illustrated in this chapter.

3.1 Introduction

Plants produce flowers as a means to ensure successful sexual reproduction. Flowers have complex morphological characteristics that provide visual and olfactory cues, as well as food rewards, for insect and animal pollinators. Once pollination and successful fertilization have been achieved, plants do not need to maintain floral organs such as petals, which serve a role in pollinator attraction. Many types of flowers are attractive to pollinators and humans because of their visual and olfactory attributes (see Chapter 2). Floral color (see Chapter 13) and form (see Chapter 10) have been well studied because they provide visual cues vital to the plant's reproductive strategy and because there is a human desire for visually stimulating plants in the environment. Floral fragrances have been studied less extensively, but due to their role in distance attraction of pollinators and their importance in the perfume industry, they are now receiving much more attention.

In the past decade our scientific understanding of floral fragrance synthesis and emission has grown steadily. We now know that floral volatiles in plants are derived from several biosynthetic pathways and that floral volatile synthesis is a highly regulated and complex process (Dudareva and Pichersky 2000; Dudareva et al. 2004;

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Pichersky et al. 2006; Schuurink et al. 2006). Floral fragrance composition and quantity both change during floral development and can be altered by pollination status (Tollsten 1993; Schiestl et al. 1997), environmental conditions (Jakobsen and Olsen 1994), endogenous circadian and diurnal rhythms, and plant hormones (Negre et al. 2003; Underwood et al. 2005). Although much of the early work in the field of floral volatile synthesis and biochemistry was conducted on plants such as *Clarkia breweri* and *Antirrhinum majus* (Dudareva and Pichersky 2000), Petunia has emerged as an excellent model system for studying the complex regulation of volatile synthesis and emission for two key reasons.

First, *Petunia axillaris* and *Petunia integrifolia*, the known progenitors of *P. hybrida*, have different visual and olfactory characteristics and different primary pollinators, even though they are indigenous to similar geographic regions in South America (Hoballah et al. 2007; see Chapter 1). The *P. axillaris* flower is white with a narrow corolla tube and synthesizes copious amounts of benzenoids and phenylpropanoids at night; it is primarily pollinated by hawkmoths. *P. integrifolia* is purple with a broad corolla tube and makes no appreciable fragrance; it is primarily visited by bees during the day (Hoballah et al. 2007). Thus, these two Petunia species have served as the basis for elegant studies on highly specific plant–pollinator interactions.

Second, Petunia Mitchell has emerged as the model of choice to study volatile benzenoid and phenylpropanoid synthesis, emission and regulation. Mitchell is an inbred colchidiploid that is easy to transform using *Agrobacterium tumefaciens* (see Chapter 19). It has a short lifecycle and produces a generous number of seeds, making it an efficient model for manipulation using antisense, co-suppression or RNAi to regulate gene expression. It has large petals and emits volatile compounds predominantly in the evening and night, the manner and composition are similar to those of *P. axillaris* (Kolosova et al. 2001; Verdonk et al. 2003). These volatiles are synthesized mostly in the corolla limb (Negre et al. 2003; Verdonk et al. 2003; Underwood et al. 2005), and emission is regulated by ethylene during corolla senescence (Underwood et al. 2005), thus making Mitchell an ideal physiological and biochemical model for studying the regulation of volatile synthesis and emission. In the past few years, a significant amount of DNA sequence has been generated for genes expressed in Mitchell flowers, and DNA microarrays have been utilized in various experiments to identify genes involved in fragrance biosynthesis and regulation by a number of factors, including the endogenous circadian rhythm (Kolosova et al. 2001; Verdonk et al. 2003; Verdonk et al. 2005) and the plant hormone ethylene (Negre et al. 2003; Underwood et al. 2005; Dexter et al. 2007).

With the help of transgenic plants in which the expression of these genes has been altered, we, as a group of scientists with collaborative interests, have been able to progress efficiently in efforts to discover the biochemical functions of genes involved in volatile benzenoid synthesis and its regulation. The remainder of this chapter is dedicated to reviewing the pertinent details of work conducted on floral volatile synthesis in Petunia. It should provide a basis to better understand the complex manner in which it is regulated and a good direction for the manipulation of fragrance characters in Petunia and other plants in the not-so-distant future.

3.2 Scent Measurement Methods

Basically, two different ways of measuring volatiles are used. The headspace of whole, intact flowers can be measured, but it is also possible to make extracts of the flowers or flower parts and analyze the endogenous scent components. Whole-flower measurements have the advantage that they represent volatiles emitted as in a biological situation; an extract of flowers could contain volatiles that are normally retained inside the petals, either as a fraction of emitted molecules, or as molecules that are not emitted at all, such as precursors.

The “live” measurements can be done on whole plants, with the flowers enclosed in a chamber of glass and Teflon. Carbon-filtered air is pumped/pulled through this chamber and the volatiles it contains are trapped on a sorbent, such as Super Q, Tenax TA, or Porapack Q, to be subsequently eluted and analyzed by gas chromatography, preferentially coupled to mass spectrophotometry (Raguso and Pichersky 1995b; Hoballah et al. 2005). These measurements can be done at different times of day and over different time intervals. The advantage of this is that the same flower can be measured throughout its lifespan. Alternatively, cut flowers can be measured to obtain higher reproducibility. Several flowers of the same plant can be put into a desiccator (Fig. 3.1A), or a glass tube (Fig. 3.1B), and the volatiles that are present in the air that is passed through are trapped on a sorbent as described above (Underwood et al. 2005; Verdonk et al. 2005). The differences in emission of a cut flower with that of a flower that is still attached to the plant have been shown to be minimal for at least the first 48 h (J. Verdonk, T. Colquhoun and D. Clark, unpublished data). Apparently, the biochemical substrates that are used for the biosynthesis of the volatile fragrance molecules must be synthesized or stored in the petals and are not transported from other parts of the plants. The trapping of volatiles on sorbents like Tenax makes it possible to quantify the amounts of volatiles that are produced, by comparing them with standards of the compounds that are measured and by including internal standards of molecules that are not produced by the flowers, such as geraniol (Raguso and Pichersky 1995a), nonyl acetate (Underwood et al. 2005), or α-terpinene (Verdonk et al. 2005).

An alternative, semi-quantitative method to measure flowers that are still attached to the plant is Solid Phase Micro Extraction (SPME). Flower buds are enclosed in a glass funnel and closed on both sides with aluminum foil during the measurement (Verdonk et al. 2003). In front of the flower, a silicone fiber coated with 100 μm polydimethylsiloxane (PDMS) is placed and pinched through the foil, exposing it directly to the headspace of the flower (Fig. 3.1C). After a fixed exposure time, the fiber is directly inserted into a GC injector for 1 min to desorb the volatiles that were trapped on the fiber. Depending on the time required for gas chromatography, a measurement can be made every half or whole hour. It has been shown that an exposure of 10 min can be enough to give a significant representation of the headspace of the flowers (Julian Verdonk, unpublished data).

Measurement of floral extracts has the advantage that the process can be automated. Flowers can be harvested at different times and subsequently frozen and stored until use. Analysis of stored volatiles of Petunia flowers has relied on SPME

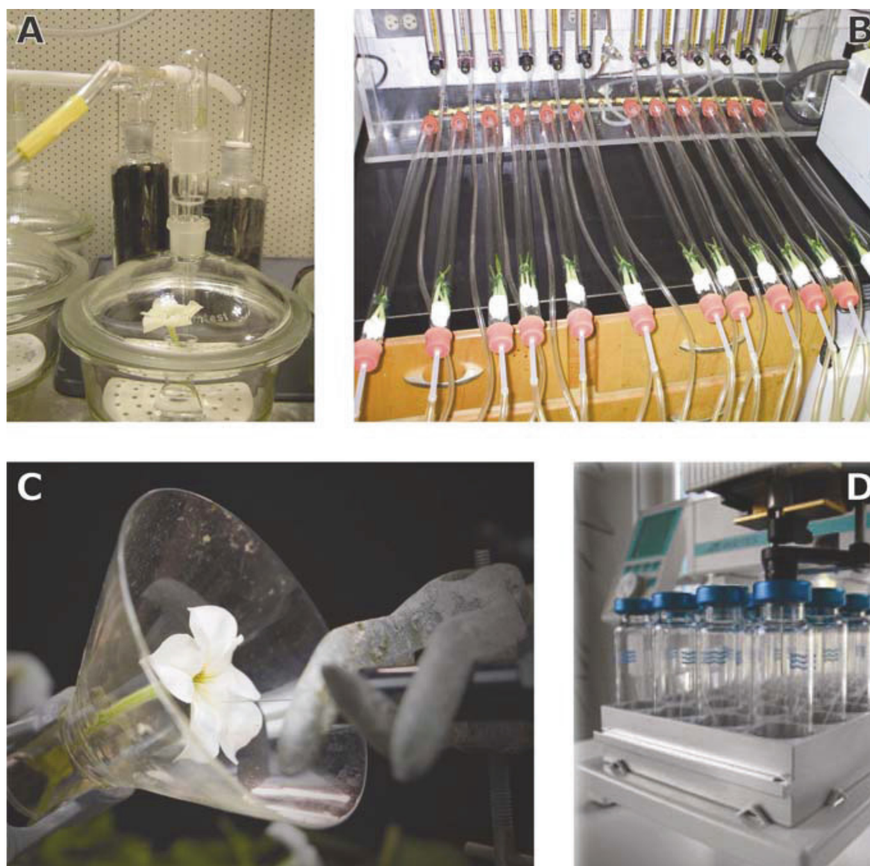

Fig. 3.1 Different methods for collecting Petunia volatiles. Flowers are enclosed in desiccators (**A**) or tubes (**B**); (**C**) a flower still attached to the plant enclosed in a glass funnel that is exposed to an SPME fiber; (**D**) setup for the measurement of $CaCl_2$ flower extracts using SPME

(Lücker et al. 2001; Verdonk et al. 2003). Frozen tissue was homogenized in a saturated (5 M) $CaCl_2$ solution (to inhibit enzymatic conversions), transferred to a vial with a septum cap, and stored on ice until use. For the measurements, the vial was heated to 50°C for 5–10 min with stirring to equilibrate (Fig. 3.1D). The mixture was then exposed to the SPME fiber at 50°C for 10–30 min and injected into a GC injector. It was demonstrated that the headspace of a $CaCl_2$ flower extract is similar to the emission of "live" flowers (Verdonk et al. 2003). The automated setup that was used by Verdonk et al. (2005) consisted of an automated sampler robot that allowed the analysis of 32 frozen samples overnight. The samples were stored in a cooling block until the robot transferred them to a heating block set at 50°C. After an incubation of 5 min, the SPME needle was injected through the septum of the vial and exposed to the headspace of the flower extract.

Schmelz et al. (2004) described a method to for Quantitative analysis of internalized volatile precursors by derivatization. Dexter et al. (2007) used this method for the measurement of coniferaldehyde and homovanillic acid, which accumulated in transgenic lines silenced for coniferyl alcohol acyltransferase (CFAT). Possibly this method can also be used to analyze the levels of other precursors, such as benzoic acid (BA) and cinnamic acid (CA). Alternatively, BA levels can be measured after extraction of flavonoids, and phenolic and benzoic acids with ethyl acetate, followed with the derivatization of the phenolic and benzoic compounds with BSTFA (*N*, *O*-bis[trimethylsilyl]trifluoroacetamide) + TMCS (trimethylchlorosilane) reagent. The trimethylsilyl derivatives formed can then be separated and quantified using GC-MS (Zhang and Zuo 2004; Verdonk et al. 2005).

While benzenoid/phenylpropanoid volatiles dominate the headspace of Petunia, other types of volatiles have also been detected. Verdonk et al. (2003) measured the headspace of flowers still attached to the plant using SPME and found two fatty acid derivatives, two aliphatic aldehydes, as well as two sequiterpenes emitted from the flowers. The fatty acid derivatives were found only when the internal volatiles were measured using the $CaCl_2$–SPME method. This is probably due to the elevated temperature at which the measurements were taken (50°C). The aliphatic aldehydes decanal and dodecanal were present in both intact flowers and the extracted flower samples. The sequiterpenes are probably emitted mostly from the sexual organs in very low concentrations; they were detected in samples of a ground stigma with the $CaCl_2$–SPME method. Furthermore, detection was limited to a brief period just after flower opening. One single report describes the detection of hexadienoic acid methyl ester and Z-9, 17-octadecadienal (Hoballah et al. 2005). Finally, the carotenoid cleavage products β-ionone (Simkin et al. 2004) and α-ionone (J. Verdonk, T. Colquhoun and D. Clark, unpublished data) have also been found.

3.3 Molecules, Pathways, and Enzymes

The floral bouquet of *Petunia hybrida* is dominated by compounds derived from the amino acid phenylalanine (Phe), with a few terpenes also observed (Verdonk et al. 2003). Volatiles derived from Phe include benzyl alcohol, benzaldehyde, methylbenzoate, benzylbenzoate, phenylacetaldehyde 2-phenylethanol, phenylethylacetate, isoeugenol, eugenol, and vanillin. Isoeugenol, one of the major components of Petunia floral scent, and its isomer eugenol, generally a minor component, have molecular structures that are still very similar to that of Phe, with a modified benzene ring and a side chain containing three carbons (C_6–C_3 compounds) (Fig. 3.2), and are thus classified as phenylpropanoids. Phenylacetaldehyde and phenylethanol are Phe-derived compounds whose side chains are two carbons long (C_6–C_2 compounds). Benzyl alcohol, benzaldehyde, vanillin, and methylbenzoate (another major component of the scent) are Phe-derived compounds whose side chains are only one carbon long (C_6–C_1 compounds) and are therefore classified as benzenoids. Some compounds are the result of further modifications and

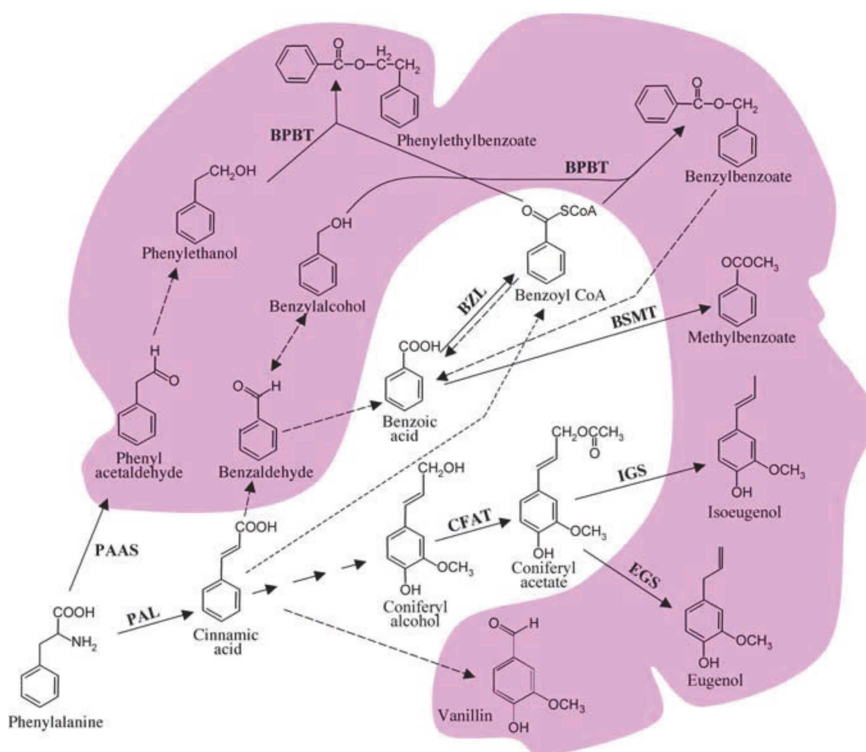

Fig. 3.2 An overview of the biochemical reactions leading to the synthesis of volatile benzenoids/phenylpropanoids emitted from Petunia flowers. Solid arrows indicate established biochemical reactions and broken arrows indicate possible steps for which enzymes have not yet been characterized. Volatile compounds are shown on a pink background. *BPBT*, benzoyl-CoA:benzyl alcohol/2-phenylethanol benzoyltransferase; *BSMT*, benzoic acid/salicylic acid carboxyl methyltransferase; *BZL*, benzoate:CoA-ligase; *CFAT*, coniferyl alcohol acyltransferase; *EGS*, eugenol synthase; *IGS*, isoeugenol synthase; *PAAS*, phenylacetaldehyde synthase; *PAL*, phenylalanine ammonia-lyase

combinations of these compounds (see below), for example, the ester benzylbenzoate (also a major scent component), which has both benzyl alcohol and benzoic acid moieties (Fig. 3.2).

While many of these compounds occur in the floral scents of other plant species as well, their biosynthesis has been extensively studied in Petunia. Many of the genes and enzymes involved in the final steps of the biosynthesis of these compounds have been characterized in detail in this species (Fig. 3.2). It has been shown that phenylacetaldehyde synthase (PAAS) catalyzes the formation of phenylacetaldehyde from Phe in one step, which involves decarboxylation and oxidative deamination (Kaminaga et al. 2006). When the expression of *PAAS* is suppressed, the emissions of phenylacetaldehyde as well as phenylethanol are reduced (Kaminaga et al. 2006), indicating that the latter is produced from the former,

although the enzyme responsible for this conversion has not yet been identified. Eugenol and isoeugenol are the end products of a long pathway starting with Phe, the initial steps of which are shared with the biosynthesis of lignin precursors but diverge at coniferyl alcohol. Coniferyl alcohol acyltransferase (CFAT) acetylates coniferyl alcohol (Dexter et al. 2007) to produce coniferyl acetate, the substrate for isoeugenol synthase (IGS) and eugenol synthase (EGS), which catalyze the formation of isoeugenol and eugenol, respectively (Koeduka et al. 2006).

The starting points in the synthesis of benzenoid volatiles are benzyl alcohol and benzyl aldehyde, which are themselves volatile, and benzoic acid. The specific reactions that give rise to these building blocks from Phe or perhaps even earlier steps in the shikimic acid pathway are still unclear, as discussed in several recent articles (Orlova et al. 2006; Wildermuth 2006). However, further modifications of these building blocks leading to the formation of volatile end products were recently discovered. A highly expressed gene in Petunia flowers, *BSMT*, encodes benzoic acid/salicylic acid methyl transferase, which transfers a methyl group from S-adenosyl-L-methionine (SAM) to benzoic acid, creating methylbenzoate (Negre et al. 2003; Underwood et al. 2005). Benzyl alcohol and benzoyl-CoA (the latter derived either from benzoic acid by the action of benzoyl:CoA-ligase [BZL] or from β-oxidation of CA, see below) are combined into the ester benzylbenzoate in a reaction catalyzed by benzyl alcohol/phenylethanol benzoyltransferase (BPBT) (Boatright et al. 2004). This enzyme also catalyzes the formation of the ester phenylethylbenzoate from phenylethanol and benzoyl-CoA (Boatright et al. 2004).

While the C_6–C_2 compounds of Petunia floral scent arise directly from Phe, the biosynthesis of C_6–C_3 and C_6–C_1 compounds occurs via *trans*-CA, which is the result of Phe deamination catalyzed by a widely distributed enzyme, L-Phe ammonia-lyase (PAL). PAL catalyzes the first committed step in the phenylpropanoid pathway and thus contributes to the control of the carbon distribution between primary and secondary metabolism. Formation of benzenoid compounds from CA requires the shortening of the propyl side chain by a C_2 unit, which can potentially occur via either the β-oxidative pathway with benzoyl-CoA as an intermediate or the non-β-oxidative pathway with benzaldehyde as a key intermediate. Recent in vivo stable isotope labeling with deuterium ring-labeled Phe ($^2H_5$-Phe) in combination with computer-assisted metabolic flux analysis of the benzenoid network in Petunia flowers revealed that both the oxidative and non-oxidative routes contribute to the formation of benzenoid compounds (Orlova et al. 2006). The contribution of these pathways to benzenoid biosynthesis is light dependent with higher input from the oxidative pathway in the light than in the dark.

Modeling of stable isotope labeling data also revealed that benzylbenzoate can be an intermediate between Phe and benzoic acid in Petunia petals. A portion of the benzoic acid pool used to synthesize benzylbenzoate is derived from benzoyl-CoA, which is synthesized primarily through the oxidative pathway (although the reverse reaction from benzoic acid to benzoyl-CoA also occurs at a low rate). The existence of the reverse flux was confirmed in feeding experiments with $^2H_7$-benzyl alcohol that led to the labeling of the benzoic acid moiety of benzylbenzoate, possible only

if a conversion of benzoic acid to benzoyl-CoA occurs (Fig. 3.2). The elimination of benzylbenzoate biosynthesis by BPBT RNAi silencing resulted in a reduction of the endogenous pool of benzoic acid and subsequently reduced emission of its immediate product, methylbenzoate (Orlova et al. 2006). These data confirm that, although benzoyl-CoA in principle could be directly hydrolyzed to benzoic acid by a thioesterase, in Petunia petals it is converted to benzylbenzoate by BPBT (Boatright et al. 2004), and a portion of this ester is then hydrolyzed to contribute to the benzoic acid pool. Comparison of fluxes within the benzenoid network in the light and dark revealed a rapid benzylbenzoate turnover, leading to its greater contribution to benzoic acid biosynthesis in the light, in contrast to a significant expansion of the benzylbenzoate pool in the dark, despite a higher rate of its emission.

Results of the labeling experiments with $^2H_5$-Phe also revealed a dilution of isotopic abundance of benzaldehyde, benzyl alcohol, and their downstream products in the dark versus light. This observation suggests that an alternative biosynthetic pathway, from a precursor other than Phe, yet to be determined, also contributes to their biosynthesis in the dark in Petunia flowers (Orlova et al. 2006). Although it is known that both oxidative and non-oxidative pathways contribute to the biosynthesis of benzenoid compounds in Petunia flowers, the genes and enzymes responsible for chain-shortening reactions have not yet been elucidated.

3.4 Functional Genomics

In the last decade high-throughput sequencing projects have generated many Expressed Sequence Tags (ESTs) for solanaceous species. The Institute for Genomic Research (TIGR) set up the first database to allow access to these data (Rensink et al. 2005). Nowadays, the Solanaceous Genome Network database hosts all the available sequence data, both genomic and EST information. For Petunia, a collection of $\pm 11{,}000$ ESTs (see Table 3.1) has been made publicly available, mainly by the efforts of David Clark, University of Florida, Gainesville, Fl, USA.

Strikingly, more than 80% of these ESTs originate from floral tissue of Petunia Mitchell, making it a rich source for floral scent research (additional EST collections are available from the laboratories of several authors of this chapter, see, e.g., https://sativa.biology.lsa.umich.edu/blast/blast.html). However, since all transposon libraries described in other chapters in this book are made from the non-fragrant Petunia W138, these cannot be directly used for floral scent research.

The availability of EST sequence resources has allowed functional genomics approaches such as microarray analysis and candidate gene identification in EST libraries followed with RNAi and silencing. Verdonk et al. (2005) showed that dedicated microarrays can be used to identify differentially regulated "scent" genes, most prominently the MYB transcription factor ODO1, encoded by *ODORANT1 (ODO1).* The microarray data also provided clues for the isolation and identification of a previously unknown biosynthetic gene, *PhIGS1*, encoding an isoeugenol

Table 3.1 Numbers of ESTs from solanaceous species available through the SGN database (http://www.sgn.cornell.edu/)

Plant species	ESTs	Unigenes
Capsicum annuum	20738	9554
Coffea canephora	55539	15721
Nicotiana tabacum	76056	25398
Petunia hybrida	11479	5135
Solanum lycopersicum		
Solanum habrochaites		
Solanum lycopersicoides	239593	34829
Solanum pennellii		
Solanum peruvianum		
Solanum pimpinellifolium		
Solanum melongena	3181	1841
Solanum tuberosum	134365	31072
Total on July 3rd 2007	540951	113996

synthase, and the subsequent identification of the homologous gene for eugenol synthase from basil, *OsEGS1* (Koeduka et al. 2006).

Other biosynthetic and regulatory genes identified by microarray experiments (Underwood et al. 2005; Verdonk et al. 2005) await characterization (Van Moerkercke, Verdonk, Clark, Haring and Schuurink, work in progress). EST-database mining for genes encoding proteins with homology to known biosynthetic enzymes resulted in the identification of *PhBSMT* 1/2 (Underwood et al. 2005), *PhBPBT* (Boatright et al. 2004), *PhPAAS* (Kaminaga et al. 2006), and *PhCFAT* (Dexter et al. 2007). Proof for the "*in planta*" activity of all the above-mentioned genes was obtained by generating RNAi or antisense transgenic plants.

Clearly, we are seeing only the beginning of the application of genomic resources in Petunia floral scent research. To the extent that the Petunia flower is a much less complex organ than the tomato fruit, we predict that much of the knowledge gained from the Petunia model can be applied to understand flavor biosynthesis in tomato fruit. The recent characterization of tomato aromatic amino acid decarboxylases involved in phenylethanol and phenylacetaldehyde biosynthesis (Tieman et al. 2006) and the identification of the *ODO1*-ortholog *SlODO1* (Kroon, Schuurink, and Haring, unpublished) point to ample opportunities for the application of comparative biology.

3.5 Regulation of Biochemical Pathways

Although the regulation of volatile phenylpropanoids/benzenoids is complex, as described below, the paradigm of precursor regulation has also been shown for Petunia Mitchell, by Kolosova et al. (2001). They demonstrated that the amount of benzoid acid (BA) oscillated during the daily light/dark cycle and showed that the

total amount of BA determines the rhythmic emission of methylbenzoate (MeBA) and most likely also that of other volatile phenylpropanoids/benzenoids. PAL activity also oscillates and peaks prior to the peak emission of MeBA (Kolosova et al. 2001). Moreover, *PAL* mRNA levels are highly regulated in Mitchell petals as well (Verdonk et al. 2003; Verdonk 2006), indicating a level of transcriptional control. From the transcriptome analyses by Verdonk et al. (2003, 2005, 2006) it is clear that several genes from the shikimate pathway (*DAHP synthase*, *EPSP synthase*, and *CM*) are also transcriptionally regulated. These shikimate pathway genes are apparently controlled by ODO1, as downregulation of *ODO1* in RNAi plants resulted in a reduction in their expression (Verdonk et al. 2005). Moreover, this also resulted in a 90% reduction of BA in the petals and a considerable drop in the emission of volatile phenylpropanoids/benzenoids, confirming the paradigm of precursor regulation.

In addition to the regulation of the shikimate pathway, the biosynthetic genes are also tightly regulated. Expression of *BSMT*, *BPBT*, *PAAS*, and *CFAT* is cyclic (Boatright et al. 2004; Underwood et al. 2005; Kaminaga et al. 2006; Dexter et al. 2007), as is that of several genes related to the SAM-cycle, such as *SAM synthetase* (Verdonk et al. 2003; Schuurink et al. 2006; Verdonk 2006). Downregulation of biosynthetic genes in RNAi plants has provided insight mostly into the metabolic flux through the various pathways (see above) but little into the regulation of gene expression. This is simply due to the fact that mRNA levels of other relevant genes were not always measured in these RNAi plants. Since downregulation of *BSMT* does not lead to any changes in the emission of volatiles other than MeBA (Underwood et al. 2005) one can ask why the absence of this most abundant volatile has such little effect. Or does downregulation of *BSMT* expression influence the expression levels of the shikimate pathway genes? Interestingly, downregulation of *CFAT* leads to diminished emission of many volatiles, but expression levels of *BSMT*, *BPBT*, *PAAS*, and *IGS* are not affected (Dexter et al. 2007). Apparently the accumulation of coniferyl aldehyde and homovanillic acid in these CFAT RNAi lines inhibits BPBT and PAAS enzyme activity, adding potentially another layer of regulation, as suggested by Kondo et al. (2007) for the isoeugenol pathway.

The interplay among the shikimate pathway genes, the biosynthetic genes, and SAM-cycle genes has, until now, been shown only by the downregulation of *ODO1* in RNAi plants. The SAM-cycle genes are not upregulated in these RNAi plants as in wild-type plants upon the onset of MeBA emission, but SAM levels are the same as in wild-type plants (Verdonk et al. 2005). Apparently this SAM cycle is tightly controlled so that no ATP is wasted. Furthermore, *BSMT* and *BPBT* are upregulated in these ODO1 RNAi plants, suggesting that expression of these genes is, at least partially, under precursor control. More insight into the interplay among these three pathways might be provided by extensive analysis of gene expression, enzyme activities, and metabolites during pollination and ethylene treatments of petals that decrease BA levels, *BSMT* expression, and concomitantly emission of MeBA (Negre et al. 2003; Underwood et al. 2005; see also the section below).

Finally, genetic analysis of recombinant inbred lines of *P. axillaris parodii* and *Petunia hybrida* W138 showed that two QTLs are predominantly responsible for the

differences in scent production by these two Petunias (Stuurman et al. 2004). Identification of these two scent loci might reveal regulatory players other than *ODO1*.

3.6 Rhythmic Emission of Floral Volatiles

In sympatric wild populations of Petunia species no hybrids have been found, suggesting that a pollination syndrome might be involved (see Chapter 2). Flowers of *P. axillaris* emit a strong fragrance in the evening, correlated with the nocturnal visits of the pollinator, a hawkmoth (Ando et al. 2001). From subsequent experiments it became clear that the flowers of the reproductively isolated sympatric species *P. integrifolia* produce very little floral scent, in a more or less constant fashion. Volatile emission from *P. axillaris* flowers appears to follow an endogenous circadian rhythm, peaking approximately after 4–6 h of darkness (Hoballah et al. 2005). Not only their emission, but also the levels of endogenously stored benzenoids follow this circadian rhythm in *P. axillaris* (Oyama-Okubo et al. 2005).

The genetically related Petunia Mitchell was used to dissect the nature of the regulation of this emission pattern. Kolosova et al. (2001) showed that emission of floral volatiles from Petunia Mitchell is rhythmic, peaking in the dark period of a 12hL/12hD rhythm. For methylbenzoate, one of the major cycling scent components, they discovered that the enzymatic activity of BSMT did not follow a cyclic pattern of activity. Instead, BSMT activity levels remained constantly high. In contrast, the enzymatic activity of PAL did follow the methylbenzoate emission pattern, peaking approximately 3 h before the maximum of scent emission. PAL is involved in the production of precursors, and indeed the levels of the principal precursor benzoic acid peaked sharply in the dark period, indicating that scent emission is regulated at the precursor level. These results were corroborated using a slightly different rhythm (16hL/8hD). In that case scent emission peaked at dusk rather than at night (Verdonk et al. 2005). The discovery that expression of a *PAL* gene correlated with the scent emission pattern boosted further research on the transcriptional regulation of benzenoid biosynthesis genes. A flower-specific *BPBT* gene was also show to be rhythmically expressed (Boatright et al. 2004). Its rhythm was ±8 h out of phase with the emission of its product, benzylbenzoate (Fig. 3.3). A similar picture emerged when emission of phenylacetaldehyde (Boatright et al. 2004) was compared with expression of the *PAAS* gene (Kaminaga et al.). For methylbenzoate emission and *PhBSMT* expression similar results were obtained, albeit using a different rhythm (18hL/6hD). The expression of CFAT paralleled the emission of its product isoeugenol (Dexter et al. 2007). In all the above cases the rhythm of gene expression was lost within one cycle when entrained plants were transferred to constant darkness.

These data suggest that a central transcriptional regulator is involved in maintaining the rhythmic emission pattern of floral volatiles. The MYB-like transcription factor ODORANT1 (Verdonk et al. 2005) appears to be involved in the regulation of precursor biosynthesis genes such as *5-enol-pyruvylshikimate-3-phosphate synthase*

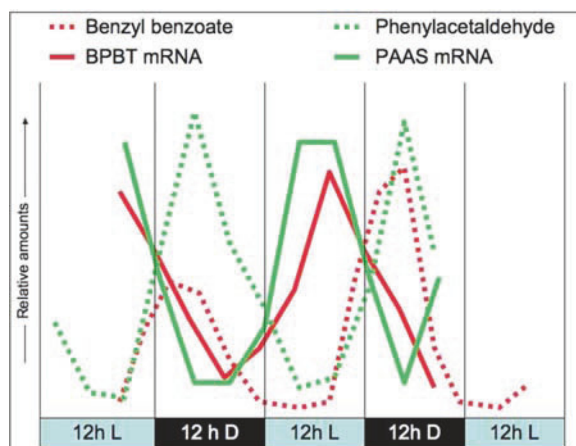

Fig. 3.3 Rhythmic scent emission and benzenoid biosynthetic gene expression in Petunia Mitchell flowers. During a 12hL/12hD cycle Petunia flowers emit benzylbenzoate and phenylacetaldehyde in a rhythmic pattern, peaking during the subjective night period. The mRNA levels of the corresponding biosynthetic genes *BPBT* and *PAAS* follow a rhythm that is ±8 h out of phase with scent emission. The graphical representation is based on data from Boatright et al. (2004) and Kaminaga et al. (2006)

(*EPSPS*). Suppression of *ODO1* strongly reduced expression of these genes and subsequently of scent emission. However, this did not downregulate the expression of biosynthetic genes like *BSMT* and *BPBT*. The crucial effect of *ODO1* suppression appeared to be a strong reduction of the scent precursor compound benzoic acid. Because expression of *ODO1* follows the floral benzenoid emission pattern, this gene appears to be regulated itself by a transcriptional oscillator. Therefore one can assume that at least two other regulatory circuits are active to maintain this circadian rhythm: one to regulate expression of *ODO1* and another to time the expression of the benzenoid biosynthetic genes.

3.7 Ethylene

The plant hormone ethylene is known to coordinate several post-pollination processes in the flowers of many plant species (van Doorn 1997). The Petunia flower has long served as an excellent system to study these processes, and in recent years Petunia Mitchell has been widely used for such studies (see Chapter 14). It has a very reproducible corolla senescence phenotype (wilting) in response to exogenous or pollination-induced ethylene. Additionally, there are ethylene-insensitive transgenic plants available that constitutively express a mutant Arabidopsis ethylene receptor (CaMV35S::etr1-1, also known as 44568) and have a significantly delayed floral senescence phenotype (Wilkinson et al. 1997). Combined, these attributes

have made this system an excellent tool for studying post-pollination processes in flowers.

In 2002, a role for ethylene in floral volatile synthesis was discovered. While conducting microarray experiments to screen for ethylene-regulated genes that were potentially involved in corolla senescence, a subset of genes whose expression was specific to the flowers, and downregulated by ethylene, was isolated (Negre et al. 2003; Underwood et al. 2005). Subsequent detailed analysis of two genes (*PhBSMT1* and *PhBSMT2*) provided clear evidence that ethylene has direct control of floral volatile synthesis during corolla senescence. Well-established models for post-pollination regulation of ethylene synthesis in Petunia indicate that ethylene is produced from the stigma/style shortly after pollination. At fertilization, a large burst of ethylene is produced by the ovary, leading to autocatalytic ethylene synthesis by the corolla and subsequent corolla senescence. In a series of elegant experiments using Mitchell and ethylene-insensitive 44568 plants, Underwood et al. (2005) demonstrated that the temporal and spatial downregulation of *PhBSMT 1/2* expression in Petunia floral organs followed the sequential pattern of post-pollination ethylene synthesis in Mitchell but not in 44568. It was also shown in this study that emission of all phenylpropanoids/benzenoids ceased in Mitchell but not in 44568 after pollination and exogenous ethylene treatments. This observation was supported by a similar downregulation of *CFAT* by ethylene (Dexter et al. 2007).

The downregulation of floral volatile synthesis is physiologically meaningful because the timing of regulation corresponds to the second major phase of ethylene synthesis, when the ovary produces ethylene as a signal to the corolla that a successful fertilization event has taken place and the corolla is no longer needed for pollinator attraction (see Chapter 14). Because much metabolic energy is necessary for benzenoid/phenylpropanoid synthesis and emission, the flowers stop maintaining the corolla and shift from a phase of pollinator attraction to one of seed production. Future work to isolate the promoters and regulatory elements of these newly discovered benzenoid biosynthetic pathway genes will further elucidate the factors necessary for the transcriptional downregulation caused by ethylene.

3.8 Is There Interplay Between Scent and Color Production?

As the pathway leading to the precursors for flavonoids (see Chapter 13) and phenylpropanoids/benzenoids is identical in petals, one could raise the question whether color production influences scent production and, vice versa, whether scent production influences color production. However, there are several lines of evidence that this is not the case in Petunia.

Hoballah et al. (2007) showed that transformation of the white *P. axillaris* (*AN2*) with *AN2* leads to a different reflectance of the petals between 500 and 600 nm without a change in the total production of benzenoids. Thus a change in color of the petals, which can only be due to the production of flavonoids, does not

lead to a reduction in the production of volatile benzenoids. Interestingly, even the fact that this *AN2* gene is under the control of the constitutive 35S-CMV promoter and not its own, highly regulated, promoter did not matter. Second, Verdonk et al. (2005) showed that downregulation of the shikimate pathway in Petunia Mitchell, and thus a reduction in the levels of precursors for volatile phenylpropanoid/benzenoid production, did not lead to a reduction in flavonoid production in Petunia Mitchell. Moreover, they also showed that downregulation of the shikimate pathway in progeny of a cross between Mitchell and the purple flowering V26 did not lead to a change in flower color in comparison to that seen in progeny with normal shikimate pathway activity (Fig. 3.4). Their explanation for this phenomenon was that the production of color and scent molecules is developmentally separated. First the flavonoids, which are stably sequestered during the lifespan of the flower, are produced. Only then, when the flower opens, the phenylpropanoids/benzenoids are produced. Third, Orlova et al. (2006) showed that reduction in benzenoid synthesis in Petunia Mitchell, through reduction of *BPBT* expression, did not lead to an increase in flavonoids in petals, unlike in seeds

Thus, all evidence so far indicates that there is no interplay between color and scent production. A recent study by Nakamura et al. (2006) indicates a close correlation between the selection of blue petals and isoeugenol production during daytime. It remains to be investigated whether there may be an as yet unidentified interaction between scent and color, perhaps acting through the *PH* genes (see Chapter 13), in these cultivars.

Fig. 3.4 Petals from two progeny of a cross between Petunia Mitchell and *P. hybrida* V26, one with normal benzenoid emission levels (*left*) and the other with reduced benzenoid emission levels (*right*) due to suppressed activity of the shikimate pathway. (©ASPB, from Verdonk et al. 2006). Their coloration is indistinguishable

3.9 Role of Floral Volatiles in Wild Accessions

A major function for floral volatiles in the natural habitat is the attraction of animal pollinators. Although flower scent is generally accepted to be a potent long-distance attractant for night-active moths (e.g., Ando et al. 2001), few field experiments clearly confirm this assumption. For Petunia, no extensive data on field studies are available, but studies with other species showed similar scent cocktails to be effective in attracting hawkmoths (Raguso and Willis 2004; see Chapter 2). This lack of information is due partly to the complex setup required for field experiments, but also to our lack of knowledge about the importance of the volatile compounds both in the plant and for the animals.

From wild accessions of *Petunia axillaris* mostly benzenoid compounds have been identified among the headspace volatile compounds (Hoballah et al. 2005). Among these, methylbenzoate, benzaldehyde, phenylacetaldehyde, and benzyl alcohol are consistently found and have emerged as candidates for major components in the Petunia volatile mix perceived by hawkmoths (see below). Other benzenoid compounds such as eugenol, isoeugenol, and vanillin as well as methylsalicylate have been detected, but may not be present in all wild plants and under all environmental circumstances. Variation in wild species is substantial and crosses subspecies classification (Kondo et al. 2006).

Electro-antennogram experiments have demonstrated that whole-flower mixtures, as well as isolated components, stimulate *Manduca sexta* antennae under controlled conditions (Hoballah et al. 2005; Fig. 3.5). While these experiments clearly indicate the importance of some compounds in alerting insects, studies in the field and choice experiments will be required to clarify the role of each compound.

Species of the *Petunia integrifolia* group display a typical bee-pollination syndrome (Galliot et al. 2006a; see Chapter 2). Benzaldehyde and related compounds are found in some accessions (Hoballah et al. 2005), especially those that have been propagated in botanical gardens. However, it is unclear to what extent *P. integrifolia* produces scent in the natural habitat and whether it requires benzenoid compounds to attract bees as pollinators.

Hummingbirds are likely to be major pollinators of *Petunia exserta*, a rare species from Southern Brazil (Stehmann 1987). The role of volatiles in hummingbird attraction may be minor, although the birds can perceive them (Kessler and Baldwin 2007). It will be interesting to study scent production in this species, as it is very closely related to *P. axillaris*.

Not all compounds detected in the floral headspace samples of Petunia are likely to serve as pollinator attractants. Instead some of them may be important for plant defense. Nicotine, for instance, has been reported to act as an insect repellent in *Nicotiana* species (Kessler and Baldwin 2007). In this context, a comprehensive description of insect species that visit the diverse Petunia species in Argentina, Uruguay, Paraguay, and Brazil, especially in respect to the control of herbivores, will be very useful.

Scent emission varies among Petunia species not only in composition of the volatiles but also in timing. *Petunia integrifolia* accessions do not alter their volatile

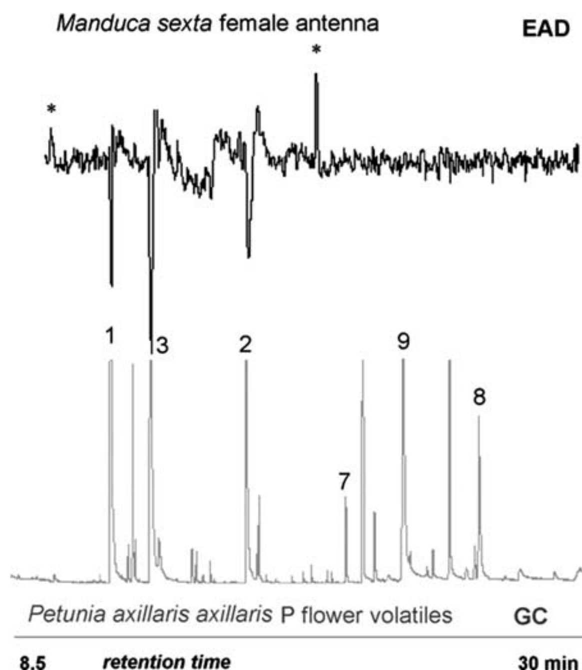

Fig. 3.5 *Petunia axillaris* floral volatiles were resolved by gas chromatography and applied to isolated antennae of *Manduca sexta* (**1**) benzaldehyde (**2**) benzyl alcohol (**3**) methylbenzoate (**7**) eugenol (**8**) vanillin (**9**) isoeugenol. *Asterisks* denote occasional losses of contact between antenna and electrode. Reproduced from Hoballah et al. 2005

emissions detectably (Hoballah et al. 2005), while all *Petunia axillaris* accessions tested begin volatile production in the evening, when night-active hawkmoths are beginning to fly (Kolosova et al. 2001; Hoballah et al. 2005; Schuurink et al. 2006). This circadian emission persists for several nights, until fertilization, which leads to a rapid decrease in volatile production accompanied by wilting of the flower (Negre et al. 2003).

As most Petunia varieties analyzed for gene expression are inbred and/or hybrid accessions, findings that hint to a paramount importance of genes such as *ODO1* (Verdonk et al. 2005) and *BSMT* (Negre et al. 2003) in specialization of bee- and moth-pollinated Petunia will need to be confirmed in wild species. Quantitative trait locus (QTL) studies (Galliot et al. 2006b) must then be translated into field experiments involving specific lines that carry small introgressions from one species in the background of another. A further interesting consideration concerns the cost of odor production, especially in relation to other means of Petunia to attract pollinators, such as color or nectar.

3.10 Conclusions and Projections

From this chapter it must have become clear that over the last decade *Petunia hybrida* has become the dominant model plant to study benzenoid/phenylpropanoid biosynthesis and emission, and it is there to stay for the foreseeable future. As

indicated in the various sections of this chapter, much remains to be discovered. Moreover, we have not even begun to touch the biophysics of emission, one of the bigger enigmas in the science of volatiles.

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Chapter 4
ADH and PDC: Key Roles for Enzymes of Alcoholic Fermentation

Judith Strommer and Freydoun Garabagi

Abstract In plants the enzymes pyruvate decarboxylase and alcohol dehydrogenase are generally associated with the alcoholic fermentation pathway, producing a diffusible, non-acidic, and relatively non-toxic end-product for anaerobic glycolysis while regenerating a small amount of NAD^+ and ATP. Work with Petunia and tobacco has provided evidence that a more critical function in pollen, and probably other organs and tissues, is to feed carbon back into general metabolism by way of a pyruvate dehydrogenase (PDH) bypass. Alcohol dehydrogenase is also linked to the biosynthetic pathway producing linear six-carbon volatiles, and perhaps some aromatic volatiles, associated with attraction of insect pollinators.

4.1 Introduction

After nearly a century of study, enzyme functions should be well established, especially for the relatively abundant enzymes lumped into the "housekeeping" category. But enzymes have been approached through diverse disciplines: biochemistry focused on reaction mechanisms and specific pathways; genetics linked phenotypes and isozymes to genes; and physiology by and large attended to large-scale systems within the organism. A view of the whole required new sets of tools, many of which became available over the past four decades through the discipline of molecular biology. With the help of appropriate model systems, we are slowly filling in the big picture for 'simple' enzyme systems like those traditionally associated with alcoholic fermentation.

Petunia, with its strong genetic grounding and easy handling, has proved an excellent system in this effort. This chapter focuses on two key enzymes of alcoholic fermentation, straight-chain alcohol dehydrogenase (ADH, EC 1.1.1.1) and

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pyruvate decarboxylase (PDC, EC 4.1.1.1), summarizing early work on diverse species and more recent studies relying on a combination of traditional and molecular approaches for the analysis of these enzymes in Petunia.

4.2 The Fermentation Enzymes and Hypoxic Stress

PDC catalyzes the first step in anaerobic fermentation, producing acetaldehyde from the pyruvate generated in glycolysis. Under normal conditions favoring respiration, pyruvate is fed through the PDH complex into the Krebs (TCA) cycle for oxidative phosphorylation. In the absence of oxygen, oxidative phosphorylation cannot proceed. Through ethanolic fermentation, requiring the sequential activities of PDC and ADH, a low level of ATP production can be maintained, together with regeneration of NAD^+.

The presence of ADH activity in plants was reported in 1943, based on a survey of dehydrogenase activities in extracts of oat coleoptiles (Berger and Avery 1943). The enzyme was of practical interest due to its inhibition of fruit softening (Biale 1946), but interest among basic researchers was bolstered by the work of Hageman and Flesher (1960), who first demonstrated the role of ADH in reducing acetaldehyde to ethanol under oxygen-limited conditions. In the 1960s, Drew Schwartz merged anaerobic and genetic studies of ADH in maize (Schwartz 1966; Schwartz and Endo 1966). His work led to a large body of genetic and physiological work in relation to *ADH* variants and their roles in the response to low oxygen, particularly in roots of maize seedlings.

The classical plant pathway for alcoholic fermentation is represented by the black solid and dashed lines in Fig. 4.1. In the 1980s, the predicted hypoxic induction of the two key enzymes acting in addition to ADH in anaerobic glycolysis, that is, lactate dehydrogenase (LDH; Hoffman, Bent, and Hanson 1986) and PDC (Laszlo and St. Lawrence 1983) was demonstrated in maize. LDH induction occurs at an early stage of oxygen deprivation in plant seedlings, as in vertebrate muscles, converting pyruvate to lactate and, in the process, lowering cytosolic pH. In plant cells, unlike muscle cells, PDC is activated by the pH drop and converts the pyruvate to acetaldehyde. The subsequent reduction to ethanol by ADH regenerates some energy potential in the forms of NAD^+ and ATP. The response, it has long been agreed, enhances the potential for survival of germinating seeds and seedlings during episodes of spring flooding, during which floodwaters displace soil oxygen (e.g., Kloeckener-Gruissem and Freeling 1987; Drew 1990).

Other organs, including pollen, fruits, and tubers were known to produce measurable levels of ADH and were reasonably argued to experience some degree of hypoxia. Their ADH activities therefore fit the pattern of an anaerobic stress response. The demonstrated induction of ADH by low temperature (Christie, Hahn, and Walbot 1991) and various other stresses, including dehydration, ozone, wounding, and SO_2 treatment (Kimmerer and Kozlowski 1982; Dolferus, Jacobs, Peacock, and Dennis 1994; Desikan, Machkerness, Hancock, and Neill 2001; Kato-Noguchi

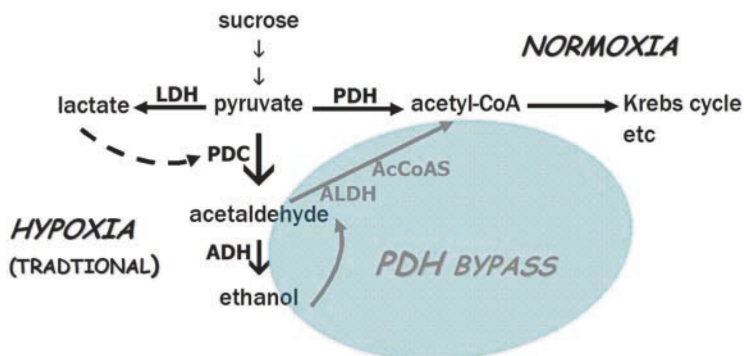

Fig. 4.1 Classical alcoholic fermentation pathway (black) with PDH bypass superimposed (gray/blue). Pyruvate is normally directed to the Krebs cycle via the PDH complex, which produces acetyl-CoA. The fermentation pathway of plants, as traditionally understood, is indicated by solid lines: when oxidative phosphorylation is prevented, pyruvate is diverted from the PDH complex by LDH (lactate dehydrogenase), which produces lactate, thereby lowering cytosolic pH. PDC, activated by the pH drop, converts pyruvate to acetaldehyde, which is then reduced to ethanol by ADH. Gray lines denote operation of the alternative PDH bypass, which provides a route for production of acetyl-CoA via acetaldehyde. In this model, ADH and ALDH (aldehyde dehydrogenase) compete for acetaldehyde. By means of the bypass, PDC and ADH essentially control delivery of carbohydrate catabolites for cellular metabolism

2001; Seki et al. 2002) were understood as extensions of the so-called anaerobic response, helping to ensure plant survival when mitochondrial activity is disrupted.

There were other refinements to the anaerobic stress story. Roberts, Callis, Wemmer, Walbot, and Jardetzky (1984), for example, provided evidence that the pH stabilization provided by the switch from lactate to ethanol production was critical for preventing cellular acidosis in roots of maize seedlings.

The finding that ADH activity in pollen was far greater than that required for maximal fermentation (Roberts, Chang, Webster, Callis, and Walbot 1988) was surprising, but it had been known for years that plants devoid of pollen ADH activity were at no discernible disadvantage except under hypoxic conditions (Schwartz 1969). The generally shared conclusions were (1) the role of PDC is to carry out the first step in fermentation, supplying acetaldehyde to ADH and protecting the plant from acidosis, and (2) ADH activity serves to detoxify acetaldehyde while providing limited energy under oxygen-limited conditions.

ADH is active as a dimer, the subunits of which are encoded by a family of *ADH* genes in all plants examined to date, with the apparent exception of Arabidopsis, in which the activity of just one gene has been demonstrated. Amino acid sequences encoded by different members of the family generally differ sufficiently to allow electrophoretic separation of the various homo- and heterodimers, which can then be visualized by a dye-linked enzymatic reaction (Fig. 4.2). Isozyme patterns from extracts of suitably treated tissue are generally agreed to reflect the combinations of polypeptides that were present in a cell at the same time. Although it was clear from numerous studies that relative amounts of the various ADH isozymes vary

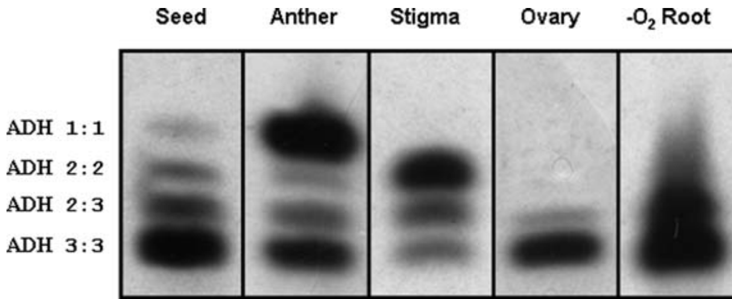

Fig. 4.2 Activity assays of Petunia tissues showing profiles of homo- and heterodimers of ADH. Protein extracts from tissues were separated on a nondenaturing acrylamide gel and stained for ADH activity with NAD^+, ethanol, and nitroblue-tetrazolium

according to the source tissue or treatment, the potential significance in terms of differentiated functions, with minor exception, seems to have escaped notice or interest. PDC enzymatic activity is less easily analyzed, which may help explain the relative paucity of early research. It is active as a homotetramer, like ADH it is cytosolic.

4.3 ADH and PDC in Petunia

Several years ago we chose Petunia as a model dicot to complement the genetic and physiological studies associated with the production of ADH isozymes in maize. The goal of this work was to try to link gene expression with enzymatic function. Two *ADH* genes were quickly identified, and it appeared from early analyses that *Petunia hybrida* used alcohol dehydrogenase genes and enzymes in ways similar to those of maize: three isozyme bands were detectable in extracts of hypoxic seedling roots, the slowest matching the electrophoretic migration pattern of the sole band of ADH activity derived from pollen. The first cloned gene, obtained from a genomic library screened with a maize cDNA probe, encoded products that migrated with the slowest band of ADH activity, and hybridizing RNA was produced at very high levels in immature anthers. The gene was designated *ADH1* and the product ADH1 (Gregerson, McLean, Beld, Gerats, and Strommer 1991). Activity of the subsequently cloned *ADH2* gene was demonstrated in other potentially hypoxic tissues, but ADH1 activity alone was present in pollen (Gregerson, Cameron, McLean, Dennis, and Strommer 1993; Strommer, Gregerson, Foster, and Huang 1993; Foster Atkinson, Cameron, and Strommer 1996). Molecular mapping and comparisons to Petunia Mitchell, however, provided evidence for a third gene and led to the identification of *ADH3* and its polypeptide ADH3, the electrophoretic behavior of which was indistinguishable from ADH1. Loci for *ADH1* and *ADH3* are closely linked on Chromosome IV; *ADH2* maps to Chromosome III (Strommer, Gerats, Sanago, and Molnar 2000, 2001; Garabagi and Strommer 2004).

With three distinct nucleotide sequences in hand and electrophoretic isozyme patterns clarified, patterns of ADH activity in Petunia were characterized by means of isozyme analyses, histochemistry, RT-PCR, and promoter–reporter fusions (Foster Atkinson 1996; Garabagi and Strommer 2004; Garabagi, Duns, and Strommer 2005), the latter limited to *ADH1* and *ADH2* promoters fused to a green fluorescent protein (GFP) coding region.

Isozyme gels, together with histochemical analysis and gene-specific RT-PCR, verified the involvement of ADH2 and ADH3, but not ADH1, in the hypoxic response. They demonstrated that hypoxic induction of ADH activity in roots of young plants spread outward from the stele, and low levels of activity of ADH activity, associated with *ADH2* and *ADH3* RNA, could occasionally be detected around the vasculature of untreated seedlings. This pattern provided direct support for hypotheses of chronic stellar anoxia (Armstrong and Beckett 1987; Thomson and Greenway 1991) based on measurements of volatiles and mathematical modeling. At the same time, it demonstrated that the ADH forms associated with the hypoxic response in vegetative tissues, ADH2 and ADH3, were distinct from that responsible for all ADH activity in pollen, ADH1.

ADH2 and ADH3 homo- and heterodimers were the only isozymes detected throughout the vegetative plant, with relatively more activity in ADH3-containing dimers. Enzyme activities ranged from undetectable to low in normoxic young plants, with many-fold induction in response to hypoxia. This response was apparent in roots, hypocotyls, epicotyls, and leaves. Products of *ADH2* and *ADH3* were also encountered at relatively high levels in anthers of flower buds, dropping to barely detectable levels in open flowers (Gregerson et al. 1991). The *ADH2::GFP* fusion verified strong *ADH2* promoter activity in these organs (Fig. 4.3A, B, and data not shown). These expression patterns fit well with the expected hypoxic response.

ADH1-based dimers and *ADH1*-RNA, on the other hand, were detectable only in pollen and seed (discussed below). As described by Gregerson et al. (1991), *ADH1* RNA and ADH1 homodimers are first detected late in bud development, with RNA levels peaking in anthers of open flowers. Separation of immature pollen from the anther verified pollen as the source of ADH1 (Foster Atkinson 1996). At pollen maturity, ADH1 levels are much higher than those seen in anthers of buds; *ADH1*-RNA, on the other hand, is no longer detectable. Reporter studies (Fig. 4.3C, 4.3D), based on fusion of the *ADH1* promoter to the *GFP* gene, showed a similar expression pattern, with high levels of GFP fluorescence in mature pollen, similar to what is seen for ADH1 activity (Garabagi et al. 2005).

The two *PDC* genes of Petunia were cloned and characterized by Gass et al. (2005). Their products exhibit minimal overlap in expression. All gene expression in floral organs and the vegetative plant, with the exception of pollen, is associated with *PDC1*; pollen expression alone relies on *PDC2*. Thus, PDC2 and ADH1 are present together in pollen; PDC1 and ADH2/3 are coproduced elsewhere in the plant. All five gene products are detectable in seeds, with relatively lower levels of PDC2 and ADH1.

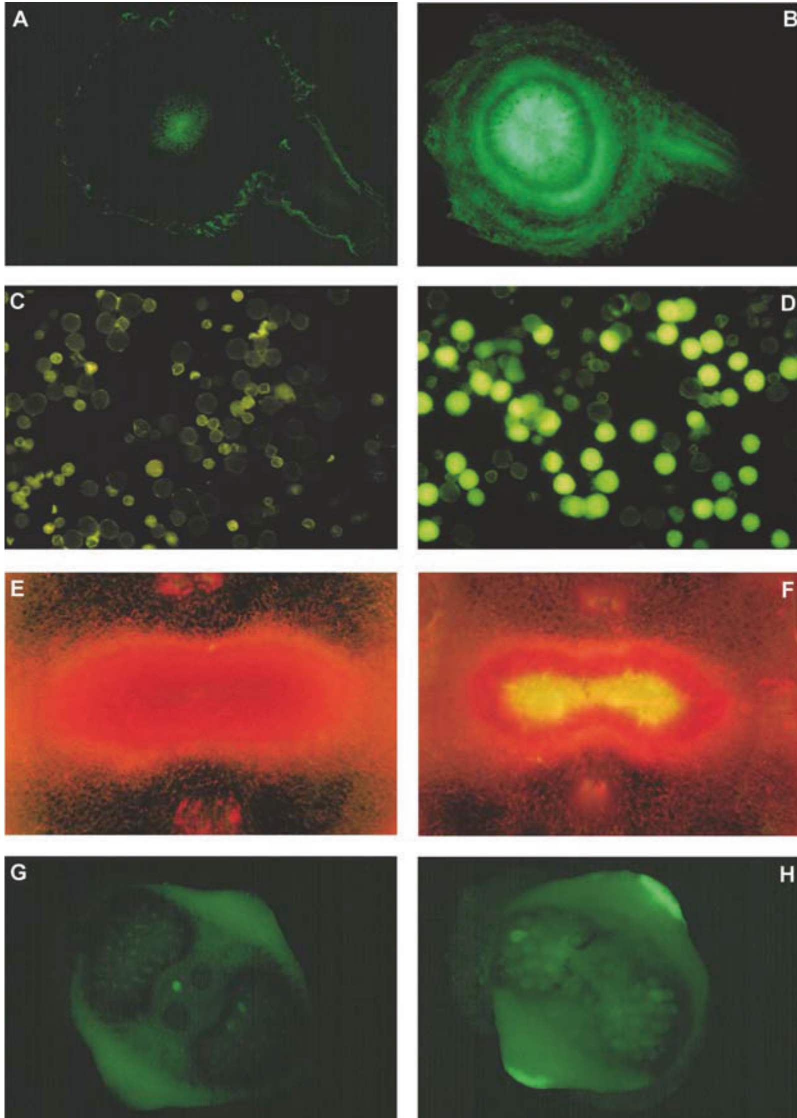

Fig. 4.3 GFP fluorescence images captured with a green-specific (500–530 nm; **A–D, G, H**) or long-pass (>500 nm; **E, F**) filter after excitation with blue light (450–490 nm). All images except **C** and **D** depict cross sections. Normoxic (**A**) and hypoxic (**B**) roots from Petunia seedlings carrying a *GFP* reporter gene fused to the *ADH2* promoter; pollen from control (**C**) and *ADH1-GFP*-transformed (**D**) plants; styles from control (**E**) and *ADH2-GFP*-transformed (**F**) plants (red is due to chlorophyll autofluorescence); base of control (**G**) and *ADH2-GFP*-transformed ovaries (**H**). Green in panel G represents weak endogenous autofluorescence of the Petunia ovary

4.4 Isozyme Functions

4.4.1 Pollen

The ubiquity of ADH in pollen, together with evidence and arguments that alcohol dehydrogenase activity in maize pollen exceeds the amount needed for measured ethanol production (e.g., Roberts et al. 1988), and the demonstration that maize pollen without ADH is at no apparent disadvantage (Johnson, Cobb, and Drew 1994), presented a paradox in relation to the role of ADH in pollen. The demonstration of a pollen-specific ADH form differing from those induced in response to hypoxia in Petunia suggested the likelihood of a distinct function for ADH of pollen. Starting with the closely related genus *Nicotiana* and a focus on PDC, Cris Kuhlemeier and colleagues provided a new perspective and a possible answer.

The critical findings from tobacco (Bucher, Brander, Sbicego, Mandel, and Kuhlemeier 1995; Tadege and Kuhlemeier 1997; Tadege, Dupuis, and Kuhlemeier 1999; Mellema et al. 2002) included the following: (1) respiration in pollen is tenfold higher than in leaves; (2) the rate of ethanol production (and *ADH* gene expression) in pollen is not a function of oxygen availability but rather sugar supply; (3) aldehyde dehydrogenase is active in pollen and, unlike ADH, required for pollen viability; and (4) radio-labeled carbon introduced in ethanol can be recovered in CO_2 and lipids. On the basis of these findings they concluded that, in relation to fermentation, pollen is more similar to baker's yeast than to vegetative plant tissues. Pyruvate, they proposed, can be channeled through a PDH bypass via PDC-dependent conversion into acetaldehyde, which is converted into acetate through the action of aldehyde dehydrogenase, and then into acetyl-CoA, which is available for gluconeogenesis, introduction into the TCA cycle, or fatty acid/lipid biosynthetic pathways, all potentially important uses of carbon in fast-growing, metabolically active cells like germinating pollen. The role of alcohol dehydrogenase, they argued, is that of a "safety valve," to protect against accumulation of toxic acetaldehyde. They suggested that in pollen ADH acts in both reaction directions: to reduce dangerously high levels of acetaldehyde, and, when metabolite levels change, to re-oxidize accumulated ethanol to acetaldehyde for entry into the PDH bypass (Fig. 4.1). The demonstration that growing pollen could cycle carbons from exogenously supplied ethanol into CO_2 and lipids provided evidence for their hypothesis.

In 2005 the group reported on results of analyses based on Petunia, for which a *PDC2* null mutant was recovered from a line with high transposable element activity (Gass et al. 2005). Whereas no *ADH*-null has been shown to affect pollen performance, the mutant pollen lacking PDC – which produced no detectable acetaldehyde or ethanol – showed clearly reduced transmission through the style. Chemical inhibition of the alternative pathway for pyruvate utilization, the PDH pathway, completely inhibited pollen tube growth in mutant, but not wild-type, pollen. Exogenously supplied ethanol rescued the germination potential of PDH-inhibited mutant pollen. This evidence confirmed that ADH can oxidize ethanol in pollen and that PDC is capable of its mobilization so as to maintain biosynthetic capacity in the absence of the standard PDH-dependent drive of pyruvate into general plant

metabolism. Essentially PDC then, not ADH, is the critical enzyme in the pathway, explaining the minimal phenotype of *ADH*-null pollen but leaving unsolved the mystery of the apparently huge surplus of ADH activity in germinating pollen.

4.4.2 Styles

Intense fluorescence, signifying strong *ADH2* promoter function, together with high levels of demonstrated ADH2 activity, are apparent in the transmitting tract, which serves as the conduit for growing pollen tubes. Fluorescence is seen in both pollinated and unpollinated pistils of Petunia (Figs. 4.3E, 4.3F). In this tissue, and no others examined, activity of ADH2 homodimers greatly exceeds that of ADH3-containing isoforms. Expression of *ADH2* and *ADH3* was confirmed by RT-PCR analysis (Garabagi and Strommer 2004; Garabagi et al. 2005). Linskens and Schrauwen (1966) had reported high oxygen tensions in unpollinated stigmas, with a wave of low oxygen tension accompanying the growing tips of pollen tubes. Consistent with their description was the report that *ADH* gene expression and ADH enzyme activity in the styles of potato flowers are induced by pollination (van Eldik, Ruiter, van Herpen, Schrauwen, and Wullems 1997).

van Eldik et al. argued that pollen-induced ADH does not act to provide energy through anaerobic fermentation in the hypoxic style (simple hypoxic induction), but to oxidize the great amount of ethanol produced by the pollen tube, measured in vitro with tobacco pollen at 100 mM (Bucher et al. 1995). The acetaldehyde, they proposed, then feeds into the PDH bypass.

Invocation of the PDH bypass in the transmitting tract is attractive, given the high level of pollen-based ethanol and its almost certain diffusion into the transmitting tract. The direct evidence for operation of the PDH bypass is so far restricted to pollen, however, in potato and Petunia it relies on distinct PDC and ADH isoforms. Choosing between the obvious alternatives – fermentation to generate energy under hypoxic conditions versus remobilization of carbons from ethanol diffusing from pollen – will require direct experimental evidence.

4.4.3 Ovaries

The *ADH2::GFP* reporter transgenic plants revealed *ADH2* promoter function in the Petunia ovary, which also exhibits ADH activity detectable by histochemical analysis (Foster Atkinson 1996; Garabagi and Strommer 2004; Garabagi et al. 2005) and *PDC1* gene expression (Gass et al. 2005). More than 40 years ago, direct oxygen tension measurements obtained by attaching a microelectrode to ovaries of the large flowers of amaryllis demonstrated the inherent hypoxia of the ovary (Linskens and Schrauwen 1966). By analogy, the observed *PDC1* and *ADH2* expression in maternal tissues of the Petunia ovary is likely to be associated with hypoxia. Lacking reporter-based information for *ADH3*, we are left with the prediction that, as

in other hypoxic tissues of the plant, ADH3 is likely to be produced in the ovary together with ADH2.

4.4.4 Nectaries

Analysis of ADH isoforms present in the ovary was complicated by the proximity of the nectaries paired at its base. These tissues produce scented attractants for pollinators (Fahn 1979). The *ADH2::GFP* transformants feature striking fluorescence of the nectary (Fig. 4.3G, 4.3H), increasing with flower development. Isozyme patterns of partially dissected ovaries, consisting of both ovary and nectary, showed significantly more activity associated with ADH3 than ADH2 (Fig. 4.2). From the fluorescence intensity in the *ADH2* reporter transgenic plants, we conclude that most of the observed activity represents nectary-based ADH. The position and function of the nectaries suggest two roles for ADH in these tissues: (1) the high level of metabolic activity and cloistered position of the nectary may foster chronic hypoxic conditions and (2) ADH may play a role in the production of volatiles contributing to scent, particularly via the lipoxygenase pathway.

The lipoxygenase pathway (Fig. 4.4), which produces a group of C6 volatile aldehydes and straight-chain alcohols from linolenic and linoleic acids (Hatanaka 1993), is specifically implicated in the production of "green note" compounds, responsible for the characteristic fresh scent of leaves. Specific scents depend in part on ratios

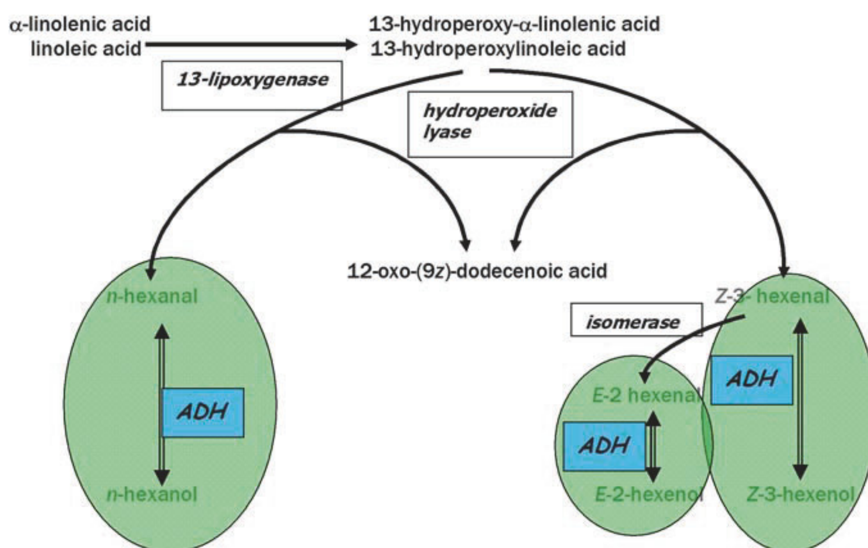

Fig. 4.4 A summary of the lipoxygenase pathway, source of C6 volatiles. Hexanal, hexenal, and hexenol have all been identified in volatiles produced by the nectaries and petals of Petunia (see text for details), arguing for the involvement of alcohol dehydrogenase activity in this pathway

of the aldehydes and alcohols, the interconversions of which are catalyzed by ADH. There is evidence that these compounds are associated with more than just green-note character: when Speirs et al. (1998) analyzed transgenic tomato plants with a range of ADH activities in developing fruit, they found ADH-dependent differences in the levels of these C6 aldehydes and alcohols, together with discernible effects on the flavor of *ADH*-overepressing tomato fruits. Indeed, in experiments directly addressing the potential role of the lipoxygenase-derived C6 volatiles as pollinator attractants, Hoballah et al. (2005) demonstrated direct excitation of the antennae of Petunia-pollinating moths by these molecules.

To test for the activity of the lipoxygenase pathway in nectaries of Petunia, we analyzed chromatographs of nectary extracts for evidence of C6 volatiles characteristic of the pathway (Garabagi et al. 2005). We were able to identify three of the four predicted C6 compounds, *n*-hexanal, *Z*-3-hexenol, and *n*-hexanol, in gas chromatograms. Confirmation of their presence leads to the assignment of at least part of the observed ADH activity of nectaries to production of C6 volatiles, in a pathway distinct from both anaerobic fermentation and the PDH bypass. Thus the product of the *ADH2* gene, and almost certainly the *ADH3* gene as well, serves in two different pathways with non-overlapping functions, the fermentation/bypass pathway and the lipoxygenase pathway.

Mitchell and Jelenkovic (1995) have demonstrated the ability of an NAD-dependent ADH from strawberries to act on a number of branched-chain and aromatic alcohols associated with strawberry aroma. By analogy, *ADH2* and/or *ADH3* of Petunia may play a role in aromatic, as well as C6, volatile production in scent-producing organs of Petunia flowers (see Chapter 3).

4.4.5 Petals

A low level of ADH3:ADH3 homodimer activity was detectable in extracts of petals, which is generally considered the primary floral scent organ (Dudareva and Pichersky 2000). The presence of *ADH3* (but neither *ADH2* nor *ADH1* RNA) provided corroboration for the production of ADH3, and GC verified the presence of C6 volatiles produced via the lipoxygenase pathway in petals (Garabagi and Strommer 2004; Garabagi et al. 2005). These results provide evidence that ADH in the petal, as in the nectary, contributes to floral scent and the ability to attract insect pollinators.

4.4.6 Seeds

Both *ADH1* and *ADH2* promoter constructs in transgenic plants displayed activity in developing Petunia seeds (Garabagi et al. 2005), which also contain both *PDC1* and *PDC2* RNA (Gass et al. 2005). Isozyme analyses provided evidence for the presence of four ADH isozymes: ADH1, ADH2, and ADH3 homodimers and ADH2:ADH3

heterodimers. The absence of ADH1-containing heterodimers indicates that production of ADH1 is separated temporally and/or spatially from that of ADH2 and ADH3. ADH2/3 and PDC1 appear to be at much higher levels than ADH1 and PDC2. The seed contains considerable endosperm and a well-formed embryo (Sink 1984). By analogy to other parts of the plant, the simple hypothesis, awaiting direct testing, is that ADH1 and PDC2 are restricted to one, ADH2/3 and PDC1 to the other.

There are multiple potential explanations for ADH activity in the seed. It may be required early in germination, an event associated with intense metabolic activity and a potentially hypoxic environment. These conditions are likely to involve a combination of ethanolic fermentation and the PDH bypass. Second, the mature seed is not metabolically inactive, and ADH may play a role in protecting the seed from effects of acetaldehyde accumulation. In a series of studies Zhang and his coworkers (Zhang, Maeda, Furihata, Nakamaru, and Esashi 1994; Zhang, Nakamaru, Tsuda, Nagashima, and Esashi 1995a; Zhang et al. 1995b; Zhang, Nagata, Miyazawa, Kikuchi, and Esashi 1997) demonstrated that seeds of a variety of species release volatiles including acetaldehyde and alcohol. They demonstrated the ability of the seeds to interconvert acetaldehyde and ethanol, and measured particularly high levels of acetaldehyde accumulation under conditions of low relative humidity and temperature. This acetaldehyde accumulation, associated with the formation of acetaldehyde–protein adducts, was correlated with reduced seed viability. Enzymatic activity of aldehyde dehydrogenase and operation of the PDC bypass in seeds have not been investigated, but the work of Zhang and his colleagues suggests that at least part of the plant, in the seed, relies directly on ADH for aldehyde detoxification.

4.5 Conclusions

PDC and ADH are closely linked metabolically, and research focused on one tends to overlap with the other. The evidence to date supports a critical role for PDC in Petunia. That role, however, is probably not the one originally assumed to operate in maize, that is, activation by low pH to ensure conversion of pyruvate to ethanol rather than lactic acid. The evidence argues that its critical role is to produce acetaldehyde, for which aldehyde and alcohol dehydrogenases then compete. Judging from mutational effects, its role in supplying acetate through the PDH bypass, at least in reproductive plant parts, is probably more important than its role in ethanolic fermentation.

Examination of *ADH* gene expression in floral organs of Petunia has been valuable in determining the metabolic functions of ADH, which in pollen, at least, appears to act as a gatekeeper balancing acetaldehyde and ethanol. This may, in fact, be its fundamental function in vegetative and reproductive plant parts, that is, in leaves, stems, hypocotyls, and roots experiencing variable degrees of hypoxia, as well as in the style and germinating pollen. In addition to its complex role in

anaerobic and aerobic alcoholic fermentation, ADH appears to play a role in the production of characteristic scent volatiles. Thus, ADH acts in pathways ranging from anaerobic fermentation to scent production and carbon remobilization, involving the fermentative glycolytic, lipoxygenase, and PDH bypass pathways, respectively.

These two enzymes and their analysis in Petunia have been valuable in extending our understanding of the strategies employed by plants to adapt to their environments. In doing so they exemplify the ability of "simple" housekeeping enzymes to fulfill disparate enzymatic functions and to connect pathways for finely tuned developmental and environmental responsiveness.

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Chapter 5
Gametophytic Self-Incompatibility in Petunia

Thomas L. Sims and Timothy P. Robbins

Abstract Gametophytic self-incompatibility (GSI), which prevents growth of a pollen tube through the style, provides a means of preventing self-pollination. Seen in most eudicot plant families, GSI in the genus *Petunia* was described by Darwin in the 19th century. By the time the first edition of this monograph was published in 1984, nearly a century later, most of the readily observable phenomena associated with self-incompatibility in the genus *Petunia* had been described and, as in a number of other plant systems, it had been demonstrated to depend on the actions of genes encoded at a single highly polymorphic S-locus. Molecular research of the past two decades has provided a depth of understanding into the mechanisms underlying the earlier observations, particularly in the identification of a number of SI-associated genes, their sites of action, and to some extent the mechanisms involved. This chapter summarizes what has been learned, with a focus on the molecular biology underlying GSI in Petunia, and highlights the major questions that remain unanswered.

5.1 Introduction

> ....protected flowers with their own pollen placed on the stigma never yielded nearly a full complement of seed; whilst those left uncovered produced fine capsules, showing that pollen from other plants must have been brought to them, probably by moths. Plants growing vigorously and flowering in pots in the green-house, never yielded a single capsule; and this may be attributed, at least in chief part, to the exclusion of moths (Darwin 1891).

Self-incompatibility (SI), the phenomenon by which plants can recognize "self" pollen and therefore prevent inbreeding, while accepting "non-self" pollen, has been the subject of study ever since Darwin first described his observations of self- and cross-fertilization in Petunia in his book *The Effects of Self and Cross Fertilization in the Vegetable Kingdom* (Darwin 1891). At the time that the first Petunia

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monograph was published (Sink 1984), much of the essential phenomenology of gametophytic self-incompatibility (GSI) had been described. Researchers such as Mather (1943), Linskens (1975), and de Nettancourt (1977) had determined that GSI in Petunia was governed by a single, multiallelic S-locus, and that recognition and rejection of self pollen was controlled gametophytically by alleles expressed in pollen. Mutations unilaterally inactivating self-incompatibility in pollen (pollen-part mutations) had been identified and associated with centric chromosomal fragments (Brewbaker and Natarajan 1960). Tetraploid plants with diploid heteroallelic pollen had been shown to be self-compatible due to "competitive interaction" in pollen. Shivanna and Rangaswamy (1969) had demonstrated that pollination of immature styles could be used to overcome self-incompatibility (a phenomenon now understood to result from high-level expression of the S-RNase late in the development of the style). Ascher (1984) had demonstrated quantitative variation in the strength of the self-incompatibility reaction, which he termed pseudo-self-compatibility. In the ensuing years, research in this area has resulted in a far better understanding of the molecular biology underlying many of the observations just described.

A variety of experimental approaches resulted in identification of the S-ribonuclease (S-RNase) as the style-recognition component of gametophytic SI, acting together with both the previously elusive "pollen-S" gene and a number of other genes that play critical, supporting, or yet unclear roles in this response. Despite the enormous progress that has been made in understanding the molecular basis of pollen recognition and rejection, many of the fundamental aspects of gametophytic self-incompatibility remain to be fully deciphered. The most widely accepted model for S-RNase-based incompatibility proposes that self and non-self S-RNase proteins are imported into growing pollen tubes. In a compatible pollination, non-self S-RNases are inhibited from acting whereas in incompatible pollinations, self S-RNases act to degrade pollen RNA and inhibit growth. Recognition of S-RNases as self or non-self is determined via the action of a pollen-expressed S-locus-encoded F-box protein, SLF. What remains elusive, however, is how haplotype recognition (determined by the S-RNase and by the SLF protein) is integrated with either the release or continued inhibition of S-RNase activity.

5.1.1 Genetics, Physiology, and Distribution of GSI

Gametophytic self-incompatibility has been estimated to occur in up to three-quarters of eudicot families (Igic and Kohn 2001; Steinbachs and Holsinger 2002). The most widespread form of GSI, as found in *Petunia hybrida*, is based on the interaction of style- and pollen-expressed allelic proteins encoded by a single, multiallelic S-locus. The style and pollen components together form a recognition "haplotype" (two or more tightly linked allelic variants). Recognition and rejection of pollen depend on whether there is a match of haplotypes between the growing pollen tube and the style. If the haplotype expressed in the pollen ("pollen-S") matches one of the two S-haplotypes expressed in the style (an incompatible cross), growth of the

pollen tube is inhibited in the transmitting tract, and fertilization rarely occurs. Conversely, if there is no match between the haplotype expressed in the pollen and that expressed in the style (compatible cross), pollen tube growth continues to the ovary, resulting in fertilization and seed set. In most cases, compatible versus incompatible crosses can be distinguished on the basis of seed capsule formation. In compatible crosses, large seed capsules that may contain up to a few hundred seeds are formed. In a fully incompatible cross, no seed capsules are formed, and no seed is produced. An alternative method of assaying pollination success is the use of fluorescence microscopy, traditionally using aniline blue as a fluorochrome, to monitor the extent of growth of pollen tubes. Aniline blue stains callose, a β–1, 3 glucan found in pollen tubes, and fluoresces with UV illumination. During incompatible pollinations, the majority of pollen tubes terminate growth in the upper third of the style, whereas in compatible pollinations pollen tubes reach the ovules.

As will be described more completely below, it is now understood that a self-incompatibility haplotype is defined by the presence of a specific S-RNase allele and a matching SLF allele linked at the same S-locus and inherited as a single unit due to a local suppression of recombination. A number of other proteins, including HT-B, the 120 kDa protein, SBP1, and SSK1, have been shown or hypothesized to play critical roles in the expression of the self-incompatibility response. The known and/or hypothesized roles of these genes and proteins are described in the sections below.

Gametophytic self-incompatibility has been well studied not only in Petunia, but also in other members of the Solanaceae, as well as in the Plantaginaceae and Rosaceae (Igic and Kohn 2001; Olmstead et al. 2001). Although this chapter focuses on the study of GSI in Petunia, much of our current understanding of this response comes from experiments involving other genera of the Solanaceae, such as *Nicotiana,* and *Solanum,* as well as from *Antirrhinum* (Plantaginaceae) and *Prunus* (Rosaceae). Experimental results from these systems will be discussed as appropriate. Where similar experiments have been performed in both Petunia and other plant systems, in most cases only the Petunia experiments are mentioned.

5.1.2 Early History of SI in Petunia

Although Darwin (1891) described the essential features of self-sterility in Petunia, it was not until later that Harland and Atteck (1933) established that a gametophytic mechanism controls the specificity of the pollen. Mather (1943) questioned the identity of the Petunia material used in some of these early studies and considered that it was probably *P. hybrida* rather than *P. violacea* (synonymous with *P. integrifolia*; see Chapter 1 and references therein). The current consensus is that *P. hybrida* has arisen from hybridization between the purple-flowered SI species *P. integrifolia* and the white-flowered self-compatible (SC) species *P. axillaris.* These interspecific crosses exhibit unilateral incompatibility (UI) and are successful only with *P. axillaris* as the female parent (Mather 1943). This is typical of UI that has been

described in other genera of the Solanaceae. A further important discovery in these early studies was the phenomenon of breakdown in SI that occurs when a diploid undergoes colchicine-induced tetraploidy (Stout and Chandler 1941).

Several authors have commented on the suitability of *P. hybrida* and its wild relatives for SI research (Linskens 1975; Ascher 1984). These features include a generally strong SI reaction leading to the absence of seed set, although this can conveniently be overcome by bud pollination. The latter technique allows homozygous stocks to be established and maintained by either bud pollination or vegetative propagation. The large floral organs facilitate controlled pollinations but are also advantageous for biochemical or physiological studies. This has allowed detailed studies of pollen tube growth rates in both compatible and incompatible pollinations (Herrero and Dickinson 1980, 1981). These indicated that the difference in growth rate occurs in the stylar transmitting tissue, consistent with what is now known about the distribution of S-RNase. For a detailed discussion of these and other cytological studies the reader should refer to Ascher (1984).

5.1.3 Pseudo-Self-Compatibility, Partial Breakdown of SI

In early studies using *Petunia hybrida,* Ascher and coworkers revealed considerable variation in the responses of different GSI plants (Ascher 1984). Detailed genetic studies have shown that self-compatibility can arise because either the maternal or the paternal aspects became nonfunctional. Ascher coined the term "non-discriminating styles" and "pollen-mediated pseudo-self-compatibility" to describe each of these conditions (Flaschenreim and Ascher 1979a, b). However, there is also a state that is intermediate between the two extremes, in which some seed set occurs. Described by Ascher as "Pseudo-self compatibility" (PSC), the phenomenon has been widely studied in a number of systems. In one such study in *Nemesia,* part of the system that causes this self-compatibility could not be described as being attributed to either the pollen or the style (Robacker and Ascher 1982). A similar intermediate level of response has also been described in *Senecio* (Hiscock 2000).

Ascher defined PSC as the ability of an otherwise self-incompatible plant to set seed when self-fertilized or crossed to other individuals bearing the same S-allele. This definition distinguished the partial breakdown of self-incompatibility found in PSC plants from the compatibility seen in plants lacking an SI system. Ascher further defined a quantitative measure of PSC, percent PSC, by taking the ratio of the number of seeds produced in an incompatible cross to that produced in a fully compatible cross using the same individual. By expressing SI behavior as % PSC, Ascher could distinguish the partial breakdown of SI from generalized effects on fertility. Because all of Ascher's studies took place prior to the identification and cloning of specific genes that govern self-incompatibility interactions, the molecular basis of PSC behavior has not been fully described. It is likely that the different levels of stylar-based PSC described (Flaschenreim and Ascher 1979b; Dana

and Ascher 1986b) resulted from reduced expression or activity of the S-RNase protein in styles. The molecular basis of pollen tube–expressed PSC (Flaschenreim and Ascher 1979a; Dana and Ascher 1986a) is yet to be elucidated, and will be an interesting area of investigation now that the pollen-recognition component of GSI has been identified.

In a study that attempted to determine whether PSC in *Petunia hybrida* resulted from the hybrid origin of cultivated Petunia, Dana and Ascher (1985) selected for the presence of PSC in *Petunia integrifolia* plants grown from seeds collected in the wild. They found that individual self-pollinated *P. integrifolia* plants were capable of expressing greater than 20% PSC (20% of the number of seeds produced in a fully compatible cross). Thus, whatever the molecular basis of the partial breakdown of self-incompatibility, it does not appear to have arisen as an artifact of the hybrid cross(es) between *P. integrifolia* and *P. axillaris* that resulted in *P. hybrida*. PSC or full SC is widespread in cultivated *P. hybrida* and in some cases appears to be associated with a particular S-allele, S_O (Ai, Kron, and Kao 1991; Robbins, Harbord, Sonneveld, and Clarke 2000).

5.1.4 Early Biochemical Studies of SI

The early characterization of S-proteins in the pistils of *Nicotiana alata* plants of defined S-genotypes paved the way for the subsequent isolation of the first cDNAs encoding S-proteins in the Solanaceae (Anderson et al. 1986). In *Petunia hybrida*, similar studies by Kamboj and Jackson (1986) identified electrophoretic variants of abundant pistil proteins that correlated with different S-genotypes. These proteins were basic (pI 8.3–8.7) but no N-terminal sequence was reported. In a subsequent study using S-alleles obtained from H. Linskens at the University of Nijmegen, a similar range of pI values (8.7–9.3) was reported (Broothaerts et al. 1989).

Table 5.1 A comparison of N-terminal S-RNase sequences reported for functional S-alleles in *Petunia hybrida and P. inflata*. The *underlined region* is the first conserved domain (C1) common to all S-RNases of the Solanaceae

Allele	N-terminal sequence	Reference
S_{1l}	D F D Y M Q L V L T W P A S F C Y R P R	Clark et al. 1990
S_{2l}	Y F E Y M Q L V L T W P P A F C H I K X	Clark et al. 1990
S_{3l}	E F E L L Q L V L T W P A S F C Y A N H	Clark et al. 1990
S_1	S F D H W Q L V L T W P A G Y C K V K G	Broothaerts et al. 1989
S_2	N F D Y F Q L V L T W P A S F C Y P K N	Broothaerts et al. 1989
S_3	N F D Y F Q L V L T W P A S F C Y P K N	Broothaerts et al. 1989
S_b	A F D H W Q L V L T W P A G Y C K I K G	Broothaerts et al. 1991
S_x	D F D Y M Q L V L T W P A S F C Y R P R	Ai et al. 1992
S_v	Y F E Y M Q L V L T W P P A F C H I K R	Robbins et al. 2000
S_{1i}	N F E Y L Q L V L T W P A S F C F R P K	Ai et al. 1990
S_{2i}	N F D Y F Q L V L T W P A S F C Y P K N	Ai et al. 1990
S_{3i}	N F D Y I Q L V L T W P A S F C Y R P K	Ai et al. 1990

S-proteins were identified that cosegregated with three S-alleles (S_1–S_3) and were shown to accumulate in the stigma and style during flower development, peaking at anthesis. Protein purification allowed for the recovery of N-terminal sequences of all three alleles, providing clear evidence of amino acid differences (Table 5.1). These S-proteins were subsequently shown to be glycosylated, and the apparent differences in MW (28–32 kD) could be accounted for by variations in the number and length of the carbohydrate side chains (Broothaerts, Vanvinckenroye, Decock, Van Damme, and Vendrig 1991). Similar patterns of glycosylation have been established for the S-proteins of *Nicotiana alata* (Woodward, Bacic, Jahnen, and Clarke 1989).

5.2 S-RNase: The Style-Recognition Component

The first cDNA sequences to be reported for S-proteins in *Petunia* were for *P. hybrida* (Clark et al. 1990) and *P. inflata* (Ai et al. 1990). These sequences revealed a similarity with the T2-type of fungal ribonucleases initially observed in *Nicotiana alata* in a work that led to the term "S-RNase." The S-RNase gene is expressed at high levels late during the development of the pistil (Clark, Okuley, Collins, and Sims 1990), and encodes a secreted protein that accumulates to high levels in the transmitting tract of the style (Anderson et al. 1989; Ai et al. 1990). Comparative sequence analysis of S-RNase genes isolated from a number of species (Anderson et al. 1989; Ai et al. 1990; Clark et al. 1990; Ioerger, Gohlke, Xu, and Kao 1991; Xue, Carpenter, Dickinson, and Coen 1996; Ishimizu, Shinkawa, Sakiyama, and Norioka 1998) demonstrated that S-RNase proteins show a regular pattern of interspersion of highly conserved amino acid sequence with more variable sequence. Conserved domains C2 and C3 contain two histidine residues, His32 and His91, that along with Lys90 make up the catalytic site of the ribonuclease (Ida et al. 2001). S-RNase proteins in the Solanaceae and Plantaginaceae contain two highly variable sequence domains, HVa and HVb (Ioerger et al. 1991; Xue et al. 1996). Gain-of-function experiments (Lee, Huang, and Kao 1994) in which a S_3-RNase of *Petunia inflata* was transferred to a plant of the S_1S_2 genotype showed that transgenic plants expressing the S_3 protein at levels comparable to endogenous S-RNase had acquired the ability to reject S_3 pollen. Lee et al. (1994) also used an antisense approach to downregulate the S_3-RNase in a S_2S_3 background. Plants with reduced levels of S_3-RNase were no longer capable of inhibiting S_3-pollen. In a subsequent experiment, McCubbin, Chung, and Kao (1997) introduced a RNase$^-$ (H93R) variant of the S_3 S-RNase of *Petunia inflata* into an S_2S_3 background. The resulting transgenic S_2S_3(S_{3H93R}) plant demonstrated a dominant-negative phenotype that affected only the S_3 allele; when self-pollinated, the transgenic plant was self-compatible. Crosses using pollen from other testers indicated that the dominant-negative transgenic plant had lost the ability to reject S_3 pollen but was unaffected in its ability to reject S_2 pollen. This result suggests that the S_{3H93R} allele somehow blocks an interaction or prevents the normal function of the S_3 allele. In all of

these experiments, only the style recognition was altered. Pollen-recognition specificity was not affected, confirming that a separate gene product from the S-RNase encoded the "pollen-S" component.

The ribonuclease activity of the S-RNase is correlated with pollen rejection. McClure, Gray, Anderson, and Clarke (1990) labeled pollen RNA in vivo by watering plants with a solution containing ^{32}P-orthophosphate, and showed that incompatible pollinations were associated with degradation of pollen-tube RNA, whereas pollen tube RNA was not degraded following compatible pollination. Both transgenic experiments (Huang, Lee, Karunanandaa, and Kao 1994) and analysis of spontaneous mutants (Royo et al. 1994) demonstrated that eliminating the catalytic ribonuclease activity of the S-RNase (e.g., by mutation of the active site histidine to asparagine), also eliminated the ability to reject pollen.

S-RNase proteins are glycoproteins, and show variability in the number, type, and fine structure of glycan chains associated with S-RNases (Woodward et al. 1989). The carbohydrate group does not appear to be essential for self-incompatibility, as elimination of the glycosylation site has no effect on the ability of a S-RNase to reject self pollen (Karunanandaa, Huang, and Kao 1994).

5.2.1 Basis of Recognition Specificity of the S-RNase

Experiments investigating the basis for allelic specificity in the S-RNase protein have generally focused on the role of the hypervariable regions. An experiment in *Solanum chacoense* appeared to provide strong evidence that the hypervariable regions were both necessary and sufficient for the specificity (Matton et al. 1997). The S_{11} and S_{13} S-RNase alleles of *S. chacoense* differ by only 10 amino acids across the entire protein, three of which are found in HVa and one in HVb. Matton et al. (1997) used in vitro mutagenesis to change the four S_{11} residues in the HVa and HVb regions to those found in S_{13}, then expressed the altered allele transgenically in an $S_{12}S_{14}$ background. Pollination with S_{11} and S_{13} pollen demonstrated that changing only these residues changed the recognition specificity of the transferred S-RNase from S_{11} to S_{13}. In an extension of this experiment (Matton et al. 1999), changing only two residues in HVa plus the HVb residue, resulted in a "dual-specificity" S-RNase that retained the ability to reject S_{11} pollen while acquiring the ability to also reject S_{13} pollen. Other experiments, however, have suggested that this may not be the outcome in all cases. Zurek, Mou, Beecher, and McClure (1997) made constructs exchanging the complete HVa and HVb domains of the S_{A2} and S_{C10} S-RNase alleles of *Nicotiana alata*. When expressed transgenically, the resulting protein had lost the ability to reject S_{A2} pollen, while not acquiring the ability to reject S_{C10} pollen, suggesting that protein regions outside of the hypervariable domains play a role in recognition.

The protein crystal structure has been determined for the S_{F11} S-RNase of *Nicotiana alata* (Ida et al. 2001) and provides support for the involvement of the hypervariable domains in allelic recognition and interaction. In *Nicotiana* the two

hypervariable regions are separated in the primary amino acid sequence but located next to each other on the surface of the tertiary structure (Ida et al. 2001). The HVa region is further characterized by a cluster of positively charged side chains, whereas the HVb region has a cluster of negative charges. Comparative sequence analysis showed that the most highly variable amino acids in the hypervariable regions are located on the surface of the S_{F11} S-RNase and readily accessible to solvent (Ida et al. 2001). These include all four of the residues equivalent to those targeted in the mutagenesis experiments of Matton et al. (1997, 1999).

5.2.2 Allelic Diversity of S-RNases

Allelic diversity at the S-locus in *Petunia hybrida* is much more limited than that of the wild species from which it is derived: *P. axillaris* and *P. integrifolia* (see Chapter 1). The number of S-alleles found in the cultivated forms of *P. hybrida* is probably fewer than 10 (Robbins et al. 2000), compared to that of the natural populations in South America, which may have 40 or more distinct S-alleles (Tsukamoto et al. 2003). This reflects the bottleneck of plant breeding in which relatively few individuals were used in the initial interspecific hybridizations. Moreover, the low allelic diversity suggests that subsequent hybridizations and introgressions from wild relatives have been relatively infrequent during the 150 years or so of *P. hybrida* cultivation.

The estimate of the minimum number of S-alleles in *P. hybrida* is most easily based on the number of S-RNase sequences rather than on phenotypic assays based on pollinations (Robbins et al. 2000). Broothaerts and coworkers reported the N-terminal sequences for purified S-RNases derived from Linsken's original three S-alleles, S_1, S_2, and S_3 (Broothaerts et al. 1989). They also reported an S-allele derived from a commercial variety which was named S_b (Broothaerts et al. 1991). Sims and coworkers were the first to report cDNA sequences for three S-RNases from *P. hybrida*, derived from stocks originally described by Ascher (Clark et al. 1990). Rather confusingly, the Ascher S-alleles were also named S_1–S_3 and yet sequence comparisons indicated that these were distinct from the Linskens alleles. Robbins et al. (2000) proposed that the Linskens S-alleles be given the suffix "L" to avoid future confusion. It is also important to distinguish these S-alleles from the S_1–S_3 alleles of the species *P. integrifolia* ssp. *inflata*.

To this initial set of seven *P. hybrida* S-alleles (S_1–S_3, S_{1L}–S_{3L}, S_b) should also be added a functional allele S_x, derived from crosses between *P. hybrida* and *P. inflata* (Ai, Tsai, and Kao 1992). The S_x allele was derived from the *P. hybrida* parent and was conditionally functional depending on background modifiers that segregated from the *P. hybrida* parent (Ai et al. 1991). One additional functional allele was identified from the self-incompatible stock V13 maintained by the Free University of Amsterdam (Harbord, Napoli, and Robbins 2000). Surprisingly, this stock had been maintained as an inbred line (perhaps by inadvertent early bud pollination), yet it was homozygous for a novel S-RNase sequence (Robbins et al. 2000). This

allele has also been identified in an independent cultivar by Entani et al. (1999). Table 5.1 presents the N-terminal regions for all nine reported *P. hybrida* S-alleles and of S_1–S_3 of *P. inflata* for comparison. The cDNA sequences are available for all alleles except S_{1L} and S_{2L}, and these alleles may no longer be available. It is possible that they are equivalent to extant alleles, as the N-terminal sequences of S_{1L} and S_x and of S_{2L} and *Sv* are identical. It follows that the number of distinct functional S-alleles identified in *P. hybrida* may be as few as seven.

The number of S-alleles identified in natural populations of Petunia has not been rigorously determined at the level of population genetics. One study of a single population of 100 individuals of *P. inflata* from Argentina identified 19 different S-haplotypes (Wang, Hughes, Tsukamoto, Ando, and Kao 2001). The cDNA sequence was obtained for 15 haplotypes maintained as bud self-homozygotes. Surprisingly, one of these was identical to the S_1 allele that has been extensively studied in transgenic plants (Ai et al. 1990). Phylogenetic analysis of the *P. inflata* S-RNase sequences revealed a trans-specific pattern of similarity with S-RNase sequences from other members of the Solanaceae, as demonstrated previously for a small sample of alleles (Ioerger, Clark, and Kao 1990). Natural populations of *P. axillaris* have been found to contain as many as 40 different haplotypes, although the situation is complicated by self-compatibility that can occur as a result of loss of style or pollen function (Tsukamoto et al. 2003).

5.2.3 RNase Mapping Studies

The S-locus has been mapped near the centromere of chromosome I in two closely related members of the Solanaceae, *Lycopersicon/Solanum esculentum* (n=12) (Tanksley and Loaiza-Figueroa 1985) and *Solanum tuberosum* (Gebhardt et al. 1991). In *Petunia hybrida* (n=7) an indirect approach was taken, using fluorescence in situ hybridization (FISH) localization of T-DNA inserts (ten Hoopen, Harbord, Maes, Nanninga, and Robbins 1998) that were known to be linked to the S-locus (Harbord et al. 2000). This approach physically mapped the S-locus in the V26 cultivar to a sub-centromeric region of chromosome III. In a more direct approach, Entani et al. (1999) also employed FISH to localize the S^{B1}-RNase gene of *P. hybrida* to a sub-centromeric region of chromosome III in a SI line “PB”.

These two results are contradicted by an independent RFLP study by Strommer, Gerats, Sanago, and Molnar (2000) who mapped the S-RNase gene to chromosome IV using a VR hybrid mapping strategy. The situation is further complicated by the RFLP mapping using a potato marker CP100 reported by ten Hoopen et al. (1998). This heterologous RFLP marker has shown consistent cosegregation with the S-locus of *P. hybrida* (Harbord et al. 2000). When mapped with the same VR mapping population used by Strommer et al. (2000), the localization of CP100 was to chromosome III rather than chromosome IV. This makes it difficult to explain the different localizations due to cultivar-specific differences in genome organization, although such variability is known to be a recurring feature of *P. hybrida*

cytological studies (Montijn, ten Hoopen, Fransz, Oud, and Nanninga 1998; see also Chapter 10).

A common feature of these cytological studies in *P. hybrida* (ten Hoopen et al. 1998; Entani et al. 1999) is a localization for the S-locus at or near the centromere. Several authors have noted that a centromeric location of the S-locus in the Solanaceae may provide tight linkage between the S-RNase and pollen-S, as this region is characterized by suppressed recombination (Round, Flowers, and Richards 1997; Copenhaver, Browne, and Preuss 1998). However, this does not appear to be a general feature, because in *Antirrhinum* the S-locus is located toward the telomere (Yang, Zhang, Li, Cheng, and Xue 2007).

Physical mapping of S-locus genes in Petunia has also been achieved by genomic cloning strategies. McCubbin, Wang, and Kao (2000a) isolated pollen cDNAs linked to the S-locus, and subsequently McCubbin, Zuniga, and Kao (2000b) and Wang and colleagues (Wang, Wang, McCubbin, and Kao 2003; Wang et al. 2004) used these cDNAs, along with the S-RNase, to screen BAC libraries for S-locus contig clones. This work suggested that the S-locus of *Petunia inflata* may span $>$4.4 Mb of chromosomal DNA. Sequencing of a 328 kb region containing the S_2-RNase revealed the presence of approximately 50 genes, one of which was a pollen-expressed polymorphic F-box gene termed $PiSLF_2$. $PiSLF_2$ was subsequently shown by a transgenic approach to be a pollen-S gene (Sijacic et al. 2004).

5.3 Non-S-Locus Stylar Factors

Although the S-RNase plays a key recognition (and cytotoxic) role in the style, other factors are required for expression of self-incompatibility. For example, using different species of *Nicotiana* for transgenic experiments, Murfett et al. (1996) showed that expression of the S-RNase gene in transgenic SC *Nicotiana plumbaginifolia* was insufficient for S-allele-specific pollen rejection, whereas expression of the S-RNase in *N. plumbaginifolia* X SC *N. alata* hybrid plants did result in S-allele-specific pollen rejection. The clear implication was that some factor(s) must be expressed in *Nicotiana alata* that is/are not expressed in *N. plumbaginifolia*, and that the factor(s) is/are required for pollen rejection. Using a differential screen based on the above observation, McClure, Mou, Canevascini, and Bernatzky (1999) cloned a small (101 amino acid) asparagine-rich protein from SC *N. alata* that they named HT. This protein was predicted to be secreted and processed to a mature form of 86 kDa. Antisense experiments in transgenic plants showed that downregulation of HT in styles resulted in the inability to reject pollen, even though the S-RNase was expressed at normal levels (McClure et al. 1999).

O'Brien et al. (2002), working in *Solanum chacoense*, extended this work to demonstrate two different isoforms of HT, which they named HT-A and HT-B. Antisense downregulation of HT-B duplicated the results of McClure et al. (1999) in that incompatible plants were converted to self-compatible plants. Downregulation of HT-A had no effect on the self-incompatibility response. The requirement of

HT-B for pollen rejection was further demonstrated by analyzing different SC and SI species of tomato as well as cultivated tomato. Kondo et al. (2002a, b) showed that SC tomato species had various defects in expression of both S-RNase and HT-B, ranging from deletion of the genes, to low expression, to mutations that prevented production of normal protein. The role of HT and S-RNase expression in SC in *P. hybrida* is unclear at present. However, there is clear evidence for SC factors that are unlinked to the S-locus (Ai et al. 1991; Harbord et al. 2000), and the mapping of the HT genes in *Petunia* will be informative.

The style expresses several proteins at high levels in addition to the S-RNase. Among these are TTS (Cheung, May, Kawata, Ou, and Wu 1993), PELPIII (Goldman, Pezzotti, Seurinck, and Mariana 1992; de Graaf, Knuiman, Derksen, and Mariani 2003), and a 120 kDa protein (Lind, Bacic, Clarke, and Anderson 1994). In affinity-gel binding assays, Cruz-Garcia, Hancock, Kim, and McClure (2005) showed that all three of these stylar glycoproteins formed high molecular weight complexes with the S-RNase. These authors hypothesized that S-RNase may be taken up into pollen tubes in the form of a complex that includes one or more of these proteins. Indeed, in a recent work, Goldraij et al. (2006) showed that S-RNase taken up into pollen tubes is sequestered in a vacuolar-like compartment that is bounded by the 120 kDa protein. The 120 kDa protein, like HT-B, is also required for S-allele-specific pollen rejection. Downregulating the 120 kDa protein using RNA interference in *Nicotiana plumbaginifolia* X *N. alata* hybrids eliminated the ability to reject S-allele specific pollen. The same RNAi plants could, however, continue to reject pollen from *N. plumbaginifolia*, whereas plants downregulated for HT-B failed to reject either S-allele-specific pollen or *N. plumbaginifolia* pollen (McClure et al. 1999; Hancock, Kent, and McClure 2005).

5.4 SLF: the Pollen-Recognition Component

‘The first cDNA encoding an S-RNase protein was reported in 1986 (Anderson et al. 1986), but it would be sixteen years before the first published cloning of a gene that would turn out to be pollen-S (Lai et al. 2002). Despite numerous attempts over the ensuing period to identify pollen-S, it was only the improvement in techniques for cloning large-insert DNA libraries along with the ability to sequence long stretches of DNA the made it possible to finally identify and clone the S-locus F-box gene subsequently shown to be pollen-S. Prior research led to several predictions for the expected properties of the pollen-S component, which formed the basis for attempts to clone this gene. First, mutants defective in style or pollen expression of GSI are often fully functional for GSI in the complementary organ, indicating that the style (S-RNase) component and the pollen component (pollen-S) are encoded by separate genes. This observation was reinforced by transgenic experiments (discussed above) in which gain-of-function or loss-of-function experiments that altered S-RNase specificity had no effect on recognition specificity in the pollen. Second, because recombination between the S-RNase and pollen-S is rarely observed, it

has been assumed that the two genes are physically linked, or may be located in chromosomal regions suppressed for recombination. Third, the pollen-S gene must be expressed in pollen, most likely in a pollen-specific fashion. Fourth, models of GSI recognition strongly suggested that pollen-S and the S-RNase must physically interact. Fifth, because S-RNase alleles are highly polymorphic (primarily in the hypervariable regions) and recombination is suppressed at the S-locus, pollen-S alleles were expected to be similarly polymorphic. As will be elaborated below, the first four of these predictions hold true, while the expectations of high levels of polymorphism for the pollen-S component are only partially supported. Finally, the observation that GSI breaks down in tetraploid plants, and in plants where the S-locus is partially or fully duplicated such that heteroallelic pollen is produced, provided a clear test for the behavior of a gene putatively identified as pollen-S.

5.4.1 The Inhibitor Model for Pollen-S

Most current evidence supports a cytotoxic model for pollen-tube rejection wherein the S-RNase acts to degrade pollen RNA, thereby inhibiting protein synthesis and further elongation of pollen tubes (Sims 2005). Cross-compatibility, therefore, must result from the absence of S-RNase activity in pollen tubes, either by preventing the initial import of S-RNase or by inhibiting the action of S-RNases inside pollen tubes. Several different experimental approaches have now demonstrated that the model of S-RNase inhibition is correct, although the precise nature of that inhibition has not yet been conclusively demonstrated.

Direct observations of pollen tubes using electron microscopy and immunogold labeling of S-RNases demonstrated that both compatible and incompatible S-RNases were imported into pollen tubes (Luu, Qin, Morse, and Cappadocia 2000). These results are inconsistent with a receptor model for pollen-S, in which it acts as a “gatekeeper” to exclude non-self S-RNases. Rather, these results support a model in which the S-RNase is imported into all pollen tubes regardless of genotype, but is specifically prevented from acting in non-self (compatible) pollen tubes. More recently, Goldraij et al. (2006) showed that S-RNases imported into *Nicotiana* pollen tubes are apparently sequestered in a vacuolar-like compartment in compatible pollen tubes and in the early stages of self-incompatible pollinations.

5.4.2 Breakdown of Incompatibility and Competitive Interactions

Prior to direct observations of S-RNase import into pollen tubes, most of the evidence supporting the hypothesis of a pollen-expressed inhibitor came from investigations of “competitive interaction” in diploid heteroallelic pollen from tetraploid plants. It is a well-established observation (Crane and Lewis 1942; Lewis and Modlibowska 1942; Brewbaker and Natarajan 1960; de Nettancourt 1977) that gametophytic self-incompatibility breaks down in tetraploid plants, provided that the

diploid parent is heterozygous at the S-locus. Under these conditions, however, the breakdown of self-incompatibility is only on the pollen side. That is, tetraploid heterozygous styles remain capable of rejecting haploid pollen with a matching S-allele, but diploid, heteroallelic pollen is self-compatible on either diploid or tetraploid styles. This phenomenon of breakdown of self-incompatibility in the pollen has been termed "competitive interaction."

Competitive interaction does not require complete duplication of genomes. Brewbaker and Natarajan (1960) showed that self-compatible mutants of *Petunia inflata* had centric chromosome fragments that presumably duplicated the S-locus. In a similar mutational analysis of the pollen component in *Nicotiana alata*, Golz and coworkers (Golz, Su, Clarke, and Newbigin 1999; Golz, Oh, Su, Kusaba, and Newbigin 2001) induced pollen-part mutations by gamma irradiation, and then characterized the genetic behavior and molecular basis of the induced mutations. All of the recovered pollen-part mutants were self-compatible and demonstrated competitive interaction in pollen with other S-alleles. All of the pollen-part mutants resulted from duplications of all or part of the S-locus, and no pollen-part mutations were associated with chromosomal deletions. Thus, pollen-part mutations induced by radiation phenocopy the tetraploid condition via duplication of the S-locus, most likely by duplication of pollen-S. These results are consistent with a model in which pollen-S is an inhibitor of self-S-RNases. According to this model, in tetraploid plants (or in plants with radiation-induced duplicated S-loci), heteroallelic pollen would possess two inhibitors, each capable of inhibiting all S-RNases except their cognate inhibitor, and therefore all S-RNases would be inhibited. According to this model, deletions in pollen-S would not be recoverable, since any pollen tube having a deleted pollen-S would be unable to inhibit the S-RNase, and would be rejected.

5.4.3 Evidence that the S-Locus F-Box Protein Is Pollen-S

Transgenic experiments that relied on the phenomenon of competitive interaction in pollen (described above) provided definitive proof that the S-locus F-box genes are pollen-S. Sijacic et al. (2004) transformed S_1S_1 *Petunia inflata* with a $PiSLF_2$ gene construct. Transgenic plants expressing both the $PiSLF_2$ transgene and the endogenous F-box gene $PiSLF_1$ were self-compatible, as would be predicted from the phenomenon of competitive interaction. The breakdown in self-incompatibility occurred only in the pollen and did not affect stylar expression of self-incompatibility. Pollen from the transgenic $S_1S_1/PiSLF_2$ plants was compatible on non-transgenic S_1S_1 plants, while S_1 pollen from the non-transgenic S_1S_1 plants was rejected by styles of the transgenic $S_1S_1/PiSLF_2$ plants. Progeny resulting from the compatible pollinations carried the $PiSLF_2$ transgene, and all were self-compatible. In a further experiment, $PiSLF_2$ was used to transform S_2S_3 *Petunia inflata.* Transgenic $S_2S_3/PiSLF_2$ plants produced a mixture of pollen genotypes: haploid S_2 and S_3 pollen (rejected via the standard self-incompatibility response), heteroallelic $S_3/PiSLF_2$ pollen, and homoallelic $S_2/PiSLF_2$ pollen. The transgenic

$S_2S_3/PiSLF_2$ plants were all self-compatible. Analysis of the progeny showed that the resulting plants were all S_2S_3 or S_3S_3. Failure to recover S_2S_2 plants in the progeny indicates that only $S_3/PiSLF_2$ was functional, as predicted, because competitive interaction should not occur in $S_2/PiSLF_2$ pollen.

In similar experiments, Qiao et al. (2004) transformed S_3S_3 *Petunia hybrida* with the *AhSLF-*S_2 pollen F-box gene together with the S_2-RNase gene from *Antirrhinum hispanicum.* Transgenic plants carrying intact genes for *AhSLF-*S_2 and the S_2-RNase and expressing both at normal levels were self-compatible, and all produced seed when used as the pollen donor to non-transformed S_3S_3 Petunia. This is the expected result from competitive interaction between different pollen-S genes expressed in the same pollen grain. In the reciprocal cross, these plants rejected non-transgenic S_3 pollen. Taken together with the results reported by Sijacic et al. (2004), these experiments demonstrate that the F-box gene encodes the pollen-recognition factor of gametophytic self-incompatibility, pollen-S. It is striking in the work reported by Qiao et al. (2004) that the transferred *AhSLF-*S_2 demonstrated functional conservation in the ability to induce competitive interaction in the pollen, despite sharing only 30% amino acid sequence identity with endogenous *Petunia* SLF genes.

5.4.4 Pollen-Part Mutants in the Rosaceae

Several *Prunus* species, in the Rosaceae, have been shown to carry *SLF* (also called *SFB*) genes linked to the S-locus (Ushijima et al. 2003; Entani et al. 2003; Yamane, Ikeda, Ushijima, Sassa, and Tao 2003). Transgenic assays to demonstrate that the *SFB/SLF* genes encode functional pollen components of GSI are not yet possible in *Prunus.* Several self-compatible mutants have, however, been identified in different species of *Prunus*, and all show various defects in SFB. Sequence analysis of S-locus F-box genes showed that the F-box motif was located at the N-terminus of the protein. The *Prunus* SFB/SLF proteins also have two hypervariable regions, HVa and HVb, located near the C-terminus of the protein. Ushijima et al. (2004) reported the characterization of two self-compatible mutants of *Prunus avium* and *P. mume*. DNA sequence analysis predicted that the HVa and HVb domains should be missing in the two mutant SFB proteins. $SFB_{4'}$ has a frame shift mutation that produces an altered amino acid sequence in the HVa region and a stop codon just upstream of the HVb region. SFB_f has a 6.8 kb insertion sequence in the region encoding the C-terminal portion of the protein. The insertion would code for 37 amino acids before a stop codon is reached; the mutant protein would lack the C-terminal 195 amino acids found in normal SFB proteins, and therefore lack both HVa and HVb regions. Sonneveld, Tobutt, Vaughan, and Robbins (2005) characterized two pollen-part mutants of *Prunus avium.* One of these was the same $SFB_{4'}$ mutant described by the previous group. A second mutant, $S_{3'}$, had a deletion that removed the entire *SFB* gene. The $S_{13'}$ self-compatible mutant in *Prunus cerasus* also shows alterations in the *SFB* gene (Tsukamoto, Hauck, Tao, Jiang, and Iezzoni 2006). In this allele, a 1 bp guanine-to-thymine substitution at position +733 produces a UAA stop codon

that truncates the SFB protein and eliminates the HVa and HVb regions. Two additional *SFB* mutations have been reported in self-compatible peach, *Prunus persica* (Tao et al. 2007). SFB_1 contains a 155 bp insertion that results in a truncated SFB protein, while SFB_2 has a 5 bp insertion that produces a stop codon in the middle of the protein, truncating the protein upstream of the HVa and HVb regions (Tao et al. 2007). Together, the identification of different pollen-part mutants in these species provides strong support for the identification of SFB/SLF as the pollen component of GSI in the Rosaceae. It is noteworthy that pollen-part mutants resulting from a deletion or mutation of SLF have not been reported in the Solanaceae.

5.5 The Role of Ubiquitination in GSI

The identification of F-box proteins as pollen-S, along with the identification of a RING-HC protein PhSBP1 (see below), suggested a role for the ubiquitin-proteosome system in self-incompatibility recognition. F-box proteins are the recognition components of multiprotein SCF-type E3 ubiquitin ligases. These complexes target proteins for ubiquitination and degradation via the 26S proteosome. The prototypical SCF complex consists of the F-box protein, SKP1, a Cullin protein and a RING domain protein RBX1 (Cardozo and Pagano 2004; Schwechheimer and Villalobos 2004).

A potential role for ubiquitination in gametophytic self-incompatibility was first suggested by the isolation of a gene encoding the RING-HC-containing protein PhSBP1 from *Petunia hybrida* (Sims and Ordanic 2001). In an attempt to identify pollen-expressed proteins interacting with the S-RNase, Sims and Ordanic (2001) screened a yeast two-hybrid library from mature pollen of *P. hybrida* with a bait construct for the N-terminal half of the *P. hybrida* S_1-RNase. This screen identified a gene, named *PhSBP1* (for *P. hybrida* S-RNase binding protein), that bound to N-terminal but not C-terminal regions of the S-RNase. Sequence characterization of *PhSBP1* indicated that it contained a C-terminal RING-HC (or C_3HC_4) protein domain. Such domains have been shown to be characteristic of E3 ubiquitin ligases, the components of the ubiquitin-proteosome system that interact with specific substrates targeted for ubiquitination and protein turnover (Freemont 2000). O'Brien, Major, Chantha, and Matton (2004) similarly screened a pollen two-hybrid library from *Solanum chacoense* with a bait consisting of the HVa and HVb domains of the *S. chacoense* S_{11} allele. This screen resulted in the isolation of a *Solanum PhSBP1* ortholog, *ScSBP1*. In a confirmation of the apparently key role of SBP1, Hua and Kao (2006) carried out a yeast two-hybrid screen using three separate bait constructs of the $PiSLF_2$ F-box gene of *Petunia inflata*. All three of these baits bound to a protein 98% identical to PhSBP1, which was named PiSBP1. Further protein interaction assays showed that PiSBP1 also bound to $PiSLF_1$ of *P. inflata*, as well as to an unrelated F-box protein PiFBP2411 (Hua and Kao 2006).

These results suggested an attractive model for how SLF/SFB, and possibly SBP1, function in recognition of self/non-self pollen and inhibition of the growth

of self pollen (Kao 2004; Sims 2005). This model can be stated as follows: during pollination, pollen grains (either self or non-self) are deposited on the stigmatic surface, germinate and produce pollen tubes that begin to grow through the transmitting tract of the style, where they encounter secreted S-RNases. Both self and non-self S-RNases are imported into pollen tubes. S-RNases recognized as non-self are recognized by a SCF^{SLF} (SCF-S-locus F-box)-E3 ubiquitin ligase complex, and targeted for ubiquitination and degradation via the 26S proteosome. S-RNases recognized as self are not ubiquitinated, retain ribonuclease activity, and act to degrade pollen tube RNA, thereby inhibiting protein synthesis and pollen tube growth. This model makes at least two predictions: first, that S-RNase should be polyubiquitinated and degraded in compatible pollinations, and second, that downregulation of *SLF/SFB* should result in complete self-incompatibility. Available evidence, however, provides only modest support for these two predictions. Experiments designed to determine if S-RNases were degraded in pollen tubes (Qiao et al. 2004) suggested a possible reduction in S-RNase levels. The observed reduction was far from dramatic however, and it was difficult to determine from the data presented whether the observed reduction was statistically significant at all time points. Goldraij et al. (2006) also assayed the level of S-RNase protein in both compatible and incompatible pollinations, and found little evidence to support large-scale degradation of S-RNase in compatible pollen. Hua et al. (Hua, Fields, and Kao 2008) have argued, however, that the failure to observe significant differences in S-RNase levels between compatible and incompatible pollen tubes cannot be used as evidence against the protein degradation model. To date there have been no reports regarding the effect of downregulation of *SLF* in transgenic plants. As discussed above, however, mutants of different *Prunus* species in which truncated SFB/SLF proteins are produced or the gene is completely deleted are self-compatible rather than universally incompatible. The study of downregulation or mutation of the *SLF* gene in Solanaceae will be an important area for future comparative studies of the S-RNase-based mechanism in these two families.

5.5.1 Evidence for a SCF^{SLF}-Like E3 Ubiquitin Ligase Complex

Although the precise role of ubiquitination and/or protein degradation in GSI remains unclear, there is strong evidence for the involvement of a SCF-type complex in self-incompatibility. It appears, however, that the proposed SCF^{SLF} complex differs somewhat from the prototypical SCF complex. The canonical SCF complex contains the core subunit SKP1; however, Hua and Kao (2006) showed that SKP1 proteins did not bind to products of *PiSLF* alleles in protein interaction assays. Similarly, Huang, Zhao, Yang, and Xue (2006) could not detect binding between SKP1 proteins in *Antirrhinum* and AhSLF. Huang et al. (2006) identified a SKP1-like protein, AhSSK1, by using a bait construct of the *Antirrhinum* F-box protein AhSLF-S_2 to screen a yeast two-hybrid library. In addition to its interaction with AhSLF, AhSSK1 interacted with another scaffold component of SCG complexes, CUL1. Huang et al. (2006) proposed that AhSSK1 may act as a bridge between AhSLF

and CUL1. The prototypical SCF complex contains a small RING protein RBX1. In the experiments of Hua and Kao, no interaction could be detected between RBX1 from *Petunia inflata* and either CUL1 or SLF. Lastly, both Sims (unpublished) and Hua and Kao (2006) found that SBP1 binds to the E2 ubiquitin conjugation protein PhUBC1. Together, these results suggest that a SCF^{SLF}-like E3 ubiquitin ligase complex may function in gametophytic self-incompatibility. Components of this complex would include SLF, SBP1 (replacing RBX1), CUL1, and SSK1 (replacing SKP1). This complex could play a role in recognizing either all S-RNases or in specific recognition and inhibition of non-self S-RNases.

5.6 Vacuolar Sequestration of S-RNase in Compatible Pollen Tubes

Two style-expressed proteins, HT-B and the 120 kDa glycoprotein, have been shown to be required for expression of self-incompatibility (McClure et al. 1999; Hancock et al. 2005). Downregulation of either renders otherwise self-incompatible plants incapable of rejecting pollen. In an examination of the role that they play in compatible and incompatible pollination, Goldraij et al. (2006) investigated the subcellular localization of these proteins, the S-RNase, and compartment-marker proteins in growing pollen tubes. S-RNase, HT-B and the 120 kDa protein were all imported into both compatible and incompatible pollen tubes. In compatible pollen tubes, and in pollen tubes at early stages of incompatible pollinations, the S-RNase appeared to be sequestered in a vacuolar compartment bounded by the 120 kDa glycoprotein. HT-B appeared to be degraded in compatible pollen tubes. In incompatible pollen tubes, late in pollination, this compartment appeared to break down. S-RNase levels persisted, as did HT-B, but the 120 kDa protein was no longer evident. In antisense HT-B plants, which were completely self-compatible, S-RNase remained sequestered (Goldraij et al. 2006).

5.7 Conclusion: Models for Pollen Recognition and Rejection

Taken together, current results support two alternative models for how S-RNase activity is inhibited in compatible pollen tubes, but released in incompatible pollinations. According to the SCF^{SLF} complex model, an E3 ubiquitin ligase complex preferentially recognizes non-self S-RNases and ubiquitinates them, most likely leading to degradation. In the sequestration model, compatibility occurs due to the continued sequestration of S-RNase in a vacuolar compartment, which is coupled to the degradation of HT-B. In an incompatible pollination, according to this model, HT-B remains intact and the vacuolar compartment breaks down, releasing the S-RNase. The challenge for researchers in this field is in how to distinguish and/or resolve these models. Particularly in the sequestration model, it is unclear what the role of SLF or the proposed SCF complex might be, and also how a vacuolar-sequestered S-RNase can interact in any fashion with pollen-S, which is cytoplasmic. It is

possible that the role of ubiquitination in this response is not to target proteins for degradation, but instead to target a specific protein substrate to the endomembrane system. Indeed, mono-ubiquitination (as opposed to poly-ubiquitination) has been shown to target proteins for endocytosis rather than degradation (D'Azzo, Bongiovanni, and Nastasi 2005). Given the significant contributions of *Petunia inflata* and *P. hybrida* as model systems for GSI research to date, Petunia will undoubtedly continue to provide insights into the mechanisms of this agronomically significant trait.

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Chapter 6
Cytoplasmic Male Sterility and Fertility Restoration in Petunia

Jason D. Gillman, Stéphane Bentolila, and Maureen R. Hanson

Abstract Cytoplasmic male sterility (CMS) in Petunia is due to an aberrant chimeric mitochondrially encoded gene designated *pcf*. Despite the ubiquitous expression of *pcf* throughout CMS Petunia plants, the primary defect is the disruption of pollen development. Sporogenous and tapetal cells in anthers of CMS lines display abnormalities during meiosis, ultimately resulting in abortion of pollen. Petunia lines carrying the CMS cytoplasm can be restored to normal male fertility by the presence of a single copy of a dominant nuclear *Restorer of Fertility* (*Rf*) gene. The *Rf* gene reduces the amount of the CMS-associated protein to near-undetectable levels. The *Rf* gene in Petunia is a member of the pentatricopeptide repeat-motif-containing gene family, a large nuclear gene family implicated in control of the expression of organellar genes. Fertility restoration in Petunia appears to involve interactions between the RF protein and *pcf* transcripts, perhaps affecting processing and/or translation.

6.1 Cytoplasmic and Genic Male Sterility in Petunia

Male-sterile phenotypes in Petunia may be encoded by either nuclear or cytoplasmic factors. The manifestations of cytoplasmic male sterility (CMS) in Petunia are a lack of pollen production, with female fertility and floral morphology usually remaining unaffected (Fig. 6.1). In certain nuclear backgrounds, alterations in floral bud development and plant morphology have also been observed. Nuclear genes interacting with the Petunia CMS cytoplasm evidently affect plant height, flower cover, flower diameter, and sometimes result in "bud blast" (Izhar and Frankel 1976). Genic male sterility (GMS), encoded by the nuclear genome, sometimes appears in inbred lines of an outcrossing species. This is thought to arise from an accumulation of deleteri-

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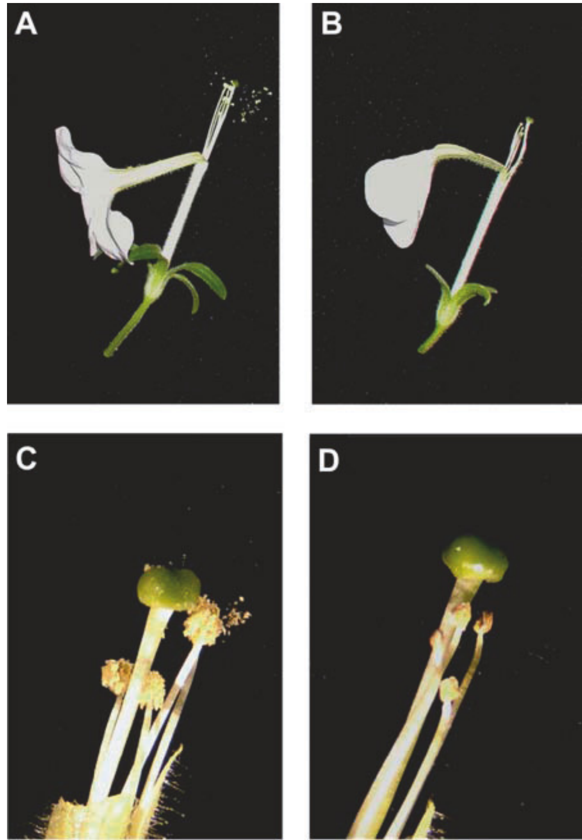

Fig. 6.1 Phenotype of CMS and fertility restoration in Petunia. (**A**) Dissected flower from *Petunia parodii* CMS line 3688 transformed with the nuclear fertility restorer allele. (**B**) Dissected flower from *Petunia parodii* CMS line 3688, with portion of petals removed to show dehisced anthers lacking pollen. (**C**) Dissected flower from CMS line 3688 transformed with the nuclear fertility restorer allele, showing dehisced anthers bearing pollen (yellow grains). (**D**) Dissected flower from *Petunia parodii* CMS line 3688, showing sterile anthers

ous nuclear mutations that impair pollen production. This multigenic form of male sterility is readily identified by its amelioration upon outcrossing (Frankel 1970; Izhar and Frankel 1973). Genic male sterility controlled by single, recessive nuclear genes has also been observed (Levan 1942; Welzel 1954; Frankel 1962).

CMS is valued for its simple manner of inheritance: all seed progeny of a plant will carry the trait, while progeny of GMS lines will segregate fertile and sterile progeny. Fertile lines carrying the CMS-encoding cytoplasm can be generated by incorporating one or more nuclear genes termed *Restorers of Fertility* (*Rf*; Pelletier and Budar 2007; see Fig. 6.1). In Petunia, and most other species that exhibit CMS, removal of *Rf* genes through segregation in sexual progeny yields male-sterile plants. Because all progeny of a CMS plant must necessarily result from cross-pollination, CMS/restorer systems are used in hybrid seed production, eliminating the need for manual removal of anthers.

Because nuclear *Rf* genes prevent a mitochondrially encoded disruption of pollen, CMS/*Rf* systems are also models for basic research into nuclear/organellar interactions. Such genetic materials are particular valuable for research in vascular plants, which are obligate aerobes unable to tolerate the loss of nuclear or mitochondrial gene functions necessary for essential mitochondrial function. While mitochondrial function mutants can be readily investigated in the facultative anaerobe *Saccharomyces cerevisiae* (Boldogh, Fehrenbacher, Yang, and Pon 2005), defects in mitochondrial genes in plants are correlated with severe developmental abnormalities and lethality (Newton, Gabay-Laughnan, and de Paepe 2004).

In Petunia, multiple *Rf* genes are known. Some genes form a polygenic restoration system, requiring multiple genes to confer male fertility. Some *Rf* genes also show temperature sensitivity, restoring fertility only at low temperatures. The best understood *Rf* locus is a monogenic, dominant, non-temperature-sensitive restorer termed *Rf1* (Edwardson and Warmke 1967). Fertility restoration is sporophytic, with the genotype of the mature plant (sporophyte) determining whether pollen is produced in CMS lines (Izhar 1978).

6.1.1 Origin of CMS in Petunia

As related by Duvick (1959), CMS in Petunia was the result of a purposeful breeding project wherein interspecific hybrids were generated, based on the successful generation of male sterility in tobacco (Clayton 1950). H.L. Everett and W.H. Gabelman, two graduate students of D.F. Jones, utilized *P. hybrida* as a male parent in crosses with an unknown female parental line (presumably *P. axillaris*, *P. integrifolia* or *P. parodii*) to yield CMS lines. This information suggests that CMS in Petunia is alloplasmic, having arisen when the nuclear genome from one species was paired with the mitochondrial genome from a different species, the combination resulting in cytoplasmic male sterility (Pelletier and Budar 2007). Unfortunately, the specific records concerning the origin of CMS in Petunia have apparently been lost or were never made, so the specific origin of CMS remains undetermined (Duvick 1959). It also remains possible that the CMS-encoding mitochondrial DNA (mtDNA) arose through spontaneous rearrangements induced by mixing nuclear genomes of one species with mitochondrial genomes of another rather than being true alloplasmic sterility (see Hanson and Conde 1985).

The Petunia CMS trait was successfully transferred into a wide variety of Petunia species by repeated backcrosses and distributed to plant breeders. In Petunia, only one type of CMS has been identified. Petunia CMS lines collected from various labs around the world were deemed to be carrying the same cytoplasmic background because they could all be restored to fertility by *Rf1* and all exhibited the same response to nuclear backgrounds that affect timing of microspore breakdown, floral development, and plant height (Izhar and Frankel 1976).

6.2 Development in CMS: Fertile and Restored Lines

The histological and ultrastructural characteristics of microspore development in CMS and fertile lines have been extensively characterized for CMS and male-fertile lines of *Petunia hybrida* (Bino 1985a, b) and for isonuclear CMS, male-fertile, and restored lines of *Petunia parodii* (Conley, Parthasarathy, and Hanson 1994). During premeiotic stages in both species, anther development of CMS, normal and restored lines is indistinguishable.

In *P. hybrida*, the first CMS-related abnormalities occur during leptotene, when cells in the tapetal layer are smaller and display unusually large vacuoles and dense cytoplasm (Bino 1985a). Organelles display unusual morphologies by late prophase: mitochondria from the sterile line appear elongated with tubular cristae, whereas mitochondria from the corresponding fertile line are ellipsoid and cristae remain plate-like. Tapetal cell abortion occurs by anaphase I, by which time plastids and mitochondria have completely degenerated. Sporogenous tissue in developing anthers of fertile and sterile lines also displays differences. Although the timing is highly variable compared to that seen in tapetal cells, vacuolar abnormalities again are the first recognizable deviation (Bino 1985b). Disorganized dense cytoplasm, disintegrated mitochondria and plastids, and abnormalities in callose deposition are apparent by the conclusion of prophase I. The CMS phenotype is highly cell specific, as vascular and epidermal tissues in anthers of CMS lines display no recognizable abnormalities (Bino 1985a; Conley et al. 1994).

In *Petunia parodii*, the early stages of meiosis are indistinguishable among CMS, male-sterile and restored-fertile lines. At no stage of meiosis can differences between male-fertile and restored-fertile lines be detected at the microscopic and ultrastructural levels (Fig. 6.2). In CMS lines, however, sporogenous tissues fail to complete meiosis normally. The first deviations occur during late meiosis, with sporogenous tissues displaying amorphous cytoplasm and reduced numbers of nucleoli (Fig. 6.2). Tapetal cells at this stage also display aberrant development, with the appearance of multiple small vacuoles and a lesser number of large vacuoles. The ultimate consequence is a failure to produce pollen (Fig. 6.2). The onset and progression of the meiotic breakdown in *P. hybrida* and *P. parodii* appear to be very similar (Conley et al. 1994).

The disruption of sporogenous and tapetal cells observed in Petunia may occur through the mechanism of programmed cell death (PCD). Fragmentation of DNA in degenerating sporogenous cells in Petunia CMS anthers has been observed by two-photon microscopy (C.A. Conley, W.R. Zipfel, and M.R. Hanson, unpublished). It is possible that a toxic protein encoded by the CMS cytoplasm triggers PCD in Petunia cells, leading to the observed death of tapetal and sporogenous cells. PCD in Petunia has been most thoroughly studied during petal senescence, when the hallmark DNA fragmentation has been observed (Xu and Hanson 2000, see Chapter 14). Mitochondria are known to play a role in signaling PCD (Lam, Kato, and Lawton 2001; Logan 2006). In a sunflower CMS genotype, premature release of cytochrome C from mitochondria within tapetal tissues results in an acceleration of PCD, ultimately resulting in premature anther degeneration (Balk and Leaver 2001). It is

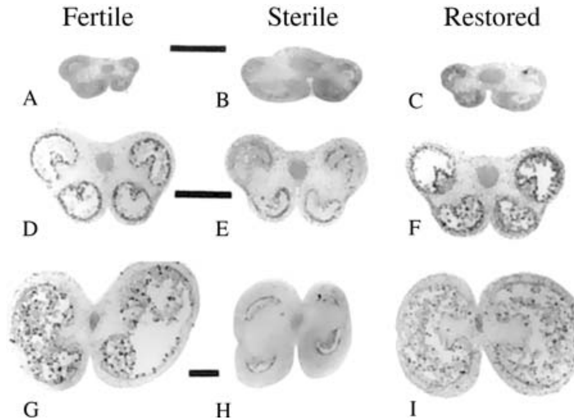

Fig. 6.2 Cross sections (1 μm) of fertile, sterile, and restored anthers imaged by light microscopy following fixation, sectioning, and staining with azure B. (**A–C**) **Stage 2**: All tissues of these pre-meiotic anthers are fully differentiated, but no aberrations in sterile development can be seen at this magnification. (**D–F**) **Stage 3**: Differences are clearly visible at this stage. Fertile (**D**) and restored (**F**) anthers contain tetrads of microsporocytes, while the sporogenous tissue of the sterile anther (**E**) has not completed meiosis. (**G–I**) **Stage 6**: In the fertile (**G**) and restored (**I**) anthers, the connective tissue between the locules has broken down and the anthers are about to dehisce. The sterile anther (**H**) has completely lost the sporogenous and tapetal cells and the connective tissue occupies nearly the whole volume of the anther. All anthers of each stage are at the same magnification. Bar=200 μm (Modified from Conley et al. 1994)

possible that degeneration observed in anthers of CMS lines of Petunia and many other species occurs through PCD; nevertheless, exactly how any CMS-encoding gene acts to disrupt mitochondrial function is still unknown (Conley and Hanson 1995; Hanson and Bentolila 2004; Chase 2007).

6.3 Physiological Aberrations in Petunia CMS Lines

Even before the Petunia CMS-encoding gene was identified, a number of attempts were made to determine why pollen development is aborted. Early studies noted a strong difference in callase activity and pH in anthers of CMS plants compared to their male-fertile counterparts (Frankel, Izhar, and Nitsan 1969; Izhar and Frankel 1971). In one CMS line, a precocious drop in pH and increase in callase activity occur during early meiotic stages. In a male-fertile line, a similar drop was not observed until much later in the meiotic process (Izhar and Frankel 1971). A similar study showed differences in the accumulation of free amino acids, an increase in the levels of asparagine, and a decrease in the levels of other assayed amino acids (Izhar 1973; Izhar and Frankel 1973). These changes in free amino acids were hypothesized to be the cause of decreased pH noted previously. The reduction in pH was also considered to engender the change in callase activity. Cultures derived from

male-sterile lines have also been noted to show lower uptake of amino acids than cultures derived from male-fertile lines (Perl, Swartberg, and Izhar 1992).

An examination of the activities of esterases (van Marrewijk, Bino, and Suurs 1986), cytochrome C oxidase activity (Bino et al. 1986b), and ATP/ADP/AMP levels (Bino, de Hoop, van Marrewijk, and van Went 1986a) revealed differences between male-sterile and fertile anthers beginning at meiosis. However, no significant differences were noted in premeiotic anthers. It was concluded that these altered activities and levels were induced by the process of degeneration, rather than causative, as they occurred after the first signs of pollen abortion.

Some reports have indicated differences in the respiratory capacity of CMS suspension batch cultures compared with male-fertile cultures. Cultures derived from a *Petunia hybrida* CMS line were noted to have lower growth efficiency, expressed in terms of conversion of glucose to dry weight, compared to cultures derived from male-fertile lines. CMS cultures also take up oxygen at approximately twice the rate of male-fertile cultures, although overall growth rate is the same for both (van der Plas, de Gucht, Bakels, and Otto 1987).

Suspension cultures of three different CMS lines were observed to differ in utilization of the alternative oxidase pathway in comparison to suspension cultures of lines containing the same nuclear background but a normal fertile cytoplasm. No differences were noted in accumulation of biomass as measured by dry weight. However, the CMS lines appeared to utilize the cyanide-insensitive alternative oxidase pathway less than the normal fertile lines. A cell line from a fertile-restored plant exhibited an increase in alternative oxidase pathway utilization relative to that seen in the CMS lines. Immature anthers of CMS plants also exhibited an altered distribution of electron flow through the two pathways, but mature anthers, ovary, and perianth tissue of CMS and fertile lines could not be distinguished in regard to respiratory activity (Connett and Hanson 1990). These observations remain unexplained and were unexpected, as the alternative oxidase pathway would have been predicted to be higher, rather than lower, in a tissue undergoing stress such as that expected in a degenerating anther. The possibility remains that the mitochondrial genomes in CMS lines of Petunia specify a defect in utilization of the alternative oxidase pathway, which, though not resulting in ATP generation, is thought to play other important roles in stress responses (Clifton, Millar, and Whelan 2006) and regeneration of reducing equivalents (Palmer 1976).

6.4 Identification of the Gene Encoding Petunia CMS

In Petunia, as in many other plants, both mitochondria and chloroplasts are predominantly inherited in a maternal manner, although a few reports indicate low-level paternal transmission in particular nuclear backgrounds (Cornu and Dulieu 1988; Derepas and Dulieu 1992). Because of the usual inheritance of mitochondrial and chloroplast genomes together in CMS lines, it was not known which of these genomes encoded CMS.

The key breakthrough needed to identify the genome and gene encoding Petunia CMS was production and analysis of somatic hybrid plants produced from the fusion of protoplasts from CMS and fertile lines (Boeshore, Lifshitz, Hanson, and Izhar 1983). The Izhar lab performed several different protoplast fusions using different CMS and fertile lines as somatic hybrid parents. The chloroplast genomes in 17 different stable sterile or fertile plants arising from fusions of three different sets of parents were characterized. Chloroplast genomes segregate in somatic hybrids and undergo recombination only rarely (Clark, Schnuabelrauch, Hanson, and Sink 1986; Morgan and Maliga 1987). Sterile somatic hybrids were found which carried the chloroplast genome of the fertile parent, and fertile somatic hybrids exhibited the sterile parent's chloroplast genome, implicating the mitochondrial genome as the source of CMS (Clark, Izhar, and Hanson 1985).

Unlike the segregation of chloroplast genomes in somatic hybrid plants, the parental mitochondrial genomes in Petunia protoplast fusions undergo many recombination events followed by segregation of novel mitochondrial genomes in somatic hybrid plants and their progeny (Hanson 1984; Rothenberg, Boeshore, Hanson, and Izhar 1985; Rothenberg and Hanson 1987, 1988). As a result, recombinational genetics can be used to track segregation of mitochondrial DNA regions with phenotypes exhibited by somatic hybrid plants. A large somatic hybrid (T_0) population resulting from fusion of CMS and fertile parents proved ideal for analysis of mitochondrial genomes. Of approximately 4000 initial somatic hybrid plants obtained, most were stably male-fertile (97%) but some were stably male-sterile (2%), or exhibited unstable fertility phenotypes (1%, Izhar, Schlicter, and Swarzberg 1983; Wolf-Litman, Soferman, Tabib, and Izhar 1992). Progeny of the unstable lines segregated for plants with sterile or fertile flowers for several generations. The analysis of the stable recombinant fertile and sterile progeny revealed that they contained novel recombinant mitochondrial genomes (Boeshore et al. 1983; Hanson, Rothenberg, Boeshore, and Nivison 1985). Therefore, it became feasible to search for a restriction fragment length polymorphism (RFLP) that could distinguish mitochondrial genomes encoding CMS vs. fertility. A single RFLP was identified that correlated with the male-sterile phenotype (Boeshore, Hanson, and Izhar 1985; Clark, Gafni, and Izhar 1988). The region detected by the RFLP was cloned and sequenced to reveal an unusual chimeric gene, termed the *Petunia CMS-associated Fused gene, (pcf*, Young and Hanson 1987) (Fig. 6.3).

The role of *pcf* in male sterility was further supported by the analysis of unstable lines derived from the original protoplast fusion experiment (Boeshore et al. 1983), which continued to segregate for male fertility and sterility. In all cases, reversion to male fertility in these lines corresponded with loss of the CMS-associated RFLP (Wolf-Litman et al. 1992). To date, all naturally occurring CMS genes identified in any species have proven to be mitochondrially encoded (Schnable and Wise 1998; Hanson and Bentolila 2004; Chase 2007). In general, CMS-encoding genes arise through recombination of normal mitochondrial genes with each other or with sequences of unknown origin, and many feature regulatory or coding sequences of subunits of the ATP synthase (Hanson and Bentolila 2004). CMS appears to arise

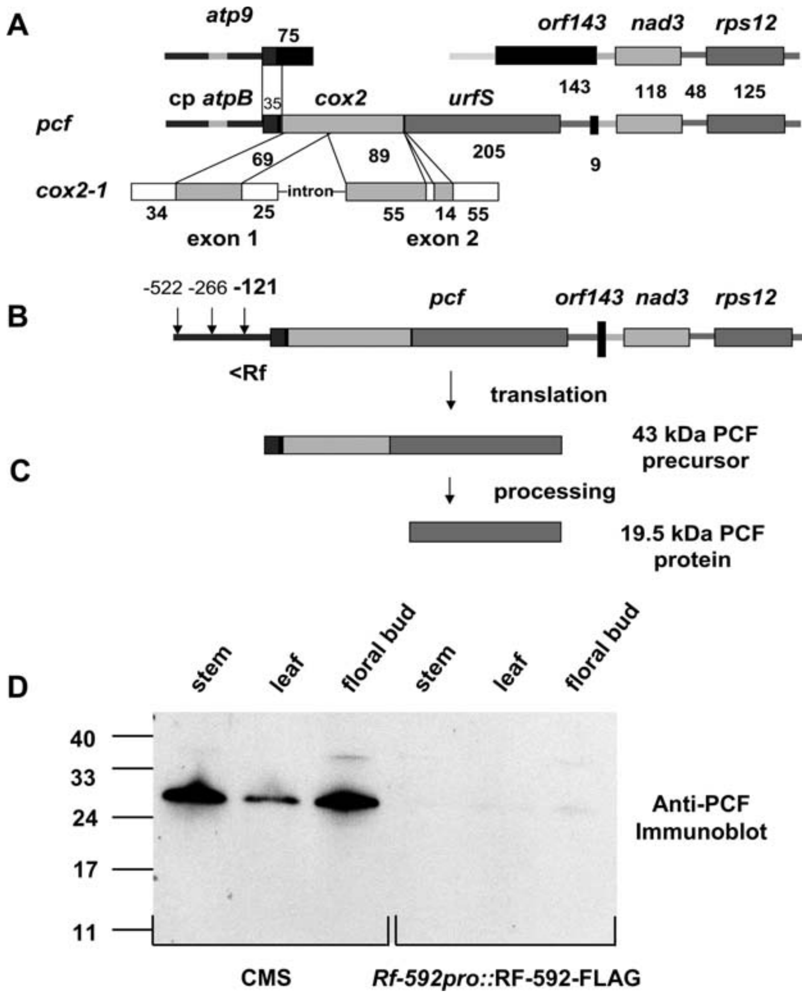

Fig. 6.3 The *Petunia CMS-associated fused* (*pcf*) gene locus. (**A**) Diagram of the *pcf* locus. *Numbers* indicate nucleotides. Specific regions of similarity are indicated by *diagonal lines*. Normal copies of *atp9* and *cox2* are present elsewhere in the mtDNA. *pcf* is co-transcribed with the only copies of *nad3* and *rps12* in the CMS genome. The mtDNA of the fertile line contains *orf143* upstream of *nad3/rps12* whereas only a small portion of *orf143* is retained in the *pcf* locus. (**B**) 5′ transcript termini identified by S1 nuclease mapping on *pcf* transcripts. One class of transcripts (−121, indicated in *boldface*) is specifically reduced in *Rf* lines. (**C**) A 43 kDa PCF precursor is processed to form a 19.5 kDa protein. (**D**) A 19.5 kDa processed PCF protein is reduced in all tissues in isonuclear lines carrying an *Rf592* transgene. Figure **D** is modified from Gillman, Bentolila, and Hanson (2007). Data supporting the gene and locus diagrams were first reported in Young, Hanson and Dierks 1986; Young and Hanson (1987); Rasmussen and Hanson (1989); Pruitt and Hanson (1989); and Hanson et al. (1989, 1999)

as a result of a gain-of-function mutation – a toxic coding region happens to come under the control of a mitochondrial promoter, and if the protein is expressed at sufficient levels to disrupt pollen production but not impair the whole plant, a male-sterile phenotype is created.

6.4.1 The Expression of pcf Is Affected by the Petunia Rf Gene

The *pcf* gene (Fig. 6.3) is composed of 35 codons of the open reading frame of *atp9*, portions of two of the exons of *cox2* (comprising 156 codons), and 207 codons of unknown origin (*urf-S,* Young and Hanson 1987). Normal copies of *coxII* and *atp9* are present elsewhere in the mitochondrial genome (Pruitt and Hanson 1989; Young et al. 1986). Sequences homologous to *urf-S* have not been detected in completely sequenced plant genomes such as Arabidopsis and rice, and may have resulted from horizontal transfer from an unknown source.

The gene is co-transcribed with the only copies of two essential genes, *nad3 and rps12* (Hanson, Pruitt, and Nivison 1989; Rasmussen and Hanson 1989) that are present in the original Petunia CMS genome. An open reading frame of unknown function, *orf143*, is upstream of *nad3* and *rps12* in the fertile line, but only a small segment of *orf143* is present near *nad3/rps12* in the CMS line. *Orf143* sequences are present elsewhere in the CMS mtDNA (Hanson, Wilson, Bentolila, Köhler, and Chen 1999). The mapping of the 5′ transcript ends of the *pcf* transcript revealed three termini, located at approximately –522, –266, and –121 nucleotides relative to the start codon (Fig. 6.3). Transcripts containing the –522 termini make up a very small fraction of the total transcript population and are barely detectable by S1 nuclease protection assay (Young and Hanson 1987). The specific proportions of these transcripts also vary among tissues. The considerable variation in the overall steady-state levels of *pcf* transcript in CMS lines with different nuclear backgrounds, regardless of the presence of *Rf*, revealed that additional nuclear genes other than *Rf* play a role in *pcf* transcript accumulation. One transcript class (with 5′ termini at position –121 relative to the start codon) was observed to consistently decrease in an *Rf*-specific manner in restored lines (Pruitt and Hanson 1991). Nevertheless, RNA from the *pcf* locus is present in both restored and male-sterile lines (Pruitt and Hanson 1991; Wolf-Litman et al. 1992). The smaller two transcripts apparently are processing products, while the largest transcript is likely to be a primary transcript, according to circular RT-PCR data (J. Gillman, unpublished data). Thus a difference in efficiency of transcription initiation is not likely to account for the altered ratios of 5′ termini in the restored vs. CMS lines (Hanson et al. 1999).

No differences in RNA editing of the *nad3* and *rps12* genes have been detected in CMS vs. fertile lines (Wilson and Hanson 1996). Transcripts of *pcf* undergo editing in regions homologous to *atp9* and *cox2,* but no editing of the *urf-S* portion has been detected (Nivison, Sutton, Wilson, and Hanson 1994).

Mitochondrial proteins in CMS, fertile (containing a normal cytoplasm), and CMS-restored lines were analyzed with antibodies against ATP9, COX2, and URF-S sequences. No difference in abundance of ATP9 or COX2 has been detected among any of these lines (Nivison et al. 1994). ATP9 and COX2 antibodies do not detect a CMS-specific signal, but the anti-URF-S antibody does detect a protein with a 25 kDa mobility on SDS gels that is absent from fertile lines. In fertility-restored lines carrying the CMS cytoplasm, the level of this protein is dramatically reduced in all tissues analyzed (Nivison and Hanson 1987, 1989).

The apparent 25 kDa protein results from processing of a precursor 43 kDa protein, the size predicted from the entire *pcf* open reading frame. N-terminal sequencing revealed the apparent 25 kDa protein is actually a 19.5 kDa product that displays aberrant mobility under SDS-PAGE (Fig. 6.3) and is entirely encoded by the *urf-S* region of *pcf*. The 19.5 kDa protein fractionates with both soluble and membrane proteins, while the unprocessed protein is present only in the soluble fraction. Membrane-associated PCF remains sensitive to protease treatment, unlike COXII protein, which suggests it is not an integral membrane protein although it is apparently loosely membrane associated (Nivison et al. 1994).

The PCF protein has been detected in both vegetative and reproductive tissue. It remains a puzzle why the aberrant Petunia protein and other abnormal mitochondrial proteins found in CMS lines of other species do not seriously impair vegetative growth and specifically affect reproductive development. Theories for this specificity include a unique role for mitochondria during pollen development, detrimental interaction of aberrant proteins with other molecules found only in developing pollen, and an increase in the abundance of the abnormal protein to particularly toxic levels in developing pollen. The latter hypothesis is presently the most attractive explanation for tissue-specific disruption in Petunia, as the PCF protein was detected at high levels in sporogenous tissue of developing anthers on tissue prints (Conley and Hanson 1994) using the cryostat technique (Conley and Hanson 1997).

The targeting of protein carrying the URF-S region present in the 19.5 kDa protein to mitochondria following expression in the nucleus does not result in a sterile transgenic plant (Wintz et al. 1995). This may be due to inappropriate localization of PCF. In transgenic lines expressing a nuclear-encoded, mitochondrial-targeted form of PCF, the PCF protein can be found only in the soluble fraction of mitochondrial extracts, in contrast to native PCF, which can be found in both soluble and membrane fractions (Wintz et al. 1995). However, it remains possible that the presence of a very small amount of the precursor protein is actually responsible for pollen disruption rather than the processed portion that accumulates. Small amounts of the precursor protein can be detected in pulse-chase experiments as well as in immunoblots that have been probed with a sensitive antibody and exposed for a lengthy period (Nivison et al. 1994; Köhler and Hanson, unpublished). The precursor PCF protein contains transmembrane domains from both ATP9 and COX2 and is toxic to *E. coli* even when expressed at low levels (Hampton and Hanson, unpublished).

6.4.2 Organization of mtDNA in Male-Sterile and Male-Fertile Lines

Physical maps of mtDNAs from two lines of Petunia have been generated, although sequence data are still limited. Hybridization and some sequencing have allowed mapping of tRNAs, putative replication origins, and a number of major protein-coding genes (DeHaas et al. 1991; Folkerts and Hanson 1989, 1991; Weber-Lotfi, Marechal-Drouard, Folkerts, Hanson, and Grienenberger 1993). The male-fertile line (3704) and the male-sterile line (3688) were chosen because they were used in the 4000-plant somatic hybridization experiment performed by the Izhar lab (Izhar et al. 1983). By analysis of cosmids, a 443 kb master circular map can be constructed for 3704 and the genome of 3688, a male-sterile line, can be organized into an 803 kb master circular map (Fig. 6.4). While the maps obtained from restriction mapping of individual cosmids are circular, and cosmids that exist indicate recombinant molecules are present at high proportions, the actual physical configuration of mtDNA of no angiosperm species is presently known.

The master circular map for male-fertile mtDNA has three recombination repeats, two of which are in direct orientation, with the third inverted. At least four isomers of the 443 kb circle are possible, and cosmids that could represent three sub-genomic molecules, consisting of 199 and 244 kb (Folkerts and Hanson 1989), were also detected.

The large circular map of the male-sterile line can be resolved into two smaller apparently sub-genomic maps, with the recombinant regions predicted to have different stoichiometries (Fig. 6.4). One sub-genomic molecule contains *pcf*; the other contains *atp9-1*. The *atp9-1*-containing sub-genomic molecule is approximately 1.5 times more abundant than the *pcf* molecule, based on hybridization signals. The recombination repeats are similar in sequence to those of the male-fertile line, but are in direct orientation. Smaller sub-genomic circles of 266, 130, and 277 kb are also predicted (Folkerts and Hanson 1991; Hanson and Folkerts 1992).

A comparison of the two physical maps reveals large-scale differences (Fig. 6.4). Although at least 10 regions within both genomes show conserved gene order and orientation on the local scale, a massive difference in organization is evident (Folkerts and Hanson 1991). Because we do not know the progenitor line for the CMS genotype and no other Petunia cytoplasms have been mapped, we do not know how recently the recombination events occurred that created the *pcf* locus. We also do not know whether fertile lines exist that may have a genome organization more similar to CMS line 3688 than the configuration of 3704.

We previously described a model for creation of genomes with three recombination repeats, as found in the line 3704 map, and another model to explain how two very large regions of a mitochondrial genome can become duplicated, as seen in the CMS line 3688 map (Hanson and Folkerts 1992). The models postulate that occasional rare recombination events between small regions with limited similarity give rise to rare molecules that sometimes recombine with more common sub-genomic molecules.

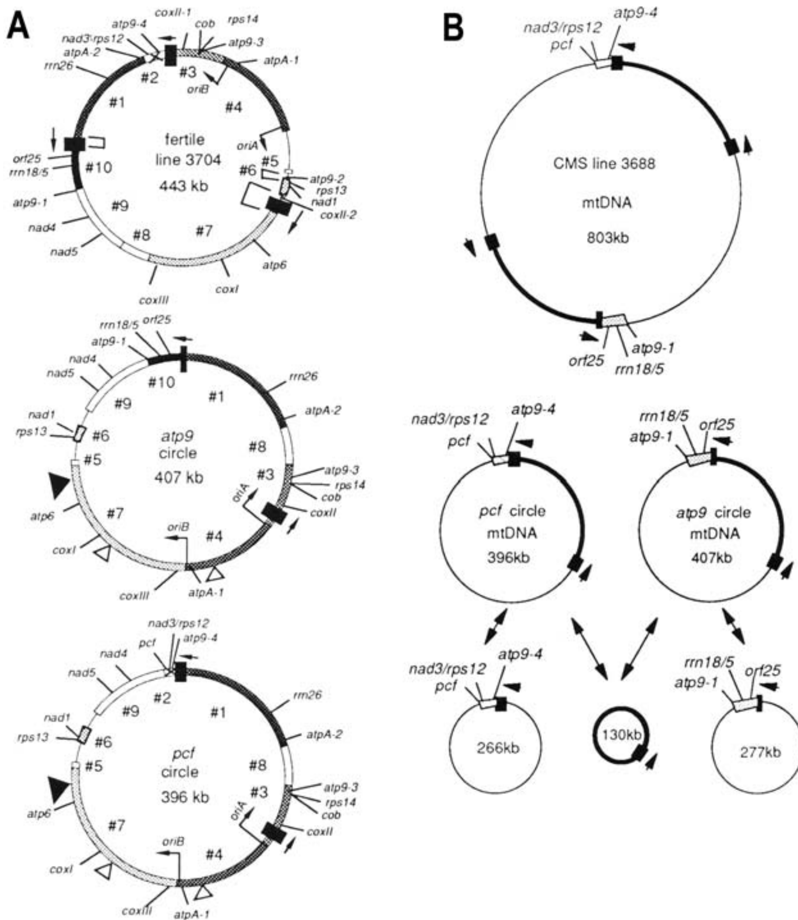

Fig. 6.4 Genomic organization of mtDNA in fertile and CMS lines. (**A**) Comparison of the structure of the fertile and sterile Petunia mitochondrial genomes. Sectors conserved between the two lines are shown by regions of similar fill. *Thin line* in the 3688 map represents regions that could not be assigned to a corresponding sector in the 3704 genome. Small deletions or insertions in the sterile genome relative to the fertile genome are indicated by *open* and *filled triangles*, respectively, outside the circle. Brackets inside the 3704 circle mark regions that are not found in the mtDNA from the CMS line. Positions and orientations of the recombination repeats are indicated by *black rectangles* flanked by filled *arrowheads*. Locations and orientations of two putative mitochondrial replication origins are shown by the *arrows* inside the circles and marked *oriA* and *oriB*. Approximate mitochondrial gene locations are shown outside the circle. (**B**) Possible mtDNA sub-genomic arrangements, derived from analysis of CMS line 3688 (From Folkerts and Hanson 1991, copyright Genetics Society of America)

We have traced the likely origin of one of these rare recombination events that could have led to the creation of *pcf*. The loss of the *cox2* intron and 75 nucleotides of exon 1 in the *cox2* portion of *pcf* can be explained by formation of a *cox2* pseudogene after use of a cryptic splice site in exon 1 rather than the normal splice site, followed by reverse transcription (Pruitt and Hanson 1989). Such a *cox2* pseudogene and normal *atp9* and *cox2* genes exhibit a small area of sequence similarity at the point where *atp9* and *cox2* are joined in the chimeric *pcf* gene (Pruitt and Hanson 1989). By a process of sorting-out, such novel genomes arising through chance recombination may become fixed, in a manner analogous to the stable recombinant genomes produced following somatic hybridization (Conklin and Hanson 1994).

6.5 Graft Transmission of CMS in Petunia

One puzzling aspect of CMS in Petunia concerns reports that the CMS trait can be transmitted by grafting. The first reported instance of such transmission was by Frankel, who utilized a fertile scion on a CMS stock plant (Frankel 1956). Upon self-fertilization of the fertile scion, several CMS progeny were obtained. Several other groups have also reported successful transmission of CMS, though differing in regard to the generation in which CMS progeny were first noted. Edwardson and Corbett (1961) reported complete sterility appearing in the F2 generation, with partial sterility found in the F1 generation. One investigator, as described in Izhar (1984), reported being able to induce CMS in the fertile scion itself. A subsequent study, utilizing more extensive controls, failed to reproduce evidence for the graft-induced transmission of CMS, and the appearance of sterile flowers was attributed to other causes (van Marrewijk 1970). At the time, one frequently cited explanation for graft transmission speculated that CMS in Petunia was due to a virus. However, efforts to treat CMS lines with conditions shown to cure viral infection failed to generate male-fertile plants (Izhar, Joseph, and Evenor 1988).

The transmission of CMS via grafting is not readily explained, given the overwhelming evidence that CMS in Petunia is due to the presence of a novel mitochondrial gene. However, transmission of CMS by grafting remains an incompletely researched phenomenon. It is unfortunate that no CMS line arising from a report of transmission to a fertile scion is available, making it impossible to directly compare the mitochondrial DNA in such a line to the mitochondrial genome of CMS line 3688.

6.6 Identification of the *Rf* Gene in Petunia

In order to isolate the *Rf* gene, both RAPD and AFLP markers, in conjunction with the bulked segregant analysis technique (Michelmore, Paran, and Kesseli 1991), were used to map the *Rf* gene roughly to a location in the Petunia genome (Bentolila, Zethof, Gerats, and Hanson 1998). Due to the very limited genetic map information available for Petunia at the time, molecular markers for chromosome two of the

tomato genetic map were exploited. Although some rearrangements between these two solanaceous species were observed, the combined Petunia and tomato information allowed mapping of the *Rf* gene to a 650 kb region on chromosome four (Bentolila et al. 1998). Further bulked segregant analysis revealed additional markers linked to *Rf*. The marker that co-segregated most closely with the *Rf* gene was used to screen a BIBAC library prepared from Petunia DNA. This approach allowed for the isolation of a 37.5 kb BIBAC clone that segregates with *Rf* (Bentolila and Hanson 2001).

Following sequencing of the BIBAC, two genes were found to encode putative mitochondrial transit sequences, as would be expected of a protein that could affect mitochondrial gene expression. Upon transformation of CMS lines, one of these genes restored normal pollen production in addition to reducing the levels of PCF protein (Bentolila, Alfonso, and Hanson 2002). This *Rf* gene contains pentatricopeptide repeat (PPR) motifs, comprising 490 out of the 592 codons (Fig. 6.5). The Petunia *Rf* gene was thus termed *Rf-PPR592*; for brevity, it will be referred to here

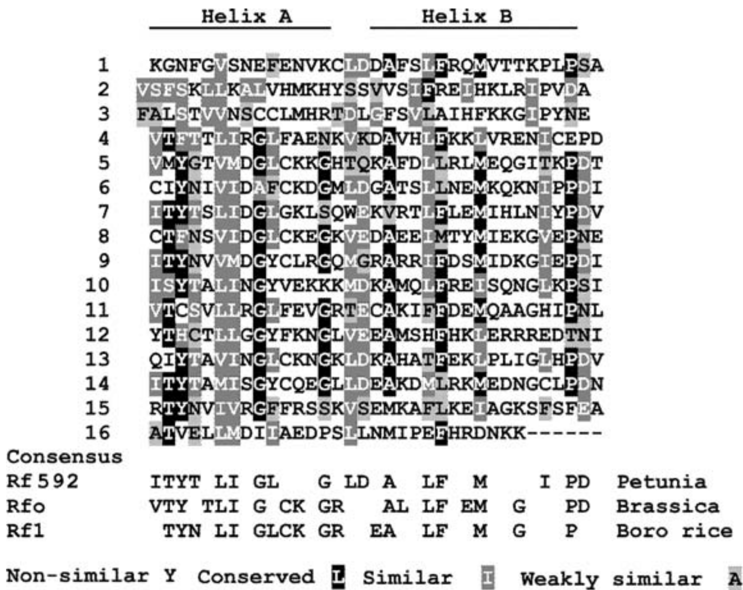

Fig. 6.5 The PPR motif and nuclear genes that modify mitochondrial gene expression; alignment of the 14 PPR motifs in the predicted Petunia RF protein with two additional degenerate PPR-derived motifs. The 14 PPR motifs were identified by MEME software (Bailey and Elkan 1994). Identical amino acids are shown as black background with white lettering; at least 7 of the 14 motifs must have an identical or highly similar amino acid for the residue to be included in the Petunia RF consensus sequence. Similar amino acids are shown in dark gray and weakly similar residues are in light gray. The locations of the two predicted anti-parallel {alpha}-helices are indicated (Small and Peeters 2000). Below the PPR motif alignment are shown the consensus sequences of the PPR motifs found in Petunia RF, Brassica/radish Rfo, and PPR8-1, the putative Boro rice RF (Modified from Hanson and Bentolila 2004)

as *Rf592*. A highly similar gene containing 591 codons is present 5′ of *Rf592*. This gene, termed *Rf591*, displays greater than 90% identity with *Rf592* at the nucleotide level (Fig. 6.5). However, *Rf591* is not a fertility restorer: CMS plants transformed with *Rf591* constructs fail to show reduced PCF and remain sterile (Alfonso 2002).

Another highly similar gene, *rf592*, was isolated from a CMS line by PCR with the same primers used to clone *Rf592*. The *rf592* gene is expressed in roots but not in floral buds. Differences between *rf592* and the genes in the *Rf* locus exist in both the coding region and putative regulatory regions (Bentolila et al. 2002). The RF592 protein consists of an N-terminal 28 amino acid region predicted to target the gene product to mitochondria, 14 tandem repeats of the pentatricopeptide repeat motif (Fig. 6.5) and a C-terminus comprised of two highly degenerate PPR motifs (Bentolila et al. 2002). RF592, RF591, and rf592 display a remarkable level of amino acid sequence identity (Fig. 6.6), although only RF592 can restore fertility (Alfonso 2002; Alfonso, Bentolila, and Hanson 2003).

Petunia *Rf592* was the first fertility restorer gene affecting the expression of an abnormal mitochondrial gene to be cloned. The first restorer gene cloned from any species, maize *Rf2,* is evidently an unusual "biochemical restorer" needed to ameliorate a defect caused by the small amount of toxic URF13 protein that remains despite the presence of the maize *Rf1* allele (Cui, Wise, and Schnable 1996). Several additional fertility restorer genes that affect mitochondrial gene expression have been cloned from other plant species, and all exhibit similarity to Petunia RF592 (Brown et al. 2003; Kazama and Toriyama 2003; Akagi et al. 2004; Komori et al. 2004; Klein et al. 2005). These restorers tend to reduce expression of the abnormal gene (Hanson and Bentolila 2004; Chase 2007), and all contain multiple PPR motifs, which are degenerate 35-amino acid repeated units similar to the 34-amino acid TPR protein motif. The PPR-motif-containing gene family was originally identified by bioinformatic analysis of the Arabidopsis genome (Aubourg, Boudet, Kreis, and Lecharny 2000; Small and Peeters 2000), and has undergone a considerable land-plant-specific expansion. Arabidopsis possesses approximately 450 PPR genes compared to only 6 in mammals and 5 in budding yeast (Lurin et al. 2004). A growing body of evidence has implicated the PPR motif in control of various aspects of organellar gene expression: genes containing the PPR motif have been shown to affect transcription, RNA stability, RNA editing, RNA cleavage, RNA splicing and translation (Andrés, Lurin, and Small 2007).

6.6.1 Expression and Function of the Rf592 Gene Product

How do *Rf592* and similar restorers act to suppress expression of abnormal genes? One characteristic of the PPR-motif-containing restorers cloned from Petunia, Brassica, and rice is that each species contains highly similar, closely linked genes, making the generation of specific antibodies potentially difficult. For further studies of *Rf592*, therefore, stable lines expressing a FLAG-tagged version of RF592 were

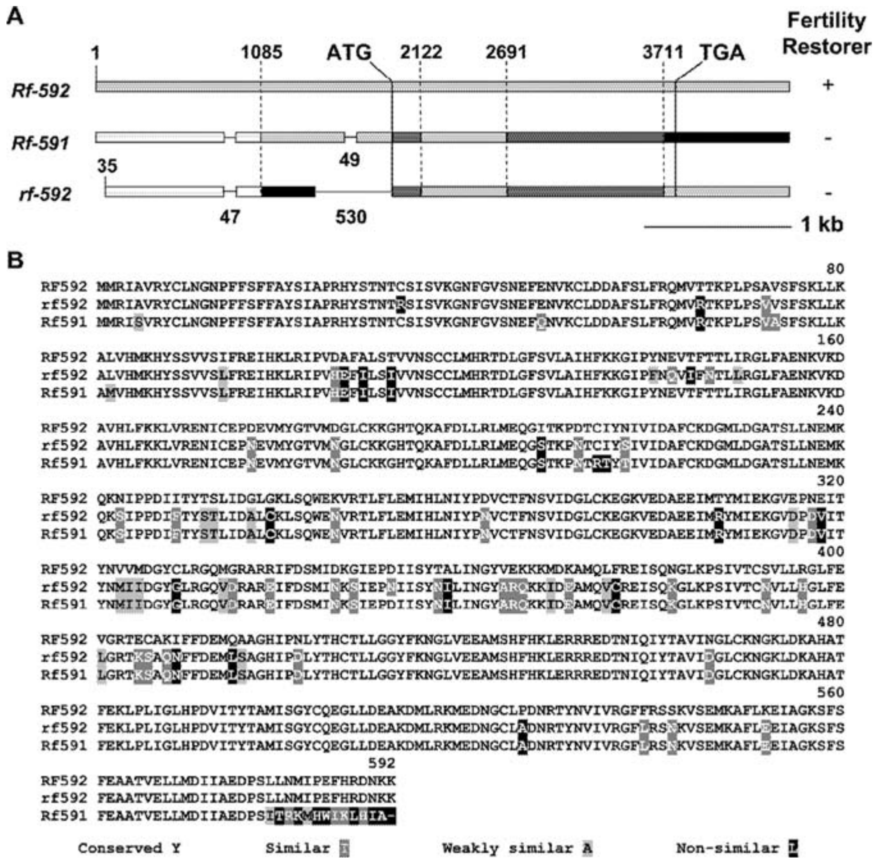

Fig. 6.6 Alignment of three *Rf*-related genes: *Rf592* is the fertility restorer, *rf592* is a non-restorer isolated from a CMS line, and *Rf591* is a non-restorer located in tandem upstream to *Rf592* within the *Rf* locus. (**A**) Alignment of *Rf592, Rf591,* and *rf592* showing putative promoter and terminator regions. Similarly shaded regions indicate >95% nucleotide identity. (**B**) Alignment of the amino acid sequence of RF592, rf592, and RF591. *Black text with white background* indicates identity, *light gray background* indicates a conservative substitution, *dark gray* indicates a less conservative substitution, and *black background* indicates a non-conservative substitution or deletion

generated (Gillman et al. 2007). When CMS lines are transformed with *Rf592-FLAG* constructs controlled by the *Rf592* promoter, plants are restored to male fertility (Fig. 6.1). The addition of a FLAG tag at the C-terminus of RF592 thus does not apparently hinder its ability to restore fertility.

Because PCF protein is expressed in suspension cultures, from which mitochondria are most conveniently isolated, we also utilized cultured CMS lines expressing RF592-FLAG under control of the CaMV 35S promoter, which results in easily detectable levels of RF592-FLAG protein. Fractionation experiments revealed that RF592 is present in a large, presumably multi-protein, complex that is tightly associated with, but not inserted into, the inner side of the inner membrane of

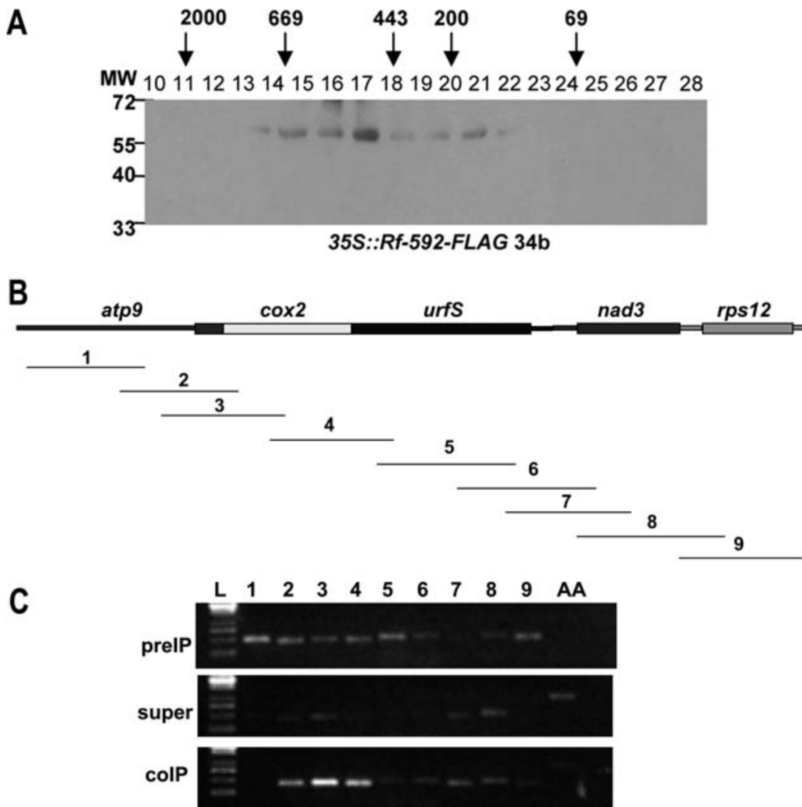

Fig. 6.7 (**A**) Immunoblot analysis of RF592-FLAG protein present following gel filtration of mitochondrial protein. Total mitochondrial protein was separated by size on a column, and fractions examined by immunoblots probed with an anti-FLAG antibody. Fractions 10–28 were analyzed (0–9 consisted of column void volume). The elution peak of each gel filtration standard is indicated by an *arrow*. MW size standards are indicated in *kDa*. (**B**) Diagram of the transcribed regions of the *pcf* locus, with coding (*larger rectangles*) and non-coding regions (*thinner rectangles*) shown. The regions amplified by each of nine different RT-PCR primers are shown as *black lines*. AA=RT-PCR using a primer pair specific to the *atpA* gene (*a control*). (**C**) Scanning RT-PCR analysis of *pcf* RNA found in the immunoprecipitate fraction following treatment of mitochondrial protein from *35S::Rf592-FLAG* plant with anti-FLAG antibody. L lane contains a 1 kb molecular ladder. (Modified from Gillman et al. 2007)

mitochondria. The RF592 complex fractionates with a predicted size between 400 and 600 kDa, in contrast to the ~60 kDa molecular weight of mature RF592 protein (Fig. 6.7). The mobility of the complex is sensitive to RNase treatment and shows co-fractionation with *pcf* RNA. RNA encoded by *pcf* is immunoprecipitated by anti-FLAG antibodies. The region of the *pcf* transcript that is most abundant in the immunoprecipitate is the 5′ untranslated region of *atp9* and/or the *atp9* portion of the chimeric *pcf* open reading frame (Gillman et al. 2007). It is likely that the complex interacts directly with this region of the mitochondrial transcripts. As this

region is likely to be critical for translation, current data are consistent with the hypothesis that RF592 acts by affecting the synthesis of PCF protein.

Although transcripts of *pcf* are also affected in restored lines, it is possible that a block in translation results in susceptibility of transcripts to degradation. Yeast mutants blocked in translation of mitochondrial transcripts have been reported to exhibit reduced amounts of the affected transcripts (Poutre and Fox 1987; Fox et al. 1988).

6.7 Conclusion

The work reviewed in this chapter makes it evident that there has been substantial progress in understanding the molecular basis of CMS and fertility restoration in Petunia. Maps of the mitochondrial genomes of a CMS and normal fertile cytoplasm illustrate the massive reorganization in mitochondrial genomes that can occur over a short evolutionary time scale. With the advent of rapid and inexpensive large-scale sequencing technology, an increasing number of mitochondrial genomes are becoming available. Sequencing of the mapped Petunia genomes as well as additional species should provide more insight into the recombinational processes that reorganize mitochondrial genomes.

The anatomical aspects of the onset and progression of the degeneration of tapetal and sporogenous tissues in CMS lines have been carefully examined and described by both light and electron microscopy. Future studies may take advantage of new fluorescent dyes, fluorescent protein labeling, and advances in fluorescence microscopy. Numerous biochemical differences between male-sterile and male-fertile anthers have been discerned, but the specific defect that leads to tapetal and sporogenous tissue degeneration remains a question for future investigation.

Petunia is one of only a handful of species in which both the CMS-associated and fertility restoration genes have been identified. Like those of many other known CMS-associated genes, *pcf* in Petunia is a chimeric fusion of a mitochondria promoter and portions of the coding region of known mitochondrial genes to an open reading frame of unknown origin. Though no sequence homologous to the unknown open reading frame has yet been found in any other organism, perhaps the source of this sequence will be revealed as the DNA database continues to grow. The PCF gene product undergoes processing to form a 19.5 kDa protein, which accumulates to a high degree in anthers of CMS lines, although it is also present in vegetative tissue. Another question for the future, also a puzzle in other CMS/restorer systems, is why reproductive development is more seriously affected by the CMS-associated protein than vegetative development.

The Petunia fertility restorer is a nuclear-encoded protein that profoundly affects the expression of a mitochondrial gene, thus serving as a model for understanding how the nucleus may regulate mitochondrial activities. The Petunia CMS-associated protein is reduced to near-undetectable levels in all tissues tested in fertility-restored lines. The identification of the Petunia fertility restorer as a pentatricopeptide repeat

(PPR) gene family member has resulted in interest in PPR-motif-containing proteins in other CMS/restorer systems as candidate restorer genes, as many important restorer genes remain to be cloned. PPR motif-containing proteins have been implicated in several different aspects of the control of both mitochondrial and chloroplast gene expression by the nucleus, including RNA processing, stability, and translation, all of which could result in the decreased accumulation of the PCF protein seen in Petunia restored lines. The fertility restorer has been shown to be part of a large multiple-protein, RNase-sensitive complex that is associated with *pcf* transcripts. The next question is whether the Petunia restorer controls *pcf* expression by affecting RNA metabolism or translation. With the genetic materials and information available for the Petunia CMS/restorer system, Petunia-based research is likely to lead the way in explaining the complex process of nuclear-mitochondrial coordination.

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Chapter 7
Development and Function of the Arbuscular Mycorrhizal Symbiosis in Petunia

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Abstract The majority of terrestrial plants live in symbiotic associations with fungi or bacteria that improve their nutrition. Critical steps in such a symbiosis are mutual recognition and subsequent establishment of an intimate association that involves the penetration of plant tissues and, in many cases, the invasion of individual host cells by the microbial symbiont. The most widespread symbiosis of plants is the arbuscular mycorrhizal (AM) symbiosis, which can improve plant nutrition and stress resistance. The AM symbiosis is controlled by intrinsic factors such as SYM symbiosis genes, and extrinsic factors such as nutrients. Important experimental systems in symbiosis research are legumes (*Medicago truncatula* and *Lotus japonicus*) and grasses (rice and maize), but Solanaceae are also catching up. In this chapter, we summarize recent advances in AM research on Petunia, which complement ongoing efforts in the AM research community.

7.1 Introduction

Plants live in association with numerous microorganisms that affect their growth in various ways. The rhizosphere, the soil material in close proximity to the root, is particularly rich in diverse microorganisms, mostly bacteria and fungi. They utilize root exudates, a complex mixture of substances including carbohydrates, organic acids and nitrogenous compounds that are continuously released by roots. Many rhizospheric microbes do not appear to affect plant growth; they are neutral. Others can cause considerable damage and are classified as pathogens. Certain microbes, however, are beneficial, promoting plant growth through improved plant nutrition or increased resistance to pathogenic microbes and abiotic stress. In a few cases, which are referred to as symbioses, the interaction between the beneficial microbe

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and the plant has evolved into an intimate association with benefits for both partners. The most prevalent symbiotic interactions involve plant roots and fungi, thus these symbioses are referred to as mycorrhiza (Greek for fungus-root). There are chiefly two types of mycorrhizal associations: ectomycorrhiza (EM), in which the fungi penetrate the root but remain between the cells, and arbuscular mycorrhiza (AM), in which the fungus invades individual cells of the root cortex and forms specialized intracellular structures, called arbuscules, for nutrient exchange (Peterson, Massicotte, and Melville 2004). Since Petunia forms only AM we focus on this symbiosis.

The AM association is found in a wide range of land plants and is probably the most widespread (Brachmann and Parniske 2006) and oldest symbiotic association of plants in general (Kistner and Parniske 2002; Redecker 2002). There are approximately 150–200 described AM fungal species, which together form the order *Glomeromycota*, a sister order of the *Ascomycota* and the *Basidiomycota* (Schüssler, Schwarzott, and Walker 2001), to which the EM fungi belong. On the contrary, the majority of land plants (>100,000 species) form AM associations. The interaction is largely nonspecific under laboratory conditions; however, under natural conditions, a certain level of selectivity and functional specialization may occur (Smith and Read 1997). Although the arbuscules gave the association its name, it should be noted that, paradoxically, arbuscules are not formed in all AM interactions (Smith and Read 1997). The *Arum*-type AM, which is characterized by the formation of intracellular arbuscules, is found in many cultivated species, including Petunia, whereas in the *Paris*-type AM, which is observed in many wild species, the fungus forms intracellular hyphal coils instead of arbuscules (Smith and Read 1997). These hyphal coils may develop fine branches and are then referred to as arbusculate coils (Peterson et al. 2004). AM and EM have little in common besides the fact that they both involve fungi. In contrast, recent genetic studies have revealed an unexpected overlap between AM and the root nodule symbiosis (RNS), formed between legumes and rhizobia (Parniske 2000; see below).

The AM symbiosis is ancient and may have facilitated the colonization of land by plants. The first fossil records of mycorrhizal plants are dated to be at least 400 million years old (Remy, Taylor, Hass, and Kerp 1994), which coincides approximately with the first fossils of land plants, dating to 475 million years ago (Wellman, Osterloff, and Mohiuddin 2003). The AM symbiosis is commonly found in all major land plant taxa, consistent with an origin dating back to the root of the different higher plant lineages (Kistner and Parniske 2002; Paszkowski 2006). Certain plant families have secondarily lost the capacity to form AM, and the distribution of these non-mycorrhizal plants in different phylogenetic clades suggests a polyphyletic origin (Brundrett 2002); non-mycorrhizal species have not been reported in the Solanaceae.

Based on the relatively recent emergence of the RNS, which evolved only about 65 million years ago, it has been hypothesized that rhizobia found a new key to open the old gate to the symbiotic gardens: they acquired the ability to generate a signal that triggered the same symbiotic pathway that had been used by AM fungi for more than 300 million years (Kistner and Parniske 2002).

In AM symbiosis the plant provides essential carbohydrates to the obligate symbiotic fungus in exchange for mineral nutrients, in particular phosphate (P_i, Bucher 2007; Javot, Pumplin, and Harrison 2007a) and nitrogen (N, Govindarajulu et al. 2005; Chalot, Blaudez, and Brun 2006), but other nutrients have also been reported to be delivered to the plant host by AM fungi (Clark and Zeto 2000; George 2000). Phosphorus and nitrogen are essential elements that are required in large amounts due to their central role as structural components of nucleic acids, proteins and lipids, and their ubiquitous occurrence in primary and secondary metabolism. In many soils, plant growth is limited by the availability of P and N.

Notably, mineral nutrients play a role in AM not only as substrates of transport but also as regulators of the interaction. In general, low nutrient levels promote AM development, whereas high levels limit the interaction. This effect has been understood as an active mechanism of the plant, allowing it to minimize the drain of carbohydrates to the fungus under conditions in which the fungus does not confer a benefit because the plant is optimally supplied with nutrients.

Traditional physiological studies on AM were carried out in various plant species including monocot and dicot crops as well as wild species (Smith and Read 1997); however, the most important emerging model systems for recent genetic studies of AM have been legume species (Peterson and Guinel 2000; Marsh and Schultze 2001; Udvardi, Tabata, Parniske, and Stougaard 2005). Recently, rice and maize, as representatives of the monocots, became important model species for AM research (Güimil et al. 2005; Paszkowski, Jakovleva, and Boller 2006). In addition, tomato and potato have been employed for molecular-genetic research on AM (Barker et al. 1998; Rausch et al. 2001; David-Schwartz et al. 2001, 2003; Nagy et al. 2005; Chen, Hu, Sun, and Xu 2007), but both species have their limitations: potato genetics is limited due to polyploidy and tomato has poorly developed reverse genetics tools. However, the Solanaceae include many important crops (tomato, potato, tobacco, pepper, eggplant), all of which can be colonized by AM fungi. It was therefore important to establish a solanaceous model with strong tools for forward and reverse genetics. Here we describe how Petunia has recently contributed to the study of the function of phosphate transporters and other genes with a suspected role in AM development or function. Furthermore, the use of Petunia as a convenient model for forward genetics to isolate mutants with defects in AM development is discussed.

7.2 Development of AM Symbiosis

7.2.1 Early Stages

AM fungi are obligate biotrophs. They can persist for years in the soil as spores, but after germination their hyphae exhibit only limited presymbiotic growth in the soil; for reproduction and long-term survival they need a plant host. Before the establishment of the symbiotic interaction, fungal hyphae emanating from germinated spores

or from neighboring roots grow through the soil until they perceive a constitutively released plant signal, strigolactone (Akiyama, Matsuzaki, and Hayashi 2005), which induces hyphal branching (Buée, Rossignol, Jauneau, Ranjeva, and Bécard 2000) and metabolic activity of AM fungi (Tamasloukht et al. 2003; Besserer et al. 2006). Strigolactone thereby promotes fungal growth and increases the chance of a physical contact with a root. In addition, AM fungal hyphae grow chemotropically toward roots (Sbrana and Giovannetti 2005), an effect that could be related to strigolactone gradients in the rhizosphere.

Once an AM fungus has reached a host root, it first colonizes the root surface and then forms appressoria (Garriock, Peterson, and Ackerley 1989). These infection structures can be triggered by physical contact with isolated cell walls of epidermal root cells in the absence of additional diffusible signals (Nagahashi and Douds 1997). During appressorium formation, but preceding the first signs of penetration, the underlying epidermal cell responds with a striking program of cellular reorganization (Genre, Chabaud, Timmers, Bonfante, and Barker 2005). First, the nucleus rapidly migrates to a position just below the appressorium, then it moves away, leaving behind an aggregation of microtubules, actin microfilaments, and ER cisternae which become organized into a finger-shaped structure, the prepenetration apparatus (PPA), which projects into the cell lumen. The PPA defines a trajectory through the cell that precisely presages the path of the invading fungal hypha (Fig. 7.1).

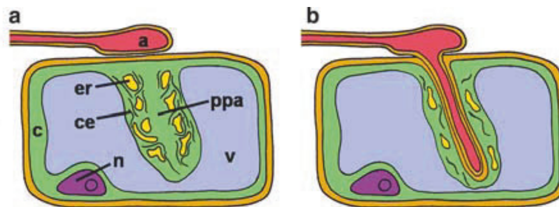

Fig. 7.1 Early stages of infection by AM fungi. (**A**) Formation of the pre-penetration apparatus (ppa) at the site of appressorium formation by the fungus. (**B**) Infection hypha follows the path traced by the ppa; **a**, appressorium; **c**, cytoplasm; **ce**, cytoskeletal elements; **er**, endoplasmatic reticulum; **n**, nucleus; **v**, vacuole; **ppa**, pre-penetration apparatus. Reproduced with permission from D. Reinhardt, Programming good relations – development of the arbuscular mycorrhizal symbiosis, Curr. Opin. Plant Biol. 10, 98–105, ©Elsevier 2007

7.2.2 Later Stages

From the first colonized cell, the hyphae grow inward either between cells of the hypodermis and the outer cortex, as in *Arum*-type associations, or intracellularly, as in some *Paris*-type associations. Within a few days of initial penetration, the fungus forms the first arbuscules or hyphal coils in cortical cells. Thereafter, intercellular hyphae are formed, promoting longitudinal expansion of the infected area. From established infections, hyphal networks emanate from the root to explore the soil

for nutrients and to infect new roots in the vicinity. Ultimately a root system can be colonized over nearly the entire root length. However, the plant has control over the fungus, limiting fungal growth to tissues outside of the endodermis (thus protecting the vasculature from invasion), and meristematic tissues at the root tip remain fungus-free. Furthermore, the plant can limit fungal proliferation when it is already colonized (Catford et al. 2003) or if it is well supplied with nutrients, a condition under which the fungus would consume carbohydrates without returning a nutritional benefit to the plant.

7.2.3 The Arbuscule: "Pièce de Résistance" of the AM Interaction

The AM fungi have invented a specialized intracellular structure, the arbuscule, for nutrient exchange with the plant. It consists of highly ramified hyphae with very fine terminal tips (Fig. 7.2a), resulting in an increased surface-to-volume ratio compared to normal hyphae (Dickson and Kolesik 1999), a feature that makes them particularly efficient for nutrient transfer. If a control cortex cell with its apparently "empty" space, filled largely by the vacuole (indeed, *vacuum* is the latin word for emptiness), is compared with an arbuscule-containing cell, the prominent increase in cytoplasmic content, organelle number, and many small vacuoles that surround the numerous fine hyphae can be easily appreciated (Fig. 7.2b).

Root cortex cells respond with a range of cellular and transcriptional adaptations during the development of an arbuscule. The nucleus becomes enlarged, a sign of active transcription, and migrates to a central position enveloped by fine branches

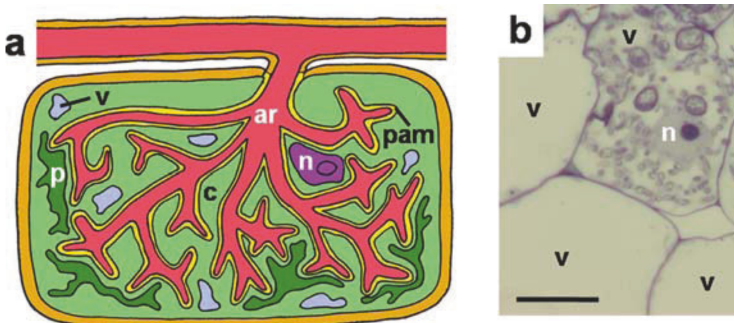

Fig. 7.2 The arbuscule: site of nutrient exchange. (**A**) Arbuscules fill most of the cell volume. They are surrounded by the periarbuscular membrane of the plant. The nucleus is embedded between the hyphae and plastids are associated with the fungus. (**B**) A semi-thin section through a Petunia root showing a colonized cell (*upper right*) and three non-colonized cells (*left and lower*). Note the large central vacuole in non-colonized cells and numerous small vacuoles in the colonized cell. **ar**, arbuscule; **c**, cytoplasm; **n**, nucleus; **p**, plastids; pam, periarbuscular membrane; **v**, vacuole. Scale bar: 25 μm. Partially reproduced with permission from D. Reinhardt, Programming good relations – development of the arbuscular mycorrhizal symbiosis, Curr. Opin. Plant Biol. 10, 98–105, ©Elsevier 2007

of the arbuscule or arbusculate coil (Bonfante-Fasolo 1984; Cavagnaro, Smith, Kolesik, Ayling, and Smith 2001). The large central vacuole is fragmented into many small vacuoles. Although the arbuscules are intracellular, they remain surrounded by a plant membrane, the periarbuscular membrane (PAM), which is continuous with the plasmalemma and lines even the finest hyphal branches (Fig. 7.2a). On the cytoplasmic side of the PAM the cytoskeleton of the host cell establishes a network around the arbuscular branches (Timonen and Peterson 2002). Ultimately, arbuscule development culminates in the establishment of a symbiotic interface comprising the PAM, the fungal membrane, and the interfacial matrix between them (Bonfante and Perotto 1995). It is at this site that nutrients and possibly signals are exchanged (Karandashov and Bucher 2005; Genre and Bonfante 2005).

Cells that are inhabited by arbuscules develop an elaborate machinery for active nutrient transfer. They produce phosphate transporters (PTs) that reside in the PAM and are likely to take up the phosphate delivered by the arbuscule (Rausch et al. 2001; Harrison, Dewbre, and Liu 2002). The PAM and the fungal membrane of the arbuscule contain H^+-ATPases for the generation of an electrochemical gradient required to energize nutrient transport (Ferrol, Gianinazzi, and Gianinazzi-Pearson 2002). Indeed, arbuscules are surrounded by an acidic environment (Guttenberger 2000), consistent with a role for H^+-ATPases in active nutrient transfer.

7.2.4 Fungal and Plant Signals Coordinate Development

The concerted cooperation between plant and fungus implies specific signals to coordinate cellular processes in both partners (Reinhardt 2007). The first word in the molecular dialogue appears to be the strigolactone from the plant (see above; Akiyama et al. 2005). A local fungal signal then informs the epidermal host cell about the exact position of the appressorium (Genre et al. 2005). A diffusible symbiosis signal from the fungus, a hypothetical analog to the nod factor from rhizobia (hence referred to as myc factor), triggers the expression of certain symbiosis-related genes in the plant (Kosuta et al. 2003), and presumably activates the symbiotic program (common SYM pathway; see below). A cell-autonomous signal triggers the induction of PTs and other genes in arbuscule-containing cells (Rausch et al. 2001; Harrison et al. 2002; Liu et al. 2003; Karandashov, Nagy, Wegmüller, Amrhein, and Bucher 2004; Drissner et al. 2007), whereas a short-range diffusible signal appears to induce gene expression in limited groups of cells in the root cortex (Liu et al. 2003).

7.3 Molecular-Genetic Regulation of AM Symbiosis

Due to their biotrophic life-style, AM fungi cannot be propagated in sterile culture. In recent years, however, so-called monoxenic cultures have been established which allow for the in vitro cultivation of mycorrhizal fungi on transformed hairy roots

under aseptic conditions (Fortin et al. 2002; Declerck, Strullu, and Fortin 2005). On the plant side, genetic studies have been hampered by the tedious screening procedures (unlike nodules, AM cannot be seen by the naked eye – they must be stained for visualization), and by the lack of an obvious strong effect of the AM on growth, which could be used for genetic screens. In contrast, both partners in the root nodule symbiosis (RNS), legumes and rhizobia, can be cultured in sterile culture, are amenable to genetic manipulation, produce easily visible symbiotic organs (the nodules), and show obvious nutritional effects under N-starvation. For these reasons, genetic studies of AM have long lagged behind RNS research. However, mutant analysis of the past decade has revealed unexpected overlaps in the regulation of these seemingly disparate symbiotic systems.

Large numbers of mutants with defects in RNS (*nod*$^-$ mutants) were collected in legume model species such as pea, alfalfa, bean, and more recently from *Lotus japonicus* and *Medicago truncatula* (Peterson and Guinel 2000; Marsh and Schultze 2001), which during the past decade became the best-developed models for endosymbiotic interactions (Udvardi et al. 2005). The *nod*$^-$ mutants were tested for defects in the AM interaction, and it turned out that approximately 50% were also *myc*$^-$ (Duc, Trouvelot, Gianinazzi-Pearson, and Gianinazzi 1989). This indicated a common regulatory pathway for the two symbiotic interactions (Gianinazzi-Pearson and Dénarié 1997). Accordingly, the mutants were called common *sym* mutants (the affected genes are the common *SYM* genes), and they define the common SYM pathway (Parniske 2004). Since the AM interaction is much older than RNS (>450 My versus <80 My), it appears that the more recent RNS evolved by incorporating components of the AM regulatory pathway (Kistner and Parniske 2002). However, the fundamental differences between AM and RNS, such as the nature of the microsymbiont, the cytology and anatomy of the respective structures, and the nutrients involved, suggest that upstream and downstream of the common SYM pathway specific components must have evolved for the two symbioses.

7.3.1 The Common SYM Pathway

The common SYM genes encode putative signaling components which suggest a signaling cascade involving a nuclear calcium signal at its center (see Fig. 7.3, Parniske 2004; Oldroyd and Downie 2006). The common SYM pathway involves at least four genetically defined signaling steps:

1) A symbiosis receptor kinase (SYMRK in *Lotus*, NORK in pea; DMI2 in *Medicago*) is thought to integrate a symbiotic signal released directly or indirectly upon contact with rhizobia or AM fungi (Endre et al. 2002; Stracke et al. 2002).
2) Predicted membrane proteins (CASTOR and POLLUX in *Lotus*, DMI1 in *Medicago*) with homology to a bacterial potassium channel may be involved in generating ion fluxes (Ané et al. 2004; Imaizumi-Anraku et al. 2005). CASTOR and POLLUX GFP-fusion proteins were initially shown to be localized to the

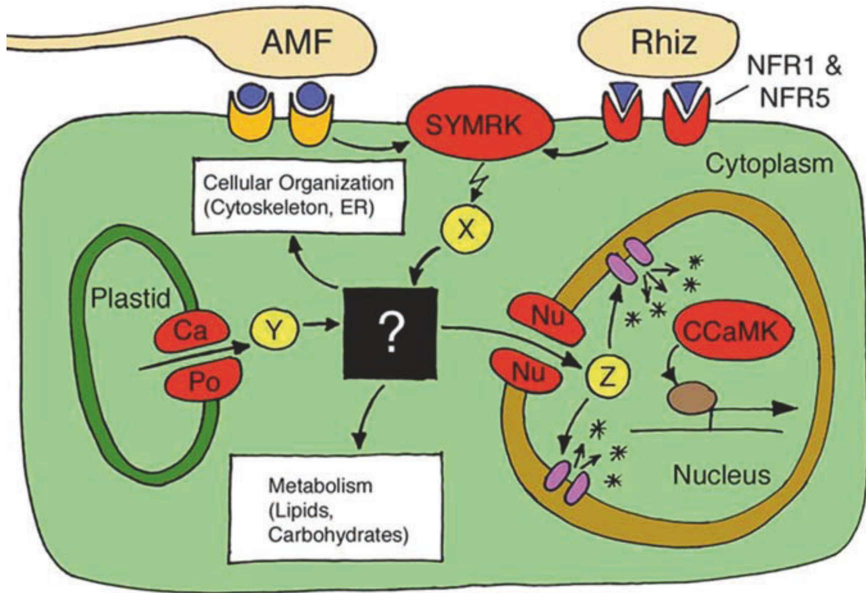

Fig. 7.3 A model of early signaling in symbiosis. Rhizobia produce nod factors (*triangles*) that are perceived by a receptor complex involving NFR1 and NFR5. An analogous pathway is predicted for the perception of hypothetical myc factors released by AM fungi (*circles*). Both signals are integrated by SYMRK and transduced via phosphorylation of an unknown substrate (**X**). Symbiotic signaling also requires a putative plastidic ion channel consisting of Castor (**Ca**) and Pollux (**Po**), which may release a plastidic factor (**Y**) required for signal transduction. Ultimately, a second messenger (**Z**) is translocated to the nucleus in a NUP133/NUP85-dependent manner (**Nu**), where it triggers calcium channels to release calcium (**stars**) from the nuclear envelope (**calcium spiking**). The calcium signal activates a calcium- and calmodulin-dependent protein kinase (CCaMK), which induces the transcription of symbiosis genes. Symbiotic signaling also leads to changes in cellular organization and metabolism. How the events at the plasmalemma, the plastid, and the nucleus are connected is unknown (*Black box*). Reproduced with permission from D. Reinhardt, Programming good relations – development of the arbuscular mycorrhizal symbiosis, Curr. Opin. Plant Biol. 10, 98–105, ©Elsevier 2007

plastids (Imaizumi-Anraku et al. 2005), whereas the *Medicago* homolog DMI1 was recently localized to the nuclear envelope (Riély, Lougnon, Ané, and Cook 2007). Whether the functions of CASTOR, POLLUX, and DMI1 are indeed associated with different subcellular compartments remains to be clarified.

3) A calcium- and calmodulin-dependent protein kinase (CCaMK in *Lotus*, DMI3 in *Medicago*) is a likely candidate for the integration of a nuclear calcium signal (Lévy et al. 2004; Mitra et al. 2004; see below). Constitutive activation by mutation of an autoinhibitory domain of CCaMK is sufficient to trigger the formation of empty nodules in the absence of rhizobia or nod factor (Gleason et al. 2006; Tirichine et al. 2006).

4) Two proteins with homology to nucleoporins, NUP133 (Kanamori et al. 2006) and NUP85 (Saito et al. 2007), may form a specific nuclear pore complex, required for symbiotic signal transduction.

A central component of the common SYM pathway is calcium. In RNS, the perception of the symbiotic signal (the nod factor) in root hairs induces a rhythmic cellular calcium oscillation (calcium spiking) within minutes (Ehrhardt, Wais, and Long 1996). The calcium spiking response is absent in plants mutated in either *SYMRK/NORK/DMI2* or *CASTOR/POLLUX/DMI1* genes, suggesting that the products act upstream of calcium spiking. In contrast, calcium spiking is not affected in *ccamk/dmi3* mutants, indicating that their gene products function downstream of the calcium spiking and may be involved in deciphering the calcium signal (Oldroyd and Downie 2006).

Homologs of the common *SYM* genes have been found in plant species outside the legumes among monocots (Paszkowski et al. 2006; Zhu, Riely, Burns, and Ané 2006) and dicots (Sekhara Reddy, Schorderet, Feller, and Reinhardt 2007; TIGR EST database), suggesting that the common SYM pathway could be conserved among all species that are able to form AM. This observation is in agreement with the hypothesis that the common SYM pathway represents the legume version of an ancient AM regulatory pathway.

7.3.2 Specificity in Symbiotic Signaling

The discovery of the common SYM pathway raised the question of how specificity is mediated in symbiosis signaling of plants. It appears that in a legume both the nod factor as well as the hypothetical myc factor (or a secondary signal) have to be transduced by the SYMRK. However, downstream events are very different in AM and RNS. So, when the calcium signal eventually reaches the nucleus, how does the cell know whether it should trigger the nodulation or the mycorrhizal pathway? In theory, there are two possible means of achieving specificity: (i) the calcium signal is nonspecific and requires an additional parallel signal, or (ii) the calcium signal itself carries specific information that could, for instance, be encrypted in the signature of the calcium spiking response (Harper, Breton, and Harmon 2004). Indeed, the calcium signal triggered by AM fungi is different from the rhythmic calcium spiking induced by the nod factor or rhizobia, arguing for the second possibility (Kosuta et al. 2008).

In contrast, some early responses of roots to AM fungi, e.g., *ENOD11* induction (Kosuta et al. 2003) and epidermal opening (Demchenko, Winzer, Stougaard, Parniske, and Pawlowski 2004), appear to be independent of the common SYM pathway, thus implicating parallel pathways in symbiotic signaling. It may therefore be that symbiotic signaling involves a combination of a specific calcium signal with specific parallel signaling pathways.

7.3.3 Analysis of Gene Expression in AM

Apart from the effects of AM fungi at the cytological level (PPA, PAM, cytoskeleton, etc.), the symbiotic program ultimately results in the activation of genes that are responsible for the progression of symbiotic development and for the function of the symbiosis. Transcript profiling has revealed major shifts in plant gene expression during AM symbiosis (Liu et al. 2003; Brechenmacher et al. 2004; Grunwald et al. 2004; Hohnjec, Vieweg, Puhler, Becker, and Kuster 2005; Frenzel et al. 2005; Kistner et al. 2005; Güimil et al. 2005). Some of the affected genes have functions in nutrient transport (Bucher 2007; Javot et al. 2007a); others may be involved in reorganization of the cytoskeleton (Timonen and Peterson 2002) or in cell wall modification (Balestrini and Bonfante 2005). A number of induced genes, among them genes coding for PTs and for putative cell wall remodeling proteins, are expressed exclusively or primarily in the cells that contain arbuscules (Balestrini et al. 1999; van Buuren, Maldonado-Mendoza, Trieu, Blaylock, and Harrison 1999; Gianinazzi-Pearson, Arnould, Oufattole, Arango, and Gianinazzi 2000; Rausch et al. 2001; Journet et al. 2001; Harrison et al. 2002; Liu et al. 2003) or branched and coiled hyphae (Karandashov et al. 2004; Nagy et al. 2005).

The early stages of AM fungal colonization are generally accompanied with a transient induction of genes encoding pathogenesis-related (PR) proteins and other markers of defense (Dumas-Gaudot, Gollotte, Cordier, Gianinazzi, and Gianinazzi-Pearson 2000) (García-Garrido and Ocampo 2002). In a conceivable scenario, this phenomenon may represent a first reaction of the plant to unspecific microbial signals (elicitors) from the AM fungus, before recognition of the myc factor triggers the switch to the symbiotic program, resulting in suppression of the defense response.

Recently, large-scale sequencing efforts have been undertaken to characterize the transcriptome of mycorrhizal Petunia roots. A normalized root library and two subtractive libraries (mycorrhizal minus control and P-treated minus control) were constructed and approximately 20,000 EST sequences were generated from this material. Sequences of many genes with a known or assumed role in AM have been identified, e.g., mycorrhiza-specific PTs and the common *SYM* genes *PhSYMRK* and *PhCASTOR*. This offers the possibility of testing the functions of these genes in AM of Petunia, and determining whether the homologs of common SYM genes are functional orthologs (see below).

7.3.4 Non-legume Species in AM Research

From the above considerations, it follows that genetic analysis has to be intensified in order to identify the specific regulatory components upstream and downstream of the common SYM pathway. In the case of RNS, the likely candidates for the nod factor receptor, which acts upstream of the common SYM pathway, have been cloned (Radutoiu et al. 2003; Madsen et al. 2003). Three putative transcription factors have been identified – NIN, NSP1, and NSP2 – that control downstream

events in the development of nodules (Schauser, Roussis, Stiller, and Stougaard 1999; Kalo et al. 2005; Smit et al. 2005). Although many mycorrhiza-specific steps have been characterized at the microscopic and physiological levels, little is known about the genetic control of AM apart from involvement of the common SYM pathway.

Despite the difficulty of screening (see above), a few mutants with a myc$^-$ phenotype have, in recent years, been identified in non-legume species. These studies were carried out in tomato (Barker et al. 1998; David-Schwartz et al. 2001, 2003) and maize (Paszkowski et al. 2006). The latter study took advantage of the fact that mycorrhizal maize roots accumulate a pigment that allows for screening without a staining step. This led to the identification of seven mutants that fall into three phenotypic classes: *no perception* (*nope*; no interaction), *taciturn* (*taci*; reduced colonization), and *precocious arbuscular mycorrhiza* (*pram*; more intense and faster colonization). Among the three tomato mutants so far described, two belong to the *pre-mycorrrhizal interaction* (*pmi*) class due to their defects at early stages of the interaction (David-Schwartz et al. 2001, 2003), while the third was called *reduced mycorrhizal colonization* (*rmc*) (Barker et al. 1998), because it was generally less colonized, like most of the common *sym* mutants.

In a genetic screen for AM-defective mutants in *Petunia hybrida*, a number of mutants with defects at various steps of the AM interaction, from early penetration into epidermal cells to arbuscule development in the root cortex, were identified (Sekhara Reddy 2007). One of them has a particularly interesting phenotype, as it is affected strongly at the level of arbuscule development (see below).

7.3.5 A Petunia Mutant Affected in Arbuscule Development

Screening of *P. hybrida* line W138 for mutants affected in the development of AM symbiosis yielded the mutant *penetration and arbuscule morphogenesis1* (*pam1*) (Sekhara Reddy et al. 2007). The *pam1* mutant exhibits defects in penetration of the epidermis and invasion of the root cortex (Sekhara Reddy et al. 2007), resulting in the early abortion of many entering hyphae and a strong decrease in general root colonization, just as in most legume mutants and in *rmc* of tomato (Barker et al. 1998). In cases where the fungus was able to invade the cortex, it colonized the root with a profuse intercellular hyphal network, but arbuscule formation was blocked. Instead, many short intracellular hyphae were formed as lateral protrusions of the intercellular hyphae (Fig. 7.4). The fact that intracellular hyphal structures were found, in both epidermal and cortical cells, suggests that the defect of *pam1* is not in the process of cellular penetration *per se*, but rather in an accommodation program that allows the fungus to form appropriate intracellular structures, i.e., coils in the epidermis and arbuscules in cortical cells. Weak mutant alleles of common *SYM* genes have also been shown to display dual defects, at the stages of root penetration and arbuscule formation (Novero et al. 2002; Demchenko et al. 2004); in these cases, however, the formation of lateral branches of intercellular hyphae in the cortex were not reported. Indeed, the *SYM* gene homologs *PhSYMRK*, *PhCASTOR*, and *PhDMI3*

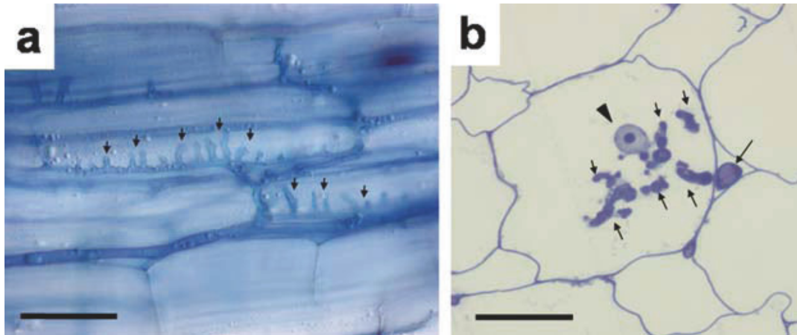

Fig. 7.4 Phenotype of the *pam1* mutant in Petunia. (**A**) *Glomus intraradices* grows along the root between cortical cells (horizontal hyphae) and forms lateral appendages instead of arbuscules (*arrow heads*). (**B**) An intercellular hypha (*long arrow*) has formed intracellular lateral protrusions (*small arrows*) instead of an arbuscule (compare with Fig. 7.2B). The nucleus (*arrowhead*) is associated with the hyphae but is not as enlarged as in a cell with an arbuscule (see Fig. 7.2B). Scale bars: 50 μm in (**A**), 25 μm in (**B**). Reproduced with permission from Sekhara Reddy et al., A petunia mutant affected in intracellular accommodation and morphogenesis of arbuscular mycorrhizal fungi, Plant J., ©Blackwell Publishing 2007

did not appear to be mutated in the *pam1* mutant (Sekhara Reddy et al. 2007). Taken together, these results show that the plant plays an active role in morphogenesis of the intracellular fungal structures, the hyphal coils and the arbuscules.

7.4 The Role of Nutrients in AM Symbiosis

The best-studied nutrient element with a role in AM is phosphorus (Bucher 2007; Javot et al. 2007a). Phosphorus is absorbed from the soil solution mainly in the form of orthophosphate (P_i). Although P_i is common in most soils, the majority of it is firmly bound in organic or mineral form, resulting in very low concentrations of P_i available for plant uptake, usually 1–10 μM (Bieleski 1973). This is >1000-fold lower than the free P_i concentration inside the cells. Consequently, transport of P_i into the root occurs against a steep concentration gradient and, in addition, against a negative membrane potential. P_i transport is therefore an energy-requiring mechanism mediated by two components of the plant cell membrane: (1) P-type H^+-ATPase, which creates a proton gradient across the membrane, and (2) P_i transporters (PTs), i.e., membrane proteins that couple the transport of protons across the membrane with a selective symport of P_i (Raghothama and Karthikeyan 2005).

7.4.1 Specific Phosphate Transporters Mediate Phosphate Uptake

A major benefit of AM to plants is improved P_i nutrition. P_i is taken up into root cells by specific PTs, which have been extensively studied during the last decade.

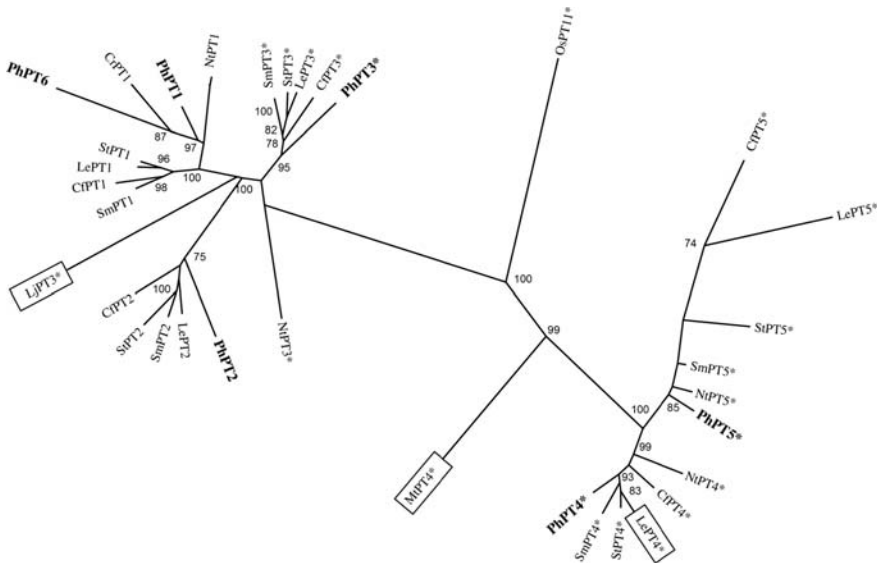

Fig. 7.5 Phylogenetic relationships of mycorrhiza-related phosphate transporters. Protein distance phylogeny between phosphate transporters of the Pht1 family isolated in Solanaceae and the recently identified Petunia members (Wegmüller et al. 2008). Some legume and rice members are included for reference. Genes with enhanced expression levels in mycorrhizal roots (*asterisks*) belong to two phylogenetically distant families. The *Medicago*, *Lotus*, and tomato genes that were tested functionally by knockdown/knockout (Nagy et al. 2005; Maeda et al. 2006; Javot et al. 2007b) are boxed. The suffices represent the following species: **Cf**: Capsicum frutescens; **Cr**: Catharanthus roseus; **Le**: Lycopersicon esculentum; **Lj**: Lotus japonicus; **Mt**: Medicago truncatula; **Nt**: Nicotiana tabacum; **Os**: Oryza sativa; **Ph**: Petunia hybrida; **Sm**: Solanum melongena; **St**: Solanum tuberosum

These P_i transport proteins contain 12 membrane-spanning domains and belong to the Major Facilitator Super-Family (MFS) of transporters, like sugar, antibiotic, ion, and amino acid transporters (Pao, Paulsen, and Saier 1998). In plants they belong to the Pht1 family, comprised of several subfamilies (Fig. 7.5; Bucher, Rausch, and Daram 2001; Mudge, Rae, Diatloff, and Smith 2002). Since their discovery in Arabidopsis and potato (Muchhal, Pardo, and Gaghothama 1996; Leggewie, Willmitzer, and Riesmeier 1997; Smith, Ealing, Dong, and Delhaize 1997), a large number of *Pht1* genes have been identified and characterized, mainly from Arabidopsis, cereals, and solanaceous species (Karandashov and Bucher 2005; Javot et al. 2007a). For several of these, complementation of yeast mutants has proved the ability of the corresponding proteins to mediate P_i transport (Muchhal et al. 1996; Leggewie et al. 1997; Daram, Brunner, Persson, Amrhein, and Bucher 1998; Rausch et al. 2001; Harrison et al. 2002; Nagy et al. 2005).

Recently, a unified nomenclature for P_i transporters has been established (Bucher et al. 2001; Mudge et al. 2002). The family is called Pht1, with the addition of a five-letter prefix for the species and an extension for the individual family members. For

example, the *Petunia PT3* is therefore referred to as *PEThy;Pht1;3*. For simplicity, we abbreviate the genes in the text and in Fig. 7.5 as follows: *PhPT3* (for *Petunia hybrida* P_i transporter 3). When a group of orthologous genes from different plant species is discussed it is referred to as the PT3 group.

7.4.2 The Mycorrhizal P_i-Uptake Pathway

In nature, plants such as Arabidopsis that absorb P_i mainly through epidermal cells via their root hairs (referred to as the "direct P_i-uptake pathway") are an exception. Most plants are associated with AM fungi, and in this case, the contribution of the so-called "mycorrhizal uptake pathway" can account for up to 100% of the total plant P uptake (Smith, Smith, and Jakobsen 2003). The mycorrhizal contribution is important in most natural soils, where P_i availability is usually so low that a P_i depletion zone is formed around the root system. In mycorrhizal roots, P_i is first absorbed from the soil by fungal P_i transporters situated in the extraradical hyphae, and then transported through the hyphae to the roots, probably in the form of polyphosphate. In the intraradical hyphal network the polyphosphate is depolymerized (Ezawa, Smith, and Smith 2002) and finally delivered to the plant at the level of the symbiotic interface, the arbuscules (see above), or coiled hyphae inside colonized root cells (Bucher 2007).

The transport of P_i at the symbiotic interface is thought to follow the same mechanism as in root hairs, involving H+-ATPases and P_i transporters expressed in colonized cells. This view is supported by the characterization of *Pht1* genes, the expression of which is specific to, or enhanced in, mycorrhizal roots in several species (Bucher 2007; Javot et al. 2007a), and furthermore by the localization of the encoded protein in the periarbuscular membrane (Harrison et al. 2002). In general, the *Pht1* genes responsible for P_i uptake in mycorrhizal roots are different from those that are expressed in non-colonized roots. This suggests functional specialization among different members of the Pht1 family in roots. The essential role of mycorrhiza-inducible PTs in symbiotic P_i uptake was recently demonstrated using *Medicago truncatula* and *Lotus japonicus* plants in which the corresponding *Pht1* genes were mutated or silenced, respectively. These plants showed a reduction in P_i uptake via AM, growth retardation, and, surprisingly, a decrease in AM colonization, suggesting that symbiotic P_i uptake is essential to maintain the interaction (Maeda et al. 2006; Javot, Penmetsa, Terzaghi, Cook, and Harrison 2007b). This indicates that AM fungal development is subject to a feedback regulation mechanism that responds to the nutrient status of plants (see below).

7.4.3 Functional Analysis of PTs in Petunia

Analysis of the *Pht1* family in several solanaceous species revealed an additional member, *PT5*, compared to the families in Arabidopsis and legume species (Nagy

et al. 2005; Chen et al. 2007). Sequence comparison suggests that *PT5* may have arisen from a duplication of *PT4*. The resulting redundancy complicates functional analysis; hence, Petunia, as an efficient system for reverse genetic analysis of the solanaceous *Pht1* family, was chosen to isolate multiple mutants of *Pht1* genes. First, the *Pht1* family of Petunia was characterized by amplifying genomic DNA sequences with primers corresponding to conserved regions of the *Pht1* genes. Six Petunia genes, *PhPT1* through *PhPT6,* were isolated (Wegmüller et al. 2008 and unpublished work). *PhPT1* through *PhPT5* are very similar to previously known solanaceous *Pht1* genes like potato *StPT1*through *StPT5*. The sixth member is closely related to *Catharanthus roseus PIT1* (Kai, Masuda, Kisuhiro, Osaki, and Tadano 1997), and represents a gene previously unknown among solanaceous and other species, including legumes.

PhPT1 and *PhPT6* are constitutively expressed in roots, whereas *PhPT2* is expressed mainly in P_i-starved roots. *PhPT3* through *PhPT5*, like their potato and tomato homologs, show enhanced expression levels in mycorrhizal roots, again suggesting different biological roles of the two groups of genes (Wegmüller et al. 2008).

To better understand the significance of functional diversity within the *Pht1* gene family of Petunia, a reverse genetic approach based on insertional mutagenesis was pursued. The identification of loss-of-function mutants unable to express either one or a combination of the *Pht1* genes was carried out by screening the Nijmegen mutant populations using gene family-specific primers (collaboration with T. Gerats, University of Nijmegen, The Netherlands). Six mutants were found showing transposon insertions in *Pht1* genes: one in *PhPT3*, three in *PhPT4,* and two in *PhPT5*. Phenotypic analysis of single, double, and triple mutants should reveal their respective functions in AM symbiosis. Furthermore, these mutants will be used to estimate the selective advantage of AM-dependent P_i-acquisition in competition experiments under controlled and natural conditions.

7.4.4 Functional Diversification and Evolution of Phosphate Transporters

Mycorrhizal *Pht1* genes cluster together in two distinct subfamilies (Fig. 7.5). The first subfamily is closely related to constitutively expressed genes like *PhPT1*. It comprises genes of only solanaceous and legume species and is represented in Petunia by *PhPT3*(see below). The corresponding proteins transport P_i with high affinity, and are expressed mainly but not exclusively in mycorrhizal roots. The second subfamily consists of genes clustering with *PhPT4* and *PhPT5*, which are not expressed at detectable levels in non-mycorrhizal roots. These genes are proposed to encode transporters with lower affinity for P_i, and, in contrast to members of the first subfamily, most of them are specifically expressed in mycorrhizal roots. Most mycorrhizal plant species, including monocots, have at least one gene of the second group. This subfamily was therefore proposed to be evolutionarily older than the first (Karandashov and Bucher 2005). In addition to this ancestral group, a novel set of mycorrhizal genes is hypothesized to have evolved more recently in

some branches of the eudicot tree, but the physiological significance of this gain is still unclear. Symbiotic P_i uptake appears to depend on only one distinct *Pht1* gene in *Medicago truncatula* and *Lotus japonicus*, as single-gene knockouts exhibit strong phenotypes (Maeda et al. 2006; Javot, Penmetsa, Terzaghi, Cook, and Harrison 2007b). In the Solanaceae, on the other hand, at least three *Pht1* genes were demonstrated to be strongly expressed in mycorrhizas and, accordingly, a single-gene knockout did not lead to reduced P_i uptake capacity of mycorrhizal tomato plants (Nagy et al. 2005). Conceivably, gene duplication allows for a fine-tuning of biochemical properties suitable in particular environments (Nagy et al. 2005). Nevertheless, evidence concerning the function of individual *Pht1* genes in solanaceous species is still missing.

7.4.5 Exploring the Transcriptional Regulation of Symbiotic PT Genes

The restriction of mycorrhiza-related PTs to cells with intracellular fungal structures (Karandashov et al. 2004) implies a cell-autonomous signal in the activation of *PT* genes. To identify this signal, extracts of mycorrhizal roots were fractionated and tested for their ability to induce a *PT*-promoter-reporter gene construct in non-mycorrhizal root cultures: the major inducing component turned out to be lyso-phosphatidylcholine (Drissner et al. 2007). To identify additional components of *PT*-promoter induction, a forward genetic approach was adopted. To establish a screen for mutants deregulated in mycorrhizal P_i transport, the Petunia Mitchell line was transformed with a bidirectional mycorrhiza-inducible *PT*-promoter construct driving the luciferase and β-glucuronidase (GUS) reporter genes. These plants were crossed with the mutator strain W138 to generate a population of transgenic transposon insertion lines, which were then screened for reduced expression of the reporter genes. One mutant candidate showed reduced expression of luciferase and GUS, as well as Petunia mycorrhiza-inducible *PTs,* while the level of expression of the other *PTs* remained unaltered (Wegmüller et al. 2008). This mutant may be affected in a signaling step that specifically triggers symbiotic *PT* gene induction.

7.4.6 Nutritional Aspects of the AM Interaction – N and Others

Due to its wide occurrence in proteins, nucleic acids, and secondary metabolites, nitrogen is needed in large amounts and frequently limits plant development. Nitrogen occurs in different forms in the soil: organic sources (amino acids) and the inorganic forms nitrate (NO_3^-) and ammonium (NH_4^+). All of them can be taken up from the soil and transferred to plants by AM fungi (Ames, Reid, Porter, and Cambardella 1983; Frey and Schüepp 1993; Johansen, Jakobsen, and Jensen 1994; Tobar, Azcon, and Barea 1994; Mäder et al. 2000; reviewed in Clark and Zeto 2000; George 2000; Javot et al. 2007a). Nitrogen taken up by extraradical hyphae of the AM fungal

network is fixed in the form of arginine, which is translocated to the roots (Govindarajulu et al. 2005). Delivery to the plant occurs in a carbon-free form, presumably as NH_4^+ (Govindarajulu et al. 2005; Chalot et al. 2006). However, transcript profiling shows that plant ammonium transporters, as well as nitrate transporters, are induced in mycorrhiza, indicating that both forms may be delivered to the plant (Frenzel et al. 2005).

7.4.7 Endogenous and Exogenous Regulators of AM Colonization

AM colonization is restricted to tissues outside the endodermis, i.e., the cortex and epidermis; the central stele and root tips (including the root meristem) remain fungus-free. This may indicate natural host barriers against over-proliferation of the fungus. In addition, the plant can restrict further colonization once an infection is established (reviewed in Vierheilig 2004a). Experiments on split root systems indicate that plants generate a mobile signal that restricts additional colonization in distant roots (Vierheilig 2004b). The same is true for RNS, in which the *HAR1* gene mediates a negative regulatory mechanism to avoid over-proliferation of nodules (Nishimura et al. 2002; Krusell et al. 2002). Interestingly, grafting experiments showed that the receptor kinase HAR1 functions in the shoot. It appears, therefore, that a root-borne signal is sensed in the shoot, which, in turn, signals back to the root to limit nodulation. Moreover, the negative pathways in AM and RNS interact: AM colonization is limited in nodulated plants and *vice versa* (Catford, Stähelin, Lerat, Piché, and Vierheilig 2003).

In addition to endogenous genetic regulation of symbiosis, nutrients have been shown to influence symbiotic development. P_i at high levels suppresses AM symbiosis (Menge, Steirle, Bagyaraj, Johnson, and Leonard 1978; Jasper, Robson, and Abbott 1979; Thomson, Robson, and Abbott 1986; Amijée, Tinker, and Stribley 1989). Although nitrogen is a substrate for mycorrhizal uptake, its role in the control of AM root colonization is less clear. At low P_i levels, nitrate may promote colonization (Hays, Reid, Stjohn, and Coleman 1982; Hepper 1983; Heijne, Dueck, Vandereerden, and Heil 1994), whereas high levels of both nutrients suppress the interaction even more strongly than P_i alone (Hays et al. 1982; Sylvia and Neal 1990; Azcon, Ambrosano, and Charest 2003). Hence, N and P_i mediate interdependent effects.

Nutritional effects may depend on the plant species involved. Petunia is particularly P_i-responsive (Fig. 7.6), more than leek, for example, and may therefore be particularly well suited for the study of nutritional effects and the complex interactions among different nutrients. Interestingly, the suppressive effect of high P_i in Petunia depends on sufficient availability of other nutrients (Fig. 7.6). When high P_i was supplied in the absence of other nutrients, it limited root colonization only weakly. When P_i was applied together with micronutrients, suppression was still compromised, but when P_i was used in combination with macronutrients, suppression of colonization was comparable to that seen in plants receiving the entire nutrient mix (F. Breuillin and D. Reinhardt, unpublished). This suggests that one or more

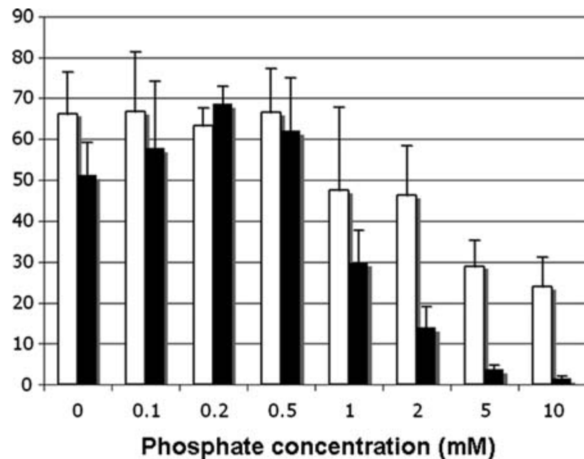

Fig. 7.6 Complex P_i-dependent nutritional regulation of AM colonization in Petunia. Phosphate (P_i) levels above 0.5 mM in the fertilizer solution lead to suppression of root colonization. This effect depends on appropriate nutrition with other essential nutrients (*black columns*). If no other nutrients are provided, colonization is much less suppressed (*white columns*)

macronutrients are essential for P_i-dependent suppression. Conceivably, the plant "evaluates" the supply of the individual nutrients, and controls colonization by the fungus accordingly. The symbiosis-promoting effect of nutrient deficiency is dominant over the suppressive effect of high nutrient levels. This mechanism ensures promotion of the symbiosis as long as just one essential macronutrient is lacking, despite high P_i levels. Future experiments should address the question of which individual macronutrient(s) is/are responsible for the interaction with the P_i-effect.

7.5 Discussion and Conclusions

A critical question in AM research is the nature of the molecular dialogue between the symbionts. Although the presence of the common SYM pathway has suggested a central role of calcium and probably the perception of a symbiotic signal by SYMRK, numerous questions remain unanswered. The nature of the symbiotic myc factor, apparently a diffusible molecule released from fungi before they attach to a root, remains elusive. Furthermore, it is not known how the plant detects the exact position of the appressorium in order to direct the migration of the nucleus and later the formation of the PPA. On the plant side, the strigolactones are probably not the only signals to control fungal development.

A second important issue is how the symbiosis is regulated by nutrients and other exogenous factors. Here, mutant screens for plants with compromised suppression at high P_i levels will help identify the genes involved. It should also be kept in mind that AM associations confer more benefits than simply nutrient acquisition; for example, mycorrhiza formation promotes stress tolerance and disease resistance (Azcon-Aguilar and Barea 1996; Fritz, Jakobsen, Lyngkjaer, Thordal-Christensen, and Pons-Kühnemann 2006). Such benefits may be lost when addition of fertilizers results in suppression of the fungus. Hence, being able to control the

nutrient-dependent repression of AM could be of interest, especially under agricultural conditions, where nutrient supply is enriched through fertilizers (He and Nara 2007). Selection for crop lines with reduced nutrient-mediated suppression of AM could thus improve general plant fitness.

The best-studied model plant, *Arabidopsis thaliana*, does not form symbiotic associations. Therefore, new species had to be established for genetic and genomic studies of root symbioses. *Medicago truncatula* and *Lotus japonicus* have become the leading model species of RNS and, after the uncovering of the common SYM pathway, of AM symbiosis (Udvardi et al. 2005). Phylogenetic arguments and the fossil record indicate that AM symbiosis evolved at least 400 million years ago, whereas nodulation emerged less than 100 million years ago. It appears therefore, that the rhizobia found a way to hitchhike on the preexisting ancient "AM SYM pathway", resulting in what we now know as the "common SYM pathway".

Based on sequence analysis, the common SYM genes are conserved among most higher plants that are able to form AM interactions, and it is expected that they have similar roles in AM development. However, it is possible that during the evolution of the RNS developmental program the original "AM SYM pathway" may have undergone certain modifications; therefore, the common SYM pathway of the legumes may not necessarily be representative of all angiosperms (or all land plants in general). Two plant groups will be particularly important for the study of the SYM pathway: cereals (in particular rice and maize) and the Solanaceae. Concerning the Solanaceae, ample sequence information has been gathered from tomato, tobacco, and potato. However, in these species, the tools for forward and reverse genetic approaches are not fully developed. Here Petunia fills a gap: due to its high mutation rate, Petunia line W138 has proved very useful for mutant screens. AM mutant screens are very laborious, hence this is a critical argument. Secondly, reverse genetics tools are well developed, resulting in the isolation of insertion mutants in all the mycorrhiza-induced members of the PT family in Petunia.

In conclusion, Petunia has recently become a useful model system of symbiosis to represent the Solanaceae, and perhaps other vascular plants. With the availability of increasing numbers of EST sequences and with ongoing mutant screens, Petunia can be expected to contribute considerably to the functional and developmental analysis of AM symbiosis in the future.

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Chapter 8
Vegetative Branching in Petunia

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Abstract Plant form is established by the response of the plant to endogenous and environmental cues. One architectural process for which genetic components have been identified is the decision for axillary buds to grow. In Petunia, a number of genes involved in the decision to branch have been identified and aspects of their functions are elucidated. The genes altered in the *dad* mutants appear to be involved in a single pathway that controls branching and to interact with auxin and cytokinins. These genes mediate the production and reception of hormones inducing and suppressing bud outgrowth. Among species there is a high degree of gene conservation in the pathway and the similarities and differences in gene functions have shown the power of using multiple plant systems. The understanding of developmental processes allows controlled modifications to be made, and the continuing research into axillary bud fate will have a significant impact on future improvements to crop species.

8.1 Introduction

The range of architectural forms present in the plant kingdom is a wonderfully diverse collection of ecological adaptations built from a relatively limited set of similar building blocks. Petunia (*Petunia hybrida* Vilm) is an exemplar for the modular development of plant form as described by Steeves and Sussex (1989). Each vegetative module (phytomer) contains an internode, a node bearing a leaf and, in the axil of the leaf, an axillary meristem. The modules are produced continuously from the shoot apical meristem (SAM) until the floral transition, when the meristem terminates in a flower. Axillary meristems may go through phases of dormancy or growth and are the source of branches.

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Vegetative branching is a plastic process: the branching structure of a particular plant is controlled at an endogenous level (the genetic components expressed during development) but it is remarkably responsive to environmental conditions. An understanding of the processes controlling the integrated response would allow for a better appreciation of how diversity is manifested. This knowledge could also lead to improvements in crop species, as well as the domestication of a broader range of plant species. The selection of favorable plant architecture has already been a feature in the domestication of plants.

Here we review the research on Petunia vegetative branching and its control at a genetic level, with particular reference to our investigation of the genes altered in the *decreased apical dominance* (*dad*) mutants of Petunia. The control of branching has been studied in a number of model systems, and we compare and integrate the results obtained in Petunia with those reported for other plant species.

8.2 Branching Architecture of Petunia

Petunia grows in a monopodial manner during the vegetative growth phase, followed with sympodial development after the transition to flowering. The early growth of the plant has a rosette appearance due to less internode elongation than that seen in later nodes. Axillary meristems are not immediately evident until the axil is two or three leaves removed from the SAM. Under most growth conditions four distinct zones of nodes can be identified along the main axis; these are classified by their potential to form axillary branches (Fig. 8.1, Snowden and Napoli 2003). Zone I includes the first few nodes produced by the plant (the cotyledonary node and first 2–3 vegetative nodes for the inbred line V26) and does not usually produce branches. Immediately acropetal to these basal nodes is a zone of nodes (Zone II) capable of branching, depending on environmental conditions. In the V26 line, Zone II consists of nodes 3–9 under long-day conditions. Branches from this zone are initiated during the vegetative growth phase, and are formed first from the older nodes, then later from younger nodes (i.e., acropetally). In wild-type plants, these primary basal branches rarely produce second-order branches, except during the transition to flowering. Zone III contains axillary meristems that normally do not grow into branches.

When Petunia undergoes the floral transition, the main shoot terminates in a flower. The main axis of growth for the plant then continues with a series of sympodial branches, which make up a cymose type of inflorescence (see Chapter 9). The first sympodial branch is produced from the axil of the leaf before the terminal flower. In addition to the sympodial system of branches, additional axillary branches are initiated from the main shoot (Zone IV in Fig. 8.1), starting from the axil of the penultimate leaf adjacent to the terminal flower. Further axillary branches from the apical portion of the main shoot are initiated in a basipetal direction.

For an individual inbred line or species of Petunia, considerable diversity can be observed in the plant architecture when plants are grown under different

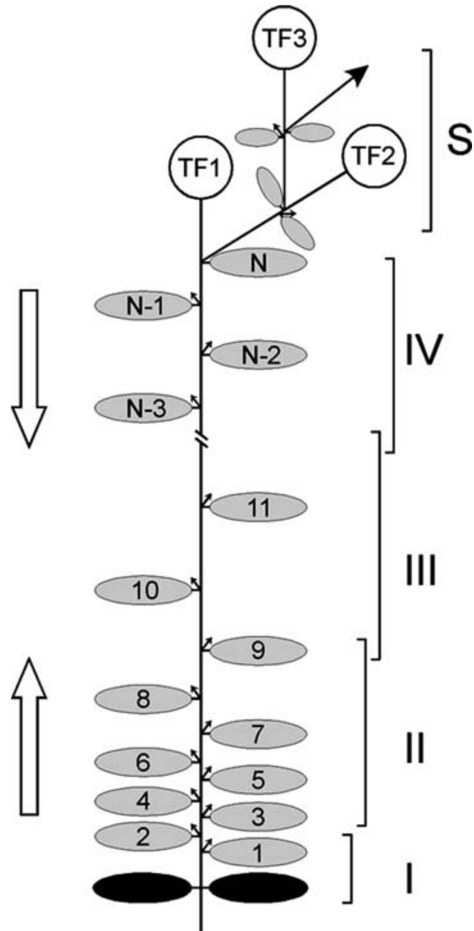

Fig. 8.1 The growth habit of Petunia (reproduced from Snowden and Napoli 2003, with permission from CSIRO Publishing). This diagram shows the positions of branching zones observed in wild-type plants grown in long-day conditions. **Zone I** comprises an initial zone of nodes where branching is suppressed in wild-type, including those at the cotyledons (shown in *black*). **Zone II** is the group of nodes where basal branching occurs in an acropetal direction, as indicated by the arrow. In **Zone III** branching is suppressed. In **Zone IV** apical branches form in a basipetal direction, as indicated by the arrow. The main shoot terminates in a flower (TF) at node N, and a series of sympodial branches (S) form

environmental conditions. Branching can be inhibited, for example, by growing plants in crowded conditions with a limited amount of space for root growth (Napoli 1996; Haver and Schuch 2001), and branching can also be affected by the nutritional status of the plants (Napoli and Ruehle 1996).

The most-studied environmental conditions with respect to their effects on Petunia architecture are photoperiod duration (daylength) and temperature. Reductions in temperature (Cathey and Campbell 1984; Adams, Pearson, and Hadley 1997) and daylength (Brunaud, Bognon, and Cornu 1977; Cathey and Campbell 1984; Adams et al. 1997; Snowden and Napoli 2003) result in increased branching. A close investigation of the difference in growth habit observed under long-day versus short-day conditions has shown that, for a range of inbred *P. hybrida* lines, not only are more basal branches produced under short-day conditions, but growth of the main shoot is also inhibited (Adams et al. 1997; Brunaud et al. 1977; Snowden and Napoli

2003). This inhibition of growth of the main shoot is often irreversible, even when the plants are subsequently transferred to long-day conditions. Branches on plants grown under long days also tend to be upright, while branches on short-day plants are more prostrate (Snowden and Napoli 2003).

In addition to environmental influences, there is an endogenous component that controls plant architecture. A mutational approach has been used to identify some of the genes involved in control of the growth habit of Petunia (see Sect. 8.4). Natural variation of plant architecture in Petunia has been characterized by Brunaud et al. (1977) who defined the architecture of 12 different inbred *P. hybrida* lines, as well as those of the species *P. parodii*, *P. axillaris*, and *P. violacea*. They reported that the overall growth of each of the characterized lines can be explained as above (Fig. 8.1), with the initial zone of nodes at the base of the plant that do not produce branches (Zone I), two zones of basal and apical branching (Zones II and IV), and the presence of the zone of axillary meristems for which branching is suppressed (Zone III). However, considerable variation among the different lines and species was also observed. For example, the percentage of nodes that produced a branch varied widely within the inbred *P. hybrida* lines characterized (27% for the Sh2 line compared with 80% for the TLh2 line, both grown in short-day conditions). In addition, when grown in a long-day photoperiod, the relative position of the basal branches in *P. violacea* is at more apical nodes than is observed for the other species.

8.3 Hormonal Regulation of Architecture

Apical dominance refers to the observation that the growing shoot apex exerts an inhibitory influence over axillary meristems (see Cline 1996). Comprehensive reviews of apical dominance can be found in Napoli, Beveridge, and Snowden (1999) and Dun, Ferguson, and Beveridge (2006), so we shall only briefly summarize a few important points here. If the shoot apex is removed from the plant (e.g., by herbivory, or by decapitation in experimental treatments), then the apical inhibition of axillary meristems is removed and branch outgrowth occurs. For the *P. hybrida* inbred line V26, usually between one and three branches will form immediately proximal to the site of decapitation (K.C. Snowden, unpublished observations).

The phytohormones auxin and cytokinin have long been thought to have roles in apical dominance (see reviews by Cline 1994 and Napoli et al. 1999). The evidence is strongest for auxin, which is produced in the shoot apex and transported basipetally, inhibiting the outgrowth of axillary meristems. Classic experiments performed in plants other than Petunia have shown that exogenous auxin can often substitute for the excised shoot tip to inhibit shoot outgrowth (Cline 1996). Auxin, however, is thought to act indirectly, as it does not enter the dormant axillary buds, but rather acts within the shoot, via an as yet unknown second messenger (Booker, Chatfield, and Leyser 2003). Cytokinins are candidates for this second messenger, as cytokinins generally promote shoot growth and levels of the two hormones appear to be linked (Nordström et al. 2004). However, the timing of cytokinin action appears

to be too slow to initiate bud outgrowth, as the increases in cytokinin content are often observed after bud outgrowth has begun (Turnbull, Raymond, Dodd, and Morris 1997).

Using transgenic plants, auxin and cytokinins have been altered in Petunia, with resulting changes in plant form. Plants overexpressing the *Agrobacterium iaaM* (tryptophan monooxygenase) gene produced a tenfold excess of indole-3-acetic acid (IAA) and exhibited very little branching, although a number of developmental abnormalities were observed (Klee, Horsch, Hinchee, Hein, and Hoffmann 1987). When the *ipt* gene (encoding isopentenyl transferase, which is involved in the production of cytokinins) from *Agrobacterium*, was expressed in transgenic Petunia under the control of a senescence-activated promoter (SAG 12), some lines exhibited increased branching (Clark, Dervinis, Barret, Klee, and Jones 2004). These results are similar to those obtained for other plant species (e.g., see reviews by Napoli et al. 1999 and Synková, Wilhelmová, Šesták, and Pospíšilová 1997). In addition, Petunia was used to isolate a cytokinin biosynthetic gene in plants (Zubko et al. 2002; see Sect. 8.4).

Ethylene has also been shown to affect branching, which may not be surprising given that perturbations of one class of hormone can lead to alterations in others (Klee 2003). Petunia plants treated with ethephon (an ethylene-producing compound) responded with a reduction in shoot IAA, and an increase in branching (Haver and Schuch 2001). In additional experiments, an increase in zeatin riboside (a cytokinin) was observed, as well as a reduction in auxin content, in ethephon-treated plants that exhibited increased branching (as increased numbers of branches, increased length of branches or both; Haver, Schuch, and Lovatt 2003).

8.4 Genes that Alter Plant Architecture

To date, eight genes have been shown to alter the axillary branching architecture of Petunia. Three *dad* mutants were described in Napoli and Ruehle (1996), where they were identified as having altered vegetative branching during a mutational screen to isolate mutants with altered architecture. More recently, the overexpression of the *Lateral Shoot-Inducing Factor* (*LIF*; Nakagawa et al. 2005) and the *Shooting* (*SHO*; Zubko et al. 2002) genes have been shown to increase vegetative branching. Four mutants of inflorescence branching have also been identified: the *sympodial* (*sym*) and *arborescent* (*arb*) mutants (Napoli and Ruehle 1996) and *extra petals* (*exp*) and *aberrant leaf and flowers* (*alf*) mutants (Souer et al. 1998). The *sym1* mutant is allelic to *exp* (R. Koes, personal communication). Genes involved in vegetative branching in Petunia and other species are listed in Table 8.1.

8.4.1 Shooting (SHO)

The *SHO* gene, encoding an IPT protein, was isolated from Petunia by activation tagging (Zubko et al. 2002). The primary line (Ph-sh) could be grown only in tissue

Table 8.1 Genes involved in axillary meristem outgrowth. Gene names have been abbreviated according to original references

Functional gene name	Orthologous genes by species: Petunia	Arabidopsis	Pea	Rice	Other	Phenotype when grafted to WT rootstock
CCD7	*DAD3*[1, 2]	*MAX3*[6, 7]	*RMS5*[15, 16, 17]	*HTD1*[22]		reverted[31, 32, 33] (ND rice)
CCD8	*DAD1*[1, 3]	*MAX4*[8]	*RMS1*[8, 16, 18]	*OsCCD8a*, *OsCCD8b*[2] *D10*[37]		reverted[8, 33, 34] (ND rice)
F-BOX (FBL7)	*PhFBL7*[2]	*MAX2*[9] *ORE9*[10, 11]	*RMS4*[16, 17]	*D3*[23]		not reverted[12, 35] (ND petunia, rice)
—	*DAD2*∗ [1]					not reverted[31]
Zinc-Finger protein	*LIF*[4]					ND
IPT	*SHO*[5]					ND
CYP450 (CYP711)		*MAX1*[9, 12]		5 genes[24] (by sequence similarity)		reverted[32] (ND rice)
TCP		*BRC1*[13] *TBL1*[14]		*OsTB1*[25, 26] *FC1*[26]	Maize: *TB1*[27, 28] Sorghum: *SbTB1*[29]	ND
—			*RMS2*∗ [16]			reverted[35]
—			*RMS3*∗ [16]			weakly or not reverted[35]
—			*RMS6*∗ [19]			not reverted[19]
—			*RMS7*∗ [20]			not reverted[20]
—			*BSH*∗ [21]			ND
AGP					Tomato: *LeAGP-1*[30]	ND

∗ indicates the gene has yet to be cloned; WT, wild-type; ND, not determined; –, unknown. [1] Napoli and Ruehle (1996); [2] KCS, unpublished data; [3] Snowden et al. (2005); [4] Nakagawa et al. (2005); [5] Zubko et al. (2002); [6] Booker et al. (1999); [7] Booker et al. (2004); [8] Sorefan et al. (2003); [9] Stirnberg et al. (2002); [10] Oh et al. (1997); [11] Woo et al. (2001); [12] Booker et al. (2005); [13] Aguilar-Martínez et al. (2007); [14] Finlayson (2007); [15] Blixt (1976); [16] Arumingtyas et al. (1992); [17] Johnson et al. (2006); [18] Apisitwanich, et al. (1992); [19] Rameau et al. (2002); [20] Morris et al. (2003); [21] Symons et al. (1999); [22] Zou et al. (2005); [23] Ishikawa et al. (2005); [24] Nelson et al. (2004); [25] Lukens and Doebley (2001); [26] Takeda et al. (2003); [27] Burnham (1959); [28] Doebley, et al. (1997); [29] Kebrom et al. (2006); [30] Gao et al. (1999); [31] Simons et al. (2007); [32] Turnbull et al. (2002); [33] Morris et al. (2001); [34] Napoli (1996); [35] Beveridge et al. (1996); [36] Beveridge et al. (1997); [37] Arite et al. (2007).

culture; however, a partly reverted line (Ph-sh2) could be grown in soil. The Ph-sh2 plants produced more branches, and featured shorter internodes, delayed senescence and flowering, and smaller flowers than seen in the wild type. In wild-type Petunia, the expression of the *SHO* gene is detectable in a range of tissues, with highest expression in the roots. Overexpression of the endogenous *SHO* gene in the Ph-sh and Ph-sh2 lines resulted in an increased production of cytokinins. This was the first description of a plant-derived *IPT* gene functioning in plants to produce active cytokinins. Perhaps most interestingly, the authors showed that the overproduction of these cytokinins in the stem was sufficient to cause the changes in phenotype.

8.4.2 Lateral Shoot-Inducing Factor (LIF)

The gene for the zinc-finger protein LIF was isolated from Petunia (Nakagawa et al. 2005) and has a role in branching that appears to be opposite to that of the genes defined by the *dad* mutants. When the gene is overexpressed in transgenic Petunia plants, an increase in basal branching is observed, including the production of secondary branches that are rarely observed on wild-type plants. This increase in branching is not due to the production of additional axillary buds. No homozygous mutant has been isolated for the *LIF* gene, and transgenic plants with a knockout of *LIF* gene expression have not been regenerated, indicating that this gene has an essential function in plant growth and development. Overexpression of the *LIF* gene affected other aspects of Petunia morphology beyond that observed for branching. Internode lengths were shortened while cell sizes were generally increased, indicating a reduction in cell number. The flower shape of *LIF*-overexpressing plants was altered due to a change in cell size on the abaxial (but not adaxial) side of the petals. Differences were also observed in various cytokinins extracted from leaf tissue, with an increase in isopentenyladenine and decreases in *trans*-zeatin and *cis*-zeatin. The LIF protein is nuclear-localized and, interestingly, the gene is expressed in nodal tissue underneath axillary buds, suggesting a role for this gene in signal transduction, perhaps by interpreting mobile signals from the stem into an effect on branching in the axillary meristems (Nakagawa et al. 2005).

8.4.3 Decreased Apical Dominance (dad) Genes

All the three *dad* mutants exhibit increased outgrowth of axillary meristems and a decreased total plant height (Fig. 8.2, Napoli 1996; Snowden and Napoli 2003; Snowden et al. 2005; Simons et al. 2007). With respect to these major effects, the *dad1-1* and *dad2* mutants are similar in phenotype, while effects of the mutation in *dad3* plants are less severe. Three *dad1* mutant alleles have been characterized (Snowden et al. 2005). The *dad1-1* mutant is the original and extreme mutant, with *dad1-2* intermediate between *dad1-1* and wild type, and *dad1-3* very similar to wild type.

Fig. 8.2 Phenotype of *dad* branching mutants compared with the wild type. From left to right, *dad1-1*, *dad2*, *dad3*, and wild-type plants. All plants are from the V26 inbred line of *P. hybrida* Vilm. Photograph taken by B. Quinn

The most detailed phenotypic characterization of a *dad* mutant has been that of *dad1-1*. The mutant shows an increased propensity for the outgrowth of axillary meristems over a wider range of vegetative nodes than the wild type. This includes the development of secondary branches (those formed from axillary meristems on primary branches), generally absent in intact wild-type plants (Napoli 1996). In proportion to the increase in branching there is a decrease in internode length, leading to overall decreased stature of the plants. Intermediate *dad1* mutant alleles show a less substantial gain in branching and a less severe decrease in height (Snowden et al. 2005).

As described above, the V26 line of Petunia shows a significant alteration in branching habit in response to daylength, a response that is presumably mediated through the phytochrome-signaling pathway. In the *dad1-1* mutant this response to daylength is largely lost, with plants grown under either short or long days producing the same number of branches and maintaining a compact form (Snowden and Napoli 2003).

The *dad* mutants are generally described as being nonpleiotropic (reviewed in Napoli et al. 1999). However, other phenotypic changes are observed in the *dad1-1* mutant, including mild leaf chlorosis, delayed leaf senescence, reduced root development in the main root mass but with significant gain in adventitious rooting from the lower stem and branches, reduced flower size, and delayed flowering time.

The interdependence of flowering time and branching has been investigated with the *dad1-1* mutant (Napoli 1996; Snowden and Napoli 2003). The additional branches present in the *dad1-1* mutant are not the cause of the increased number of

nodes to flowering. Using an early flowering mutant (*sym1*), it was shown that *dad1-1/sym1* double mutants had a flowering time restored to wild type, but remained significantly more branched, demonstrating that increased branching in *dad1-1* was not a response to delayed flowering.

The branching potential and flowering time have also been measured in *dad2* and *dad3* mutants (Snowden and Napoli 2003). Each of the three mutants could be distinguished from wild type and one another in long-day conditions; the degree of branching is *dad1-1*>*dad2*>*dad3*>wild type. Experiments carried out under different conditions have failed to distinguish *dad1-1* and *dad2* mutants on the basis of axillary branch numbers alone (Simons et al. 2007). In an earlier paper it was noted that under short-day conditions the phenotypes of these two mutants converged (Snowden and Napoli 2003). The *dad1-1* plants are completely daylength-insensitive. In contrast, *dad2* and *dad3* plants respond to short-day conditions by increasing the number of axillary branches. Despite the alteration in branch number, the *dad2* and *dad3* plants are still somewhat daylength-insensitive, as they do not alter their branch angle to the prostrate form seen in wild type, but rather remain upright and bushy in appearance like *dad1-1* (Snowden and Napoli 2003; unpublished data). Because the *dad* mutants show signs of daylength insensitivity, the branching-control pathway responding to daylength must be, at least in part, mediated by the *dad* genes. The *dad2* and *dad3* mutants also differ from *dad1-1* in flowering time, with both the *dad2* and *dad3* genotypes having a number of nodes to flowering similar to that seen in wild type (Snowden and Napoli 2003).

The levels of auxin and cytokinin in the three single mutants have been measured and compared with those of wild-type Petunia. The auxin levels measured in the *dad* mutant shoot apices were somewhat lower than those of wild-type Petunia, while the auxin levels in the stem tissue of *dad* mutants were approximately the same or higher than those of wild type (Table 8.2). Although these data indicate that decreases in upper stem/apex auxin levels are unlikely to be the cause of the *dad* increased-branching phenotype, auxin levels in the axillary meristem or in adjacent tissue at the node are yet to be measured.

In all the *dad* mutants, the levels of cytokinins in xylem sap were significantly decreased in comparison with those of the wild type (Table 8.3). Similar results have been reported in pea, where the increased branching *ramosus* mutants *rms1*, *rms4*, and *rms5* all have decreased levels of sap cytokinin (Beveridge et al. 1997; Morris et al. 2001). Only one of the *rms* mutants (*rms2*) has levels of xylem sap cytokinin that are comparable with those of wild-type pea (Beveridge 2000). This has been interpreted as the effect of a feedback mechanism which is disrupted in the *rms2* mutant. Given that cytokinins can promote branch outgrowth when applied to axillary buds (e.g., Pillay and Railton 1983), the decreased levels in *dad* mutant xylem sap indicate that it is unlikely that they are the graft-transmissible branching signals controlled by the *DAD1* and *DAD3* genes. Instead, this decrease in xylem sap cytokinins could be a secondary effect of the increased branching phenotype.

Although the commonly held belief is that cytokinins exported from the roots of plants affect apical dominance, recent research has indicated that cytokinins are synthesized in tissues other than roots (Nordström et al. 2004; Tanaka, Takei, Kojima,

Table 8.2 Auxin content in wild-type and *dad* mutant (*P. hybrida* var. V26)

Genotype	Experiment[a]	IAA in shoot apex (ng/g)[b]	IAA in upper stem (ng/g)
wild-type	I	29.2	18.0
	II	22.8	14.9
dad1-1	I	22.0	16.9
	II	ND	18.4
dad2	I	19.3	56.8
	II	19.7	22.5
dad3	I	12.0	12.5

[a]Two independent sets of plants (Experiments I and II) were grown for auxin analysis (Snowden et al. 2005). [b]Shoot apices and stem sections were harvested from 6-week-old plants and snap frozen in liquid nitrogen, ground to a fine powder and spiked with known concentrations of deuterated IAA ([$^2H_5$] indole-3-acetic acid). The endogenous and deuterated auxins were extracted with methanol and filtered through C18 SepPak cartridges (Waters Corp., USA). The extracts were incubated with diazomethane to methylate the IAA, and then partitioned with diethyl ether. Auxin measurements were carried out by GC-MS-SIM by J. Ross (University of Tasmania) as described by Ross (1998). Endogenous and deuterated IAA were quantified as described by Morris et al. (2001); IAA is expressed in ng.g fwt^{-1}. All samples were pools of at least six plants. ND, sample levels were not determined.

Table 8.3 Cytokinin content of xylem sap from wild-type and *dad* mutant *P. hybrida* var. V26

Genotype	Experiment[a]	DHZ9G[b]	ZR	DHZR	iPA	Total
wild-type	I	N	0.731	0.151	0.085	0.968
	II	N	0.629	0.035	N	0.664
dad1-1	I	N	0.068	N	N	0.068
	II	N	0.136	N	0.048	0.184
dad2	I	N	0.146	N	N	0.146
	II	N	0.213	N	N	0.213
dad3	I	0.073	0.065	N	N	0.138

[a]Two independent sets of plants (Experiments I and II) were grown for cytokinin analysis as in Snowden et al. (2005). Xylem sap was harvested from 12-week-old plants by decapitation of the plants approximately 1 cm above soil level and vacuum treatment for 1 h. During vacuum treatment the apparatus was covered in order to protect any light-sensitive compounds. The xylem sap was snap frozen and then freeze dried. Dried samples were resuspended in methanol and spiked with known concentrations of deuterium-labeled cytokinin standards. Samples were filtered through C18 SepPak cartridges and a 45 μm pore filter, concentrated and resuspended in methanol, then dried down completely in glass inserts for storage. Cytokinin analysis was performed by electrospray tandem mass spectrometry (LC-MS-MS) by C. Ngo (University of Queensland), largely as described in Prinsen et al. (1995). [b]Cytokinins measured were dihydrozeatin-9-glucoside (DHZ9G), zeatin riboside (ZR), dihydrozeatin riboside (DHZR), isopentyladenosine (iPA), zeatin (Z), *cis*-zeatin (cisZ), dihydrozeatin (DHZ), zeatin-9-glucoside (Z9G), *cis*-zeatin riboside (cisZR), and isopentyladenine (iP); all amounts are in pmol. g fwt^{-1}. N, the cytokinin was not detectable at the limits of the equipment. Z, cisZ, DHZ, Z9G, cisZR, and iP were below the level of detection in all samples.

Sakakibara, and Mori 2006). There may be a need to measure their levels at the site of cytokinin action in the control of branching (stem nodes and axillary buds). Tanaka et al. (2006) have demonstrated that cytokinins are produced at the point where bud outgrowth is controlled and the levels in these tissues may be more directly relevant to control of branching.

8.5 Interactions of the *DAD* Genes

Genetic and grafting approaches have been used to investigate the functions of the *DAD* genes. In one approach the three possible *dad* double mutants have been analyzed for their effects on plant architecture (Simons et al. 2007). Petunia is easy to use in controlled crosses, and so is highly amenable to analysis of mutant traits by this type of methodology. The *dad1-1/dad2* double mutant is indistinguishable from the single mutants, while the *dad2/dad3* double mutant has a phenotype like that of *dad2*. The *dad1-1/dad3* double mutant has a branching phenotype similar to that of *dad1-1* but reduced height compared with either single mutant and, in addition, a delayed flowering time (Simons et al. 2007). The observation that one of the double mutants is additive, while the other two are not suggests, that the interaction of the *DAD* genes is more complex than a simple linear pathway (see Sect. 8.8).

Interactions of the genes have also been studied at the whole-plant level using micrografting (Napoli 1996). Results are summarized in Fig. 8.3. This grafting method allows the joining of plants cut through the hypocotyl; grafts can also be performed at higher internodes. Grafts of like-genotype to like-genotype plants produced no alteration in any of the measured phenotypic traits (Napoli 1996; Simons et al. 2007). Wild-type rootstocks were able to revert either *dad1-1* or *dad3* mutant scions to a near-wild-type phenotype but did not alter the branching on *dad2* scions (Napoli 1996; Simons et al. 2007). The *dad1-1* plants also reverted to near-wild-type as a result of interstock grafts, in which a small section of wild-type stem was inserted at the hypocotyl of an otherwise mutant plant (Napoli 1996). Mutant plants with wild-type interstock grafts at internodes above the cotyledons showed reversion to the wild-type phenotype only at nodes above the wild-type stem (Simons et al. 2007). The authors suggested that a graft-transmissible signal originating in the roots moves acropetally to alter axillary branch development. If *dad1-1* scions grafted to wild-type roots were allowed to form adventitious roots above the graft

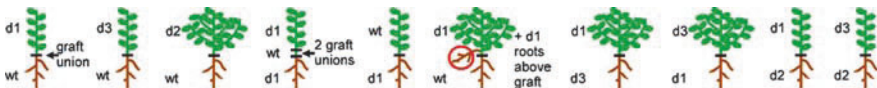

Fig. 8.3 Diagram of grafts involving *dad* mutants showing the scion branching phenotype in *P. hybrida*. The genotype of the scion (*green*) and the rootstock (*brown*) are identified as follows: d1=*dad1-1*, d2=*dad2*, d3=*dad3*, wt=wild type. For clarity the phenotype is shown as either branched or not-branched

union then the *dad1-1* scion failed to revert to wild-type branching (Napoli 1996), indicating the presence of a branch-inducing signal coming from the *dad1-1* roots. The reciprocal grafts involving *dad1-1* and *dad3* were unable to alter the phenotype of the scion, which indicates that the DAD1 and DAD3 proteins are required in the same tissue to produce the signal (Simons et al. 2007). However, *dad2* rootstocks were able to decrease the observed branching of both *dad1-1* and *dad3* scions (Simons et al. 2007). Grafting of wild-type scions to *dad* mutant rootstocks produced less consistent results. In some experiments, the *dad3* rootstocks slightly increased the number of axillary branches in wild-type scions (Simons et al. 2007); in most experiments no effect was observed in wild-type scions grafted to rootstocks of any *dad* mutant.

The expression of the *DAD1* gene has been investigated in both wild-type plants and the *dad* mutants. *DAD1* is predominantly expressed in the roots and, at a lower level, in the stems of wild-type plants (Snowden et al. 2005). This is consistent with the concept of a graft-transmissible signal originating in the roots. In each of the *dad* mutants, the level of expression of the *DAD1* gene is higher in the stem tissue (but not the roots) than in the wild type, suggesting that a feedback control mechanism is acting in the stem tissue.

Grafting with the *dad1-1* and *dad2* mutants and subsequent quantitative RT-PCR analysis confirmed that expression of the *DAD1* gene correlated with the phenotype of the scion rather than the genotype (Simons et al. 2007). For example, reduced *DAD1* expression was detected in the reverting scions of the *dad1-1* mutant when grafted over wild-type or *dad2* roots, compared with the high levels of *DAD1* expression in the branched *dad1-1* scion grafted onto its own roots. This result suggests that the expression of the genes is responding in some way to the outgrowing branches (e.g., the number of active meristems).

8.6 The Identity and Biochemical Function of DAD1 and DAD3

The genes mutated in the *dad1* and *dad3* Petunia plants have been cloned (Snowden et al. 2005; unpublished data) and are both members of the carotenoid cleavage dioxygenase (CCD) protein family (PFAM03055). This relatively large family of proteins is present in bacteria, plants, and animals, but no members have been found in fungi to date. Orthologs of *DAD1* are *More Axillary Growth4* (*MAX4*) from Arabidopsis (Sorefan et al. 2003) and *RAMOSUS1* (*RMS1*) from pea (Sorefan et al. 2003), and orthologs of *DAD3* are *MAX3* (Booker et al. 2004) and *RMS5* (Johnson et al. 2006). These Arabidopsis and pea genes regulate branching in much the same manner as *DAD1* and *DAD3* in Petunia, that is, the mutants have a phenotype of increased axillary branching and decreased height. These genes were originally named for the mutant phenotypes; however, we suggest the genes be renamed to reflect their positions within the CCD phylogeny (Snowden et al. 2005), with the *DAD1* orthologs becoming *PhCCD8*, *AtCCD8*, *PsCCD8*, and the *DAD3* orthologs renamed *PhCCD7*, *AtCCD7*, and *PsCCD7*.

A variety of functions has been identified for the CCD proteins. In bacteria, a subgroup of enzymes cleave lignostilbene (Kamoda and Saburi 1993a, b); in animals, one member isomerizes an apo-carotenoid (Moiseyev, Chen, Takahashi, Wu, and Ma 2005), and in all three groups, that is, bacteria, plants, and animals, some of the enzymes have been shown to cleave carotenoids or apo-carotenoids (e.g., Kamoda and Saburi 1993a; Schwartz, Tan, Gage, Zeevaart, and McCarty 1997; von Lintig and Vogt 2000). As yet no biochemical activity for *PhCCD7/DAD3* or *PhCCD8/DAD1* has been shown in Petunia, and, although *AtCCD7/MAX3* and *AtCCD8/MAX4* have been shown to cleave carotenoids in a bacterial system (Schwartz, Qin, and Loewen 2004; Auldridge et al. 2006), the activity of the proteins *in planta* awaits discovery.

8.7 Comparative Aspects of Vegetative Bud Outgrowth

Much of the work investigating vegetative branching has been done in model species (Petunia, pea, and Arabidopsis) with increased branching mutants (Table 8.1). The relatively nonpleiotropic *dad1–dad3*, *rms1–rms7* (pea), and *max1–max4* (Arabidopsis) mutants all have normal levels of axillary bud formation and most of the genes appear to control the same decision path in bud fate. However, there is less certainty about whether *RMS6* and *RMS7* are part of the pathway, and although *RMS2* is considered part of the pathway, this gene's interactions with the others are at times difficult to explain. While the level of gene conservation is striking, some differences in branching are apparent between Petunia, pea, and Arabidopsis. Do these differences reflect differences in initial architecture or differences in the functions of the genes in the control of axillary branching?

The three model systems are all herbaceous and produce a phytomer with an axillary meristem in each node (pea also has two accessory meristems per node). Most of these meristems remain dormant. The branching that is induced in the *dad/rms/max* mutants is constrained by the growth forms of the plants. Petunia and pea are long-shoot plants and Arabidopsis is a short-shoot plant until flowering. When Arabidopsis makes the floral transition, the apical meristem and axillary meristems that are released grow out as long shoots. In the Petunia mutants the extra branches come from an expanded and over-activated Zone II, where basal branching normally occurs, and branching proceeds in an acropetal manner (Snowden et al. 2005). In the pea mutants, the most basal (1–3) nodes grow out to produce branches (Beveridge et al. 1996), although in most research of *rms* mutants these branches are removed to allow a more obvious pattern of extra branching to occur at the apical nodes. Finally, in the Arabidopsis mutants the extra axillary growth is limited to the production of axillary leaves (short shoots) in the basal nodes until flowering, when both acropetal and basipetal branching (producing long shoots) are increased (Stirnberg et al. 2002). Interestingly, the increase in apical branching (equivalent to Zone IV in Petunia) after flowering is limited to Arabidopsis. This may be an effect of the short vegetative stem, which leads to an architecture in which even the most distal nodes are only at short distance from the root.

Another difference among the model systems involves the effect of daylength on wild-type versus mutant plants. In all three species there is limited branching before flowering in long-day conditions, with short days producing some branching at the base of the plant. The Petunia mutants are partly insensitive to daylength (see Sect. 8.4.3). That there is little change in the number of branches may be due to constraints on the number of branches that the plant is able to support (Snowden et al. 2005). However, in pea the effects of short-day conditions on the *rms1–rms5* mutant plants are significant, leading to a further increase in branch number and outgrowth (Arumingtyas et al. 1992). This suggests that the integrator of the daylength and the *RMS* signals is downstream of the *rms1–rms5* mutants. Arabidopsis *max* mutants also produce more branches in short days but this change is due, at least in part, to the greatly increased number of nodes from which branching can occur.

One of the most interesting aspects of the Petunia *dad* mutants is the presence of a graft-transmissible branch-inducing signal (Napoli 1996; see Sect. 8.5). There are no published reports of such an effect in either pea or Arabidopsis, where hypotheses focus on a branch-suppressing signal. However, research on the branching of *Trifolium repens* strongly suggests that there is a root-derived branch-inducing signal in that species (Thomas and Hay 2007). This signal appears to have a limited movement along the stem, and acts to make buds both proximal and acropetal to roots (main or nodal) competent to grow. Buds retain their outgrowth potential over long time frames even if the activating roots are subsequently removed (Thomas and Hay 2007). Whether the same signal is present in Petunia and *T. repens* is a matter for further study.

8.8 A Model for Axillary Branch Outgrowth

Given that branch growth is one of the major processes that can be used by the plant to regulate form in response to changes in the environment, it is not unexpected that the axillary meristem is able to integrate and respond to a range of stimuli. For example, removal of the SAM by herbivory results in outgrowth of axillary meristems, allowing the plant to continue growth through flowering and reproduction despite loss of the part of the plant that normally executes these functions. The identification of mutations affecting branching and the cloning of the genes involved in branching in Petunia and other species is allowing for the development of a molecular genetic model for the control of branch outgrowth.

8.8.1 In the Roots and/or Stem

The presence of a positive graft-transmissible branching signal in Petunia is confirmed by grafting studies, which demonstrate that *dad1-1* scions grafted over wild-type rootstocks revert to wild type unless *dad1-1* roots form above the graft union (Napoli 1996; Snowden et al. 2005). The branched mutant phenotype maintained in these grafts indicates that a signal from the *dad1-1* roots is able to promote

branching even in the presence of any inhibitory signal from the wild-type roots. By contrast, the presence of a graft-transmissible inhibitory signal is inferred from Arabidopsis genetics and grafting experiments using reciprocal grafts, which show that the *MAX1* (*CYP450*) gene acts downstream of *AtCCD7/MAX3* and *AtCCD8/MAX4* (Booker et al. 2005). The simplest interpretation of these results is that an inhibitory signal from *AtCCD7* and *AtCCD8* is processed by CYP450, ultimately leading to production of a graft-transmissible inhibitor. An obvious interpretation integrating the data from both species is that the substrate of CCD7 and CCD8 is a promoter, and the product an inhibitor, of branching. However, it seems more likely that CCD7 and CCD8 act at a step in a pathway that processes polyenes, and that mutation of these enzymes alters flux in that pathway. The observation of other changes in phenotype in the *dad1-1* mutants (Snowden et al. 2005) suggests that more than one compound in the pathway may have effects at a distance.

Reciprocal grafting and double-mutant analysis in Petunia show that the PhCCD7 and PhCCD8 proteins must act in the same tissue either as a dimer or as sequential enzymes on an intermediate that cannot move from tissue to tissue (Snowden et al. 2005). Bioinformatic analysis and uptake assays in pea chloroplasts and tobacco cell line plastids suggest that both CCD7 and CCD8 are plastid-localized (Booker et al. 2004; Auldridge et al. 2006). In bacterial expression systems, both Arabidopsis enzymes have been shown to act on polyenes, specifically carotenoids (Booker et al. 2004; Schwartz et al. 2004; Auldridge et al. 2006). This has been confirmed for the PhCCD7 and PhCCD8 enzymes in our laboratory (unpublished data). Taken together, these data suggest that compounds in the pathway are processed or produced in the plastids, and that one or more of the compounds is transported acropetally from the roots to the stems. The observation that a small interstock graft of wild-type tissue between *dad1-1* mutant rootstock and scion is sufficient for reversion suggests that during transport the compounds can be processed by plastids, which in turn suggests transport via cell-to-cell movement rather than bulk flow in the xylem or phloem.

8.8.2 At the Node and/or Axillary Meristem

Mutant and grafting studies suggest that at least two genes associated with branching have their effects in the shoot. *DAD2* is shoot specific, suggesting that either the DAD2 protein is active in the signal reception/transduction pathway or DAD2 is involved in the production of a precursor that moves from the shoot to the roots to be acted upon by the CCD7/CCD8 pathway. In pea the *rms3* mutant is also non-graft revertible and may be orthologous to *DAD2* (see Table 8.1). AtFBL7 (*MAX2*, a leucine-rich repeat type F-Box protein) is also shoot specific and postulated to play a role in signal transduction (Stirnberg et al. 2002). The involvement of F-box proteins in protein turnover makes it tempting to postulate that FBL7 directly degrades a (transcription) factor that promotes branching.

Two genes, *LIF*, a zinc-finger transcription factor (see above; Nakagawa et al. 2005), and *TEOSINTE BRANCHED 1* (*TB1*), which is also thought to be a transcription factor (Doebley, Stec, and Hubbard 1997; Kebrom et al. 2006;

Aguilar-Martínez et al. 2007; Finlayson 2007), have been shown to be expressed at, or very close to, the axillary bud. Plants overexpressing LIF appear to have a phenotype similar to that of *dad1-1* plants, suggesting that LIF may directly promote branching, making it a possible target of FBL7. By contrast, TB1 appears to be a repressor of branching at the meristem. In sorghum, *TB1* is repressed by $PhyB^{fr}$, which is deactivated by far-red light (Kebrom et al. 2006), indicating that TB1 provides a direct connection between the shade-avoidance response and branching. Analysis of *tb1/max* double mutants and transcriptional downregulation of *TB1* in *max3* and *max4* mutants (Aguilar-Martínez et al. 2007) and *max2* mutants (Finlayson 2007) suggest that the CCD7/8-derived signal may be integrated by TB1 in the meristem and that the signal transduced by the F-box protein (FBL7) is also integrated by TB1.

The role of auxin in branching is still not fully understood. Decapitation experiments and classical apical dominance theory suggest that auxin inhibits axillary meristem outgrowth. However, auxin levels measured in plants with mutant *CCD7* and/or *CCD8* genes are typically elevated or unchanged. Recent work in Arabidopsis (Bennett et al. 2006) shows that auxin levels are increased in the stems in *max1*, *max2*, *max3* and *max4* mutants. *PIN* gene expression and hence auxin transport rates appear to be increased in *max1* and *max3* mutants, and *pin1/max1* and *pin1/max3* double mutants have a reduced-branching phenotype compared with the *max* mutants. These results suggest that auxin transport (leading to increased auxin levels at or near basal nodes) is required for the increased branching seen in *max* mutants. Whether the changes in auxin transport lead to increased or decreased auxin levels in the axillary meristem is not clear.

Integration of the data into a single model in one species is not straightforward. As yet some results have been seen in only a single species and some results are difficult to integrate with others. However, cloning of some key genes in Petunia and study of the interrelationships between some of the more recently discovered elements of the branching pathway should allow construction of a working model of branching.

8.9 Current and Future Directions

Identification of the branching signal molecule(s) is a key focus of research worldwide, with many research groups now working in cooperation. The variety of model systems used by individual groups is allowing for the strengths of each system to be harnessed, hopefully speeding up the final identification. When the signal molecule is known, new experimental approaches will be possible. Biochemical methods might be used to identify possible precursors and mutational experiments used to confirm predictions. Knowing how the signal is linked to the metabolism of the plant might suggest alternate points of control in the overall development of the plant and its responses to environmental conditions. If the signal or its precursors can be synthesized and labeled, tracing the flux of the components through the plant could provide evidence for the mode of interaction of the signal with the axillary buds.

The ongoing discovery of genes involved in the decision of axillary buds to grow will allow further identification of similarities and differences between species. In the introduction we commented on the diversity of plant architecture. Understanding the relative contributions of different architecture controls (e.g., phyllotaxy, axillary meristem formation, internode elongation, root/shoot balance, bud growth) will be key to establishing how the observed diversity is generated. Comparative analyses using Petunia, pea, and Arabidopsis, and the genes identified in Table 8.1, will clarify the importance of the bud outgrowth control system on plant form.

We have noted that the Petunia *dad* mutants have altered phenotypes for characters other than axillary bud outgrowth. Although we have taken a reductionist approach in our research, we appreciate that plants are complex systems. Hence the connectivity of this pathway to other control systems is not surprising, particularly given the multiple effects of plant hormones. Whether the connections we see are part of wild-type plants or are only an effect of the mutant state is unknown. In either case, appreciating the linkages may allow us to identify some of the biochemistry underlying the control system.

The knowledge of branching control gained in model species will be transferred to crops to improve varieties and domesticate new species. As we highlighted in the introduction, the architecture of plants has been an important trait for selection in plant breeding efforts. *TB1* orthologs may prove to be very useful tools, as the *TB1* gene has already been involved in one of the major phenotypic alterations leading to domesticated maize (Doebley, Stec, and Gustus 1995). Ascertaining the genes most appropriate for selection or transgenic modification in crop species may be difficult without direct experimentation in the target species. The genes altered in the *dad/rms/max* mutants are possible targets for alteration in crops; however, the secondary effects will need to be assessed in target species.

Petunia has proved to constitute a valuable system to study the control of axillary meristem outgrowth, having attributes that make it straightforward to investigate subtle changes in plant architecture. Cross-species comparisons with Petunia have led to questions that might not otherwise have been asked about the control of branching. Continued work in Petunia and other systems will result in a better understanding of the developmental and environmental controls of branching.

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Chapter 9
Development of the Petunia Inflorescence

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Abstract Angiosperm species display an amazing variation in the timing and position of flower formation. Comparative genetic analysis in species with different inflorescence architectures, like Arabidopsis and Petunia, provides insight into the genetic alterations underlying these anatomical differences. The picture that emerges is that distinct inflorescence types are controlled by genes encoding conserved proteins, and that the observed diversity results from substantial changes in their expression patterns and regulatory interactions.

9.1 Introduction

A key question in biology is how molecular changes in genomes during evolution have led to the diversity that is seen today in the morphology and body architecture of animals and plants. One of the early and most important findings in molecular developmental biology was that the development of animals with very different body plans relies on deeply conserved "toolkit" genes, such as the well-known homeobox (HOX) genes. This finding seemed at first a paradox and raised the question of how conserved genes can specify organisms with widely different morphologies. One possibility, which was put forward very early (King and Wilson 1975), is that evolution of morphology proceeds primarily via alterations in gene regulation and gene expression patterns rather than from changes in coding regions of genes. This view has been particularly strongly advocated by some researchers in evolutionary developmental biology (evo-devo), in both scientific and popular literature (e.g., Carroll 2005a, b), and has more recently been narrowed down to mutations in *cis*-acting gene elements that control transcription (e.g., Prud′homme, Gompel, and Carroll 2007; Wray 2007). However, others have pointed out that innovation or

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modification of gene products (proteins) has played an important role as well and argued that many of the cases cited as evidence for evolution via regulatory (*cis*) elements do not exclude alternative explanations (Hoekstra and Coyne 2007). Thus, the issue is far from settled.

Most evo-devo research relies, by nature, on a comparative analysis of distinct species, and arguments that a certain genetic difference was responsible for an anatomical change often rely on transgenic experiments. A major problem lying at the heart of the means-of-evolution controversy is that transgenic experiments with nonmodel animal species are rarely possible. Plants, like animals, display an enormous variation in body architecture. Because a decent number of higher plant species are amenable to forward genetic analyses and/or transformation, they offer unique possibilities for evo-devo studies, possibilities that have so far hardly been explored.

Species of flowering plants (Angiosperms) display an astonishing variation in architecture of the plant body. They are built of very similar organ types, such as leaves, stems, petals, and stamens, but differ in the numbers of each organ and the positions where they arise on the plant body. This is seen most dramatically when the plants flower. Angiosperm species differ, for example, with respect to the season and plant age at which they switch from vegetative growth to flowering, and flowers can be arranged on the plant body in a variety of patterns (e.g., Weberling 1989; Coen and Nugent 1994; Benlloch, Berbel, Serrano-Mislata, and Madueno 2007). Flowers may occur as solitary structures at the end of a stem or a branch, or they can be organized into inflorescences (branched structures bearing multiple flowers) in a variety of patterns (Fig. 9.1). Inflorescences can be divided into three major classes: panicles, racemes, and cymes. In panicles, all axes terminate in flowers. In racemes flowers arise from lateral meristems, resulting in a straight (monopodial) axis. In cymes each axis terminates by forming a flower and a new axis forms in

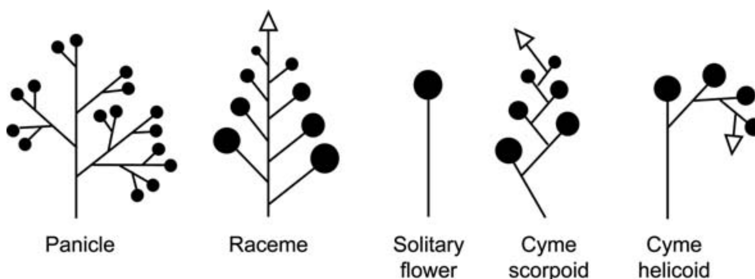

Fig. 9.1 Diagrams comparing two major types of inflorescence architecture, monopodial and sympodial. In species with monopodial growth, the (indeterminate) apical meristem maintains its meristematic characteristics, forming flowers or new inflorescence shoots on its flanks; two examples are panicles and racemes (e.g., Arabidopsis and Antirrhinum). In species with sympodial growth, the apical meristem terminates in a flower (determinate), which can be solitary (e.g., tulip), or a cymose inflorescence bearing many flowers (e.g., Petunia). *Circles* indicate flowers and *triangles* the apical meristem

a lateral position that repeats this pattern. Each of these inflorescence types can be further divided into subclasses based on differences in, for example, phylotactic patterns and lengths of internodes.

Recently a theoretical model was put forward for the development and evolution of distinct inflorescences (Prusinkiewicz, Erasmus, Lane, Harder, and Coen 2007). In this model inflorescences develop as a series of metamers, each consisting of a stem section bearing an apical meristem and a new lateral meristem. Meristems initially have a vegetative (i.e., nonfloral) identity, which enables them to generate a new metamer, but in time they lose their vegetative identity (or acquire floral identity) and terminate by forming a flower. If "vegetativeness" decreases with similar kinetics in apical and lateral meristems a panicle will be formed, but if it decreases more rapidly in apical or lateral meristems a cyme or raceme, respectively, will be formed. Thus, changes in the spatio-temporal control of floral or nonfloral meristem identity may, at least in theory, account for the three main inflorescence architectures (Prusinkiewicz et al. 2007).

Genetic analysis in Arabidopsis and Antirrhinum, both of which generate racemose inflorescences, has led to the identification of a set of genes that determine where and when flowers are formed (for reviews see Jack 2004; Krizek and Fletcher 2005; Smyth 2005; Blazquez, Ferrandiz, Madueno, and Parcy 2006). The genetic control of other inflorescence types (i.e., cymes and panicles), however, has not been studied in much detail, and hence the mechanisms that led to diversification of inflorescence architecture remain poorly understood (Benlloch et al. 2007; Koes 2008).

As Petunia has a cymose inflorescence, like most other *Solanaceae*, and is amenable to forward and reverse genetic analysis, it offers an excellent opportunity to address this question. Here we briefly review the progress that has been made in the genetic analysis of inflorescence development in Petunia.

9.2 The Switch from Vegetative Growth to Flowering

The aerial plant body is generated from a group of stem cells located in the apical shoot meristem. All higher plants, and Petunia is no exception, pass through a vegetative growth phase during which they generate leaves and axillary meristems that develop into side branches (see Chapter 8) before they switch to the reproductive, or flowering, phase. However, the timing of this switch can differ substantially among species. Distinct species may flower in different periods of the year (seasons) or at different plant ages. Some species flower the same year in which they germinate (annuals) and then die; others flower in the second year, after a cold winter period (biannuals); while trees, for example, may take several years to flower and from then on flower every year (perennials).

To determine whether it is time to flower, plants measure a number of environmental and endogenous parameters and somehow integrate these signals. It was realized early on that day length, or photoperiod, is an important parameter by which plants measure progression through the seasons (Garner 1922; Garner 1933).

That is, some plants flower only when the light period (day) is longer than a critical threshold value (long-day plants); others when day length is shorter than the threshold (short-day plants); and yet others independently of day length (day-neutral plants). Many plants species require in addition a cold period, that is, winter, before they are capable of flowering, a phenomenon known as vernalization.

Not surprisingly, the molecular mechanisms that control flowering are best understood in Arabidopsis (for reviews see Mouradov, Cremer, and Coupland 2002; Simpson and Dean 2002; Parcy 2005). Genetic analysis identified four distinct pathways: the photoperiod and vernalization pathways, which mediate environmental signals (day length, cold period, ambient temperature), and the gibberellin (GA) and autonomous pathways, which mediate hormonal and other endogenous cues. Acting at the nodes where signals from distinct flowering pathways converge are genes that are designated "floral pathway integrators," which sum (or subtract) the signals received by distinct pathways. Ultimately, these integrators activate floral meristem identity genes such as *LEAFY* (*LFY*) and *APETELA1* (*AP1*), which promote the floral fate of meristems (see Sect. 9.4.1 below).

The three main floral pathway integrators in Arabidopsis are *FLOWERING LOCUS T (FT), SUPPRESSOR OF OVEREXPRESSION OF CONSTANS* (*SOC1*, also known as *AGAMOUS-LIKE 20*), and *LEAFY* (*LFY*). *FT* is expressed in all organs and is regulated by the photoperiodic pathway and by ambient temperature (Kardailsky et al. 1999; Kobayashi, Kaya, Goto, Iwabuchi, and Araki 1999; Balasubramanian, Sureshkumar, Lempe, and Weigel 2006). *FT* encodes a mobile protein that can move from leaves, where photoperiod is perceived, to the apex, where it promotes the formation of flowers (Corbesier et al. 2007; Jaeger and Wigge 2007; Mathieu, Warthmann, Kuttner, and Schmid 2007). Within the apex FT binds to the transcription factor FD and promotes transcription of the floral meristem identity gene *AP1* (Abe et al. 2005; Wigge et al. 2005). *SOC1* mediates signals from the GA and photoperiod pathways. It is expressed in the shoot apex and floral meristems and encodes a MADS-box transcription factor that activates *AP1* (reviewed by Parcy 2005). *LFY* encodes a unique plant-specific transcription factor and integrates signals from the GA and photoperiod pathways (Weigel, Alvarez, Smyth, Yanofsky, and Meyerowitz 1992; Parcy, Nilsson, Busch, Lee, and Weigel 1998; Blazquez and Weigel 2000). *LFY* acts both as an integrator of flowering signals and as a floral meristem identity gene, as flowers in *lfy* mutants are partially transformed into shoot-like structures (Weigel et al. 1992; see also Sect. 9.4.1 below).

The control of flowering time in Petunia has not been studied in much detail and, consequently, our knowledge is rather fragmentary. As Petunia cultivars or lines do not require a cold period for flowering, a vernalization pathway is lacking. Several studies, using different Petunia cultivars or inbred lines, showed that when flowering time is measured in days – which is of interest to commercial growers – or as number of nodes preceding the first flower – which is more relevant for developmental studies – flowering occurs much earlier in long days than in short (Cathey and Campbell 1984; Adams, Pearson, Hadley and Patefield 1999; Snowden and Napoli 2003; Roobeek, Kusters, Castel and Koes, unpublished). Mutants of the *DWARF7* gene (*DW7*) remain very compact during the vegetative phase (i.e., with very short

internodes), but can be readily and fully rescued by spraying with GA, suggesting a defect in GA synthesis. With few exceptions *dw7* plants fail to form flowers unless they are sprayed with GA, suggesting a role for GA in the onset of flowering. Thus, flowering in Petunia seems to respond at least in part to cues that are similar to those used in Arabidopsis.

Molecular genetic experiments have identified several genes that affect flowering time in Petunia and hence may be involved in perception, mediation, or integration of flowering signals. However, no attempt has been made to analyze how these genes interact and cooperate, and thus any regulatory interactions and the complete pathways remain obscure. Nevertheless, data suggest that even though flowering in Petunia and Arabidopsis depends on similar environmental cues, the network that recognizes and integrates these cues in Petunia has diverged substantially from that of Arabidopsis on several points.

Over the years many random mutagenesis screens have been performed using either EMS or endogenous transposons as a mutagen. These screens yielded a wealth of mutants in inflorescence architecture (see Sect. 9.4 below) and other aspects of the plant (e.g., flower color, leaf pigmentation, dwarfing, etc.), but to our knowledge only one flowering-time mutant, *veggie*, was recovered. The *veggie* mutants not only flower much later than wild type (Fig. 9.2; Roobeek and Koes, unpublished) but also have thicker stems and altered inflorescence architecture (see Sect. 9.4.2 below). *VEGGIE* was recently isolated and a detailed molecular analysis of its role in flowering is underway.

Fig. 9.2 Phenotypes of the late flowering *veggie* mutant and a wild-type plant. *Arrows* indicate the floral transition points. The inset in the *bottom left corner* shows a detail of the veggie inflorescence. Note that the lateral shoot emerging at the periphery of the flower generates leaves instead of a flower

Reverse genetic analyses of an extensive set of Petunia genes encoding MADS box proteins identified two genes, *UNSHAVEN* (*UNS*, also known as *FBP20*) and *PETUNIA FLOWERING GENE* (*PFG*, also known as *FBP10*) that appear to promote the onset of flowering. Ubiquitous expression of *UNS*, which is highly similar to *SOC1* from Arabidopsis, resulted in early flowering (Ferrario et al. 2004). Removal of an N-terminal domain including the MADS box resulted in a protein that failed to enter the nucleus, indicating lack of activity. Expressed ubiquitously in Petunia, the truncated *UNS* acts as a dominant negative inhibitor and causes a slight delay in flowering time.

In Arabidopsis, flowering signals ultimately activate the MADS box meristem identity gene *AP1*, and ubiquitous expression of *AP1* driven by the viral *35S* promoter is sufficient to trigger flowering even in the absence of LFY (Mandel and Yanofsky 1995). The Petunia genome contains at least three genes – *FBP26*, *FBP 29*, and *PFG* – that encode AP1-like MADS box proteins, although none of them appears to be a true ortholog of *AP1* or of *SQUAMOSA* from Antirrhinum (see Chapter 10). Ubiquitous expression of *AP1* had no effect in Petunia (Rebocho 2007). Downregulation of *PFG* by RNA interference, in contrast, severely inhibited the switch from vegetative growth to flowering. In plants with a strong RNAi phenotype flowering was completely blocked, while lines with a weaker phenotype produced occasional flowers and then reverted to vegetative growth (Immink et al. 1999). Because a *dTph1* transposon insertion that interrupted the coding sequence of *PFG* did not cause a change in phenotype (Vandenbussche et al. 2003), it appears that the *PFG* RNAi phenotype is due to downregulation of multiple (off-target) genes, a common problem associated with RNAi. Indeed, *FBP26* expression is abolished in *PFG* RNAi plants (Immink et al. 1999), but on its own this cannot explain the phenotype, as an *fbp26 dTph1* insertion mutant is phenotypically normal (Vandenbussche et al. 2003). Although these findings suggest that the onset of flowering in Petunia requires one or more *AP1*-like MADS box genes, which may be partially or fully redundant, the critical genes remain to be identified.

In Arabidopsis, transcription of the floral pathway integrator and meristem identity gene *LFY* is upregulated during the late vegetative phase in response to distinct flowering signals (Blazquez, Soowal, Lee, and Weigel 1997; Hempel et al. 1997), and 35S promoter-driven ubiquitous expression of *LFY* suffices to trigger precocious flowering in Arabidopsis (Weigel and Nilsson 1995). Ubiquitous expression of *LFY* also causes early flowering in distantly related species such as aspen and citrus trees, suggesting that its role as a mediator and integrator of flowering signals is widely conserved (Weigel and Nilsson 1995; Pena et al. 2001). However, in Petunia the *LFY* homolog *ABERRANT LEAF AND FLOWER* (*ALF*) is already strongly expressed in leaf primordia during the early vegetative phase, and ubiquitous expression of *ALF* or *LFY* has no effect, indicating that flowering signals in Petunia are not mediated by transcriptional activation of *ALF* (Souer et al. 2008). It appears that in Petunia flowering signals are mediated by activation of a distinct meristem identity gene, *DOUBLE TOP* (*DOT*), because ubiquitous expression of *DOT* or its Arabidopsis homolog *UNUSUAL FLORAL ORGANS* (*UFO*) in Petunia results in

extremely early flowering (Souer et al. 2008). *DOT* encodes an F-box protein that is thought to activate ALF by a post-translational mechanism (see Sect. 9.4.1 below). Thus to induce flowering, Arabidopsis upregulates LFY activity by transcriptional activation of *LFY*, while in Petunia induction is achieved by transcriptional activation of *DOT* followed by post-translational activation of ALF.

9.3 Architecture and Development of the Petunia Inflorescence

The Petunia inflorescence has a typical cymose architecture and consists of an indefinite number of metamers (or anthoclades), each composed of a flower, a shoot that bears the next metamer, and two leaf-like organs named bracts or prophylls, with dormant shoots in their axils (Fig. 9.3; Napoli and Ruehle 1996; Souer et al. 1998). The two meristems in the bract axils (#2 and #3 in Fig. 9.3B) have a vegetative identity (i.e., upon outgrowth they form a number of leaves before switching to flowering) and both are dormant, though to slightly different extents: the vegetative meristem in the axil of the α bract (meristem #2 in Fig. 9.3B) forms a small shoot several millimeters in size before it becomes arrested, while vegetative shoot #3 in the axil of the β bract remains smaller, not readily evident to the unaided eye. We refer here to the meristem that forms the next anthoclade as a “lateral” rather than a “sympodial” meristem, which is the term used by Napoli and Ruehle (1996). The terms are largely synonymous, but we have noticed that “sympodial” is easily confused with the sympodial meristem of tomato, a vegetative meristem arising in the axil of the leaf below the inflorescence that forms 2–4 leaves and the next inflorescence. Moreover, the term “lateral” meristem fits more easily with the nomenclature in a unified theoretical model for inflorescence development (Prusinkiewicz et al. 2007).

There is some controversy in the literature regarding interpretation of the different elements of the Petunia inflorescence. Napoli and Ruehle (1996) described the macroscopic structure of the inflorescence from the inbred Petunia line V26, much taller than the W138 line that was analyzed by others (Souer et al. 1998; Maes et al. 2001), with generally longer internodes. In the mature V26 inflorescence, the two bracts (α and β) are separated by a small stem section of several millimeters, which was interpreted by Napoli and Ruehle (1996) as a compressed internode. Napoli and Ruehle (1996) view shoot #1 as an axillary shoot that is equivalent to that in the axil of the α bract, but with a different identity (“sympodial” rather than vegetative) and growth rate (accelerated). Consequently, they view the vegetative shoot #3 in the axil of the β bract as an “accessory” meristem and omitted this shoot from their inflorescence diagrams.

Subsequent analysis of the ontology of distinct meristems and gene expression patterns suggested a somewhat different view and revealed an essential difference between the lateral IM (#1) and the vegetative meristems #2 and #3 in the time and place of their initiation. Initiation of the lateral IM #1 takes place within the apical meristem dome, and is accompanied by the expression of *NO APICAL MERISTEM* (*NAM*) as a stripe that marks the border of the incipient floral meristem (FM) at the

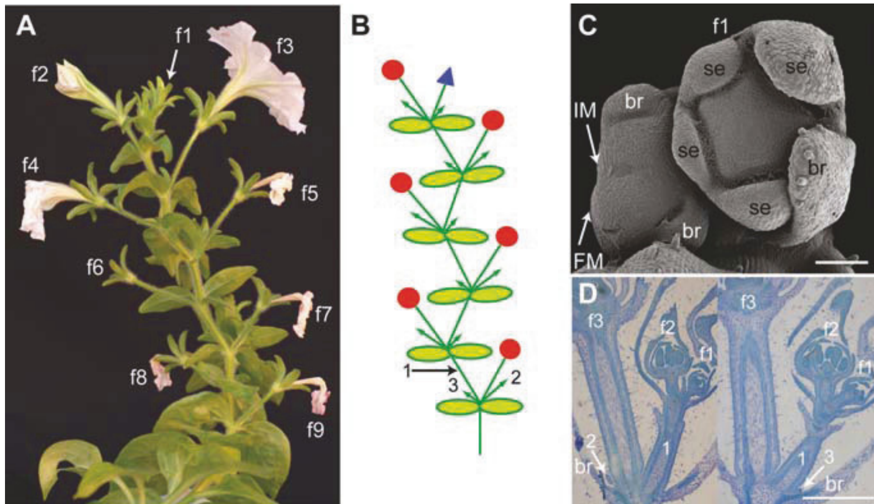

Fig. 9.3 The inflorescence architecture of Petunia. (**A**) The Petunia cymose inflorescence; f1–f9 designate flowers, from young to old. (**B**) Schematic representation of the Petunia inflorescence. In the cymose Petunia, the apical meristem forms two bracts and terminates into a flower. The continuity of the inflorescence depends on a lateral meristem formed on the flank of the apical meristem that forms the next inflorescence shoot (**#1**) reiterating the process. In the axil of each bract an axillary meristem is formed; that on the flower side (**#2**) is less dormant than that on the inflorescence shoot side (**#3**). *Circles* indicate flowers; *triangles*, the apical meristem. (**C**) Scanning electron micrograph of a inflorescence apex, showing a flower (f1) in which all the sepals (se) have already formed and the apex bears two bracts (br) supporting the apical floral meristem (FM), which will form the next flower, and the lateral inflorescence meristem (IM), which will develop the next inflorescence shoot (scale bar 100 μm). (**D**) Two sections through an inflorescence apex showing three flowers from young to old (f1–f3) and the two bracts (br) that support the older flower and the inflorescence shoot (**#1**). On the axil of the bract supporting the flower the less dormant axillary meristem forms the first leaves (**#2**), while on the axil supporting shoot **#1**, the axillary meristem forming the dormant vegetative shoot **#3** is just visible (scale bar 1 mm)

apex and the lateral IM (Souer, van Houwelingen, Kloos, Mol, and Koes 1996). At this stage the floral meristem identity gene *ABERRANT LEAF AND FLOWER* (*ALF*) is expressed in the incipient floral meristem and bract primordia, while the incipient IM is seen as a zone that lacks *ALF* expression (Souer et al. 1998). Slightly later, the lateral IM becomes visible as a separate dome next to the somewhat larger FM (Fig. 9.3C; Souer et al. 1998; Maes et al. 2001). At this stage the FM has not yet initiated floral organ primordia and still expresses *TERMINATOR* (*TER*), the homolog of *WUSCHEL* from Arabidopsis and a marker for meristem activity (Ferrario, Shchennikova, Franken, Immink, and Angenent 2006). Thus, development of the lateral IM is well underway before the apical FM terminates. Initiation of the lateral IM is in many ways similar to the initiation of floral meristems in a racemose inflorescence, like that of Arabidopsis and Antirrhinum, which is consistent with the hypothesis that diversification of inflorescence types has resulted largely from changes in the spatio-temporal control of meristem identity (Prusinkiewicz et al. 2007).

Vegetative meristems #2 and #3 in the bract axils initiate much later than the lateral IM. In the axils that are near the apex their initiation cannot be detected by SEM (Fig. 9.3C; Souer et al. 1998; Maes, Van Montagu, and Gerats 1999; Reinhardt and Kuhlemeier 2002) or by expression of marker genes such as *NAM* (Souer et al. 1996). Initiation of these vegetative meristems first (and simultaneously) becomes evident some 2 weeks after the bracts are formed, by which time the apex has already generated several additional flowers (Fig. 9.3D). The zone where these vegetative meristems initiate is so far below the apex that it is not typically visible in micrographs of the inflorescence apex. Shortly after their initiation, meristems #2 and #3 in turn initiate primordia for several leaves and then are arrested in growth. The dormancy of these meristems is at least in part due to auxin, which is synthesized in more apical regions. Manual removal of the inflorescence apex or mutation of *FLOOZY*, which encodes a flavin mono-oxygenase involved in auxin synthesis, results in precocious outgrowth of meristems #2 and #3 (Tobeña-Santamaria et al. 2002). Thus, the development of meristems #2 and #3 resembles in many ways that of the meristems in the axils of leaves.

9.4 Genetic Control of Inflorescence Architecture

Correct development of the Petunia inflorescence depends critically on several developmental processes. First it requires that the apical meristem acquire floral identity. This acquisition of floral identity involves changes in the pattern in which new organ primordia are formed (whorled instead of spiral) as well as in their identity (floral organs instead of leaves or bracts, Blazquez et al. 2006). Second, it requires initiation of a lateral meristem and tight control of its identity. That is, floral identity of this meristem should be transiently repressed, or delayed, to enable the formation of a secondary lateral IM before it acquires floral identity, which would result in the formation of a panicle instead of a cyme (Prusinkiewicz et al. 2007).

By mutational analyses several genes have been identified that are involved in (i) identity of the apical FM or the lateral IM or (ii) initiation and outgrowth of meristems and primordia. It is noteworthy that several genes that were initially classified as meristem identity genes based on their mutant phenotypes turned out to be involved in meristem initiation or proliferation and vice versa. Below the roles of these genes are discussed in more detail.

9.4.1 Genes Specifying the Identity of the Floral Meristem

Several mutants have been identified in which the apical FM fails to acquire floral identity and instead develops as a lateral IM that will initiate two bracts and an additional lateral meristem. The endless reiteration of this program results in a bushy inflorescence that contains only bracts and meristems but lacks flowers. The mutants *aberrant leaf and flower* (*alf*) and *double top* (*dot*) were identified by random

transposon mutagenesis and display a nearly complete transformation of flowers into inflorescence shoots (Doodeman, Gerats, Schram, de Vlaming, and Bianchi 1984; Souer et al. 1998; Souer et al. 2008). EMS mutagenesis in line V26 yielded a mutant, *arborescent* (*arb*), with a very similar phenotype, but it remains unknown whether it represents an allele of *ALF* or *DOT* or an additional gene (Napoli and Ruehle 1996). Molecular analysis showed that *ALF* is an ortholog of *FLORICAULA* from Antirrhinum and *LEAFY* (*LFY*) from Arabidopsis (Souer et al. 1998). LFY is a transcription factor that binds to *cis*-regulatory elements in A-, B-, and C-type organ identity genes to activate their expression (for review see Lohmann and Weigel 2002; Blazquez et al. 2006). *ALF* and *LFY* encode proteins with highly similar sequences that are exchangeable in functional transgenic assays, indicating that ALF acts in the same way (Maizel et al. 2005; Souer et al. 2008).

The expression patterns of *LFY* and *ALF* are, however, widely divergent, resulting in partially distinct functions in the spatio-temporal regulation of floral meristem identity. Inactive during early vegetative development, *LFY* is activated by distinct pathways that mediate flowering signals (Blazquez et al. 1997; Blazquez, Green, Nilsson, Sussman, and Weigel 1998; Blazquez and Weigel 2000). Within the inflorescence, activation of *LFY* in lateral FMs specifies their floral fate, while expression in the apical IM is repressed by *TERMINAL FLOWER1* (*TFL1*, Bradley, Ratcliffe, Vincent, Carpenter, and Coen 1997). If *LFY* is activated in the apical IM, as in *tfl1* mutants or in *35S:LFY* transgenics, the inflorescence terminates with a solitary flower (Alvarez, Guli, Yu, and Smyth 1992; Weigel and Nilsson 1995). In Petunia, however, *ALF* is already expressed early in the vegetative phase in leaf primordia, and in the inflorescence it is expressed in the apical FM but not in the emerging lateral IM (Souer et al. 1998). The divergent patterns of expression of *ALF* and *LFY* in the inflorescence are in accord with the hypothesis that racemes and cymes diverged through alterations in the spatio-temporal control of meristem identity (Prusinkiewicz et al. 2007; Koes 2008). However, because ubiquitous expression of *ALF* or *LFY* in Petunia has no phenotypic effect, the altered *LFY* and *ALF* expression patterns are at best only part of the story (Souer et al. 2008).

Molecular analysis showed that *DOT* encodes an F-box protein and is orthologous to *FIMBRIATA* of Antirrhinum and *UNUSUAL FLORAL ORGANS* (*UFO*) of Arabidopsis (Souer et al. 2008). Arabidopsis work suggested that *UFO* is a partially dispensable coregulator of *LFY* that is required for activation of B-type organ identity genes, responsible for specifying the identity of petals and stamens (Lee, Wolfe, Nillson, and Weigel 1997). The *ufo* mutants display defects in petal and stamen development (Levin and Meyerowitz 1995; Wilkinson and Haughn 1995); ubiquitous expression of *UFO* leads to the formation of flowers with supernumerary petals and stamens and leaves with serrated margins (Lee et al. 1997). Work in Petunia, however, suggested that *UFO* and *DOT* have a much wider function, which is partially masked in Arabidopsis due to redundancy of *UFO* (Souer et al. 2008). First, in *dot* mutants FMs are transformed into IMs, indicating that *DOT* is fully required to specify floral meristem identity. Second, constitutive expression of *DOT* in Petunia leads to extremely early flowering, and converts the cymose inflorescence into a solitary enlarged flower. This suggests that if the lateral meristem (anlage)

acquires floral identity precociously, the entire apical dome, including the lateral IM anlage, turns into a single flower. Furthermore, ubiquitous expression of *DOT* results in ectopic expression of floral organ identity genes and various homeotic transformations, including partial conversion of leaves into petals. Thus, transcriptional activation of *DOT* appears to be a major factor determining where and when flowers will be made in Petunia.

The *35S:DOT* phenotype in Petunia is in many ways comparable to that of *35S:LFY* or *35S:ALF* in Arabidopsis. Apparently, the limiting factor that determines where and when flowers are formed in Petunia is the expression of *DOT*, and in Arabidopsis that of *LFY*. This regulatory switch in the spatio-temporal regulation of flower formation appears to be largely due to alterations in the expression patterns of *ALF/DOT* and *LFY/UFO*. In Arabidopsis, *UFO* is expressed in the apical meristem throughout the vegetative phase (Lee et al. 1997; Long and Barton 1998) and hence activation of *LFY* is sufficient to induce the precocious formation of flowers (Fig. 9.4). In Petunia, on the other hand, *ALF* is expressed throughout the vegetative phase and *DOT* is inactive; hence flower formation is limited by the transcriptional regulation of *DOT*.

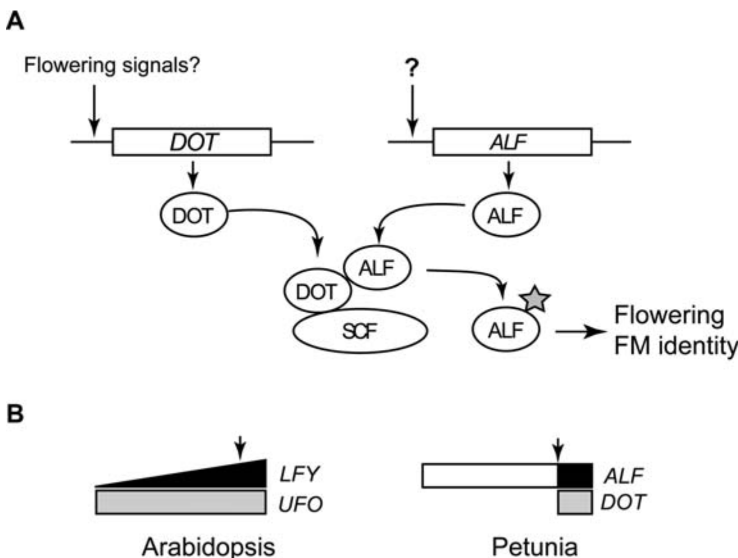

Fig. 9.4 Model for the functions of ALF and DOT. (**A**) *DOT* expression is triggered by unknown flowering signals and *ALF* regulators are still unknown. When ALF and DOT proteins are in the same cells, they interact via a SCF E3-ubiquitin ligase complex, which results in the post-transcriptional activation of ALF, possibly via ubiquitination (*star* attached to ALF), resulting in floral induction and specification of floral meristem identity. (**B**) Although *LFY* and *ALF* (and *UFO* and *DOT*) are homologs, their patterns of expression, and therefore roles in floral induction (indicated by arrow), differ. In Arabidopsis *UFO* is expressed throughout the vegetative phase, and activation of *LFY* induces the floral transition. In Petunia, *ALF* is produced from early in the vegetative phase, requiring *DOT* function for floral induction

F-box proteins such as DOT and UFO are the adaptor (target-specifying) components of SCF complexes, which act as E3 ubiquitin ligases. This is supported by the findings that DOT and UFO interact in yeast two-hybrid assays with SKP1-like proteins, which are core components of SCF complexes (Samach et al. 1999; Souer et al. 2008). Moreover, biochemical and genetic evidence indicate that UFO acts in vivo within the context of a SCF complex that is associated with the COP9 signalosome (Zhao, Yang, Solava, and Ma 1999; Wang et al. 2003; Ni et al. 2004). Ubiquitination generally leads to proteasome breakdown, which initially suggested that *UFO* might be involved in targeting a repressor of floral organ identity genes for degradation. However, yeast two-hybrid and biochemical experiments show that UFO and DOT can physically interact with ALF and LFY (Souer et al. 2008; Chae, Tan, Hill, and Irish 2008). Given that DOT activity is fully dependent on ALF and vice versa, this suggested that the role of DOT is to *activate* ALF by a post-translational mechanism.

Experiments in yeast have shown that the transcription activation domains of several transcription factors are positively regulated by specific F-box proteins (for review see Muratani and Tansey 2003; Kodadek, Sikder, and Nalley 2006). Although the mechanistic details are largely unclear, the available evidence suggests that transcriptional activation is tightly linked to ubiquitination and degradation of the transcription factor, which is presumed to occur within the chromatin. Chromatin immunoprecipitation assays indicated that UFO binds in vivo to the promoter of the B-gene *APETALA3*, in a LFY-dependent manner (Chae et al. 2008). Furthermore, Chae et al. (2008) claimed that UFO induces the formation of high molecular weight isoforms of LFY in Arabidopsis, some of which react with anti-ubiquitin antibodies. However, because the abundance of these protein species is only partially reduced in the absence of UFO, and because they were not seen in Petunia seedlings that ubiquitously express ALF and DOT or LFY and UFO (Souer et al. 2008), the significance of this finding remains to be established.

9.4.2 Genes Specifying Identity of the Lateral Inflorescence Meristem

Several mutations that disrupt development of the lateral IM or compromise its identity have been identified. In the mutants *sympodial* (*sym*), *extrapetals* (*exp*), and *hermit* (*her*), the cymose inflorescence is reduced to a solitary flower (Napoli and Ruehle 1996; Souer et al. 1998, Castel and Koes, unpublished). Molecular and genetic analyses indicate that *sym*, which was identified by EMS mutagenesis in the line V26, and *exp*, which was obtained by random-transposon mutagenesis in W138, are allelic (Procissi and Koes, unpublished).

The inflorescence of *alf exp* double mutants consists of a straight unbranched stem bearing an unlimited number of green leaf- or bract-like organs (Souer et al. 1998). This structure was at first (incorrectly) interpreted as being an inflorescence that lacks flowers (due to *alf*) and lateral shoots (due to *exp*), and suggested a role for

EXP in the initiation of the lateral IM. However, recent molecular work has shown that this interpretation of the *alf exp* phenotype and, therefore the inferred function of *EXP*, is incorrect and strongly suggested that the role of *EXP* is to repress floral identity in the lateral IM (Procissi, Castel, Kusters, and Koes, unpublished). Thus, the solitary flower phenotype of *exp* seems due to precocious activation of floral identity in the lateral IM, similar to the defect associated with *35S:DOT*. A role for *EXP/SYM* as a repressor of FM identity is consistent with the observation that *sym* mutants flower earlier than wild type (Snowden and Napoli 2003).

The loss-of-function phenotype suggests that *VEGGIE* acts, in part, as an antagonist of *EXP*. In *veggie* mutants, the lateral IM initiates normally, but instead of generating the next flower and lateral IM, it develops as a vegetative shoot that generates up to 10 leaves before terminating with the formation of a flower and a new lateral IM (Fig. 9.2; Roobeek and Koes unpublished). The *veggie* phenotype is remarkably similar to that of weak *PFG* RNAi mutants. Because RNAi inhibition is variable in time, Immink et al. (1999) interpreted this phenotype as a "reversion" from reproductive to vegetative growth. However, as the same is seen in stable *veggie* mutants, we favor a different explanation, viz. at each cycle lateral IMs of wild-type plants undergo a switch from vegetative to floral fate similar to that of the primary apical meristem.

9.4.3 Genes Specifying Initiation and Outgrowth of the Lateral IM

Thus far two genes, *HERMIT* (*HER*) and *EVERGREEN* (*EVG*), that are involved in the initiation and/or the proliferation of the lateral IM have been identified. Curiously, their loss-of-function phenotypes resemble those of *exp* and *alf*, which have defects in the specification of IM and FM identities, respectively, underscoring that gene function is not always easily inferred from a macroscopic loss-of-function phenotype alone.

In *her* mutants the cymose inflorescence is converted into a solitary flower, similar to what is seen in *exp/sym* mutants (Fig. 9.5C), although *her* flowers display various aberrations, such as nonfused petals, petaloid anthers, and malformed carpels, which are not seen in *exp/sym* flowers. Molecular and genetic evidence indicates that the *HER* gene plays a role in the initiation of the lateral IM similar to that which was initially (and incorrectly) proposed for *EXP* (Castel and Koes, unpublished).

The phenotype of *evg* resembles that of *alf* in that flowers are missing and bracts and meristems over-proliferate (Fig. 9.4F; Rebocho et al. 2008). An important difference is that in *evg* the two inflorescence stems that form at each node often remain fused and form a single fasciated stem. Initially *evg* was thought to be a floral meristem identity mutant not much different from *alf* or *dot*, and the observed fasciation was considered to be a secondary effect. However, more detailed analysis of the *evg* phenotype and molecular analysis of the *EVG* gene indicated that it is, in fact, the other way round: fasciation is a primary effect of the mutation, while the loss of FM identity is a secondary, indirect effect (Rebocho et al. 2008).

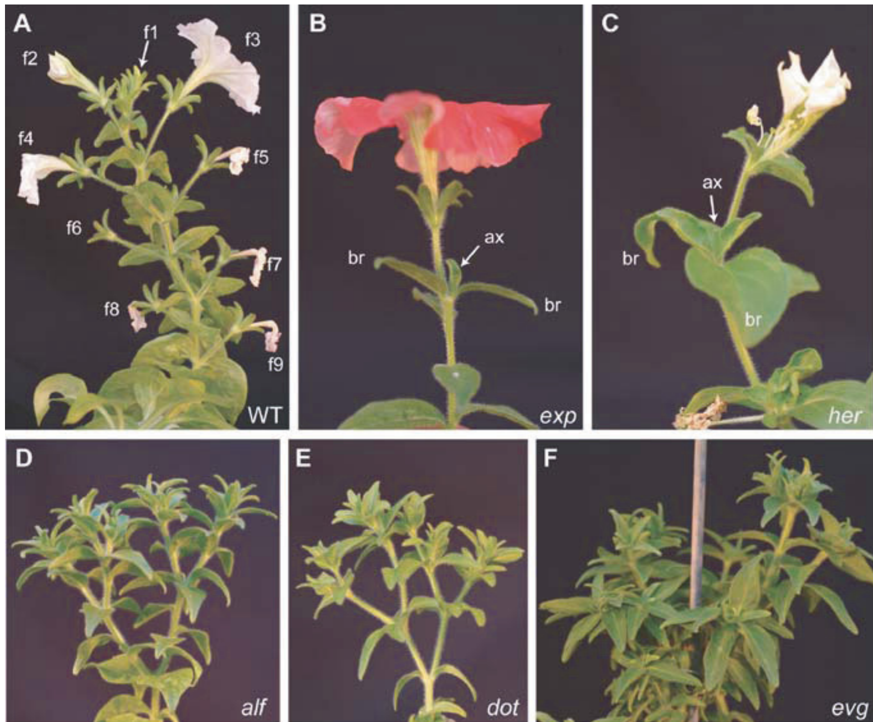

Fig. 9.5 Phenotypes of Petunia inflorescence mutants in a W138 genetic background. (**A**) Wild-type inflorescence; flowers from young to old (f1–f9) are indicated; (**B**) *exp* phenotype. The inflorescence has two bracts (br) supporting only one flower. The next flower will be formed from the axillary meristem (ax) and will be again solitary; (**C**) *her* phenotype, similar to that of the *exp* mutant. Bracts (br) support only one flower and the axillary meristems (ax); (**D**) *alf* phenotype. All the apical FMs have been converted into inflorescence meristems that behave like lateral meristems, which give rise to the next shoot and two new inflorescence meristems, thereby creating a bushy phenotype; (**E**) *dot* phenotype, similar to that of *alf*; (**F**) *evg* phenotype. In addition to the transformation of flowers into inflorescence shoots, inflorescence stems of an *evg* mutant fail to separate properly and grow fasciated

Molecular isolation and characterization of *EVG* showed that it encodes a transcriptional factor with a WUSCHEL-RELATED HOMEOBOX (WOX) domain (Rebocho 2007). The most closely related Arabidopsis genes, *STIMPY* (*STIP*) and *STP-LIKE* (*STPL*), are expressed in all Arabidopsis meristems and promote cell division and proliferation of stem cells (Wu, Dabi, and Weigel 2005; Wu, Chory, and Weigel 2007). In the Petunia inflorescence *EVG* is expressed exclusively in the early IM anlage before it becomes visible as a separate meristem dome (Rebocho et al. 2008). Moreover, the meristem domes in *evg* inflorescences are enlarged and often contain multiple domains that express *TER*, which marks the organizing center of meristems (Stuurman, Jaggi, and Kuhlemeier 2002; Ferrario et al. 2006), and *ALF* or *EVG*, which mark cells with FM and IM identities, respectively. This suggests

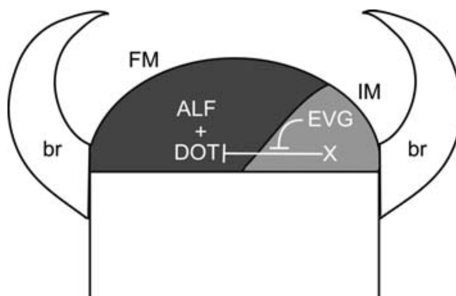

Fig. 9.6 Model for function of EVG in the inflorescence apex. The new lateral inflorescence meristem cells (IM, *light gray*) express *EVG* and a mobile X factor that prevents the expression of *DOT* in the apical floral meristem (FM, *dark gray*), where *ALF* is also expressed. Once *EVG* promotes the separation of the lateral meristem from the apical meristem, movement of X from the IM to the FM is reduced and *DOT* is expressed in the apical FM to specify, together with ALF, floral fate

that *evg* meristem domes consist of multiple fused FMs and IMs, and that the role of *EVG* is to promote the proliferation of a lateral IM shortly after its initiation.

The loss of FM identity in *evg* mutants could be largely attributed to a failure to activate *DOT* (Rebocho et al. 2008): in *evg* mutants *DOT* expression is strongly reduced, and forced expression of *DOT* from the viral 35S promoter is sufficient to restore the formation of flowers. Multiple lines of evidence suggest that *EVG* promotes *DOT* expression indirectly. First, *EVG* is expressed within the emerging IM, while *DOT* is expressed within the apical FM. Second, the *exp* and *her* mutations fully suppress the FM-identity defect in *evg*, and consequently *exp evg* and *her evg* mutants exhibit a phenotype (solitary flowers) similar to that of *exp* and *her* single mutants. Thus *EVG*, in contrast to *ALF* and *DOT*, is not needed for flower formation in a solitary-flower mutant background (*exp*, *her*).

Figure 9.6 presents a model that explains the role of *EVG*. This model assumes that the incipient IM synthesizes an unknown factor "X" that inhibits the specification of floral identity. "X" is likely to be mobile, able to move into the apical FM region and repress *DOT* expression. It need not necessarily be an excreted signal molecule, as other proteins like transcription factors have been shown to move between meristem cells (Sessions, Yanofsky, and Weigel 2000; Wu et al. 2003). It is assumed that *EVG* somehow blocks the inhibitory effect of "X" on the FM and thereby enables expression of *DOT*. EVG may act by promoting proliferation of the IM, resulting in physical separation of the FM and IM. However, if initiation or identity of the IM is compromised, as in *her* and *exp* mutants, synthesis of "X" does not take place and *EVG* is no longer needed to enable *DOT* expression and specification of floral identity in the apical FM.

9.5 Concluding Remarks

The picture emerging from a comparison of gene functions in Petunia and Arabidopsis is that their divergent cymose and racemose inflorescence architectures are controlled by homologous "toolkit" genes. The proteins encoded by these genes seem to have undergone few changes in function, as they are functionally exchangeable in

most experiments. It is, rather, the gene expression patterns and regulatory interactions that have undergone major changes; these in turn are most likely to have been caused by alterations in *cis*-acting regulatory elements within these genes, although that remains to be proved. To reconstruct how and when these genetic alterations occurred cannot be inferred from a simple comparison of Arabidopsis and Petunia (or tomato), in part because they are not closely related, and will require analysis of a wider variety of species that diverged more recently. The extent to which the genes and alterations identified thus far can account for the evolution of distinct inflorescence structures can, in the end, be tested only by recreating the steps one-by-one in either a racemose (Arabidopsis) or cymose (Petunia) background. Such a test will comprise a fair evaluation of the extent to which these changes account for the observed anatomical divergence.

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Chapter 10
Evolution and Development of the Flower

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Abstract The development of the angiosperm flower has been an important morphological innovation in plant evolution. Through studying the molecular basis of flower development in different model species we are offered insights into the diversification of developmental networks that underly the vast array of angiosperm floral morphologies. The evolution of the MADS-box transcription factor family appears to play a pivotal role in this process.

10.1 Introduction

Plant evolution has produced a range of morphological innovations, including the development of vascular tissue, the emergence of the seed, and the appearance of flowering plants. Flowering plants, or angiosperms, are presently the most successful group of land plants, and a large part of their success is thought to be due to their unique reproductive structures, the flowers. Flowers enabled the angiosperms to develop insect-mediated pollination, which may have become a driving force in their rapid radiation. Flower development and its evolution together are therefore a major field of research in evolutionary developmental biology ('evo-devo', e.g., Scutt, Theissen, and Ferrandiz 2007). By comparing developmental processes between different species, evo-devo aims at determining the evolutionary relationships between organisms and understanding the evolution of developmental processes.

The molecular basis of flower development was initially studied extensively in the model species *Arabidopsis thaliana* and *Antirrhinum majus* (Schwarz-Sommer,

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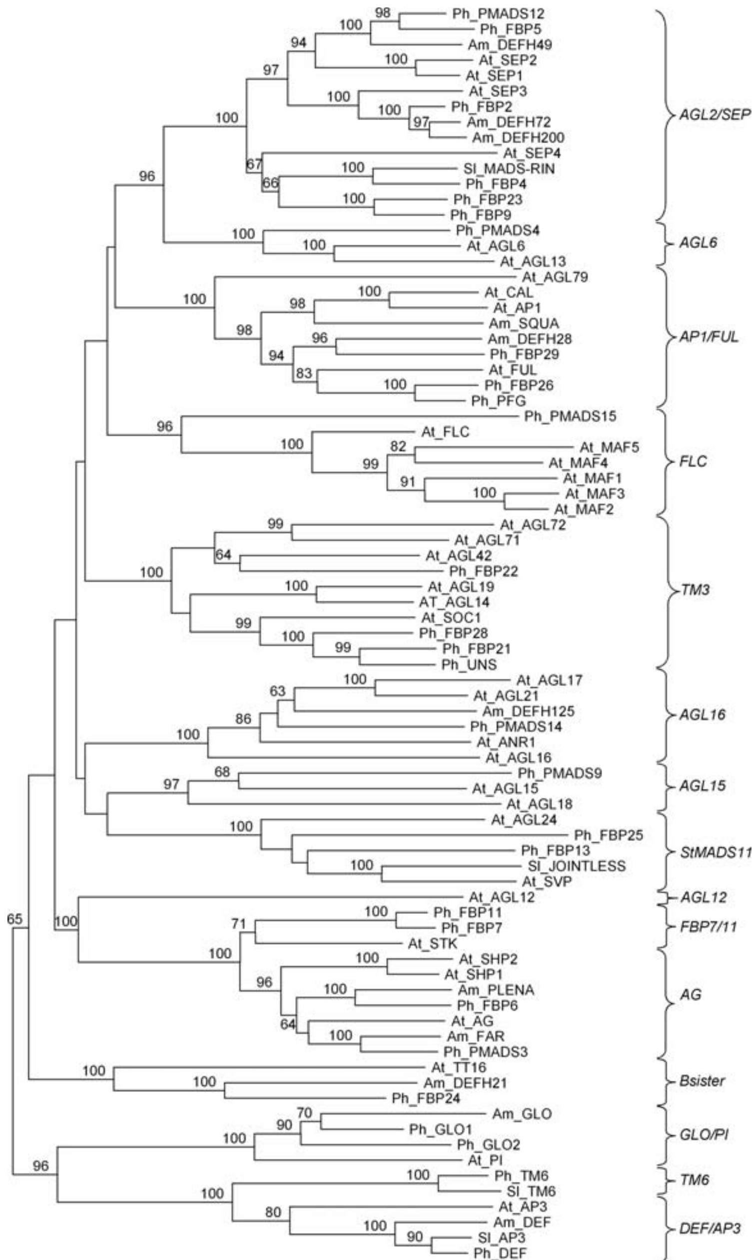

Fig. 10.1 Neighbor-joining tree of MIKC-type MADS-box genes from Petunia, Arabidopsis, and selected other species. One thousand bootstrap samples were generated to assess support for the inferred relationships. Local bootstrap probabilities (in percentages) are indicated near the branching points for branches with >60% support. Partial sequences available for Petunia genes *pMADS6*, –10, –16, and –17, and *PhFL* were not included. The tree was obtained using methods described previously (Vandenbussche et al. 2003b)

Huijser, Nacken, Saedler, and Sommer 1990; Coen and Meyerowitz 1991; Ma 1994; Davies, Cartolano, and Schwarz-Sommer 2006; Zahn et al. 2006). Research on these species soon indicated that a number of players in floral developmental pathways belong to the MADS-box gene family. All MADS-box transcription factors contain a highly conserved MADS domain at or near the N-terminus, involved in DNA binding (Ma, Yanofsky, and Meyerowitz 1991; Riechmann, Wang, and Meyerowitz 1996a).

A plant may harbor more than a hundred MADS-box genes, belonging to a range of functionally diverged subfamilies. Many of the MIKC-type MADS-box genes (Fig. 10.1) play an essential role in the determination of floral meristem and floral organ identity. Because MADS-box genes are homeotic selector genes – genes that determine how the parts of an organism develop – they are especially important in generating morphological novelty. The evolution of this gene family thus almost certainly played a central role in creating the enormous diversity in floral morphologies (Rijpkema et al. 2007).

Only by performing in-depth analyses on floral development in different species can we learn the extent to which developmental pathways controlling the formation of different floral forms have diversified or been conserved. Additional model species for molecular research on flower development are the monocots *Oryza sativa* and *Zea mays*; gymnosperms *Gnetum gnemon* and *Picea abies*; the dicot *Gerbera hybrida*, a member of the Asteraceae; and of course our favorite plant: *Petunia hybrida* (e.g., Tandre, Albert, Sundås, and Engström 1995; Winter et al. 1999; Becker, Saedler, and Theissen 2003; Gerats and Vandenbussche 2005; Furutani, Sukegawa, and Kyozuka 2006; Kater, Dreni, and Colombo 2006; Malcomber, Preston, Reinheimer, Kossuth, and Kellogg 2006; Teeri, Elomaa, Kotilainen, and Albert 2006a; Whipple and Schmidt 2006; Vazquez-Lobo et al. 2007).

10.2 The Petunia Flower

Petunia forms a cymose inflorescence and, with the transition to flowering, exhibits sympodial growth (Souer et al. 1998; Angenent et al. 2005; see Chapter 9). At the base of each flower two bracts are formed, each with a dormant (vegetative) meristem in its axil. A wild-type Petunia flower consists of five sepals, five petals, five stamens, and two carpels arranged in four concentric whorls. The five petals are fused. Stamen filaments are partly fused to the tube of the flower. The flower is zygomorphic in all floral whorls (Knapp 2002). Ovules have a single integument (Angenent et al. 1995a). The fruit is a conical capsule containing different numbers of seeds for different Petunia species (Gunn and Gaffney 1974; Sink and Power 1978). The mature fruit is surrounded by an enlarged, glandular hairy calyx composed of five lobes (the sepals) which are equal to or longer than the capsule, depending on the species (Gunn and Gaffney 1974). The genus *Petunia* features extensive floral diversity in color, petal tube length, and scent, and these differences are related to diverse pollination syndromes within the Petunia clade (see Chapters 2, 3 and 13).

10.3 Molecular Studies on Flower Development

In the early 1990s the ABC model of flower development was formulated based on mutants of Arabidopsis and Antirrhinum (Coen and Meyerowitz 1991), although in the first version of the Antirrhinum model no A-function was described (Schwarz-Sommer et al. 1990). As proposed in the well-known ABC-model, A-function genes alone specify sepal identity; A- plus B-function genes together control petal identity; B- plus C-function genes together control stamen identity; and the C-function gene alone specifies carpel identity. Moreover, A-function genes repress the expression of the C-function gene and vice versa (Coen and Meyerowitz 1991; Weigel and Meyerowitz 1994). While the formulation of the ABC model has boosted research on flower development enormously, the classical model by now seems outdated, as only B and C functions turn out to be truly conserved. Thus, a model without A function should be reconsidered (Schwarz-Sommer et al. 1990; Davies et al. 2006; Litt 2007). Moreover, within a few years of the postulation of the ABC model, research on Petunia led to the extension of the model with a D function, necessary for ovule specification (Angenent et al. 1995b; Colombo et al. 1995: see Chapter 11); this function was later also described for Arabidopsis (Favaro et al. 2003; Pinyopich et al. 2003).

Another important addition to the ABC model was the recognition of a subfamily of MADS-box genes, required for the formation of all floral organs, named E-function genes. The first indications for the existence of such a function came from research on Petunia and tomato (Angenent, Franken, Busscher, Weiss, and van Tunen 1994; Pnueli, Hareven, Broday, Hurwitz, and Lifschitz 1994; see below and Chapter 11). Over the years the majority of the Petunia MIKC-type MADS-box genes have been isolated, and many of them are analyzed functionally (see Chapter 11). An overview of all identified Petunia MADS-box genes and their known functions is given in Table 10.1.

Table 10.1 Overview of all isolated Petunia MADS-box genes with accession codes, functions, and references

Subfamily	GeneName	Genbank Accession	Function	Ref.
AP1/FUL	*PFG* (*FBP10*)	AF176782, AY370518	promote floral transition maintain IFM identity	a,b
	FBP26 (*pMADS5*)	AF176783, AY370512		a,b
	FBP29	AF335245		b
	PhFL	AY306170		c
DEF/AP3	*PhDEF* (*GP, pMADS1*)	X69946, DQ539416	petal, stamen development	d,e,f,g
	PhTM6 (*FBP8*)	AF230704, DQ539417	stamen development	g,h
GLO/PI	*PhGLO1* (*FBP1*)	M91190, AY532265	petal, stamen development	h,i,j,k,l
	PhGLO2 (*pMADS2*, *FBP3*)	X69947, AY370521	petal, stamen development	d,e,h

Table 10.1 (continued)

Subfamily	GeneName	Genbank Accession	Function	Ref.
Bsister	*FBP24* (*pMADS7*)	AF335242, AY370514	ovule, seed development	m,n
FBP7/11	*FBP7*	X81651, U90137	ovule development	o,p,q,r
	FBP11	X81852, EF179142	ovule development	n,o,p, q,r,s,t
AG	*FBP6*	X68675, AY370525		p,t,u,v
	pMADS3 (*FBP14*)	X72912, AB076051	stamen, carpel development FM determinacy	v,w,x,y
AGL2	*FBP2* (*PhSEP3*)	M91666	petal, stamen, carpel development, FM determinacy	f,i,p,t,u,z
	FBP4	AF335234		b,f,z
	FBP5 (*PhSEP1*)	AF335235	petal, stamen, carpel development, FM determinacy	b,f,p,z
	FBP9	AF335236		b,f,p,z
	FBP23	AF335241		b,f,z
	pMADS12	AY370527, AY370515		f,z
AGL6	*pMADS4* (*FBP18*)	AB031035, AY370517		b
TM3	*UNS* (*FBP20*)	AF335238	promote floral transition	b,aa
	FBP21	AF335239		b
	FBP22	AF335240		b
	FBP28 (*pMADS8*)	AF335244		b
	pMADS10	AY370522		
	pMADS16	AY370520		
AGL15	*pMADS9*	AY370526, AY370510		
AGL17	*pMADS14*	AY370528, AY370513		
pMADS6	*pMADS6*	AY370524		
	pMADS15	AY370529 AY370516		
	pMADS17	AY370523		
StMADS11	*FBP13*	AF335237		b
	FBP25	AF335243		b
	OPU9-2	AF315464	promote adventitious shoot	bb

Immink et al. (1999); Immink et al. (2003); Litt and Irish (2003); Kush et al. (1993); van der Krol et al. (1993); Vandenbussche et al. (2003); Rijpkema et al. (2006); Vandenbussche et al. (2004); Angenent et al. (1992); Angenent et al. (1993); Angenent et al. (1995a); Canas et al. (1994); de Folter et al. (2006); Nougalli Tonaco et al. (2006); Angenent et al. (1995b); Colombo et al. (1995); Colombo et al. (1997); Cheng et al. (2000); Immink et al. (2002); Ferrario et al. (2006); Angenent et al. (1994); Kater et al. (1998); Tsuchimoto et al. (1993); Kapoor et al. (2002); Kapoor et al. (2005); Ferrario et al. (2003); Ferrario et al. (2004); Prakash et al. (2003).

Instead of providing a complete summary of all the work performed on Petunia floral transition and floral organ patterning, we have chosen to focus on some clear examples of how the study of Petunia flower development has led to new insights in our understanding of the evolution and development of the eudicot flower.

10.4 Two Ways to Suppress C-Function Genes in the Perianth Whorls

In Arabidopsis two genes are generally considered to represent the A function (responsible for specification of sepal and petal identity and setting the boundary for C-function gene expression): the MADS-box gene *APETALA1* (*AP1*) and *APETALA2* (*AP2*), the only non-MADS-box gene in the ABC model (Haughn and Somerville 1988; Bowman, Smyth, and Meyerowitz 1989, 1991; Kunst, Klenz, Martinez-Zapater, and Haughn 1989; Irish and Sussex 1990; Bowman, Alvarez, Weigel, Meyerowitz, and Smyth 1993; Jofuku, Boer, Montagu, and Okamuro 1994). However, there has been increasing debate on whether the A function and its role in perianth identity as described in the ABC model actually exists, and questions about the universality of the role of *AP2* in C-function gene regulation (Maes et al. 2001; Davies et al. 2006; reviewed in Litt 2007). Recently published studies on Petunia and Antirrhinum mutants showing ectopic expression of C-function genes demonstrate that, at least for these two species, there is a different way to prevent C-function expression in the perianth whorls (Cartolano et al. 2007).

In this section, we shall first explain the actions of the originally designated Arabidopsis A-function genes, demonstrating why we might have to reconsider the classical A function responsible for specification of sepal and petal identity. We shall then focus on the role of the Arabidopsis *AP2* gene in C-function gene regulation and illustrate how Petunia and Antirrhinum employ different machineries to suppress C function in the perianth whorls.

Arabidopsis *ap1* mutants fail to develop sepals and petals; instead the outer two whorls of the flower are homeotically transformed into bract-like structures, occasionally with carpelloid features at higher temperatures (Irish and Sussex 1990; Bowman et al. 1993). Secondary flowers arise in the axils of these first whorl bracts. From these secondary flowers tertiary flowers may arise in the same manner and so on, thus resulting in a complex branched structure (Irish and Sussex 1990; Bowman et al. 1993). Both the homeotic change from sepals to bract-like organs and the formation of secondary flowers are consistent with a function for *AP1* in the transition from a branched inflorescence to a determinate flower. *AP1* achieves its role in promoting floral meristem identity by repressing *AGAMOUS LIKE24* (*AGL24,* a promoter of inflorescence fate) in the corpus of the meristem and in the developing sepal and petal primordia, together with *LEAFY* (Yu, Xu, Tan, and Kumar 2002). In *ap1* mutants it is the overexpression of *AGL24* that is responsible for many aspects of the *ap1* floral phenotype, including defects in the development of first and second whorl floral organs. Some floral organ defects of *ap1-1* mutants, especially the absence of petals, can be partly rescued by the absence of *AGL24* in an *ap1/agl24* double mutant (Yu, Ito, Wellmer, and Meyerowitz 2004). The expression domain of the C-function gene *AGAMOUS* (*AG*) is not affected in the *ap1* mutant, indicating that *AP1* has no role in C-regulation. Thus, even though at first look the mutant phenotype suggests a function for *AP1* in sepal and petal formation, its role is rather in floral transition and determination of the identity of the floral meristem.

The other Arabidopsis A-function gene is *AP2*, an *AP2/ERF* transcription factor (Jofuku et al. 1994; Kim, Soltis, Wall, and Soltis 2006). The *ap2* mutant phenotypes vary among alleles and with temperature, but all show disrupted sepal and petal development (Komaki, Okada, Nishino, and Shimura 1988; Kunst et al. 1989; Bowman et al. 1991; Jofuku et al. 1994). At normal temperatures the two outer floral whorls of the weak *ap2-1* mutant are transformed into leaf-like structures, while at higher temperatures the first whorl organs acquire more carpelloid characteristics as the second whorl organs become stamenoid, indicating ectopic expression of the C-function gene *AG* (Bowman et al. 1989; Mizukami and Ma 1992). These phenotypes point to two functions for *AP2*: determining floral meristem identity to enable the formation of floral organs, and suppressing action of the C-function gene *AG* in the first two floral whorls so that the perianth, consisting of sepals and petals, can be formed. Recently, *AP2* has also been implicated in floral transition, control of seed size, and shoot meristem maintenance (Okamuro, Szeto, Lotys-Prass, and Jofuku 1997; Jofuku, Omidyar, Gee, and Okamuro 2005; Ohto, Fischer, Goldberg, Nakamura, and Harada 2005; Wurschum, Gross-Hardt, and Laux 2006). The expression of *AP2* itself is regulated by a microRNA, *AtmiR172*. The accumulation of *AtmiR172* in the inner two whorls of the flower causes the repression of *AP2* through translational inhibition (Chen 2004).

In conclusion, the Arabidopsis A-function genes *AP1* and *AP2* are both required for determining floral meristem identity. Without functional *AP1* and *AP2*, sepals and petals fail to develop; instead, outer whorl organs develop with a vegetative or inflorescence character (bract/leaf-like organs and secondary flowers). This means a discrete perianth identity function, as described in the classical ABC-model (Coen and Meyerowitz 1991), is not required and we should reconsider the alternative model for genetic specification of floral organ identity, as this model is consistent with the available data (Schwarz-Sommer et al. 1990; Davies et al. 2006).

10.4.1 Regulation of C-Function Genes

Another important function of *AP2* is to restrict expression of the C-function gene *AG* to the inner two floral whorls, thus enabling perianth formation. While originally the functioning of *AP2* in C-function gene regulation was considered universal, in recent years contradictory data have accumulated. In Petunia three *AP2* genes have been isolated, and one of them, *PhAP2A*, is a molecular ortholog of the Arabidopsis *AP2* gene, as it is able to complement the Arabidopsis *ap2-1* mutant phenotype. However, Petunia *phap2* mutants do not exhibit a mutant phenotype in floral development; this is unlikely to be a case of redundancy, as the nearest paralog, *PhAP2B* belongs to the *AINTEGUMENTA* clade (Maes et al. 2001; Kim et al. 2006). Thus *PhAP2A*, the Petunia ortholog of the Arabidopsis *AP2* gene, seems not to be involved in C-function gene regulation. The two Antirrhinum *AP2* orthologs have also been shown not to be involved in C-function gene regulation (Keck, McSteen, Carpenter, and Coen 2003).

Classical mutants of both Petunia and Antirrhinum exhibiting features of ectopic C-function gene expression, however, have been known for many years (Vallade, Maizonnier, and Cornu 1987; Tsuchimoto, van der Krol, and Chua 1993; McSteen, Vincent, Doyle, Carpenter, and Coen 1998; Motte, Saedler, and Schwarz-Sommer 1998). Several candidate gene approaches were tried to identify the mutation which causes the petal-to-stamen conversion of the Petunia *blind* (*bl*) mutant, without success (Kater et al. 1998; Tsuchimoto, Mayama, van der Krol, and Ohtsubo 2000; Maes et al. 2001; Mayama, Ohtstubo, and Tsuchimoto 2003). As shown in Fig. 10.2, *bl* flowers display a homeotic conversion of the corolla limb into antheroid structures. The phenotype is quite variable, and can, under certain conditions, even show homeotic conversions of the tip of the sepals into carpelloid tissue. The petal tube, on the other hand, is never affected; only the petal limb is converted into antheroid tissue (Vallade et al. 1987).

The *bl* mutant phenotype is caused by ectopic expression of the C-function genes *PETUNIA MADS BOX GENE3 (PMADS3)* and *FLORAL BINDING PROTEIN6 (FBP6)* in the first two floral whorls (Tsuchimoto et al. 1993; Kater et al. 1998). This also explains why ectopic expression of *pMADS3* in transgenic Petunia leads to a phenocopy of the *bl* mutant, that is, the formation of antheroid structures on petal limbs and carpelloid tissue on sepals (see Fig. 10.2 and Tsuchimoto et al. 1993; Kater et al. 1998).

The discovery of a transposon insertion mutant with the *bl* mutant phenotype gave a long-sought entry into the molecular background of this mutant. The *bl* mutants and the Antirrhinum *fistulata* (*fis*) mutants, which have similar phenotypes, are recessive alleles of two homologous miRNA-encoding genes (Cartolano et al. 2007). *BL* and *FIS* encode miR169 family genes; *miRBL* is required to restrict C-function gene expression to the inner two floral whorls. The repression

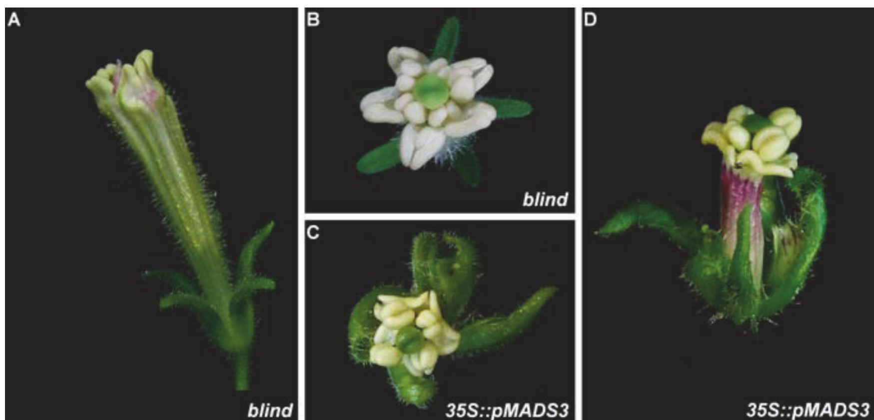

Fig. 10.2 Phenotypes of *blind* mutants and *pMADS3* overexpressors. Both *blind* mutant flowers **(A+B)** and flowers of *35S::pMADS3* overexpressors **(C+D)** show conversion of petals to stamens, although the petal tube remains unaffected. 35S::*pMADS3* flowers exhibit a more severe phenotype, with sepals displaying more carpelloid characteristics

of C function genes is not direct however, as C-function transcripts lack the miR169 microRNA recognition site that can bind to the short complementary sequence of the microRNA itself, thereby targeting the transcript for cleavage or translational inhibition. These microRNA recognition sites can be found, however, in members of the NF-YA gene family. Cartolano et al. (2007) propose a model in which *BL* mediates partial repression of these NF-YA transcription factors, which are potentially needed for upregulation or maintenance of C-function gene expression. Feedback regulation of the *miRBL* gene by the C-function genes is postulated to keep C-function gene expression at a desired level in the inner two whorls. Fine tuning can be achieved by a feedback loop between the microRNA and its direct targets (NF-YA factors, Cartolano et al. 2007).

In *bl* and *fis* mutants the microRNA-governed fine-tuning control is impaired and early C-function gene expression increases primarily within the domain where C-function genes are normally active. Lateral extension of the C-function gene domain would result from transmission of excessive C gene products to daughter cells during cell divisions, reinforced by threshold-dependent autoregulation (Cartolano et al. 2007). Further investigations are necessary to reinforce this model for C-function gene regulation by *miRBL* in Petunia and *miRFIS* in Antirrhinum acting through the NF-YA genes.

In any case it appears that in Arabidopsis on the one hand, and in Petunia and Antirrhinum on the other, independent mechanisms have evolved to serve the same function. Remarkably, the elements of the miR169-NF-YA machinery are also present in Arabidopsis, while the AP2-miR172 elements can be found in Antirrhinum and Petunia. It will be interesting to find out if these complementary mechanisms have lost some or all function, and/or acquired another.

10.5 Molecular Evolution of Genes for the Petal Identity Program

One of the key innovations in plant evolution has been the petal, tightly associated with a transformation in pollination mechanisms that involved the recruitment of insects as pollen carriers (Crepet 2000; see Chapter 2). It remains unclear whether the core eudicot petals derived from bracts, like sepals, or from sterile stamens (e.g., Ronse de Craene 2007). The molecular and functional evolution of genes encoding these organ identity programs in relation to the evolution of the flower during the radiation of the angiosperms is therefore of particular interest. The genes encoding petal and stamen identity programs are called B-function genes. Their functions were first elucidated by studies on Arabidopsis and Antirrhinum. In both species there are two B-function genes, *APETALA3* (*AP3*) and *PISTILLATA* (*PI*) in Arabidopsis, and *DEFICIENS* (*DEF*) and *GLOBOSA* (*GLO*) in Antirrhinum, that in combination are involved in determining stamen and petal identity.

Phylogenetic analysis of *AP3* and *PI* orthologs from a wide variety of plant species has enabled reconstruction of the evolutionary history of B-function genes.

The *DEF/AP3* and *GLO/PI* gene lineages are thought to have arisen from a gene duplication that has been dated from 260 MYA (Kim et al. 2004) to 290 MYA (Hernandez-Hernandez, Martinez-Castilla, and Alvarez-Buylla 2006), thus predating the origin of the angiosperms and the divergence of angiosperms and gymnosperms. The fact that extant gymnosperms possess *AP3/PI* homologs with sequence motifs that suggest an ancestral preduplication form confirms this dating (Winter et al. 2002). Within the angiosperms, the *DEF/AP3* lineage has undergone another gene duplication that resulted in two paralogous lineages, eu*AP3* and *TOMATO MADS BOX GENE6 (TM6)* (Kramer and Irish 2000). This duplication coincides with the radiation of the core eudicots, and is estimated to have happened about 92 MYA (Hernandez-Hernandez et al. 2006).

The two types of DEF/AP3-like proteins resulting from the diversification that followed the eu*AP3/TM6* duplication can easily be distinguished by their completely different C-terminal motifs, the so-called paleoAP3 and euAP3 motifs. Proteins containing a paleoAP3 motif can be found throughout the basal angiosperms, monocots, magnoliid dicots and lower and core eudicots, while euAP3-motif-containing proteins are found only in the core eudicots. Some core eudicots have both a eu*AP3* gene copy (eu*AP3* lineage) and the original paleo*AP3* gene copy (*TM6* lineage), while other eudicots – such as Arabidopsis and Antirrhinum – have lost the paleo*AP3* gene. The euAP3 C-terminal motif presumably originated from the paleoAP3 motif by a simple frameshift mutation, due to a single nucleotide deletion in one of the copies of the ancestral paleo*AP3*-type gene (Vandenbussche et al. 2003a; Kramer, Su, Wu, and Hu 2006). It has been hypothesized that eu*AP3* genes then acquired a novel function, most likely in petal development (Kramer, Dorit, and Irish 1998).

Arabidopsis and Antirrhinum both carry one eu*AP3*-lineage gene (*AP3* and *DEF*, respectively) and one *GLO/PI* gene (*PI* and *GLO*, respectively). Both *ap3* and *pi* single mutants in Arabidopsis and *def* and *glo* single mutants in Antirrhinum show the same homeotic transformations of petals to sepals and stamens to carpels. This is in accordance with the activity of the encoded proteins, DEF and GLO in Antirrhinum (Trobner et al. 1992) and AP3 and PI in Arabidopsis, as obligate heterodimers (Goto and Meyerowitz 1994; Jack, Fox, and Meyerowitz 1994; Krizek and Meyerowitz 1996a; McGonigle, Bouhidel, and Irish 1996; Riechmann, Krizek, and Meyerowitz 1996b; Yang, Xiang, and Jack 2003a; Yang, Fanning, and Jack 2003b). The expression of either of the B-function genes is initiated independently (Honma and Goto 2000; Lamb, Hill, Tan, and Irish 2002), but the maintenance of high levels of DEF and GLO or AP3 and PI depends upon the presence of the heterodimeric protein complex (Schwarz-Sommer et al. 1992; Jack et al. 1994). This occurs by autoregulation: the AP3/PI heterodimer binds CArG boxes in the AP3 promoter to promote AP3 expression (Tilly, Allen, and Jack 1998), and the PI protein, though not directly binding its own promoter, is also involved in such a positive regulatory circuit (Honma and Goto 2000). In the case of *AP3*, the auto-regulatory loop is required only in stamens: *AP3* expression is still maintained in the sepal-like organs that replace petals in the *pi-1* mutant (Jack, Brockman, and Meyerowitz 1992). In Antirrhinum, in contrast, the *DEF* and *GLO* gene products are required

to positively regulate *GLO* and *DEF* gene expression, respectively, in both whorls 2 and 3 (Schwarz-Sommer et al. 1992; Zachgo et al. 1995).

10.5.1 Petunia B-Function Genes

While eudicot species like Arabidopsis have lost the paleo*AP3* gene copy, solanaceous species seem to have retained both the paleo*AP3/TM6* and the eu*AP3* gene copies (Rijpkema et al. 2006). So far, Petunia and tomato are the only species in which the functions of both the eu*AP3* and paleo*AP3* (*TM6*) genes have been studied. Functional analyses of these two genes in Petunia (*PhDEF* and *PhTM6*) and tomato (*TAP3* and *TM6*) show clearly diverged functions (de Martino et al. 2006; Rijpkema et al. 2006). Petunia and tomato also have two *GLO*/*PI* homologs, the result of a more recent gene duplication, possibly in the lineage leading to the Solanaceae (Hileman et al. 2006; Rijpkema et al. 2006). The functions of the four Petunia B-function genes were determined using transposon insertion mutants (for *PhGLO1*, *PhGLO2*, and *PhTM6*) and an EMS mutant (for *PhDEF*), and studying their phenotypes and the phenotypes of different double mutant combinations. Figure 10.3 provides an overview of the phenotypes of the Petunia B-function mutants and the most informative mutant combinations.

Together the two Petunia *GLO/PI* genes function in the same way as their Arabidopsis and Antirrhinum orthologs *PI* and *GLO* (Vandenbussche et al. 2004). The two genes act in largely redundant fashion in petal and stamen formation, consistent with their probably recent origin. Only the *phglo1 phglo2* double mutant shows full conversion of petals to sepals and stamens to carpels. Unique functions of PhGLO1 become visible, however, with careful study of the *phglo1* single mutants. As shown in Fig. 10.3, petal midveins of *phglo1* mutants are greenish (sepaloid) and stamen filaments are not fused to the petal tube as in wild-type Petunia flowers. This indicates that *PhGLO1* uniquely controls the formation of the petal midvein and fusion of stamens to the petal tube (Vandenbussche et al. 2004). Consistent with their functions, the two *PhGLO* genes are expressed in a typical B-function manner, specifically in the second and third floral whorls throughout floral development.

PhDEF expression is also confined mainly to the second and third floral whorls, although low levels of *PhDEF* mRNA can also be detected in the first and fourth whorls (Angenent et al. 1992; van der Krol et al. 1993; Tsuchimoto et al. 2000; Vandenbussche et al. 2004; Rijpkema et al. 2006). The *phdef* mutant, which displays a conversion of petals to sepals while the stamens remain unaffected, originally prompted the suggestion that the Petunia eu*AP3* function is different from that of its orthologs in other species (van der Krol et al. 1993; Weigel and Meyerowitz 1994). The *phdef phtm6* double mutant, however, exhibits full conversion of petals to sepals and stamens to carpels, demonstrating that this is not the case. It is rather the redundant role of *PhTM6* in stamen development that results in the single-whorl homeotic conversion seen in the *phdef* single mutant (Rijpkema et al. 2006). Thus *PhDEF* displays all characteristics that are typically associated with normal eu*AP3* gene function as described for *DEF* and *AP3*.

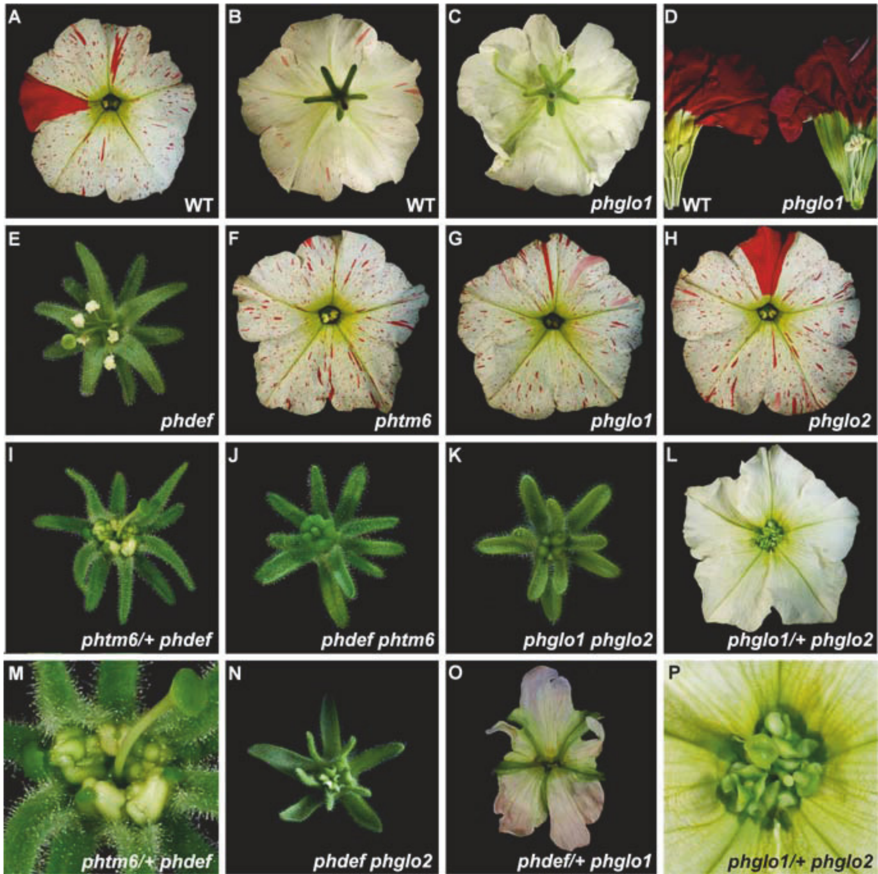

Fig. 10.3 Phenotypes of *P. hybrida* B-function mutants and mutant combinations. (**A**) and (**B**): Top (**A**) and bottom (**B**) views of wild-type W138 flower. (**C**) to (**H**): Top view of *phdef* (**E**), *phtm6* (**F**), *phglo1* (**G**), and *phglo2* (**H**) flowers; bottom view of *phglo1* flower showing green petal midveins (**C**); and (**D**) detail of *phglo1* flower showing freestanding stamen filaments (*right*), in contrast to the wild-type flower with stamens fused to the petals (*left*). (**J**), (**K**), and (**N**): Flowers of *phdef phtm6* (**J**), *phglo1 phglo2* (**K**), and *phdef phglo2* (**N**) double mutants displaying full homeotic conversion of petals to sepals in the second whorl and of stamen to carpels in the third whorl. Development of the central pistil is reduced in the *phglo1 phglo2* (**K**) and *phdef phglo2* (**N**) double mutants. (**I**) and (**M**): Top view (**I**) and detail (**M**) of a *phdef/+, phtm6* flower showing proliferated anther tissue terminating in short stigma/style-like structures in the third whorl. (**L**) and (**P**): Top view (**L**) and detail (**P**) of a *phglo1/+, phglo2* flower showing conversion of third whorl stamens to multiple carpelloid organs. (**O**): Flower of a *phdef/+, phglo1* mutant with clear sepal-like petal midveins and asymmetrically reduced petal tissue between these midveins

PhTM6 is expressed mainly in whorls three and four throughout flower development. Initially it is also expressed in the petal primordia, but expression fails to be strongly upregulated in later stages of development (Vandenbussche et al. 2004). The *phtm6* single mutant produces wild-type-like flowers. However, from the

phenotype of the *phdef phtm6* double mutant it is clear that *PhTM6* fulfills a function in stamen development that is redundant with that of *PhDEF* (Rijpkema et al. 2006). Moreover, it is obvious from the phenotypes of the *pdef* and *phtm6* single mutant and the *phdef phtm6* double mutant that *PhDEF* and *PhTM6* have divergent functions: the eu*AP3* gene *PhDEF* is involved in petal and stamen formation, while the paleo*AP3* gene *PhTM6* is involved only in stamen formation. The strong expression of *PhTM6* throughout ovule development suggests additional (redundant) functions in the fourth whorl, although this needs to be investigated. Yeast two-hybrid studies and analysis of the *phdef phglo2* double mutant uncovered more diversification in functions of Petunia B-function proteins (Vandenbussche et al. 2004). In the *phdef phglo2* double mutant (Fig. 10.3 N) stamens are fully replaced by carpels, indicating that the two remaining B-function proteins PhGLO1 and PhTM6 are insufficient to confer stamen identity. Yeast two-hybrid studies clearly show that, although PhDEF can interact with both PhGLO1 and PhGLO2, PhTM6 interacts specifically with PhGLO2 and to a much lesser degree, if at all, with PhGLO1. The absence of PhTM6-PhGLO1 heterodimers explains the lack of stamens in the *phdef phglo2* double mutant (Vandenbussche et al. 2004).

Knocking out either both DEF/AP3 partners or both GLO/PI partners leads to a full conversion of petals into sepals and stamens into carpels. Interestingly, in mutant-heterozygote combinations like *phtm6*/+, *phdef*; *phglo1*/+, *phglo2*; and *phglo1, phglo2*/+ obvious gene dosage effects are visible (Fig. 10.3). In addition the *phtm6*/+, *phdef* and *phglo1*/+, *phglo2* mutants give new functional information, as in both these mutant combinations a partial loss of determinacy can be observed in the third floral whorl. In *phtm6*/+, *phdef* flowers anthers develop as brain-like folded structures consisting of proliferating antheroid tissue and terminating in a short style/stigma-like structure (Fig. 10.3I and 10.3 M). In *phglo1*/+, *phglo2* flowers, on the other hand, the anthers are converted into proliferating carpelloid tissue (Fig. 10.3L, and 10.3P). The partial loss of determinacy in the third floral whorl of these mutants offers further support for roles of B-function genes in cell proliferation and determinacy (Bowman et al. 1992; Trobner et al. 1992; Jack et al. 1994; Sakai, Medrano, and Meyerowitz 1995; Krizek and Meyerowitz 1996a).

10.5.2 Regulatory Interactions

PhDEF expression is autoregulated and requires the presence of at least one of the *GLO*/*PI* homologs to maintain its own expression. The same applies to *PhGLO1* and *PhGLO2*; they too need the expression of at least one *DEF*/*AP3* gene. As *PhTM6* is not expressed at a high enough level in the second whorl, the *GLO*/*PI* genes cannot be maintained in this whorl in a *phdef* mutant background; they are expressed, however, in the third whorl of *phdef* mutant flowers due to complex formation of PhGLO2 with PhTM6 (Vandenbussche et al. 2004).

Interestingly, the maintenance of *PhTM6* expression does not depend on activity of the other B-function gene; nevertheless there is a connection between *PhTM6*

expression and *BLIND* (*miRBL*) function. In a *bl* mutant background *PhTM6* is strongly upregulated in the second whorl and, to a lesser extent, in the first. It remains to be determined whether *PhTM6* expression, like that of C-function genes, is repressed in the Petunia perianth organs by *BL*, or if *PhTM6* expression is actually dependent on the expression of C-function genes. The latter hypothesis is especially attractive, as Gomez-Mena et al. (2005) found that maintenance of *AP3* expression in Arabidopsis requires, in addition to the presence of a GLO/PI protein, either AG (stamens) or AP1 (petals). It could well be that upregulation of *PhTM6* expression after the initial activation in petal, stamen, and carpel primordia is possible only within the domain of C-function gene expression.

10.5.3 The Origin of Functional Diversity

Functional diversification during or after a gene duplication event can arise as a result of a change in the function of the protein encoded by one of the gene copies or through altered expression of a gene through a change in its promoter sequence. The clearly divergent expression patterns of *PhDEF* and *PhTM6* suggest that an important change in regulatory sequences must have taken place during or after the duplication that gave rise to the *TM6* and eu*AP3* lineages, at least in the evolutionary path leading to Petunia and tomato. A comparison of the promoter sequences of the *TM6* and eu*AP3* genes of Petunia, tomato, and several other eudicot species indicated that the *TM6*- and eu*AP3*-type genes contain conserved but different domains in their respective promoters (Rijpkema et al. 2006). A key question for future research is how and when during evolution eu*AP3* genes acquired the eu*AP3* 5′ regulatory element or, alternatively, *TM6* genes lost it.

Another important question is whether these promoter differences and the resulting divergent expression patterns of *PhDEF* (petals and stamen) and *PhTM6* (stamen and carpels) alone can account for the functional differences between the two genes. At the protein level, the most obvious difference between euAP3 and paleoAP3 is found in the completely distinct C-terminal motifs, which have been highly conserved within both lineages (Kramer et al. 1998). A study by Lamb and Irish (2003) pointed toward the indispensability of the C-terminal motifs, as constructs encoding truncated AP3 and PI proteins lacking these motifs were unable to rescue *ap3* or *pi* mutants when expressed in Arabidopsis. Using domain-swap experiments they also showed that the Arabidopsis euAP3 motif and the paleoAP3 motif of *Dicentra eximia* are not interchangeable, as the paleoAP3 motif retained a stamen-promoting activity but was unable to promote petal formation in that experimental situation (Lamb and Irish 2003). This suggested a possible role in evolution for the C-terminal motifs: could the new eu*AP3* gene have acquired a novel function in petal development through its new C-terminal motif? In contrast, another study demonstrated that a *PI* ortholog in pea that does not encode the PI motif is still capable of rescuing *pi* mutants when expressed in Arabidopsis (Berbel et al. 2005). Krizek and Meyerowitz had shown already in 1996 that a chimeric protein in which the AG

C-terminal domain was replaced by the AP3 C domain was normally functional as AG (Krizek and Meyerowitz 1996b). A recent thorough study by Piwarzyk, Yang, and Jack (2007) finally shed light on the situation: the euAP3- and PI-derived C-terminal motifs are not required for AP3 function in petal and stamen formation in Arabidopsis, and the previous contradictory results (Lamb and Irish 2003) are most likely due to suboptimal levels of the truncated AP3 and PI proteins (Piwarzyk et al. 2007). However, because C-terminal motifs are highly conserved it would be surprising if they had no function, even if they are apparently not crucial for the function of the Arabidopsis AP3 and PI proteins. They may, for example, enhance activities like protein complex formation, for which their presence is not essential, but which take place much more efficiently in their presence (Lamb and Irish 2003; Tzeng, Liu, and Yang 2004; Piwarzyk et al. 2007).

As Petunia harbors both paleo*AP3* and eu*AP3* genes it offered a highly attractive system for investigating the function of the paleoAP3 and the euAP3 motifs. The approach taken was to overexpress *PhTM6* under control of the 35S promoter in a *phdef* mutant background to determine if the presence of paleoAP3-type protein PhTM6 in all floral whorls could complement the eu*AP3* mutant *phdef* (Rijpkema et al. 2006). As shown in Fig. 10.4, *PhTM6* is capable of producing petals when overexpressed. In the *phdef* mutant background, *PhTM6* overexpression can almost completely rescue the *phdef* mutant phenotype, although the petals formed in these transgenic plants are not entirely normal. As the petal-promoting capacity of the paleo*AP3* type PhTM6 protein on its own seems not to differ qualitatively from that of the eu*AP3*-type protein PhDEF, the functional difference between *PhDEF* and *PhTM6* might be attributable mostly to differences in patterns of gene expression. This would be in agreement with the finding of Piwarzyk et al. (2007) that the eu*AP3* motif of AP3 is not essential for its function in petal and stamen formation. It will be interesting to perform both promoter and C-terminal domain swap experiments between *PhTM6* and *PhDEF* to analyze the relative influences of promoter and protein evolution on their functional differences.

Even though Petunia has four B-function genes, unlike the two of both Arabidopsis and Antirrhinum, the universal picture of regulation of petal and stamen formation by B-function genes is conserved. The DEF/AP3 and GLO/PI functions in Petunia are also interdependent. If either both *GLO/PI* representatives or both *DEF/AP3* representatives are nonfunctional, the result is a full homeotic conversion of petals to sepals and stamens to carpels, just as in the Arabidopsis and Antirrhinum *glo/pi* or *def/ap3* mutants. Nonetheless, the Petunia studies offer interesting new information on evolution, functional diversification, and on functions that cannot be studied in Arabidopsis. One example of this is the finding that PhGLO1 controls the fusion of stamens to the petal tube, a role for B-function genes which could not be seen in a species like Arabidopsis, which has freestanding stamens.

It remains unclear whether the roles played by the eu*AP*3 genes *PhDEF* and *TAP3* in petal development, in contrast to the lack of a role for the ancestral paleo*AP3* genes *PhTM6* and *TM6*, illustrate a case of neofunctionalization and, if so, whether this represents a Solanaceae-specific or a more general phenomenon. The other major remaining question addresses the origin of functional differences

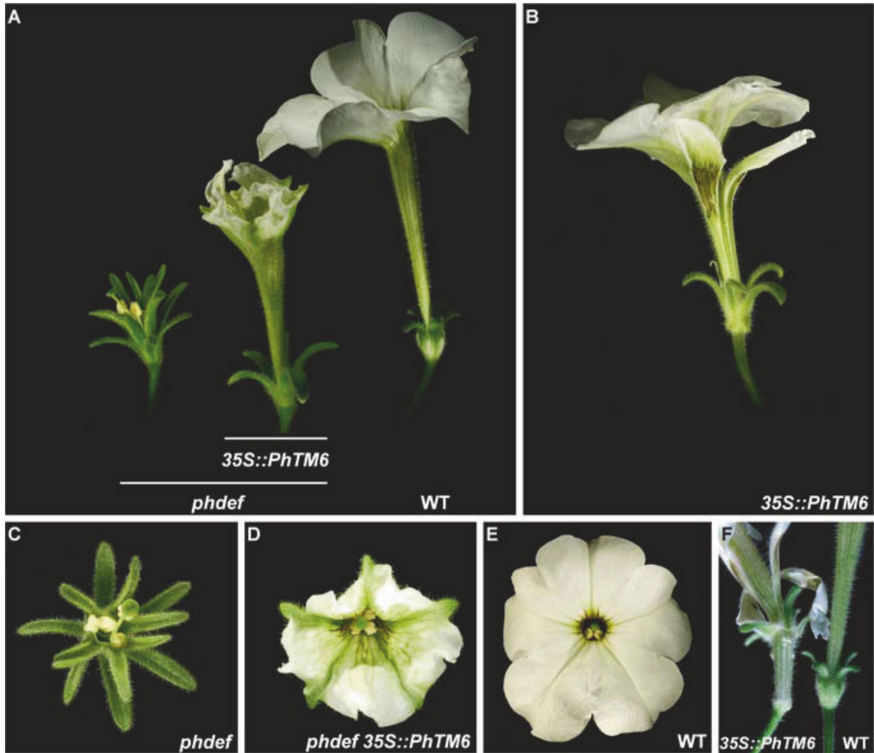

Fig. 10.4 *PhTM6* overexpression phenotypes in wild-type and *phdef* mutant backgrounds. (**A**) to (**D**): *35S::PhTM6* overexpression in a *phdef* mutant background shows almost complete complementation of petal development in the second whorl. The main petal veins retain sepal identity and stay green. (**E**): 35S::*PhTM6* overexpression in a wild-type background leads to the development of ectopic petals fused to the outer surface of the petal tube. These ectopic petals exhibit the typical venation pattern of the inside of normal petal tubes. (**F**): Detail of the first whorl organs of *35S::PhTM6* plants displaying a partial conversion to a structure similar to that of a petal tube. This petal-like structure at the base of the sepals senesces with the petals, whereas the green sepals on the top remain green throughout fruit development

between the Petunia *PhDEF* and *PhTM6* genes: can the promoter sequence difference alone account for this or do the divergent C-terminal motifs also play a role?

10.6 *Sepallata*: Redundancy and Functional Diversification

The *SEPALLATA* (*SEP*) MADS-box gene subfamily (previously called the *AGAMOUS Like2*/*AGL2* subfamily) is monophyletic (Fig. 10.1), and angiosperm-specific. The ancestor of the *SEP* genes may have existed in the common ancestor of the angiosperms and gymnosperms and been lost in the gymnosperm ancestor, as extant gymnosperms do not appear to carry *SEP* subfamily genes. Multiple *SEP*

homologs are present in distant angiosperm lineages, which suggests that the *SEP* subfamily experienced several early gene duplication events. There are two major clades, *AGAMOUS-LIKE9* (*AGL9*, *SEP*) and *AGL2/3/4* (*SEP1/4/2*), most likely the result of a preangiosperm duplication, as representatives of these two clades are already present in one of the most basal angiosperms, *Amborella* (Zahn et al. 2005). In the *AGL2/3/4* clade at least two gene duplications occurred after the origin of the eudicots but before diversification of the core eudicots. These duplication events resulted in three distinct clades: *AGL2* (containing both *SEP1* and *SEP2*), *AGL3* (*SEP4*), and *FBP9* (Zahn et al. 2005). Within the *AGL9* clade a single gene duplication seems to have occurred before the origin of the Asteraceae or during its diversification (Zahn et al. 2005). The redundancy caused by the many gene duplications has provided the *SEP* subfamily with a set of genes among which sub- or neofunctionalization could occur.

The three eudicot groups in which *SEP* genes have been most thoroughly studied using mutants and transgenics are Arabidopsis, Gerbera, and Petunia. From studies on these systems it became clear that, besides A-, B-, and C-function genes, proper flower development requires *SEP* gene expression. Petunia and tomato were the first species in which indications for a *SEP* gene function were presented, with the publication of a report on *SEP* gene cosuppression lines (Angenent et al. 1994; Pnueli et al. 1994). The subfamily name came, however, from Arabidopsis studies published several years later. The Arabidopsis triple *sep1 sep2 sep3* (*agl2 agl4 agl9*) mutant produces sepals in all floral whorls (hence the subfamily name *Sepallata*) and shows loss of meristem determinacy in the center of the flower (Pelaz et al. 2000). *SEP4* (previously known as *AGL3*) is the fourth *SEP* gene acting redundantly, and the quadruple mutant shows an even stronger phenotype than the *sep1 sep2 sep3* triple mutant. In *sep1 sep2 sep3 sep4* quadruple mutants all floral organs are replaced by leaves (Ditta, Pinyopich, Robles, Pelaz, and Yanofsky 2004). Thus, only the quadruple mutant exhibits a complete loss of floral organ identity. When expressed together with A-, B-, and C-function genes, *SEP* genes promote the conversion of Arabidopsis leaves into floral organs (Honma and Goto 2001).

While the Arabidopsis *SEP* genes have been reported to affect all floral whorls in an almost completely redundant fashion, the two described Gerbera *SEP* genes show clear subfunctionalization: *GERBERA REGULATOR OF CAPITULUM DEVELOPMENT1* (*GRCD1*) has a unique function in whorl three, while *GRCD2* has a unique function in whorl four (Kotilainen et al. 2000; Uimari et al. 2004). *GRCD1* is involved in stamen formation, as transgenic downregulation of *GRCD1* converts the sterile staminodes of the ray florets into petals (Kotilainen et al. 2000). Besides being required for the determination of carpel identity, *GRCD2* also plays a role in maintenance of floral meristem identity and the control of determinacy of the inflorescence meristem (Uimari et al. 2004; Teeri et al. 2006b).

Petunia harbors six *SEP* subfamily genes: *FLORAL BINDING PROTEIN2* (*FBP2*), *FBP4*, *FBP5*, *FBP9*, *FBP23* and *PETUNIA MADS BOX GENE12* (*pMADS12*) (Angenent et al. 1994; Ferrario et al. 2003; Immink et al. 2003; Vandenbussche et al. 2003b). The first functional studies performed on Petunia *SEP* genes used an *FBP2* cosuppression line (Angenent et al. 1994) in which *FBP2* and

FBP5 were both downregulated (Ferrario et al. 2003). These *FBP2* cosuppression plants exhibited a transformation of petals to sepaloid organs (much smaller than normal petals and completely green). The stamens were also replaced by green sepaloid structures and the placenta and ovules were affected or even fully absent and replaced by a new inflorescence (Angenent et al. 1994; Ferrario et al. 2003). Transposon insertion mutant analyses later demonstrated that the phenotype of *fbp2 fbp5* double mutants is less severe than that of *FBP2* cosuppression plants, indicating that in the latter at least one other MADS-box gene in addition to *FBP2* and *FBP5* must have been downregulated (Vandenbussche et al. 2003b).

So far a detailed functional analysis using transposon insertion mutants has been performed for only *FBP2* and *FBP5* (Fig. 10.5). The *fbp2* mutant displays a single mutant phenotype: the normal shaped petals exhibit a green hue, which is strongest in the areas surrounding the main veins and at the distal parts of the petals, indicating a petal-to-sepal conversion in these areas (Vandenbussche et al. 2003b). In addition, flowers of *fbp2* single mutants occasionally produce secondary flowers originating from the third whorl, suggesting a loss of floral meristem determinacy. Overexpression of *FBP2* in the Arabidopsis *sep1 sep2 sep3* mutant can almost completely complement the mutant phenotype (Ferrario et al. 2003). The *fbp5* single mutant is morphologically indistinguishable from wild type. The *fbp2 fbp5* double-

Fig. 10.5 Phenotypes of *P. hybrida SEPALLATA* mutants and mutant combinations. (**A**) Wild-type W138 Petunia flower. (**B**) *fbp2* mutant flower showing a green hue on the outer corolla due to a petal-to-sepal conversion in that area. (**C**) *fbp2 fbp5* double mutant flower showing an increased petal-to-sepal transformation and development of a huge pistil in the fourth floral whorl. Inside this pistil, leaf-like organs emerge from positions normally occupied by ovules. The anthers in the third whorl show a partial conversion to sepal-like organs

mutant flowers compared with *fbp2* flowers show a clearly enhanced phenotype, indicating functional redundancy, as observed in Arabidopsis. Petals of *fbp2 fbp5* mutants show an increased petal-to-sepal conversion compared with *fbp2* petals, and sepal-like structures covered by trichomes developed on top of the anthers. In the fourth whorl a huge pistil-like structure lacking transmitting-tract tissue develops, these structures covered with trichomes and consisting of two or more carpels that remain unfused at the top. Inside the carpels, leaf-like organs replace ovules, supporting a function for *FBP2* and *FBP5* in ovule development (Vandenbussche et al. 2003b). *FBP2* and *FBP5* functions in petal, anther, and carpel development are also largely redundant. In *fbp2 fbp5* double mutants the third whorl indeterminacy is not enhanced compared to that seen in *fbp2* single mutants (Vandenbussche et al. 2003b), demonstrating that *FBP2* has a unique function in maintaining determinacy in the third whorl. For the remaining four Petunia *SEP* genes diverse expression patterns and protein–protein interactions suggest more divergence in function (Ferrario et al. 2003; Immink et al. 2003).

10.6.1 Control of Meristem Determinacy

During the formation of floral organs, the center of the flower is kept in an indeterminate meristematic state, which is marked by expression of the meristem maintenance gene *WUSCHEL* (*WUS*). *WUS*, together with *Leafy* (*LFY*), induces *AG*. Subsequently, *AG* represses *WUS*, and failure to do so is reflected by continuous meristematic activity (indeterminacy) in *ag* mutants (Lenhard, Bohnert, Jurgens, and Laux 2001; Lohmann et al. 2001). In Petunia and Gerbera, downregulation of C-function genes (*pMADS3* and *GAGA2*, respectively) also leads to a loss of determinacy at the flower meristem level (Yu et al. 1999; Kapoor et al. 2002). The same loss-of-determinacy phenotypes are encountered in *SEP* mutants. Secondary flowers appear in Petunia *fbp2* mutants and downregulation of the Gerbera *GRCD2* gene results in reversion of ovaries to inflorescences (Vandenbussche et al. 2003b; Uimari et al. 2004). Besides C-function genes, the *SEP* or E-function genes thus seem to be involved in terminating the floral meristem. Ferrario and colleagues proposed that, in addition to C- and E-function genes, D-function genes might also be involved in control of the stem cell population in the floral meristem by downregulating WUS (Ferrario et al. 2006; see Chapter 11).

10.7 Concluding Remarks

A comparative analysis of floral development in a range of evolutionary well-positioned model systems can teach us how gene functions have developed, as well as provide information on the degree and mode of diversification in the regulation of flower development in the plant kingdom. Petunia flower development has been studied since the early 1990s, and research has uncovered quite

surprising divergence in the mechanisms involved. Moreover, Petunia is an ideal model species specifically for studying the molecular evolution of genes involved in petal identity programs. Performing a thorough functional analysis of the relevant MADS-box gene subfamilies in Petunia and other model species will certainly provide us with exciting information on the evolution of floral developmental networks.

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Chapter 11
Combinatorial Action of Petunia MADS Box Genes and Their Protein Products

Gerco C. Angenent and Richard G.H. Immink

Abstract During the last two decades enormous progress has been made in our understanding of the genes that control the identity of floral organs. These genes appear to be members of a large family of MADS box transcription factors that are well conserved across angiosperms. Research using Petunia as a model plant has contributed substantially to the discovery of novel MADS box gene functions and to our understanding of how these MADS box transcription factors act. The proteins function together in dimeric and possibly larger protein complexes to control the expression of target genes. This combinatorial action forms the basis of the ABC model for floral organ development and underlies many other developmental processes.

11.1 Introduction

Plant development is characterized by the formation of differentiated groups of cells originating from stem-cell containing meristems that are maintained throughout the life span of a plant. This differentiated group of cells acquires a specific identity, and subsequently the combinatorial action of cell division, cell growth, and direction of growth determines the final shape of an organ. The three major steps in plant development can be summarized as stem-cell maintenance, identity specification, and differentiation.

The pluripotent cells in the root apical meristem (RAM) are responsible for the growth of the underground root systems, while the above-ground structures are initiated by the shoot apical meristems (SAM). Both meristems are formed during embryogenesis. The SAM from Petunia produces leaves on its flank in an orderly spiral manner, positioned about 137° from each other according to the Fibonacci rules. The stem cell population in Petunia is maintained by the action of

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the homeobox transcription factor TERMINATOR (TER), which is the homolog of Arabidopsis WUSCHEL (WUS) (Stuurman, Jaggi, and Kuhlemeier 2002). When the transition from vegetative to generative growth has occurred, the vegetative SAM converts into an inflorescence meristem (IM), which generates the floral meristems (FM). In Petunia, the IM bifurcates, producing the FM and the remaining and more laterally positioned indeterminate IM (see Chapter 9). This architecture, with a terminal apical flower and a lateral indeterminate inflorescence, is referred to as a "cymose" inflorescence structure. The FM, which typically produces the floral organs arranged in concentric whorls, eventually ceases its meristematic activity to allow for differentiation of a pistil, formation of gametes, and completion of the life cycle. Termination of the FM is the result of the loss of stem-cell promoting activity, which is controlled by TER.

The identity of meristems and floral organs is determined by the so-called homeotic genes, which were first uncovered in Drosophila in the 1980s (Akam 1983; Gehring and Hiromi 1986) and later, in the early 1990s, in plants (Sommer et al. 1990; Yanofsky et al. 1990). Mutations in homeotic genes lead to conversions in identity. A classical example of a homeotic meristem identity mutant of Petunia is *aberrant leaf and flower (alf*, Gerats, Kaye, Collins, and Malmberg 1988; Souer et al. 1998), the floral meristems of which retain an inflorescence identity. A characteristic floral organ homeotic mutant is the *green petals* (*gp*) mutant (Vallade, Maizonnier, and Cornu 1987; van der Krol, Brunelle, Tsuchimoto, and Chua 1993), with petals homeotically transformed into sepals.

The majority of these homeotic genes belong to one particular family of transcription factors, the MADS box family. The name refers to the initials of the four founding members of this family: *MCM1* (Passmore, Maine, Christ, and Tye 1988), *AGAMOUS* (Yanofsky et al. 1990), *DEFICIENS* (Sommer et al. 1990), and *SERUM RESPONSE FACTOR* (Norman, Runswick, Pollock, and Treisman 1988). During the past two decades many MADS box genes have been isolated from various species, including Petunia, and forward and reverse-genetics approaches have revealed considerable conservation in gene function among angiosperm species. On the other hand, diversification in regulation of gene expression, patterns of protein interaction, and neofunctionalization of gene products have led to enormous variation in morphology. The formation of the flower and the role of MADS box genes are ideal research topics for studying evolutionary aspects of plant morphogenesis. These aspects are highlighted in Chapter 10. The roles of MADS box family members in plant developmental processes and their combinatorial action will be discussed in detail in this chapter.

11.2 MADS Box Genes and Their Protein Products

The MADS box family is one of the largest families of plant transcription factors, consisting of 107 members in Arabidopsis (Parenicova et al. 2003) and probably a similar or slightly higher number in Petunia. They can be subdivided into two lineages: type I, which are more closely related to the *SRF*-type of factors from ani-

mals (Alvarez Buylla et al. 2000), and type II factors, also referred to as MIKC type, which share similarities with the *MYOCYTE ENHANCER FACTOR 2 (MEF2)*-like genes. Regulatory proteins show in most cases a modular structure with domains that mediate interactions with other molecules such as DNA, other transcription factors, or cofactors.

MADS box transcription factors have the MADS box domain in common, which is located at the N-terminus of the protein and interacts with DNA. In addition to the MADS domain, MIKC-type proteins contain a highly variable intervening (I) region, which is important for protein interaction selectivity (Krizek and Meyerowitz 1996; Riechmann, Krizek, and Meyerowitz 1996; Yang, Fanning, and Jack 2003; Yang and Jack 2004), along with a more conserved keratin-like K-domain region and a C-terminus that supports the formation of higher-order protein complexes and may serve as a transcription activator or suppressor domain. The formation of these higher-order protein complexes among Petunia MADS box proteins is discussed in Sect. 11.7 of this chapter. The K-domain consists of three α-helical structures, with a hydrophobic site formed by leucine or isoleucine residues. This amphipathic site facilitates interaction with another MADS box molecule, leading to formation of either homo- or heterodimers. In addition to a DNA-binding domain, the MADS box domain also contains a nuclear localization signal and a conserved threonine phosphorylation site (RXX[S/T]). Despite conservation of the phosphorylation site in all MIKC proteins, it is not known whether phosphorylation at this site is essential for proper functioning of the protein. Studies by Immink (2002) revealed, however, that replacing the conserved threonine residue with alanine in the Arabidopsis APETALA1 (AP1) protein, and thereby destroying the phosphorylation site, abolished the capacity of *AP1* to complement the *ap1* mutant.

Although they are able to dimerize, type I MADS-box genes lack a K-box domain (De Folter et al. 2005). In Arabidopsis, the type I genes can be subdivided into three subclasses, Mα, Mβ, and Mγ (De Bodt et al. 2003), all of which have a simple genomic structure consisting of a single or a maximum of two exons. This is in contrast to the MIKC genes, which generally contain multiple exons.

Strategies that have been used to identify the majority of the Petunia MADS genes are cDNA library screens using conserved MADS box regions as probes (Angenent, Gusscher, Franken, Mol, and van Tunen 1992; Immink et al. 2003) and PCR-based methods using family-specific primers (Vandenbussche et al. 2003). A large number of MIKC MADS box genes have been isolated from Petunia using such approaches (for a complete list, see Chapter 10), and the encoded products possess the general features of MIKC proteins as described above. In contrast to the MIKC genes, no type I genes from Petunia have yet been reported. However, unpublished results (Bemer, Vandenbussche, and Angenent) revealed that α- and γ-types exist in the Petunia genome, although the total number and the extent of duplication may differ from what is seen in Arabidopsis.

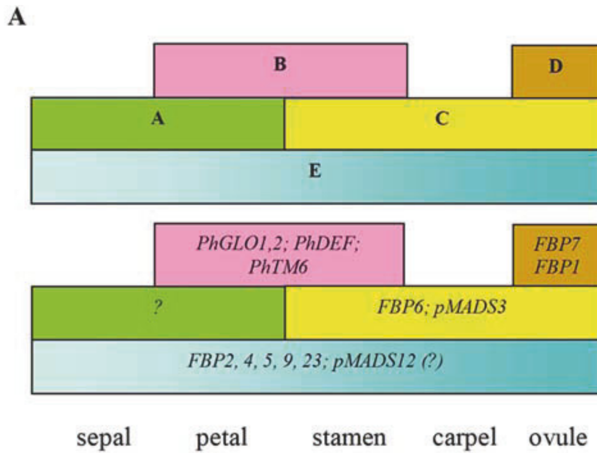
MIKC-type genes from many plant species, including Petunia, have been thoroughly investigated. They are involved in many developmental processes, such as the transition to flowering (Immink et al. 1999; Ferrario et al. 2004), identity specification of floral organs (Angenent, Franken, Busscher, Colombo, and van Tunen

1993; van der Krol et al. 1993; Kapoor et al. 2002; Vandenbussche et al. 2003; Vandenbussche, Zethof, Royaert, Weterings, and Gerats 2004; Rijpkema et al. 2006), ovule identity (Angenent et al. 1995b), and floral meristem determinacy (Ferrario, Shchennikova, Franken, Immink, and Angenent 2006). How these MADS box genes establish the flower in a combinatorial fashion will be described in the next section.

11.3 Floral Organ Identity Genes and the ABC Model

Genetic and molecular analyses carried out on several floral homeotic mutants of Antirrhinum and Arabidopsis pointed to MADS box transcription factors as key regulators of floral morphogenesis. In 1990, the research groups of Meyerowitz (Yanofsky et al. 1990) and Sommer (Sommer et al. 1990) reported isolation of the first plant MADS box genes from Arabidopsis and Antirrhinum, respectively. Mutations in *AGAMOUS* (*AG*) in Arabidopsis led to the homeotic conversion of stamens to petals and of the pistil to new reiterating floral buds, demonstrating that this Arabidopsis gene is responsible for identity specification of the reproductive organs in the inner two floral whorls and determination of the flower. Similar mutants were identified in Antirrhinum and, surprisingly, all were affected in two adjacent floral whorls and could be clustered into three distinct groups of mutants. These observations led to the proposal of a genetic model incorporating three functions, A, B, and C, which together specify the identities of the floral organs (Coen and Meyerowitz 1991). The A function in this so-called ABC model is responsible for the formation of sepals; A and B together lead to the specification of petal identity; the combinatorial expression of genes belonging to the B and C classes gives rise to stamen formation; and the C-class genes alone terminate the flower through formation of the carpel (Fig. 11.1).

The A function in Arabidopsis is represented by the transcription factor gene *AP2* (Jofuku, de Boer, van Montagu, and Okamuro 1994) and the MADS box gene *AP1* (Mandel, Gustafson-Brown, Savidge, and Yanofsky 1992). Petunia genes that are homologous to these Arabidopsis genes have been identified; corresponding mutants, however, have never been found. Maes and colleagues (2001) reported the isolation of three *AP2*-like genes and characterization of Petunia plants mutated for these genes, but no aberrations in perianth development were observed. Nevertheless, a Petunia mutant that resembles an A-function mutant exists: *blind.* Mutants have sepals with carpelloid tissues on their tips in the first whorl and antheroid structures instead of petal limbs in the second whorl (Vallade et al. 1987). Recently, the molecular basis of this mutant was uncovered, revealing that ectopic expression of the C-class genes leads to homeotic conversions in the outer two whorls of *blind* mutants (Cartolano et al. 2007). In Arabidopsis, C-gene expression in the perianth is suppressed by the A function, which is also required for sepal and petal identity. In Petunia, the spatial expression of the C genes is controlled by a miRNA that restricts the expression of the C genes in the reproductive whorls, while the mystery of a true A-function gene in Petunia remains unsolved.



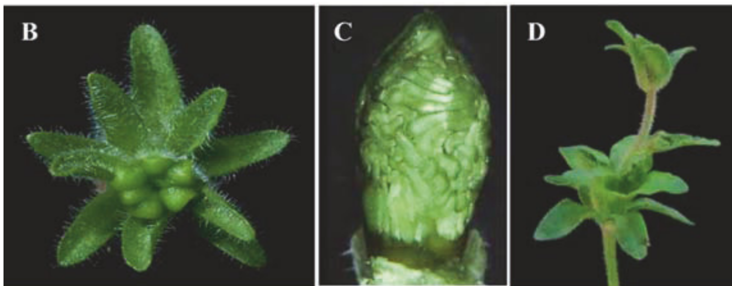


Fig. 11.1 The ABC model for Petunia and some floral organ mutants. (**A**) The ABC model for floral organ development. The combinatorial action of the five functions A–E determines the identities of sepals, petals, stamens, carpels, and ovules. The candidate Petunia genes for the homeotic functions are indicated in the bottom model. (**B**) Class B mutant with petals and stamens converted into sepals and carpels, respectively. The mutant was obtained by *dTPh1* transposon insertions in *PhGLO1* and *PhGLO2* (Vandenbussche et al. 2004). (**C**) Transgenic mutant with ovules transformed into carpels. This mutant was generated by co-suppression of *FBP11* and represents a class D mutant (Angenent et al. 1995b). (**D**) Downregulation of *FBP2* (and possibly other MADS box genes) in a co-suppression plant resulted in the conversion of petals and stamens into sepalloid organs and the pistil into a new inflorescence (Angenent et al. 1994)

Both in Arabidopsis and Antirrhinum, the B function is controlled by a pair of MADS box genes. Mutations in either of the two genes results in the conversion of petals into sepals and stamens into carpelloid organs. In Petunia, these genes have been duplicated into four distinct genes: *PhDEF*, *PhTM6*, *PhGLO1*, and *PhGLO2* (for former names of these genes, see Table 10.1, Chapter 10). Despite duplication of the *DEF* lineage, giving rise to the *PhDEF* and *PhTM6* genes, these paralogs are not fully identical and appear to have undergone partial neofunctionalization (Rijpkema et al. 2006). In contrast to the expression pattern of most B-class genes (petals and sepals), the expression of *PhTM6* is confined mainly to the third and

fourth whorls. In the third whorl it fulfills part of the B function, but its role in the fourth whorl remains unresolved. More information about these Petunia B genes and how they evolved in the angiosperm lineages is provided in Chapter 10.

The C-class lineage in Petunia has also undergone duplication, which appears to be more the rule than the exception in angiosperms. In Arabidopsis, the activity of a single gene, the *AG* MADS box gene, is essential for specification of stamens and carpels (Yanofsky et al. 1990), whereas in Petunia two *AG*-like genes, *FLORAL BINDING PROTEIN6* (*FBP6*, Angenent et al. 1993) and *pMADS3* (Tsuchimoto, van der Krol, and Chua 1993) have been identified. Both Petunia genes are expressed in stamen and carpel primordia, which is in line with a C-class function. However, only *pMADS3* overexpression (Kater et al. 1998) and downregulation (Kapoor et al. 2002) in transgenic Petunia plants resulted in homeotic conversions consistent with a function in floral organ identify. Kapoor et al. (2002) reported the phenotypic effect of antisense inhibition of *pMADS3*, which led to the formation of petaloid organs in the third whorl, while the fourth-whorl pistil appeared to be less affected. A loss-of-function mutant phenotype for the *FBP6* gene is not available, but it may be functional in combination with *pMADS3* in the carpels, where it shows its highest expression.

After the formation of the two carpel primordia during Petunia flower development, the small proportion of the floral meristem that is located between the carpel primordia remains. In a subsequent developmental step, this meristem becomes determinate and placental tissue is formed. Ovules, harboring the female gametophytes, develop from this placental tissue and can be regarded as a separate distinct floral organ based on their evolutionary origin in ancient seed plants (for review, see Angenent and Colombo 1996). A separate class of MADS box genes, referred to as the D-class homeotic genes, is required for identity specification of the ovules. In Petunia, this class is represented by two genes, *FBP7* and *FBP11*, both expressed from the early stages of placental development. At later stages, *FBP11* is expressed predominantly in the single integumental layer of the ovule, and after fertilization, in the seed coat (Angenent et al. 1995b; Colombo et al. 1997). Downregulation of these D-class genes by co-suppression in transgenic Petunia plants demonstrated that these genes specify the identity of the ovules, because carpellaid organs developed in positions where ovules normally appear. Overexpression of these genes appears to be sufficient to induce ovule formation on the perianth organs of the flower (Colombo et al. 1995). Single *fbp7* and *fbp11* mutants did not reveal a mutant phenotype, indicating that these genes are redundant (Vandenbussche et al. 2003).

After fertilization these genes are involved in maintaining the endothelial layer in the seed coat (Colombo et al. 1997). This layer connects the maternal tissue with the growing embryo and is thought to be analogous to the tapetum in the anther. Downregulation of *FBP7* and *FBP11* had a dramatic effect on development of the endothelial layer, and precocious degeneration was observed shortly after seed initiation. Although genetic proof is missing, it is likely that the D-class genes fulfill their role in combination with class C genes, first because *FBP6* and *pMADS3* are also expressed in ovules, and second because D- and C-class proteins are likely to exist in a multimeric protein complex (Ferrario et al. 2006).

In 1994, Angenent and colleagues reported the isolation of a Petunia MADS box gene, *FBP2*, that did not comply with the traditional ABC homeotic gene model, in the sense that it is expressed in three floral whorls rather than two adjacent whorls. Furthermore, downregulation of this and homologous Petunia genes by a co-suppression approach resulted in the homeotic conversion of the inner three organs into sepals (Fig. 11.1D). Strikingly, the mutant flowers developed new inflorescences, indicating that determinacy of the flower was also affected. This floral phenotype could not be explained by the action of the ABC genes and was therefore ascribed to the effects of a new class of genes, the E-class organ identity genes (Pelaz, Ditta, Baumann, Wisman, and Yanofsky 2000). In Arabidopsis, the E function is represented by the partially redundant *SEPALLATA (SEP)1-4* genes (Pelaz et al. 2000; Ditta, Pinyopich, Robles, Pelaz, and Yanofsky 2004), whereas the inner three floral whorls are specified by the action of *SEP1,2,3* in combination with the ABC MADS box genes. The fourth *SEP* gene (*SEP4*) appears to be more important for floral meristem identity and determination of sepal identity (Ditta et al. 2004). In Petunia, several *SEP*-like genes are also known, although their full complexity in relation to function and redundancy has yet to be resolved. In the *fbp2* co-suppression mutant, which fully phenocopies the triple *sep1,2,3* knockout mutant in Arabidopsis, *FBP5* was also downregulated (Ferrario et al. 2003). However, the double loss-of-function mutant *fbp2fbp5*, obtained by transposon insertion, showed a less severe phenotype compared to that of the *fbp2* co-suppression mutant (Vandenbussche et al. 2003). This double mutant had petal-to-sepal conversions, sepal-like structures on top of the anthers, and replacement of ovules in the ovary by leaf/sepal-like organs. This phenotype demonstrates that other Petunia *SEP-like* genes are essential for the proper specification of floral organs. Candidates for the E function in Petunia, in addition to *FBP2* and *FBP5,* are *FBP4, FBP9, pMADS12*, and *FBP23* (Ferrario et al. 2003; Immink et al. 2003; Vandenbussche et al. 2003).

11.4 Other Floral Functions Controlled by MADS Box Genes

11.4.1 Floral Determinacy

In contrast to the shoot, which theoretically can produce leaves indefinitely, the floral meristem (FM) forms only a limited number of floral organs before it terminates. In Petunia, the flower becomes determinate when the carpel primordia are formed, and the remaining FM cells differentiate into placental tissue. Mutant analyses have revealed that several MADS box genes are important for determinacy of the flower, as their mutants exhibit indeterminacy (Fig. 11.1D; Angenent et al. 1994; Kapoor et al. 2002). Impaired E function, which was observed in *FBP2*-co-suppression plants, resulted in production of new inflorescences in the center of the flower, indicating that the formation of flowers had been reiterated. This function of the E genes has been confirmed in Arabidopsis (Pelaz et al. 2000). The phenotype of the

Arabidopsis *ag* mutant also points to a function of this C-class gene in termination of the flower.

Because the homeobox gene *WUSCHEL* (*WUS*, Mayer et al. 1998) is responsible for maintenance of the stem cell population in the FM, it was proposed that *AG* suppresses *WUS* (Brand, Fletcher, Hobe, Meyerowitz, and Simon 2000; Schoof et al. 2000). The role of the C-class genes from Petunia in floral determinacy has not been resolved completely. Kapoor and colleagues (2002) reported that silencing of *pMADS3* led to indeterminacy in the third whorl but surprisingly, not in the center of the flower. These mutant flowers develop secondary inflorescences, which emerge alternating with whorl three organs. This phenotype indicates that *pMADS3* is involved in floral determinacy in the third whorl, while it may act redundantly with *FBP6* in determination of the fourth whorl (Kapoor et al. 2002).

Yet this is not the complete story, because class C and E genes are active long before the suppression of *WUS* occurs. More clues came from a recent report (Ferrario et al. 2006) in which control of the Petunia *WUS* homolog *TERMINATOR* (*TER*, Stuurman et al. 2002) was studied in Petunia plants ectopically expressing different combinations of organ identity MADS box genes. This study demonstrated that the simultaneous activation of *FBP11* (a D-class gene) and *FBP2* (an E-class gene) resulted in termination of the SAM and loss of *TER* expression. In a wild-type flower *FBP11* could be responsible for the timing of *TER* downregulation in the center of the flower, because the induction of *FBP11* coincides with the suppression of *TER* and the loss of floral meristematic activity (Fig. 11.2). Ectopic expression of

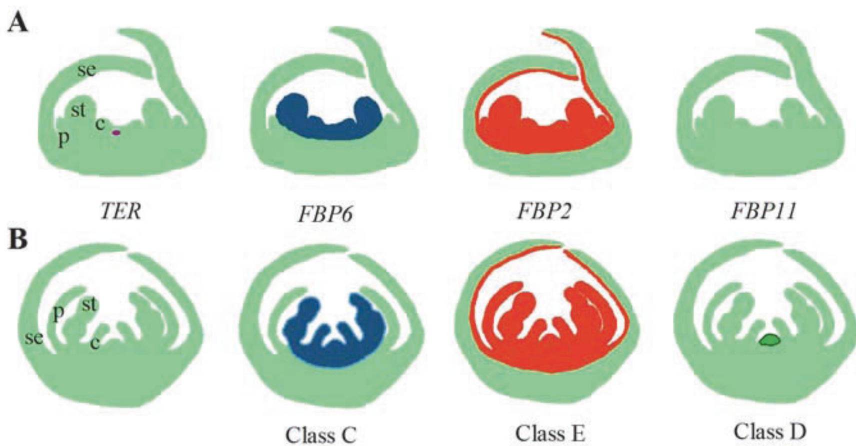


Fig. 11.2 Schematic representation of MADS box gene expression during two stages of Petunia flower development. At stage 1 (**A**) the floral organ primordia (se, sepals; p, petals; st, stamens; c, carpels) emerge from the floral meristem. At a later stage (**B**) the floral meristem is determinate and forms the placental region in the center of the flower. The expression patterns of the C, E, and D genes, *FBP6*, *FBP2*, and *FBP11*, respectively, are indicated by distinct colors. The meristem maintenance gene *TER* is expressed in the floral meristem at stage 1, but is downregulated when the meristem becomes determinate and *FBP11* expression begins (Ferrario et al. 2006)

FBP2 and *FBP11* caused significant upregulation of the C-class gene *FBP6*, supporting the hypothesis that these three genes act together to terminate the FM. In line with this hypothesis, it was observed in a yeast-three-hybrid assay (Fig. 11.3B) that the three gene products FBP2, FBP11, and FBP6 are able to form a protein complex in yeast. It was therefore proposed that such a complex, composed of these three proteins, which are all present when the placenta, becomes apparent and when *TER* is downregulated, is responsible for floral meristem determinacy (Fig. 11.2; Ferrario et al. 2006).

11.4.2 FBP24 Controls Endothelium Development in Ovules

Closely clustered in the phylogenetic tree of MIKC MADS box genes are the class B genes (Fig. 11.1) and the so-called B sister (Bs) genes (see Chapter 10). It has been suggested that the B and Bs gene lineages were generated by a duplication of an ancestral gene before divergence of the gymnosperm and angiosperm lineages approximately 300 million years ago, but after the separation of the fern lineage 400 million years ago (Becker et al. 2002). Despite the similarity in sequences, the B and Bs genes have distinct expression patterns. Whereas B genes are predominantly expressed in the second and third whorl organs, Bs genes are expressed in the ovules (Becker et al. 2002; De Folter et al. 2006). A full-length Bs gene from Petunia was identified by a yeast two-hybrid screen using FBP11 as bait and designated *FBP24*. The *FBP24* gene is specifically expressed in ovules, and in a phylogenetic tree it groups next to the B genes (de Folter et al. 2006). A co-suppression strategy was followed to downregulate the gene, resulting in aberrations in seed development. Due to the strategy used in this study, it cannot be excluded that other homologous MADS box genes were downregulated as well. Proanthocyanidins, which accumulate in the seed coat, were absent, and the ovules containing the silencing transgene degenerated shortly after fertilization. Detailed analysis of the mutant ovule demonstrated that the endothelium, the most inner layer of the single integument, was impaired. This mutant phenotype is comparable to that of the knockout mutant of the Arabidopsis *Bsister* (*ABS*) gene (Nesi et al. 2002).

11.5 Interdependency Among Petunia MADS Box Genes

Developmental processes require a robust regulatory mechanism. The expression of genes can be maintained as needed during a given developmental period, but can also decline rapidly when transition to another phase occurs. These regulatory mechanisms are often controlled by members of the same gene family or by the gene product itself. Such auto-regulatory feedback loops are also common in the MADS family. A well-known example is that of the B-class genes, the expression of which is maintained by complexes composed of the B proteins (Angenent, Busscher, Franken, Dons, and van Tunen 1995a; Rijpkema et al. 2006). The D-class genes

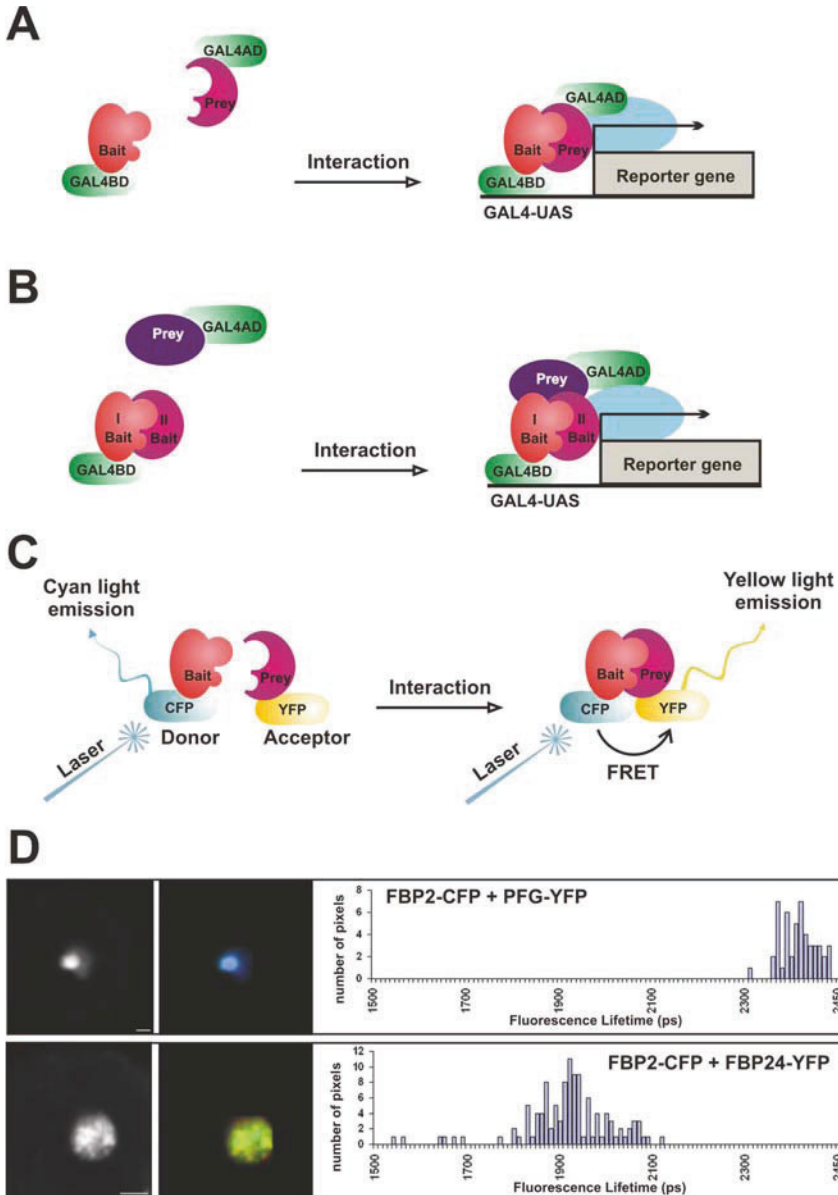


Fig. 11.3 Commonly used methods to determine protein–protein interactions (**A**) The yeast GAL4 two-hybrid system with a bait protein fused to the yeast GAL4 binding domain (BD) and the prey protein fused to the GAL4 activation domain (AD). Interaction between bait and prey proteins restores the GAL4 transcription factor that activates the reporter gene. The reporter gene either encodes a protein that produces a colored product (e.g., LacZ) or complements an autotrophic marker (e.g., adenine or histidine). (**B**) The yeast GAL4 three-hybrid system. The system is based on the two-hybrid system shown in A. In this case a dimer is used as bait and the reporter will be activated when all three proteins form a complex. Note that this system works only when the

also appear to be controlled by an auto-regulatory mechanism: constitutive overexpression of *FBP11* under the control of the 35S CaMV promoter, in combination with *FBP2* overexpression, resulted in ectopic expression of both *FBP7* and *FBP11* (Ferrario et al. 2006), a result suggesting that these paralogous genes are regulated in a similar way. In the same study, Ferrario and colleagues revealed that the C-type gene *FBP6* can be ectopically activated by the co-overexpression of *FBP11* and *FBP2* as well, indicating that these MADS box transcription factors may control the expression of *FBP6* directly. Surprisingly, the expression of the other class C gene *pMADS3* was not affected, a result which demonstrates that, while *FBP6* and *pMADS3* are closely related, they have diverged with respect to their regulation. Their gene products, moreover, appear to act differently, because overexpression of the two genes driven by the 35S promoter in transgenic Petunia plants did not result in the same phenotypes (Kater et al. 1998).

The MADS box transcription factors are able to bind a consensus DNA-binding sequence known as the CArG box. This box represents the sequence $CC(A/T)_6GG$, although there are strong binding sites known that show mismatches in this consensus sequence (for review see De Folter and Angenent 2006). Virtually no information is available about target genes of the Petunia MADS box genes and little MADS box transcription factor promoter sequence has been obtained. Therefore, no conclusions can be made about the presence of these putative binding sites in MADS box gene loci or in the Petunia genome in general. Two exceptions are provided by the promoters of *FBP7* (Colombo et al. 1997) and *PhGLO1* (*FBP1*; Angenent et al. 1993), genes which are thought to be bound by MADS box proteins and for which sequence information is available. Both promoters showed activity comparable to that of the endogenous gene when fused to a GUS reporter gene. A short genomic sequence located upstream of the start codon of *FBP6* is also available, but its activity has not been tested. Sequence analysis revealed the presence of CArG-like boxes in all three promoters, although none of them matches the consensus sequence exactly.

Fig. 11.3 (continued) "bait I" and prey protein are unable to interact and activate the reporter as a dimer. (**C**) Fluorescence Resonance Energy Transfer (FRET) between the fluorescence proteins CFP and YFP. FRET occurs only when the CFP and YFP molecules come into very close proximity, which will be the case only when bait and prey proteins interact. (**D**) FRET is monitored by Fluorescence Lifetime Imaging Microscopy (FLIM). The fluorescence lifetime is the time that the CFP fusion protein (donor) is in the excited energy state, which will drop when energy is transferred from donor to acceptor. Consequently the fluorescence lifetime of the donor will decrease when the proteins of interest interact. FBP2-CFP and PFG-YFP are unable to interact, resulting in a fluorescence lifetime of ~2.4 ns for the FBP2-CFP donor. The lifetime of the FBP2-CFP fusion drops from about 2.4 ns to 1.9 ns when it interacts with FBP24-YFP

11.6 MADS Box Protein Localization

Transcription factors are expected to be found in the nucleus, where they act directly on the DNA. Methods have been used to localize MADS box transcription factors in situ. Wittich and colleagues (1999) used a monoclonal antibody raised against the C-terminal part of FBP11 to monitor accumulation of the D-class protein FBP11 in ovules. These immunolocalizations exhibited strong signals in nuclei from the integument; the protein was absent from the nucellus and embryo sac. Although these immunolocalizations give an insight into the behavior of the native protein and provide high-resolution information, they require highly specific antibodies. Scientists therefore switched to the use of protein fusions using fluorescent tags once genes for Green Fluorescence Protein (GFP) and its derivatives became available (Prasher, Eckenrode, Ward, Prendergast, and Cormier 1992). Use of these fusion proteins and their localization in Petunia cells were reported by Immink et al. (2002) for the MADS box proteins FBP11 and FBP2. Protoplast transfections revealed that FBP2-CFP (Cyan version) was localized exclusively in the nucleus but, surprisingly, the FBP11-CFP fusion was observed only in the cytoplasm, with no evidence for its presence in the nucleus. In a subsequent experiment by Immink et al. (2002), Petunia protoplasts that expressed both the FBP11-CFP and a FBP2-YFP (Yellow version) fusion protein were analyzed, revealing nuclear localization of both proteins. These two MADS box proteins were expected to interact, as had been observed in a yeast two-hybrid assay (Immink et al. 2003). Apparently a dimer is needed for translocation of FBP11 into the nucleus. This appears to be a general hallmark for transcription factors, also observed for the root-specific Arabidopsis proteins Scarecrow (SCR) and Shortroot (SHR). SHR moves from the stele cells to neighboring cells, where it interacts with SCR and becomes trapped in the nucleus (Cui et al. 2007). Another example of a Petunia MADS box protein that relies on co-expression for nuclear transport is that of Unshaven (UNS, Ferrario et al. 2004), which is transported to the nucleus only when co-produced with the interacting protein FBP9. FBP9 and the other E-class protein FBP2 were both localized in the nucleus even when expressed alone in leaf protoplasts. This may be due to their potential to form homodimers (Immink et al. 2002), while FBP11 and UNS lack this capacity.

Nuclear localization is dependent on the presence of both a dimerization partner and a Nuclear Localization Signal (NLS), the latter also found in the MADS box domain. The conserved bipartite NLS consensus sequence can be deleted, thereby blocking translocation to the nucleus. Double transfections with a truncated MADS box protein lacking the NLS (FBP11ΔN-CFP) and a full-length interacting protein (FBP2-YFP) resulted in the co-localization of CFP and YFP signals in the cytoplasm exclusively (Immink et al. 2002). These experiments demonstrated that a NLS is needed on both interacting partners for efficient translocation. They also pointed to the importance of dimer formation of the MADS box transcription factors not only for DNA binding but also for nuclear import. Thus, information about the possible dimer combinations of Petunia MADS box proteins is essential in order to understand the molecular functioning of these transcription factors. The following section deals with these protein interactions.

11.7 MADS Box Protein Interactions

Transcription factors are active as protein complexes comprised not only of proteins of the transcriptional machinery, such as the RNA polymerases, but also of other DNA binding proteins or co-factors. MADS box transcription factors bind DNA as dimers, but the current model invokes the formation of multimeric complexes, most likely composed of four MADS box proteins (Egea Cortines, Saedler, and Sommer 1999; Theissen and Saedler 2001). The formation of multimeric complexes provides a mechanism for increasing the functional diversity of possible DNA-binding proteins, which may lead to more specificity in target gene selection. The K-domain of approximately 80 amino acid residues plays an important role in the physical interactions between MADS box proteins. It folds into three amphipathic α-helices that facilitate hydrophobic interaction with the partner molecule (Angenent et al. 1992). Studies in Arabidopsis have revealed that other parts of the protein, in particular the C-terminal part of the MADS box and the I-region, also help to determine the specificity of interactions (Krizek and Meyerowitz 1996; Riechmann et al. 1996).

The most commonly used method for identifying interactions between MADS box proteins is the yeast two-hybrid system (Fig. 11.3A; Fields and Song 1989). Two different yeast systems have been used to identify Petunia MADS box interactions (Immink et al. 2003; Rijpkema et al. 2006). The GAL4 system is a transcriptional system and, therefore, transcription factors containing an intrinsic transcriptional activation domain may activate the yeast reporter gene in the absence of an interaction with the "prey" protein. Roughly 15% of the MADS box proteins contain a transcription activation domain that is active in yeast. This "auto-activation" is overcome in the CytoTrap system (Aronheim, Zundi, Hennemann, Elledge, and Karin 1997), which is based on cytoplasmic interactions and complementation of a yeast mutant. Among the drawbacks of this system are that it leads to a substantial number of false positives and the assay is performed at a relatively high temperature (37°C). Immink and colleagues (2003) reported the results of a large yeast two-hybrid experiment in which the dimerization capacity of 21 Petunia MADS box proteins was determined. Two proteins, FBP4, and FBP13, were able to form homodimers in this yeast screen, while the rest of the MADS protein family formed one or a few different dimers (Fig. 11.4).

The MADS box family expanded considerably during plant evolution, most likely through a combination of whole-genome duplications early in angiosperm evolution and later small-scale events (De Bodt et al. 2003; Nam et al. 2004). This has led to the presence of paralogs that are still functionally indistinguishable. Functional redundancy is also a common feature of the Petunia genome. For example, the *FBP7/FBP11* genes (D genes) and *FBP2/FBP5* genes (E genes) possess largely overlapping functions. Because proteins with the same function are expected to have the same interaction partners, the identification of protein–protein interactions provides an additional tool to help determine the degree of functional redundancy among MADS box proteins. Indeed, the yeast experiments revealed that FBP7 and FBP11 have very similar interaction patterns, as do FBP2 and FBP5. The full complement of E-function family members in Petunia includes, besides FBP2 and FBP5,

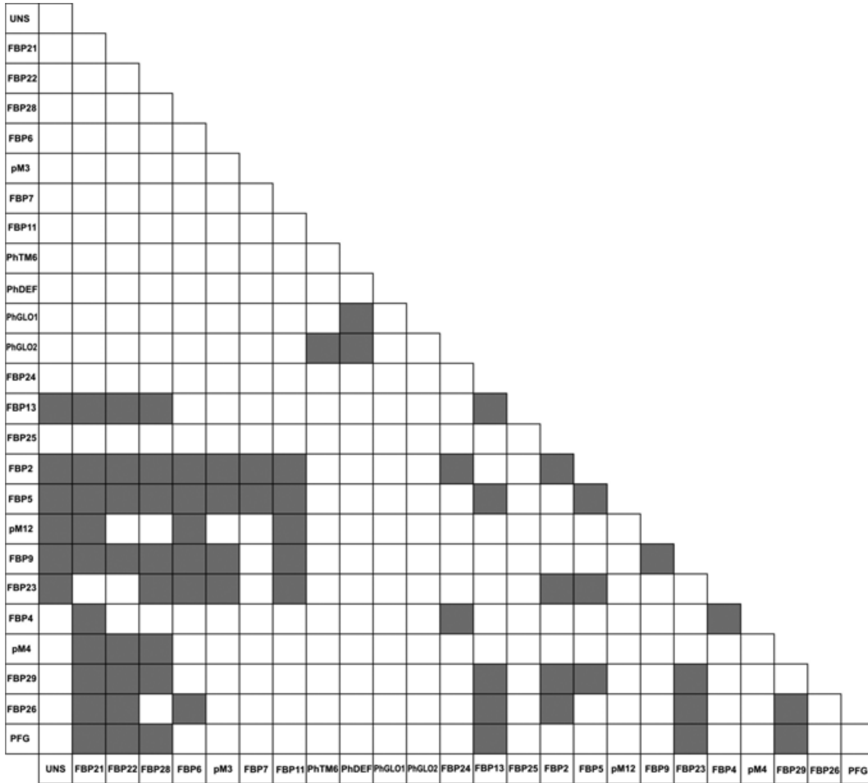


Fig. 11.4 Overview of protein interactions among Petunia MADS box proteins. Interactions between Petunia MADS box proteins were determined in a matrix-based approach using the yeast two-hybrid GAL4 and CytoTrap systems. In addition, interaction data has been obtained for a few Petunia MADS box proteins from cDNA expression library screenings and by the yeast two-hybrid GAL4 system, as well as from analyses in living plant cells. Proteins with similar interaction patterns are grouped; interactions are indicated in *gray*. Data are collected from Kapoor et al. (2002), Ferrario et al. (2003), Immink et al. (2003), Ferrario et al. (2004), De Folter et al. (2006), Rijpkema et al. (2006), Tonaco-Nougalli, Borst, de Vries, Angenent, and Immink (2006)

additional candidates such as FBP4, FBP9, FBP23, and PMADS12 (Ferrario et al. 2003; Immink et al. 2003; Vandenbussche et al. 2003). Because the interaction patterns of the additional candidates differ substantially, they are likely to be only partially redundant, having evolved separate subfunctions of the basic E function. This is in agreement with the observations by Vandenbussche et al. (2003), who studied *fbp2* and *fbp5* single and double mutants. The double mutants exhibited an enhanced phenotype when compared to that of the single *fbp2* mutant, but their phenotype was clearly less severe than that of FBP2 co-suppression plants (Fig. 11.1D, Angenent et al. 1994), suggesting that additional E-class genes exist in Petunia.

Interactions between MADS box proteins have been determined from various plant species, including Petunia (see Fig. 11.4), Arabidopsis (De Folter et al. 2005),

Gerbera (Kotilainen et al. 2000; Uimari et al. 2004), Chrysanthemum (Shchennikova, Shulga, Immink, Skryabin, and Angenent 2004), Antirrhinum (Davies, Egea Cortines, Silva, Saedler, and Sommer 1996; West, Causier, Davies, and Sharrocks 1998; Davies et al. 1999), and rice (Moon, Jung, Kang, and An 1999a; Moon et al. 1999b; Lim, Moon, An, and Jang 2000; Fornara, Marziani, Mizzi, Kater, and Colombo 2003; Lee et al. 2003; Fornara et al. 2004).

These studies have revealed that dimer formation between specific MADS box proteins is highly conserved. In particular, the floral homeotic proteins share very similar interaction patterns across species (Immink and Angenent 2002; De Folter et al. 2005). In a heterologous two-hybrid assay, it was demonstrated that the Arabidopsis protein SEPALLATA3 (SEP3) can interact with a number of Petunia proteins that are also interaction partners of the orthologous proteins from Petunia (FBP2/FBP5, Immink and Angenent 2002; Ferrario et al. 2003), confirming the functional similarity of proteins from the two species.

The yeast studies give information about the capacity and specificity of dimer formation, but they may not represent the behavior of the proteins in a plant cell environment. Therefore, in planta studies were performed using a protein interaction method based on FRET (Immink et al. 2002; Tonaco-Nougalli et al. 2006). In these studies, Petunia leaf protoplast cells were transfected with constructs encoding Petunia MADS box proteins that were tagged with either Yellow Fluorescence Protein (YFP) or Cyan Fluorescence Protein (CFP). Interaction between the MADS box proteins was monitored by FRET-FLIM, whereby an interaction causes a reduction in fluorescence lifetime of the donor (Fig. 11.3D).

Immink et al. (2002) reported interaction between FBP11 and FBP2 in plant cells, while Nougalli Tonaco et al. (2006) studied different combinations of the ovule-expressed proteins FBP2, FBP11, and FBP24. *FBP24* is the ortholog of the Arabidopsis B sister gene *ABS*, which is involved in integument and seed coat development (De Folter et al. 2006). FBP24 interacted with FBP2 in yeast and their interaction was confirmed in the FRET-FLIM assay. In contrast, no interaction was observed between FBP24 and FBP11 in yeast, but a reduction of fluorescence lifetime was observed in protoplast cells, suggesting that these proteins are able to form a heterodimer. This result demonstrates that the FRET-FLIM approach is more sensitive than the yeast two-hybrid studies. This was confirmed with respect to homodimerization of FBP2, FBP5, and FBP9, which could be detected by the in planta FRET assays, while these homodimers were never scored in yeast.

Besides the formation of dimers, MADS box proteins are thought to form higher-order complexes. The first evidence for the formation of these multimeric complexes came from a modified yeast two-hybrid screen using a dimer as bait (Egea Cortines et al. 1999). It was shown that a third Antirrhinum MADS box protein (SQUAMOSA) interacts via the C-terminal domain with the dimer composed of the two B-class proteins GLOBOSA and DEFICIENS. This type of three-hybrid screen was also performed for Petunia MADS box proteins (Ferrario et al. 2003; De Folter et al. 2006; Ferrario et al. 2006; Rijpkema et al. 2006). It was shown that for several combinations of MADS box proteins a third component in a ternary complex stabilizes the dimer. This was, for example, demonstrated for the combination FBP24

and FBP11, which interacted only when FBP2 was added in a yeast three-hybrid assay (Tonaco-Nougalli et al. 2006). Also in Petunia plant cells, FBP2 was able to strengthen the interaction between FBP24 and FBP11, providing evidence that higher-order complexes are formed in planta (Tonaco-Nougalli et al. 2006).

The ability of MADS box proteins to form multimeric complexes suggests that they are active in a combinatorial manner. Based on genetic information and the yeast *n*-hybrid experiments, Theissen and Saedler (2001) proposed the “Quartet model,” which postulates that two MADS box dimers associate into a quaternary molecule and each dimer binds to a specific DNA sequence. This model was integrated into the ABC model and predicts which multimeric complexes of MADS box proteins are responsible for the specification of floral organ identity. Figure 11.5 shows some of the currently known “Quartets” of Petunia MADS box proteins involved in floral organ development.

An intriguing observation was obtained by Ferrario et al. (2003), who studied complex formation of the various MADS box proteins belonging to the B, C, and

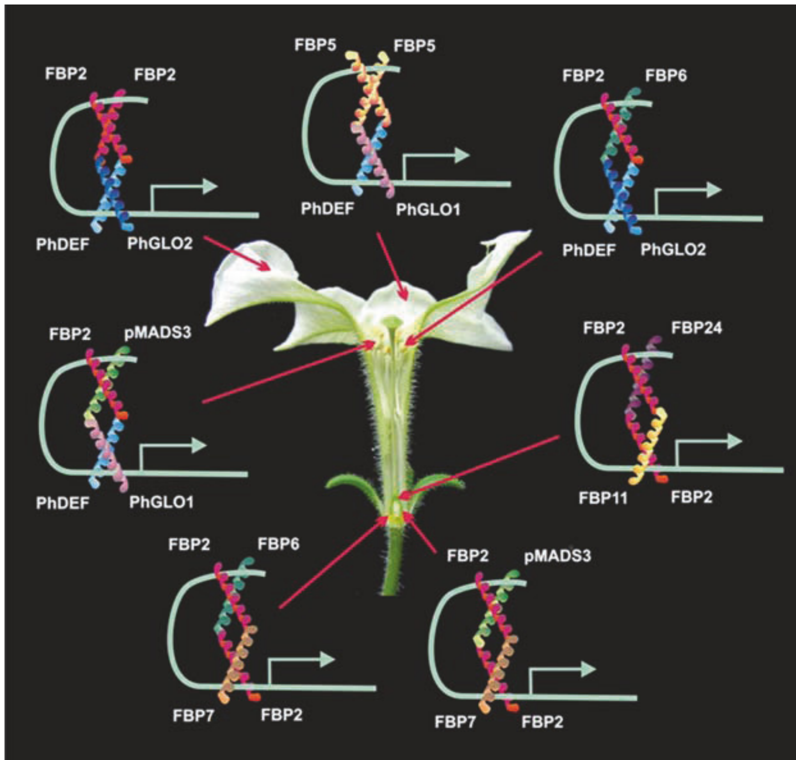


Fig. 11.5 Quartet model for Petunia. The Quartet model predicts that a double dimer of MADS box proteins interacts with target DNA sequences and controls the identity of floral organs in a combinatorial manner. Using yeast three- and four-hybrid experiments the various quaternary complexes can be tested

E classes. Both C-class proteins FBP6 and pMADS3 were able to interact with the E-class protein FBP2 in a similar manner, however they formed different complexes when combined with the paralogous B-class proteins. pMADS3 appeared to be present in a complex with the B protein PhGLO1 specifically, while FBP6 required the presence of PhGLO2. Therefore, for the formation of stamens at least two distinct multimeric complexes might be responsible. In addition, the paralogous proteins FBP2 and FBP5, which are indistinguishable in two-hybrid screens, behave differently in complex formation: only FBP2 interacts with the B dimer PhDEF-PhGLO2 (Ferrario et al. 2003; Rijpkema et al. 2006). These observations and the genetic studies reported by Vandenbussche et al. (2003) and Rijpkema et al. (2006) suggest that the duplicated floral homeotic genes have evolved slightly, leading to partial redundancy. This diversification is apparent at the level of protein complex compositions, however it remains unclear whether these highly homologous complexes have been recruited for different functions within the flower identity program.

11.8 Concluding Remarks

The formation of specific MADS box transcription factor protein complexes is an essential event in developmental signaling. The importance of dimerization for plant MADS box protein functioning was recognized already in the early 1990s. While large protein–protein interaction data sets are now available for members of the Arabidopsis MADS box family, and the dimerization capacities for all of them have been determined, the first large-scale analysis for dimerization had been carried out a few years earlier with Petunia MADS box proteins. To obtain a complete understanding of the molecular mode of action for MADS box proteins, and to comprehend how these proteins orchestrate plant development, however, knowledge about the behavior of MADS box transcription factors in planta is essential. In this respect, research in Petunia has been at the forefront, as the first interactions between plant MADS box proteins in living plant cells were reported for members of the family from Petunia. Intriguing questions remain: what are the targets of the MADS box genes and to which DNA sequences do these transcription factors bind? This field is emerging fast, in particular catalyzed by currently available deep-sequencing facilities that can provide the required genomic sequences. Identifying the target genes will be the next step in unraveling the regulatory pathway that begins with the MADS box homeotic genes and controls floral organ formation.

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Chapter 12
The Role of Expansins A in Petunia Development

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Abstract Expansins, a diverse set of proteins found in plants and some other organisms, appear to play a key regulatory role in cell expansion, thereby serving critical functions in plant morphogenesis, development, and adaptation to stress. We have isolated a number of expansin genes from Petunia. Their ongoing functional analysis provides evidence for their involvement in cell wall functions, including cellulose metabolism, disruption of noncovalent cellulose/glycan bonds, and separation of the cell wall matrix during cell expansion.

12.1 Introduction

Morphogenesis in development refers to the formation of intricate shapes and structures in developing tissues. In animals, the formation of cell and tissue structures is strongly dependent on cell–cell interactions, particularly the gain and loss of cell adhesion. This allows cells to move in relation to one another either as whole tissues or as individual migrating cells. In contrast, the rigid plant cell wall and the way cells are tightly cemented together means that such mechanisms can hardly be used in plants (Twyman 2003). Changes of shape and structure in the developing plant are therefore mediated by alternative mechanisms, such as control of the location, rate, plane and symmetry of cell division, and the regulation of cell expansion (Meyerowitz 1997). A complex surveillance system monitors the correct and productive integration of these two processes by regulating the activity and availability of key factors (Gutierrez 2005).

The extent of cell enlargement is a critical factor in the ultimate determination of cell shape and size, and this in turn depends predominantly on the extensibility of the primary cell wall (Kotilainen et al. 1999; Martin, Bhatt, and Baumann 2001;

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Smith 2003). Several cell wall proteins have been implicated in the control of extension but much of the evidence now indicates that expansins are the key regulators of this process (Brummell, Harpster, and Dunsmuir 1999; Cosgrove 1999; Choi, Lee, Cho, and Kende 2003). Their mechanism of action is not fully understood, but it appears to involve the disruption of hydrogen bonds between cellulose microfibrils and cross-linking glycans, thus allowing cellulose fibers to slide relative to one another and thereby facilitate cell expansion (McQueen and Cosgrove 2000). To better understand the role of cell expansion in Petunia development we have focused on the identification, characterization, and functional analysis of expansin genes in Petunia (*Petunia hybrida*).

12.2 Plant Cell Growth: Loosen and Slide

Plant cells enlarge via the longitudinal expansion of their cell walls, a process involving the careful regulation of "polymer creep" (Cosgrove 2005). The growing cell wall has a fiberglass-like structure with crystalline cellulose microfibrils embedded in a matrix of hemicelluloses and pectins. Hemicelluloses are cellulose-binding polysaccharides, which together with cellulose form a network that is strong yet resilient. Pectins are complex polysaccharides that have several functions: they form hydrated gels that push microfibrils apart, thus allowing them to slip over one another during cell growth; they are important determinants of cell wall porosity and thickness; and they provide the adhesive that binds plant cells together. Pectins are also the primary targets for attack by invading microbes.

Cellulose is synthesized by a large membrane-bound complex that extrudes microfibrils from the cell surface. In contrast, matrix polysaccharides are synthesized in the Golgi apparatus and are packaged into tiny vesicles that fuse with the plasma membrane and thereby deliver their cargo to the wall. Matrix polysaccharides then become integrated into the wall network by noncovalent physical interactions as well as enzymatic ligation and cross-linking reactions. The detailed structure of the cell wall is not precisely understood, and this remains a key issue for research into how the cell wall expands. Xyloglucan is believed to crosslink microfibrils either through the formation of direct molecular tethers or through some form of indirect linkage. The integration of newly secreted matrix polysaccharides into the existing network could also be mediated by endotransglycosylases/hydrolases, which cleave glycans and ligate them together. Molecular modeling data suggest that some members of the xyloglucan endotransglycosylase/hydrolase (XTH) family could target arabinoxylan and (1,3;1,4)-β-D-glucan, hemicelluloses that are notably abundant in grass cell walls (Cosgrove 2005). The complexity of the cell wall network provides many potential sites where loosening and expansion could be initiated.

Polymers in the cell wall are placed under stress and therefore undergo stretching as they resist turgor pressure, and this provides the mechanical energy required for expansion. The wall can stretch without bursting if polymer creep is carefully

controlled through the selective loosening and shifting of load-bearing linkages between cellulose microfibrils. In this way the matrix yields, allowing the cellulose microfibrils to move apart. To prevent fatal thinning, cell wall expansion must be balanced by the synthesis and integration of new cellulose and matrix polysaccharides. However, these two processes appear to be only loosely coupled in most plant cells.

"Wall loosening" refers to a molecular modification of the wall network that reduces wall stress without substantially changing its thickness. Candidate wall-loosening agents include XTH, endo-(1,4)-β-D-glucanase, hydroxyl radicals, and expansin. It is useful to distinguish between so-called primary and secondary wall-loosening agents. Primary agents catalyze stress-relaxation reactions directly in isolated cell walls, whereas secondary agents modify wall extension indirectly, by amplifying the physical effects of primary agents.

12.3 Are Expansins the Key Factors Controlling Cell Expansion?

At least four lines of evidence support a key role for expansins in the regulation of cell wall enlargement in growing cells. First, when the acid-growth behavior of isolated cell walls is eliminated by heat or protease treatment, it can be almost fully restored by the addition of purified expansin proteins (McQueen-Mason, Durachko, and Cosgrove 1992). This indicates that expansins alone are sufficient to restore extensibility to isolated cell walls. Second, the addition of exogenous expansins to growing tissues can stimulate their growth (Fleming, McQueen-Mason, Mandel, and Kuhlemeier 1997). Third, the modification of expansin gene expression also affects cell wall growth – ectopic expression stimulates growth, whereas suppression by gene silencing inhibits it (Cho and Cosgrove 2000; Pien, Wyzykowska, McQueen-Mason, Smart, and Fleming 2001; Choi et al. 2003; Zenoni et al. 2004). Finally, endogenous expansin gene expression correlates with the onset, increase, and cessation of cell growth, that is, the genes are expressed at the right time and in the right place to cause the observed morphological processes (Brummel et al. 1999; Wu, Thorne, Sharp, and Cosgrove 2001; Cho and Cosgrove 2002).

Although genetic studies provide compelling evidence that expansin plays an important role in cell expansion, little is known about the precise mechanism of action. Expansins appear to increase polymer mobility in the cell wall, allowing microfibrils to slide apart (Darley, Forrester, and McQueen-Mason 2001). However, studies of expansin activity have generally involved the use of quite crude extracts, which reveal little in the way of molecular detail. It has proved difficult to produce active recombinant expansins.

Most of the available information about expansin activity reflects studies carried out with cucumber CsEXP1A, which appears to induce cell wall extension by disrupting hydrogen bonds between cellulose fibrils and cross-linking glycans (McQueen-Mason and Cosgrove 1995, 2000). Sequence analysis of the expansin family reveals the presence of a conserved N-terminal domain (~15 kDa) with

distant homology to the catalytic domain of GH45 endoglucanases, and a C-terminal domain (~10 kDa) homologous to a family of grass-pollen allergens of unknown function. The crystal structure of a native EXPB1 purified from maize pollen was solved, revealing two domains (Yennawar, Li, Dudzinski, Tabuchi, and Cosgrove 2006). Domain 1 resembles a GH45 glycoside hydrolase, while domain 2 has an Ig-like beta sandwich with aromatic and polar residues potentially forming a polysaccharide-binding surface. Despite the presence of an endoglucanase-like domain, no catalytic activity has been detected that accounts for the action of expansins on the cell wall.

Expansins can weaken paper, which is a hydrogen-bonded network of cellulose fibrils, but this does not involve cellulose hydrolysis. Expansins also act very quickly. They induce extension within seconds of addition to wall preparations, but they do not affect the plasticity or elasticity of the cell wall (Yuan, Wu, and Cosgrove 2001). Furthermore, expansins synergistically enhance the hydrolysis of crystalline cellulose by cellulase, perhaps indicating that expansins promote the release of glucans on the surface of the cellulose microfibrils, making them available for enzymatic attack. Finally, expansins stimulate extension immediately after entering the wall, and subsequent removal restores the wall to an inextensible state. This indicates that expansin does not alter the gross wall structure or the degree of cross-linking, but may dissociate the polysaccharide complexes that link microfibrils together.

12.4 The Expansin Gene Family in Plants

Genome-wide searches in *Arabidopsis thaliana* and rice have revealed large families of expansin genes that can be organized into four subfamilies. The expansin A subfamily (*EXPA)* has the most members, with 26 genes in Arabidopsis and 34 in rice. The expansin B subfamily (*EXPB)*, originally known as the group 1 pollen allergens (Cosgrove, Bedinger, and Durachko 1997), has 6 members in Arabidopsis and 19 in rice. The mature EXPB proteins show much greater sequence divergence than the EXPA proteins. The remaining subfamilies lack some of the canonical features of the true expansins and are defined as expansin-like. The expansin-like A subfamily (*EXLA*) has three members in Arabidopsis and four in rice, whereas each species has only a single expansin-like B (*EXLB*) gene (Choi, Cho, and Lee 2006). All of the expansin proteins predicted from these sequences contain N-terminal signal peptides of about 20 amino acids that show no significant sequence conservation, but imply that all of the proteins are targeted for secretion into the cell wall.

Following the signal peptide, the EXPA and EXPB proteins share a series of conserved cysteine residues, suggesting that expansins may share a tertiary structure based on the formation of disulfide bonds. Another notable feature is the His-Phe-Asp (HFD) box in the central region, which may be a catalytic motif based on similarity to the catalytic core of microbial endoglucanases. The distal sequences of the EXPA and EXPB proteins share a series of conserved tryptophan residues, whose

position and spacing resemble those in the cellulose-binding domain of some cellulases. The EXLA proteins are highly conserved (84% sequence similarity among the Arabidopsis proteins, 73% in rice) but they lack the central HFD motif found in the EXPA and EXPB subfamilies. The EXLB proteins have conserved cysteine and tryptophan residues but also lack the central HFD domain.

Genomic DNA alignments have confirmed the presence of seven introns at conserved positions within the expansin gene sequences (Choi et al. 2006). Phylogenetic analysis indicates that the *EXPA* and *EXPB* subfamilies had already split by the time vascular plants and mosses diverged, and that they have continued to grow and diversify in different plant lineages (Sampedro, Lee, Carey, dePamphilis, and Cosgrove 2005). The authors also estimated the number of expansin genes in the last common ancestor of eudicots (including Arabidopsis) and monocots (including rice), and on this basis proposed that the four angiosperm expansin families should be divided into 17 clades (Sampedro et al. 2005).

12.5 Nonplant Expansins

Expansin proteins have been identified in several nonplant species. The N-terminal GH45-like domain is found in diverse expansin-like proteins from many organisms, such as the slime mold *Dictyostelium discoideum*, fungi, mussels (Xu, Janson, and Sellos 2001), and nematodes (Kudla et al. 2005). This polyphyletic group of nonplant expansins can be termed expansin-like family X (EXLX). In *Dictyostelium*, there are at least five EXLX genes and these sequences show by far the closest similarity to plant expansins. *Dictyostelium* expansins may lubricate the movement of cellulose microfibrils during individual cell growth, wall extension, and extracellular cellulosic matrix production, and/or they may serve to maintain the fluid state of the multicellular slug cell wall. The existence of expansin-like sequences in nonplant species is intriguing and appears to be restricted to organisms that infect plants and need to digest plant cell walls.

There are many differences in gene structure between plant and nonplant expansins, indicating separate evolutionary lineages. Their divergence might predate the origin of land plants, or alternatively the nonplant expansins could have been acquired from plants more recently by horizontal gene transfer.

12.6 Expansins in Development and Adaptation to Stress

A growing number of reports provide evidence that expansins play diverse roles in plant growth, development, and adaptation to stress. For example, expansins have been shown to play key roles in the early development of leaf primordia, fruit softening, internodal growth, sexual reproduction, and cell wall disassembly during fruit abscission (Cosgrove et al. 1997; Cho and Cosgrove 2000). Expansins are also involved in adaptations to drought and flooding, and in the extensive structural

remodeling that occurs during symbiotic microbial colonization. Some of these roles are discussed in more detail below.

12.6.1 Production of Leaf Primordia

Fleming et al. (1997) showed that topical application of expansins to discrete regions on the flanks of tomato vegetative meristems led to induction of ectopic leaf primordia, although these did not ultimately produce normal leaves. They proposed that expansins induced changes in the cell wall that modified the physical stress pattern in the meristem, causing aberrant tissue bulging, which led to the acquisition of primordium identity in cells at ectopic sites. A role for endogenous expansin in leaf initiation was indicated by in situ hybridization analysis. Expansin genes are not only expressed in the apical meristem but mRNA levels are highest in the bulging cells of the leaf primordia. More recently, Pien et al. (2001) showed that the localized induction of expansin transgene expression on the flanks of tobacco vegetative meristems not only induced the appearance of leaf primordia but reiterated the entire process of leaf development and produced phenotypically normal leaves.

12.6.2 Fruit Softening

Several reports have shown that expansins are expressed abundantly in softening fruit, for example, strawberry (Civello, Powell, Sabehat, and Bennett 1999), tomato (Rose, Cosgrove, Albersheim, Darvill, and Bennett 2000), pear (Hiwasa, Rose, Nakano, Inaba, and Kubo 2003), and banana (Trivedi and Nath 2004). Overexpression or downregulation of fruit-specific expansins was reported to influence fruit texture and juice viscosity in tomato (Brummel et al. 1999; Powell, Kalamaki, Kurien, Gurrieri, and Bennett 2003). Expansin genes are differentially regulated during fruit development, with the expression of some genes increasing in concert with fruit size, and the expression of others increasing specifically during the fruit-ripening stage. It is likely that different expansins with distinct expression patterns participate at different stages of fruit development. The role of expansin in fruit softening might be to facilitate the breakdown of glucans in the cell walls.

12.6.3 Abscission and Tissue Differentiation

In addition to cell elongation, expansins may also be involved in organ abscission and wall disassembly. Cho and Cosgrove (2000) showed that β-glucuronidase accumulated at the base of petioles in transgenic Arabidopsis plants when expressed under the control of the *AtEXP10* promoter, and the alteration of *AtEXP10* expression resulted in pedicel abscission as well as leaf growth. Increased expansin activity was observed in tissue undergoing cell separation during leaflet abscission in blue

elderberry (*Sambucus nigra*), indicating that expansins may be involved in abscission (Belfield, Ruperti, Roberts, and McQueen-Mason 2005). Chen and Bradford (2000) showed that an expansin is closely associated with endosperm weakening during the germination of tomato seeds. This process involves wall breakdown and weakening rather than growth. At least six different expansin genes are expressed in differentiating tracheary elements in *Zinnia* cell cultures. The expansin described by Im, Cosgrove, and Jones (2000) appears to be associated with the intrusive growth of protoxylem elements in *Zinnia* stems.

Cotton fiber has been used as a model experimental system because fiber elongation requires extensive cell wall loosening. Six *EXPA* genes have been identified and characterized in upland cotton. Expression analysis suggests that *GhEXP1,* which is abundantly expressed specifically in the fiber, plays an important role in cell wall loosening during fiber elongation (Harmer, Orford, and Timmis 2002).

12.6.4 Sexual Reproduction

Expansin activity has also been linked to important roles in pollination and the development of floral organs. Seven *EXPA* and three *EXPB* genes were analyzed during the development of *Mirabilis jalapa* flowers, and were shown to be regulated differentially during cell expansion and senescence (Gookin, Hunter, and Reid 2003). Proteomic analysis of a maize gametophytic male-sterile mutant *gaMS-2,* comparing the profiles of anthers and immature pollen grains in heterozygous mutants and wild-type plants, showed that Zea m 1, a group I pollen allergen, was greatly depleted in sterile pollen from the heterozygous mutant (Wang et al. 2004). The association of Zea m 1 with the sterile phenotype suggested that Zea m 1 could have two distinct roles: binding to pectins in order to facilitate cell wall deposition in pollen grains, or softening stigmatic tissues in order to facilitate penetration by the pollen tube. A tobacco *EXPB* gene is strongly expressed at the stigmatic surface during pollination, suggesting that expansins may facilitate pollination in dicots and grasses (Pezzotti, Feron, and Mariani 2002).

12.6.5 Response to Abiotic and Biotic Stress

Drought imposes a major abiotic stress that severely limits plant growth. *Craterostigma plantagineum*, the resurrection plant, is able to survive periods of drought by extensive folding of the cell wall, which maintains the integrity of connections between the plasma membrane and cell wall even when water loss results in cell shrinkage (Jones and McQueen-Mason 2004). Expansin activity increases in the leaves of this plant during the early stages of both dehydration and rehydration, providing evidence that expansins are associated with cell wall folding and desiccation tolerance. Expansins also appear to be involved in the response to flooding-induced stress. In the semi-aquatic species *Rumex palustris*, differential expansin expression

was detected in the roots in response to low O_2 levels (Colmer et al. 2004). This is consistent with the observation that expansin expression in deepwater rice correlates with internodal growth (Lee and Kende 2001, 2002).

Interactions between plants and microbes can involve extensive tissue remodeling, for example, during the formation of root nodules and mycorrhizae, and expansins have been shown to feature prominently in these processes. For example, Balestrini, Cosgrove, and Bonfante (2005) investigated the expression profiles of two cucumber *EXPA* genes, *CsEXPA1* and *CsEXPA2*, in uncolonized roots and in roots colonized with a mycorrhizal fungus. Immunoblot analysis using antibodies against CsEXPA1 and CsEXPA2 showed the presence of expansin specifically in the colonized roots, with CsEXPA1 localized at the interface zone, which is characteristic of endomycorrhiza, and CsEXPA2 localized within the cell wall. Therefore it appears that some expansins participate in the formation and maintenance of the interface, whereas others function as cell wall loosening agents to achieve the fungus-induced enlargement of cortical cells.

Giordano and Hirsch (2004) looked at the expression of *MaEXP1* (an *EXPA* gene) in developing root nodules of sweet clover during its interaction with the nitrogen-fixing bacterium *Sinorhizobium meliloti*. In situ hybridization showed that following inoculation *MaEXP1* was induced in both roots and nodules, suggesting an integral role for the gene product in the structural modifications that accompany nodule formation.

12.7 Isolation and Characterization of Petunia Expansin A Genes

Petunia hybrida is a useful model system for the analysis of expansins due to its well-characterized morphology and the availability of a large number of insertional mutants (see Chapter 17). The first Petunia expansin cDNA was isolated by screening a *P. hybrida* ovary cDNA library, yielding a 1250-bp clone with a 780-bp open reading frame that predicted a 25 kDa protein of 260 amino acids (Zenoni et al. 2004). Comparison with other expansin sequences placed this protein in the EXPA subfamily, and the gene was duly named *PhEXP1A* (Zenoni et al. 2004). Southern blot analysis with a probe hybridizing to the unique 3′-untranslated region of the sequence showed that *PhEXP1A* is a single-copy gene in the Petunia genome. A subsequent Southern blot was carried out using a probe hybridizing to a region of the *PhEXP1A* cDNA fragment that is highly conserved in *EXPA* genes (Cosgrove 2000). After high-stringency washing, five bands were identified by this probe, suggesting the presence of multiple *EXPA* genes in Petunia.

A semi-permanent *dTph1* insertional library (Gerats et al. 1990) was screened by PCR, combining a transposon-specific primer with two *EXPA*-specific primers, the latter based on the sequences of the two highly conserved expansin boxes. A total of 4096 plants from the Juliet library were screened, sampled in 3D array (see Chapter 17; Vandenbussche et al. 2003). Three genomic DNA fragments and the

corresponding insertional mutants were identified. In a parallel experiment, an ovary cDNA library was screened to extend the sequences identified in the transposon library and to isolate specific sequences for which primers were available (Kuhlemeier, personal communication). Using these combined approaches, we identified four additional Petunia expansin A cDNA sequences, which were named *PhEXP2A* (1198 bp), *PhEXP3A* (1171 bp), *PhEXP4A* (1151 bp), and *PhEXP5A* (1099 bp).

All the genomic expansin sequences contained the I-1 and I-3 introns characteristic of *EXPA* genes (Lee, Choi, and Kende 2001) at the correct sites, with lengths of the introns varying from 107 to 1300 bp. *PhEXP1A* and *PhEXP4A* had the longest introns – 1143 bp for I-1 and 1300 bp for I-3. Such large introns, comparatively rare in plants, are considered likely sites for enhancers and other regulatory elements.

Multiple alignment of amino acid sequences predicted from the five expansin cDNA sequences confirmed that they are all members of the *EXPA* gene family (Fig. 12.1). All the deduced amino acid sequences contain key EXPA features such as the two N-terminal GACGYG motifs and a C-terminal NWGQNWG motif. The predicted N-terminal signal peptides are diverse in sequence but the N-termini of the mature polypeptides are highly conserved. The amino acid sequences of all five expansins contain four conserved tryptophan residues (+) that may facilitate interaction with cellulose, eight cysteine residues (∗) that may participate in disulfide bonds, and the HFD domain (boxed), deduced from its homology to the catalytic site of GH45 glycosyl hydrolases (Cosgrove 2000), which may carry out the catalytic function of the EXPA proteins. The central region of each protein also contains a so-called a-insertion, a motif of about 14 residues that contains the highly conserved sequence GWCN at its 3′ end.

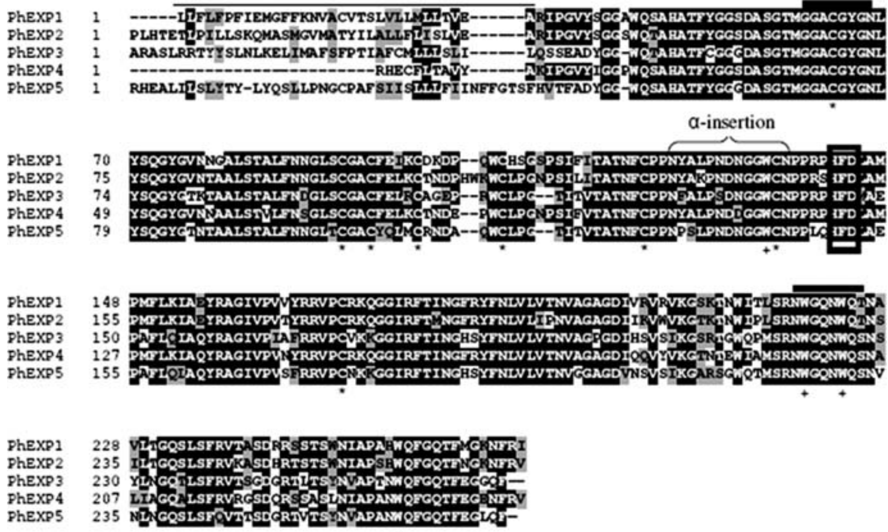

Fig. 12.1 Alignment of the predicted sequences for five *P. hybrida* expansins

Phylogenetic analysis was carried out to place the five new Petunia expansin A genes among the sequences already considered by Sampedro et al. (2005). *PhEXP3A* and *PhEXP5A* were placed within EXPA clade III, whereas *PhEXP1A*, *PhEXP2A*, and *PhEXP4A* clustered in clade XIII (Fig. 12.2). The new sequences were also classified according to an earlier phylogenetic study that divided the EXPA subfamily into four further subgroups (A, B, C, and D; Link and Cosgrove 1998). Within this system *PhEXP1A*, *PhEXP2A*, and *PhEXP4A* aligned with subgroup A, members of which are characterized by the presence of a conserved methionine residue near the HFD motif, a conserved RIPGV sequence immediately after the predicted signal peptide, and a conserved C-terminal sequence KNFRV. Subgroup A contains clades EXPA-IV and EXPA-XIII, and includes genes from diverse plant species such as corn (*ZmEXP4*) and pine (*pinusEXP2*). It has been suggested that conservation of EXPA sequence motifs within each clade may reflect conserved functions and/or the ability to respond to similar upstream signals (Link and Cosgrove 1998). The two characteristic motifs in this subgroup could be important for post-translational modification, targeting to specific compartments of the cell wall, or for interaction with particular substrates. There is some evidence for a functional association of subgroup A expansins with cell wall disassembly during germination and fruit ripening (Harmer et al. 2002).

The remaining Petunia expansins, *PhEXP3A* and *PhEXP5A*, cluster within subgroup B (clade EXPA-III). This contains many EXPA proteins that are expressed in

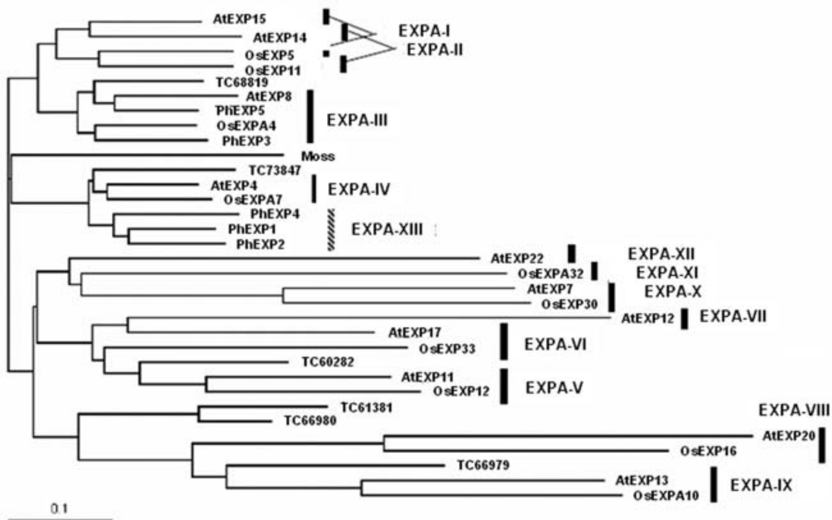

Fig. 12.2 Phylogenetic tree showing positions of cloned *P. hybrida* expansins (Ph) relative to expansin A protein sequences from *Arabidopsis thaliana* (At), *Oryza sativa* (Os), *Pinus* species (TC), and *Physcomitrella patens* (moss). The tree was calculated by the neighbor-joining method and bootstrap analysis (1000 replications) using PHYLIP. Proposed clades (Sampedro et al. 2005) are indicated at *right*

rapidly growing tissues, like elongating cotton fibers and elongating hypocotyls in a variety of species (e.g., *CsEXP1*, *CsEXP2*, *LeEXP2*, and *OsEXP4)*. We have yet to isolate Petunia expansin genes from subgroup C (clade EXPA-I), whose members have heterogeneous expression patterns, and subgroup D (clade EXPA-V), whose members are also expressed in rapidly growing tissues.

The expression profiles of *PhEXP1A*, *PhEXP2A*, *PhEXP3A*, *PhEXP4A*, and *PhEXP5A* were analyzed by semi-quantitative real-time reverse transcriptase (RT) PCR, using gene-specific primers annealing to the unique 3′-untranslated regions of each gene (Fig. 12.3). *PhEXP1A* is strongly expressed in petals, ovaries, and elongating stems; there are intermediate levels in sepals, roots, styles, and stigmata, and low levels in leaves and anthers. *PhEXP2A* is strongly expressed in leaves but only weakly expressed in sepals, ovaries, stems, petals, and roots. The *PhEXP2A* transcript is barely detectable in anthers, styles, and stigmata. *PhEXP3A* is expressed strongly and specifically in styles and stigmata, whereas low expression levels are

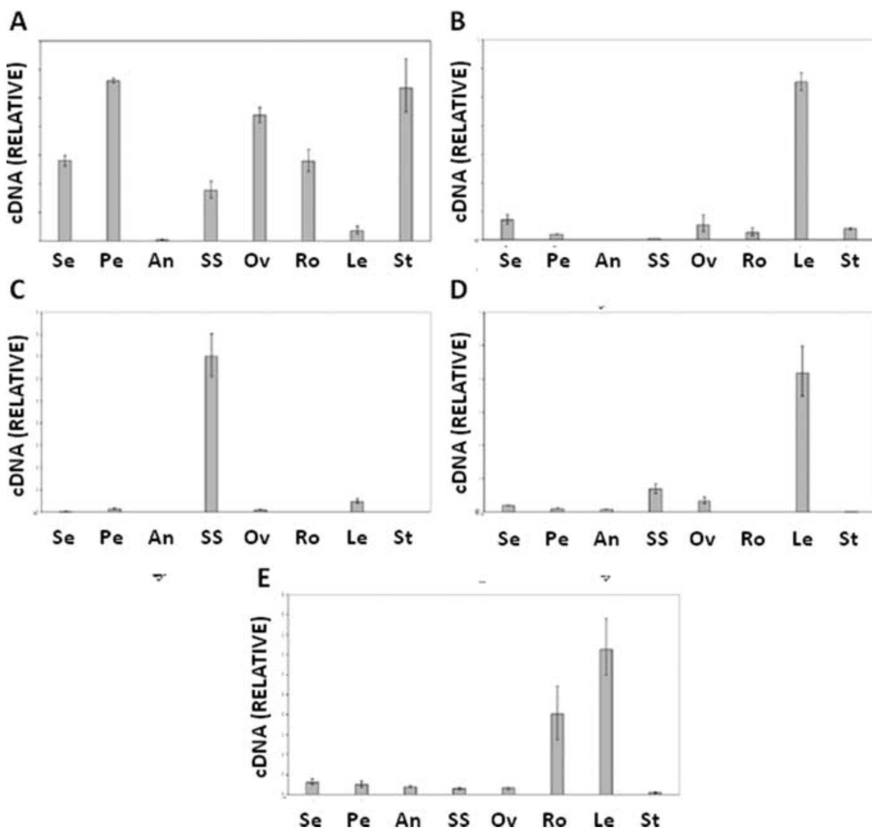

Fig. 12.3 Organ-specific expression patterns for (**A**) *PhEXP1A*; (**B**) *PhEXP2A*; (**C**) *PhEXP3A*; (**D**) *PhEXP4A*; and (**E**) *PhEXP5A*. Data were derived from analysis of RT-PCR products based on amplification from gene-specific primers. Se, Sepal; Pe, Petal; An, Anther; SS, Stigma and Style; Ov, Ovary; Ro, Root; Le, Leaf; and St, Stamen

found in other tissues. *PhEXP4A* is expressed strongly in leaves, and at lower levels in sepals, styles, stigmata, and ovaries, whereas no *PhEXP4A* can be detected in anthers, roots, and stems. *PhEXP5A* is expressed at high levels in leaves and roots, but at low levels in all other organs.

To assess whether EXPA proteins in the same clade may be functionally related, we compared the phylogenetic classifications of the five new Petunia *EXPA* genes with the expression profiles. *PhEXP1A*, *PhEXP2A*, and *PhEXP4A*, which group together (Fig. 12.2), were strongly expressed in petals and leaves, tissues in which cell enlargement is characterized by anisotropic growth. *PhEXP3A*, expressed specifically in the style and stigma, and *PhEXP5A*, expressed in leaves and roots, also group together phylogenetically (Fig. 12.2); in both style and root, isotropic cell growth contributes to the determination of organ morphology. On this basis, there does seem to be evidence for some functional significance in the phylogenetic profile of the Petunia EXPA subfamily.

12.7.1 Functional Analysis

The functions of individual genes can be established by the creation of mutants, but in many systems including Petunia it is easier to create phenocopies by interfering with target gene expression. The expression of antisense RNA is one such approach, and this was used to knockdown *PhEXP1A* gene expression in order to observe the impact on petal morphogenesis (Reale et al. 2002). Semi-quantitative RT-PCR showed that *PhEXP1A* is expressed throughout petal development, with peaks at stages 7 and 10.

Stage 7 marks the onset of cell expansion in the mesophyll of petal limbs, while stage 10 involves the expansion of epidermal cells that control flower unfolding. This suggested a correlation between *PhEXP1A* transcript levels and petal cell enlargement during petal development. We therefore focused our analysis on *PhEXP1A* expression in the petal tube and limb from stages 7 to 13. Because *PhEXP1A* expression in the limb is 3–5-fold higher than in the tube, we proposed that *PhEXP1A* is required for limb cell growth.

To test this hypothesis we produced transgenic Petunia plants in which *PhEXP1A* mRNA levels were reduced by antisense RNA expression (Zenoni et al. 2004). Four independent transgenic lines were recovered showing aberrant petal morphology (Fig. 12.4A), which correlated with the downregulation of *PhEXP1A* expression. Homozygous plants showed a significant reduction in petal length, corresponding to an average 75% reduction in the levels of *PhEXP1A* mRNA in petals. The length of the petal limb, but not that of the tube, was reduced, correlating with a significant reduction in *PhEXP1A* levels in the same flower part during development (Fig. 12.4B). The total limb surface area was reduced to approximately one-third that of the wild type, and this was attributable to reduced cell surface areas in the adaxial and abaxial epidermal limb. There were no significant differences in cell number between transgenic and wild-type plants.

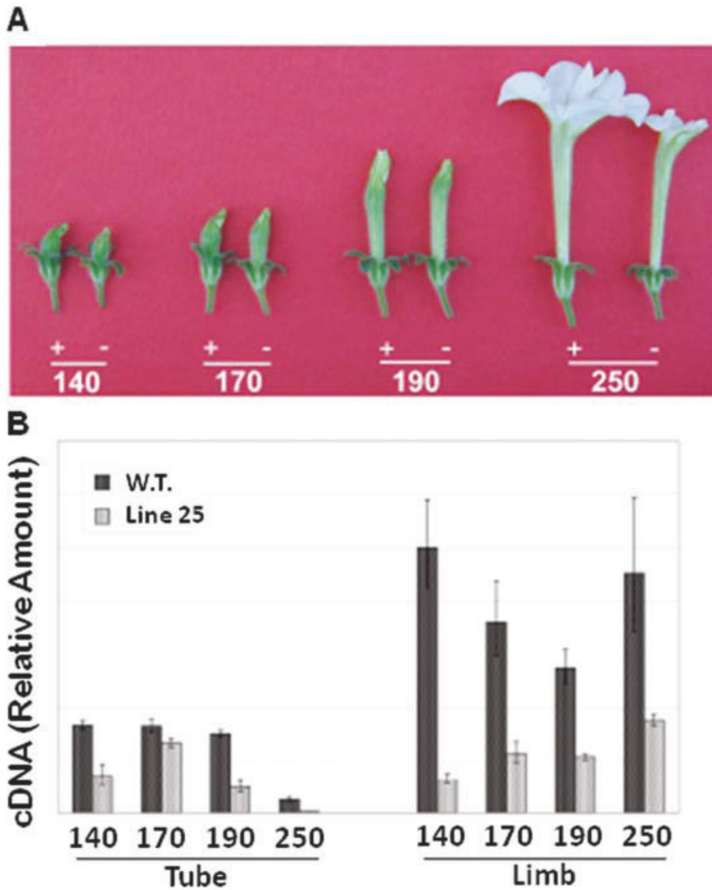

Fig. 12.4 Effect of antisense suppression of *PhEXP1A*. (**A**) Morphological changes during petal development at 140, 170, 190, and 250 h AFBA (after flower bud appearance). (**B**) *PhEXP1A* expression in tube and limb of wild-type and mutant petals at 140, 170, 190, and 250 h AFBA

Morphological analysis of epidermal limb cells (Fig. 12.5) revealed that cells in transgenic plants were smaller and had a less pronounced conical tip – the overall shape was irregular and the characteristic lobes were reduced or absent. Tangential and radial cell walls appeared thinner in the transgenic lines. Fourier transform infrared (FTIR) spectroscopy showed that the reduction in cell wall thickness in the transgenic plants could be attributed to a deficiency in crystalline cellulose. Indeed, the walls of petal epidermal cells in the transgenic lines had a higher protein content and were depleted in cellulose compared to wild-type cells.

On the basis of these results we propose that expansins may fulfill two functions in Petunia petal development: first, they may help to disrupt noncovalent bonds between cellulose microfibrils and cross-linking glycans, thereby promoting

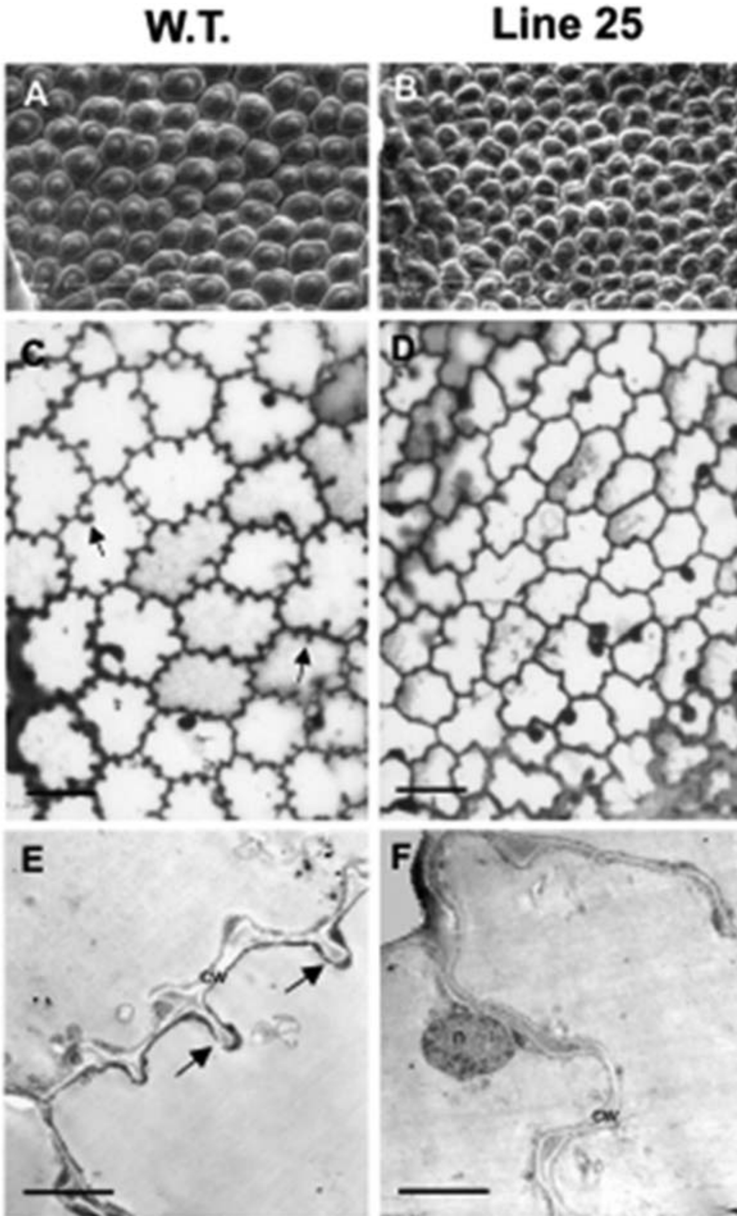

Fig. 12.5 Morphological comparison of epidermal limb cells in wild-type and *PhEXP1A*-downregulated Petunia. (**A, B**) scanning electron micrographs; (**C, D**) tangential semi-thin sections; (**E, F**) transmission electron micrographs

polymer creep as in the model proposed by Cosgrove (2000); and second, they may help to prepare the cell wall for new cellulose deposition during creep by separating the wall matrix during expansion, although a mechanism for the latter has yet to be established.

12.7.2 Identification and Analysis of Insertional Mutants

It can be difficult to create specific knockout mutants in many plant species; an alternative strategy is to first generate random libraries of mutants by saturation insertional mutagenesis and then isolate mutants in interesting genes by PCR, using primers specific for the insertional DNA construct and the target gene. A Petunia *dTph1* insertional library was screened using specific primers matching the 5′ and 3′ ends of the *PhEXP1A* cDNA sequence together with a primer specific for the *dTph1* transposon, resulting in the isolation of four *PhEXP1A* insertional mutants, one with the insertion in the first exon and three with insertions in the first intron (Fig. 12.6).

Insertional mutants for the other four expansin genes were sought by screening the same library with two family-specific primers (designed around conserved motifs in EXPA genes) together with the *dTph1* primer. No insertions were found in *PhEXP2A*, and single insertions were identified for the remaining three genes – in the third exon of *PhEXP3A*, the second intron of *PhEXP4A*, and the second exon of *PhEXP5A* (Fig. 12.6).

Lines with exon insertions were chosen for further analysis, as these were deemed most likely to show loss-of-function phenotypes. However, no morphological anomalies were observed in homozygous insertional mutants for *PhEXP1A*, *PhEXP3A*, or *PhEXP5A*. This is consistent with results obtained with equivalent

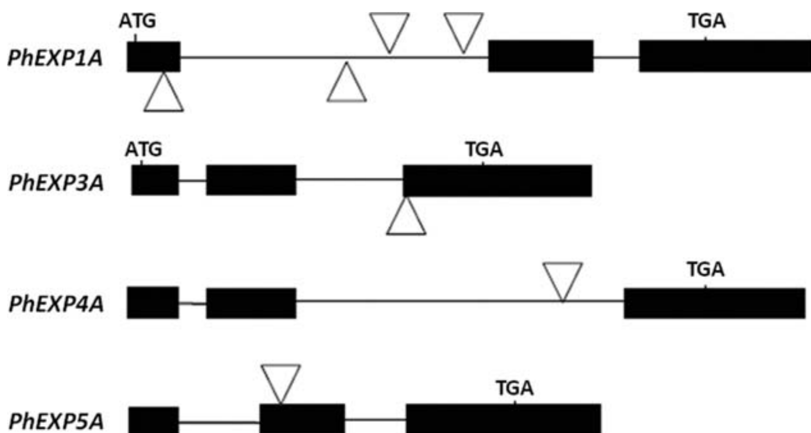

Fig. 12.6 Positions of transposable element insertions in *P. hybrida* expansin genes recovered from a *dTph1* insertional library

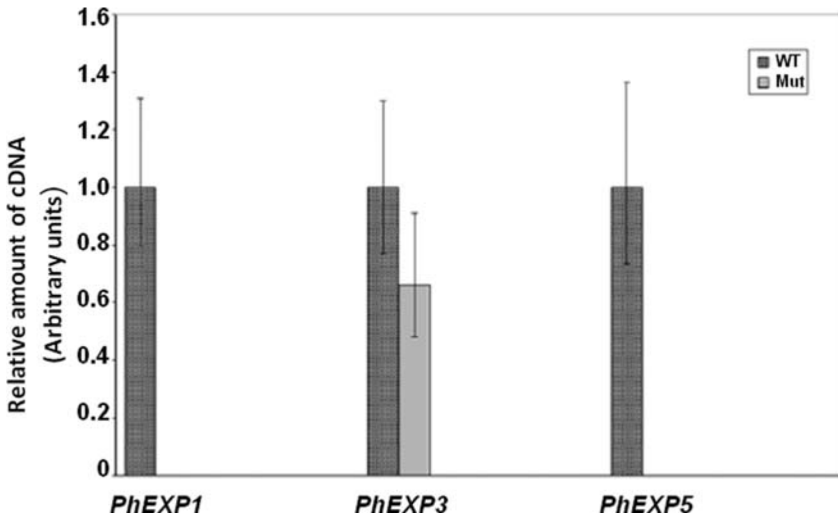

Fig. 12.7 RT-PCR-based analysis of gene expression in tissues normally expressing *PHEXP1A* (*petal*), *PhEXP3A* (*style and stigma*), and *PhEXP5A* (*leaf*), comparing levels of cDNA recovered from wild-type plants and the triple mutant

T-DNA insertion lines in Arabidopsis, perhaps indicating functional redundancy among the related genes (Cosgrove et al. 2002). To verify this hypothesis, we created three double mutants (*PhEXP1A-PhEXP3A*, *PhEXP3A-PhEXP5A*, and *PhEXP1A-PhEXP5A*) and one triple mutant (*PhEXP1A-PhEXP3A-PhEXP5A*). These plants, too, showed no sign of morphological changes compared to wild-type plants.

To confirm that the triple mutants lacked the capability to produce functional transcripts, real-time RT-PCR analysis was carried out in organs where the wild-type transcript should be expressed strongly. Thus, *PhEXP1A* was analyzed in petals, *PhEXP3A* in styles and stigma, and *PhEXP5A* in leaves. *PhEXP1A* and *PhEXP5A* transcripts were undetectable in homozygous plants but *PhEXP3A* expression persisted, albeit reduced by about 40% compared to that of the wild type (Fig. 12.7). This residual *PhEXP3A* expression in mutant plants presumably reflects the position of the *dTph1* insertion (Fig. 12.6), which might interfere with splicing of the second intron yet allow the production of some still-functional transcripts.

12.8 The Future: Expansion Beyond Expansin

Our functional analysis of *PhEXP1A* suggests a novel biological role for the expansin A subfamily. The reduction of crystalline cellulose in *PhEXP1A* mutants suggests PhEXP1A involvement in cellulose metabolism, which is a rate-limiting step in cell expansion. The enlargement of cells in development and in other processes could be blocked until this step is completed. Alternatively, plant cells could

overcome the limited availability of particular expansins by relying on the expression of genes with related functions.

In order to monitor global transcriptional changes occurring during the development of mutant petals with defective limb cell expansion, we used a cDNA-AFLP approach to compare transcript profiles in antisense *PhEXP1A* lines and wild-type plants. Messenger RNA was isolated from petal limbs at the same four developmental stages used in the *PhEXP1*A expression profiling experiments described above, and a transcriptional profile was created using 32 primer combinations, surveying about 6100 mRNAs. This analysis revealed 370 cDNA fragments (expressed sequence tags, ESTs) ranging from 50 to 500 bp, whose expression patterns differed between antisense and wild-type plants. The ESTs were isolated, re-amplified, and cloned into a TOPO® vector (Invitrogen, Carlsbad, CA) and sequenced, resulting in 190 sequences that could be used in BLAST searches against sequence databases (Fig. 12.8). Further data analysis and clustering showed that a majority of the ESTs encode proteins with metabolic functions (39%) or roles in signal transduction (17%).

One attractive theory is that the reduction of crystalline cellulose in the *PhEXP1A* antisense line causes a compensatory shift in carbohydrate metabolism to enhance cellulose synthesis. In addition, about 8% of the ESTs encode proteins known to be involved in the assembly and development of cell walls, while 10% are nucleic acid binding proteins, 5% are chloroplast proteins, 4% are defence related, and 2% are components of the cell cycle. The differential expression of these sequences was confirmed by real-time RT-PCR (data not shown).

The global transcript profile in the antisense line was also analyzed in the context of sequence ontology. The genes involved in cell wall metabolism generally

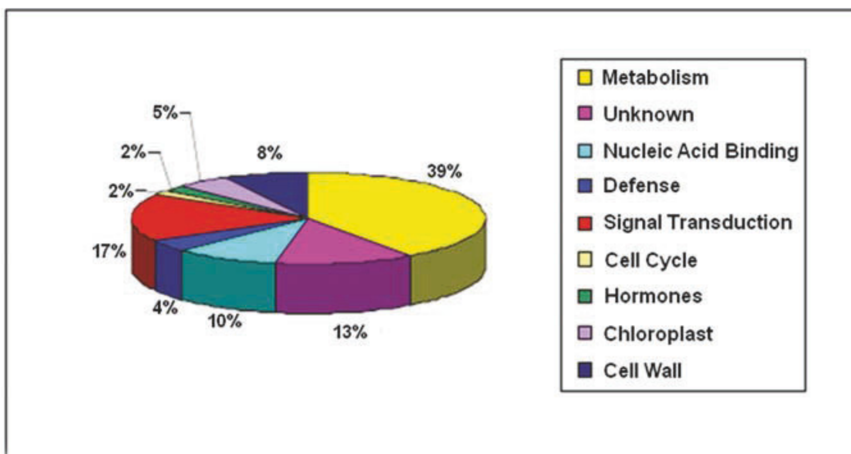

Fig. 12.8 Categorization of genes with altered expression in the *Ph1EXP1A* mutant. The analysis was based on ESTs recovered from transcript profiling of RNA from wild-type and *Ph1EXP1A* mutant petals harvested at four developmental stages

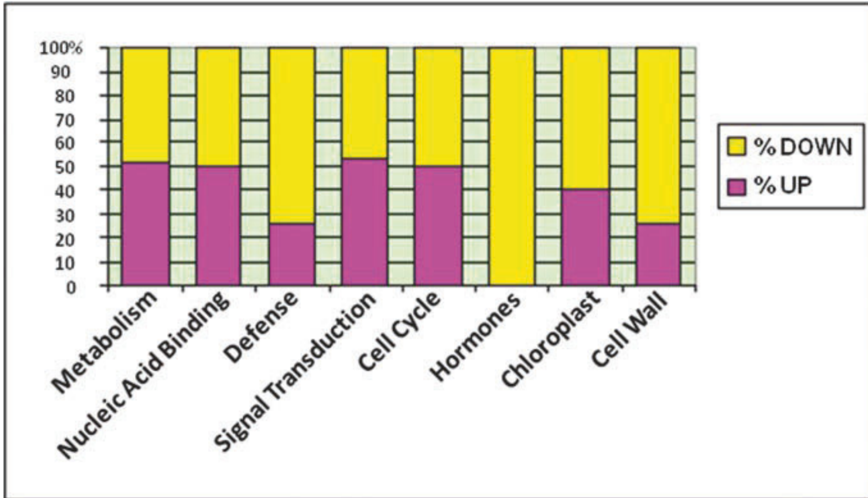

Fig. 12.9 Global modification of gene expression in petals of the *PhEXP1A* mutant, illustrating patterns of upregulation and downregulation

showed a strong reduction in expression, an average of 75% below wild-type levels (Fig. 12.9). This group contained endoglucanases, pectin methylesterase, and one additional expansin A gene. These data indicate that *PhEXP1A* may act upstream of the cell-enlargement process, regulating the expression or activity of other cell wall loosening agents. This is consistent with the idea that the expansins play an important regulatory role during cell wall extension, hence their description as primary rather than secondary wall-loosening agents. Expansins could be responsible for the critical modifications in the cell wall network, making the cell wall molecules available as substrates for other enzymes, such as endoglucanases. The additional expansin identified in this group is very similar to *LeEXP11A*, and is strongly inhibited. This suggests that some expansins can influence the activity and expression of others in a complex regulatory hierarchy that is still largely a black box despite many years of diligent research.

12.9 Conclusions

We identified five members of the *EXPA* gene family in *P. hybrida* and carried out detailed expression profiling in different tissues, revealing that *PhEXP1A* is temporally regulated during development. The single, double, and triple insertional mutants for *PhEXP1A*, *PhEXP3A*, and *PhEXP5A* had normal morphologies, which suggests that multiple knockouts will be required to avoid the effects of functional redundancy in order to establish gene function unambiguously. Antisense knockdown of *PhEXP1A* supported the involvement of this expansin in the regulation of cell wall extension. We also propose a new role for PhEXP1A: preparing the

growing cell wall for new cellulose deposition. Expansins could be also involved in regulating the expression of secondary cell wall loosening agents. Major questions remain regarding the specific wall polysaccharides targeted by expansin and the molecular mechanisms underlying cell wall loosening.

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Chapter 13
The Genetics of Flower Color

Giambattista Tornielli, Ronald Koes, and Francesca Quattrocchio

Abstract With nearly a century of excellent research on the biochemistry and inheritance of color, and the corresponding development of incredible genetic resources, Petunia has offered perhaps the best genetic system for molecular analysis of flower color. The knowledge and materials available to the Petunia geneticist, together with the tools of genetic engineering, have allowed for the isolation and characterization of a large number of genes affecting flower color, including those encoding biosynthetic enzymes, regulators of their expression, and vacuolar function. Here we summarize current knowledge about the genes responsible for the amazing diversity of colors and color patterns observable in the genus *Petunia* and propose some evolutionary implications of these findings.

13.1 Introduction

The history of Petunia research is tightly connected to the study of flower color genetics. The reason is easy to imagine after walking around for not more than two min in the greenhouse of the Vrije Universiteit in Amsterdam, where a large collection of Petunia mutants has made its home for some 25 years. Many of these mutants involve changes in flower color, resulting in an impressive exhibition of colors and patterns.

In the 1980s, a number of mutants affecting mainly flower color were identified and characterized at the University of Amsterdam and at INRA in Dijon. This work included the assignment of many of the mutations to one of the linkage groups on the seven choromosomes of Petunia and the study of allelic series for some of the unstable mutants (Maizonnier and Moessner 1979; Gerats, Farcy, Wallroth, Groot, and Schram 1984; see Chapters 15 and 17).

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Almost any step of the pathway leading to the biosynthesis of anthocyanins, the main pigments accumulating in Petunia petals, has its mutant, with the obvious consequence that an impressive number of genes involved in this process have been cloned and characterized. This includes genes encoding the enzymes of the anthocyanin pathway and regulators that control their expression, genes involved in the formation of color patterns, genes specifying the cell shape in the epidermis, and genes controlling the environment in the vacuolar lumen where the pigment molecules are stored.

Thanks to the abundance of existing mutants and the continuous appearance of new transposon-induced pigmentation mutants both in lab strains and in the fields of Petunia breeders, Petunia is at the moment the best described genetic model for factors contributing to petal color.

13.2 Factors Influencing Flower Color in Petunia

The color of the Petunia flower corolla results from the effects of several factors. In the first place, it requires the synthesis, modification, and accumulation of anthocyanin pigments, as any mutation affecting the structure of the final product of the anthocyanin pathway results in a change in petal color. Most pigmented cells contain colorless co-pigments, in Petunia primarily flavonols, that shift the color of the anthocyanin pigments. Complex formation with metal ions (Mg^{2+}), in combination with flavones (Hondo et al. 1992), has been shown to affect the final color of the corolla in *Commelina communis*, but evidence for such a phenomenon in Petunia has not yet been reported.

The shape of the epidermal cells of the petals, where the pigments accumulate, determines their optical characteristics and therefore affects the color of the corolla. A few mutants in which a clear change in color correlates with changes in cell shape have been isolated in Petunia (van Houwelingen, Souer, Spelt, Kloos, and Koes 1998). One of these mutants was shown to be the result of a transposon insertion into a MYB-like gene homologous to *MIXTA* from snapdragon (Mur 1995), which is also involved in epidermal cell shape determination (Noda, Glover, Linstead, and Martin 1994; Baumann et al. 2007).

Another factor that strongly affects flower color is the pH of the vacuolar lumen where the anthocyanin molecules are stored. Because their absorption spectra change as a consequence of shifts in the pH of the solvent, anthocyanins act as pH indicators. In wild-type flowers the vacuolar lumen is weakly acidic (~pH5), resulting in reddish anthocyanins that shift to bluer colors in mutants with less acidic vacuoles.

In this chapter, we shall go into the details of what is known about the control of some of these factors influencing flower color in Petunia. In particular, we shall report on genes involved in pigment and co-pigment biosynthesis and those affecting vacuolar acidification in petal epidermal cells.

13.3 Structural Genes of the Pathway in Petunia

The wealth of knowledge accumulated in the last several decades about the biochemistry, genetics, and molecular biology of the flavonoid pathway has made Petunia the species of choice for the study of flower pigmentation (Holton and Cornish 1995; van Houwelingen et al. 1998; Koes, Verweij, and Quattrocchio 2005).

Many structural genes encoding the enzymes of the anthocyanin pathway in Petunia flowers have been characterized with regard to function, expression pattern, and regulation (Fig. 13.1). Structural genes can be grouped into two broad classes that differ with regard to position in the pathway, regulation of expression, and evolutionary history. Early biosynthetic genes, such as those encoding chalcone synthase (*CHS*), chalcone-flavanone isomerase (*CHI*), and flavonoid 3-hydroxylase (*F3H*), are required for the synthesis of precursors of multiple classes of flavonoids. In contrast, late biosynthetic genes, such as *DFR* and genes acting thereafter, are required for synthesis of anthocyanins and proanthocyanidins only. Early and late biosynthetic genes presumably appeared sequentially during evolution, as evidenced by the facts that Bryophytes (mosses) already produce chalcones, flavanones, and flavonols, while ferns are the first to produce proanthocyanidins (and therefore to have a *DFR* gene); and anthocyanins appear only in higher vascular plants (Gymnosperms and Angiosperms), together with genes required for anthocyanin modification (*RT*, *3GT*, etc., Koes, Quattrocchio, and Mol 1994).

During the development of petals and anthers, early and late biosynthetic genes show similar, if not identical, spatial-temporal expression profiles that correlate with pigment accumulation. Nevertheless genetic evidence indicates that early and late biosynthetic genes are controlled by distinct transcriptional regulators (see below). The split of the pathway into two independently regulated units allows for independent synthesis of anthocyanins and flavonols, with different spatial-temporal patterns.

13.3.1 From Phenylalanine to 4-Coumaroyl-CoA

Little is known about the genetic control of the very first part of the phenylpropanoid pathway in Petunia, and few genes controlling the enzymatic steps that convert phenylalanine to 4-coumaroyl-CoA have been identified. A cDNA fragment encoding phenylalanine ammonia-lyase (PAL) provides the only sequence available for this enzyme in Petunia (Quattrocchio, Wing, Leppen, Mol, and Koes 1993).

The poorly characterized locus *AN10* presumably encodes, or controls, the expression of 4-coumaroyl:CoA-ligase (4CL), as *an10* mutant flowers contain little or no anthocyanins or flavonols and accumulate coumaric acid. Pigmentation of *an 10* flowers can be restored by external feeding of naringenin (van Houwelingen et al. 1998).

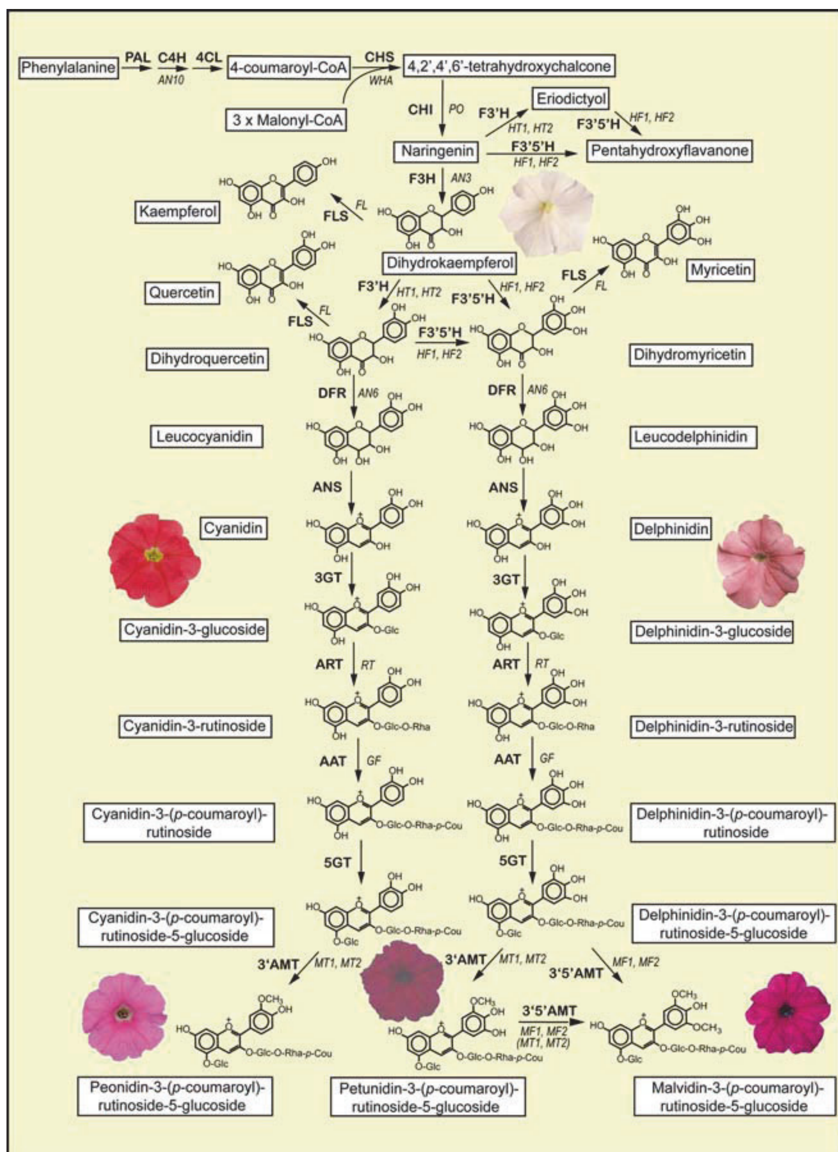

Fig. 13.1 Scheme of the flavonoid pathway in Petunia. Enzymatic activities are indicated in *bold*, genetic loci in *italics*, and major intermediates are shown in *outlined boxes*. Petunia flowers accumulating different classes of products: line W80, dihydroflavonols; R27, cyanidin; VU6, delphinidin; M1, peonidin; V30, petunidin and malvidin; F1 M1×V30, malvidin. Abbreviations: PAL, phenylalanine ammonia lyase; C4H, cinnamate-4-hydroxylase; 4CL, 4-coumaroyl:CoA-ligase; CHS, chalcone synthase; CHI, chalcone flavanone isomerase; F3H, flavanone 3-hydroxylase; FLS, flavonol synthase; F3′H and F3′5′H, flavonoid 3′ and 3′5′ hydroxylase; DFR, dihydroflavonol 4-reductase; ANS, anthocyanidin synthase; 3GT and 5GT anthocyanidin 3 and 5 glucosyltransferase; ART, anthocyanidin 3-glucosyde rhamnosyltransferase; AAT, anthocyanidin 3-rutinoside acyltransferase; 3′AMT and 3′5′AMT, anthocyanidin 3′ and 3′5′ O-methyltransferase

13.3.2 Synthesis of Flavonoids

CHS is the first enzyme in flavonoid biosynthesis. It catalyzes the condensation of three molecules of malonyl-CoA with 4-coumaryl-CoA to yield naringenin-chalcone (tetrahydroxychalcone). *CHS* genes comprise a medium-sized family in all Petunia species and cultivars analyzed. The line V30 contains some eight complete *CHS* or *CHS*-like genes as well as some gene fragments encoding only the first or second CHS exon. Several of these genes constitute subfamilies of genes that display high sequence similarity and tight genetic linkage. The number of genes in these subfamilies differs between the ancestral species from which *Petunia hybrida* originates, and consequently also among different *P. hybrida* cultivars and lines (Koes, Spelt, Mol, and Gerats 1987). Together this indicates that these subfamilies represent recently duplicated paralogs.

Only two *CHS* genes (*CHS-A* and *CHS-J*) are active in floral tissues and under UV stress conditions in seedlings. Many cultivars contain a subfamily of two *CHS-A* genes, both of which are expressed, and thus harbor three *CHS* genes active in flowers. Two other *CHS* genes (*CHS-B* and *CHS-G*) are active at a low level in UV-stressed seedlings only (Koes, Spelt, and Mol 1989a). Expression of *CHS-A* and *CHS-J* in floral tissues is coordinate, light dependent, and developmentally controlled. However, the expression of *CHS-J* (but not that of *CHS-A*) is under the control of regulators for the late biosynthetic genes (*AN1*, *AN2*/*AN4* and *AN11*, Quattrocchio et al. 1993), but its contribution to corolla pigmentation is marginal, as the level of expression is approximately 10-fold lower than that of *CHS-A* (Koes, Spelt, Van den Elzen, and Mol 1989b; Koes, Van Blokland, Quattrocchio, van Tunen, and Mol 1990).

In contrast to other plant species in which both spontaneous and induced *CHS* mutations have been described (Dooner, Robbins, and Jorgensen 1991), most attempts to isolate such mutants in Petunia were unsuccessful even after mutagenesis (Reif, Wiesbach, Deumling, and Saedler 1985), probably due to gene redundancy. A new pigmentation mutant, *white anthers* (*wha*), which might be a *chs* mutant (Napoli, Fahy, Wang, and Taylor 1999), was found by EMS mutagenesis in the V26 line. V26 plants contain a single *CHS-A* gene (Stam 1997) and an inactive *an 4* allele that blocks transcriptional activation of *CHS-J* in anthers. Thus, a mutation in *CHS-A* may account for the *wha* phenotype. Consistent with this hypothesis, the *wha* phenotype could be rescued by expression of a *CHS-A* cDNA from the viral 35S promoter. Whether *wha* contains sequence alterations in *CHS-A*, however, has not been established, and thus it cannot be ruled out that *WHA* encodes a regulator of *CHS-A* expression.

Nearly complete inactivation of all active *CHS* genes has been achieved by RNA interference (RNAi, van der Krol et al. 1988; Napoli, Lemieux, and Jorgensen 1990; van der Krol, Mur, Beld, Mol, and Stuitje 1990; Tsuda et al. 2004). The RNAi silencing of *CHS* in transgenic Petunias produces either white flowers or, more commonly, flowers with an array of pigmentation patterns (Stam, Mol, and Kooter 1997; Que, Wang, and Jorgensen 1998). Petunia breeders had already created Petunia varieties with color patterns similar to those of *CHS* RNAi lines by means of traditional

crosses (Mol et al. 1983). Such Petunia varieties produce flowers that exhibit alternating white and colored sectors (Red Star) or a white rim on the corolla (Picotee). In the white petal tissue of these varieties *CHS-A* and *CHS-J* mRNAs are strongly reduced by a post-transcriptional mechanism that is very similar to RNAi (Stam 1997). The mRNA levels of other structural anthocyanin genes in white tissue are similar to, or higher than, those in the pigmented parts of the flowers (Koes 1988; Koseki, Goto, Masuta, and Kanazawa 2005).

Transgenic plants in which *CHS* expression in anthers was silenced were also instrumental in identifying an essential role for flavonols in pollen (Mo, Nagel, and Taylor 1992; Taylor and Jorgensen 1992; Ylstra et al. 1994). The lack of CHS protein in both Petunia and maize anthers results in white pollen that is devoid of all flavonols and is unable to germinate or to produce a functional pollen tube in self-pollinations (Mo et al. 1992; Pollak, Vogt, Mo, and Taylor 1993).

13.3.3 Conversion of Chalcones

The presence of chalcones should confer yellow pigmentation to petals and anthers. However, accumulation of chalcones in plant tissues is rare: chalcone is rapidly isomerized by the enzyme chalcone flavanone isomerase (CHI), leading to the synthesis of colorless naringenin-flavanone. This isomerase activity is responsible for establishing the proper stereo configuration of the products required by the subsequent enzymes of the pathway. Even in the absence of CHI, chalcones with free 2′- and 6′-hydroxyl groups, the only type synthesized in Petunia, can spontaneously isomerize to flavanones. Spontaneous isomerization is, however, much slower than the CHI-catalyzed reaction and yields two stereoisomers (Stafford 1990). Because only one of the isomers is used for the synthesis of anthocyanins, the lack of CHI activity results in reduced anthocyanin accumulation.

Reduction of the 6′-hydroxyl group by chalcone reductase (CHR) yields 6′-deoxychalcones, which spontaneously isomerize at a much slower rate than their 6′-hydroxyl counterparts. The 6′-deoxychalcones are, however, not naturally found in Petunia, which lacks CHR activity. By introducing a *CHR* cDNA from *Medicago sativa* into Petunia, two effects were reported (Davies, Bloor, Spiller, and Deroles 1998): in acyanic backgrounds stable 6′-deoxychalcones accumulated and yellow coloration of the flower was observed, and in cyanic backgrounds paler flower colors were observed due to competition between CHR and CHI for common substrates. The accumulation of 6′-deoxychalcones in *CHR*-transgenic plants with no reduction in overall flavonoid levels strongly suggests that Petunia CHI cannot use 6′-deoxychalcones as a substrate. This confirms results from in vitro enzyme showing that the specificity of CHI from different species, in regard to the 6′-hydroxyl, is related to the type of flavonoid substrates produced by the plant (Dixon, Blyden, Robbins, van Tunen, and Mol 1988; Heller and Forkmann 1988).

The V30 Petunia genome carries at least two distinct genes encoding CHI, one of which (*CHI-B*) is expressed only in immature anthers. The role of *CHI-B* is unclear, as its mRNA expression does not correlate with the expression of CHI enzymatic activity. It is possible that *CHI-B* encodes not CHI, but a related enzyme that catalyzes a distinct reaction. The other *CHI* gene (*CHI-A*) is expressed in petals, immature anthers and, as a longer mRNA, mature anthers (van Tunen et al. 1988; van Tunen, Hartman, Mur, and Mol 1989; van Tunen et al. 1990). A combination of different approaches provided definitive proof that *CHI-A* is transcribed from tandem promoters (designated P_{A1} and P_{A2}) that are differentially used. The downstream P_{A1} promoter (giving rise to a 1.0 kb mRNA) is active in limb, tube, young anthers, seed, and at lower levels in sepal, leaf, and stem, whereas the upstream P_{A2} promoter (giving rise to a 1.5 kb transcript) is active in mature anthers, more precisely in pollen grains. The function of the long *CHI-A* transcript is still obscure because its expression is not coordinate with that of *CHS* or *DFR* genes, and no CHI enzymatic activity can be detected in anthers at this developmental stage (van Tunen et al. 1988).

The *CHI-A* gene is located at the genetically defined locus *PO* (van Tunen et al. 1990). In *po* mutants, expression of the 1.0 kb *CHI-A* mRNA is abolished in anthers, resulting in the accumulation of yellow chalcones. However, this mutation must lie outside the *CHI-A* coding region because the *CHI-A* gene from a *po* line encodes a functional CHI enzyme in petals (van Tunen et al. 1988). Experiments with promoter-GUS fusions showed that the *po* mutation specifically abolished the activity of the CHI-A P_{A1} promoter in anthers (van Tunen et al. 1988).

13.3.4 Synthesis of Dihydroflavonols

Naringenin is converted to dihydrokaempferol by flavanone 3β-hydroxylase (F3H), a soluble 2-oxoglutarate-dependent dioxygenase whose biochemical properties have been well characterized in Petunia (Britsch and Grisebach 1986). An *F3H* cDNA was identified following purification of F3H (Britsch, Ruhnau-Brich, and Forkmann 1992). Southern blot analyses suggested the presence of only one *F3H* gene in the Petunia genome (Britsch et al. 1992; van Houwelingen et al. 1998).

The conversion of naringenin into dihydrokaempferol is controlled by the *AN3* locus (Froemel et al. 1985; Britsch et al. 1992). That *AN3* contains the structural gene for *F3H* was shown by the analysis of transposon insertion mutants (van Houwelingen et al. 1998). Flowers of plants homozygous for the recessive allele *an3* are normally nearly white because anthocyanin synthesis is strongly inhibited. Furthermore they produce only traces of flavonols and accumulate flavanones. The nature of the flavanones (naringenin, eriodictyol, pentahydroxyflavanone) is determined by the flavonoid B-ring hydroxylation genes, controlled by the loci *HT1*, *HT2*, *HF1*, and *HF2* (see below). All *an3* mutants, even null mutants in which the entire *F3H* gene has been deleted, show weak residual pigmentation of the corolla. The residual pigmentation indicates that another hydroxylase might account for the partial accomplishment of this step.

13.3.5 Hydroxylation of Dihydroflavonols

The hydroxylation pattern of the B-ring of dihydrokaempferol is of central importance in determining petal color. When hydroxylation occurs at the 3′ position, dihydroquercetin is produced and this leads to the synthesis of the red/magenta cyanidin-based pigments. Alternatively, when hydroxylation occurs at both the 3′ and 5′ positions, dihydromyricetin is produced, leading to the synthesis of the blue/purple delphinidin-based pigments. This main pathway branchpoint also has major effects on the flavonol composition of the petals as described below.

The key enzymes that determine the hydroxylation pattern are flavonoid 3′-hydroxylase (F3′H) and flavonoid 3′5′-hydroxylase (F3′5′H), which are both membrane-bound microsomal cytochrome P450-dependent monoxygenases. Plant species such as rose and carnation lack F3′5′H activity and are, therefore, unable to generate purple or blue flowers.

In flowers of Petunia, the genetic loci *HT1* and *HT2* control F3′H activity, while *HF1* and *HF2* control F3′5′H activity. *HT1* and *HF1* are phenotypically expressed in the limb and tube of the corolla, whereas *HT2* action is restricted to the tube and *HF2* action is restricted to the limb. These four genes show complex epistatic relations that have been studied in great detail:

- The gene *HF1* is the most important, as it appears epistatic to *HF2*, *HT1*, and *HT2*. Therefore, if the dominant allele *HF1* is present, *HF2*, *HT1*, and *HT2* have no visible effect on flower color and only delphinidin derivatives are present (Gerats, de Vlaming, Doodeman, and Schram 1982). However, the flavonol composition in *HF1* flowers is still dependent on the *HT1* and *HT2* genotype.
- In *hf1 HF2* individuals, dihydrokaempferol and/or dihydroquercetin precursors are only partially converted to 3′5′-hydroxylated anthocyanins; the result is a mixture of cyanidin and delphinidin derivatives.
- In an *hf1 hf2* double mutant, the dominant alleles of *HT1* and *HT2* stimulate the synthesis of cyanidin derivatives and of quercetin. *HT1* is epistatic to *HT2*.
- Homozygous recessives for *ht1*, *ht2*, *hf1*, and *hf2* produce pale lilac flowers that accumulate kaempferol and very low levels of delphinidin-based pigments, due to the leaky nature of the *hf1* mutation. The strong reduction in anthocyanin synthesis is due to the inability of Petunia DFR to convert dihydrokaempferol into the corresponding anthocyanin (pelargonidin).

In Petunia, two different *F3′5′H* genes expressed in flowers have been isolated and characterized (Holton et al. 1993b). The study of insertional mutants has proven that one of these clones corresponds to the *HF1* locus (Snowden and Napoli 1998; Matsubara, Kodama, Kokubun, Watanabe, and Ando 2005). The following experimental evidence strongly suggests that the other one corresponds to the *HF2* locus: (i) restriction-fragment length polymorphism mapping in a segregating population, (ii) complementation of *hf1 hf2* mutant Petunia lines with a construct driven by a constitutive promoter, (iii) matching of tissue-specific expression (*HF2* is active only in the limb), and (iv) functional analysis in yeast (Holton et al. 1993b).

The recessive alleles *hf1-2* and *hf1-3* have been characterized in detail and found to result from the insertion of copies of *dTPH9* and *rTPH1*, respectively (Matsubara et al. 2005; see Chapter 17). All analyzed commercial varieties accumulating cyanidin derivatives harbor one of these two alleles. Because these alleles are not present in the ancestor species of the garden petunia (*P. integrifolia, P. inflata*, and *P. axillaris*), these two insertions must have happened independently during the breeding of commercial Petunia varieties. The ancestral white-flowering species *P. parodii* and *P. axillaris*, and a number of *P. hybrida* lines, harbor the weak allele *hf1-1*. This allele, which is dominant to null *hf1* alleles, determines the accumulation of wild-type amounts of delphinidin or petunidin in the tube and smaller quantities of delphinidin derivatives in the limb (de Vlaming et al. 1984). The *hf1-1* mutation has not been characterized at the molecular level.

Yeast expression studies have shown that a cDNA isolated from Petunia corollas encodes an enzyme with F3′H activity. The study of Petunia families segregating for a transposon insertion at the *HT1* locus and of the transcripts in mutant and revertant sectors in unstable *ht1* flowers provided strong indications that this clone originates from the *HT1* locus (Brugliera, Barri-Rewell, Holton, and Mason 1999).

While F3′H activity is independent from the regulatory genes *AN1*, *AN2*, and *AN11*, the expression of transcripts for F3′5′H has been reported to be controlled by this set of regulators (Brugliera et al. 1999). This is in agreement with the absence of dihydromyricetin and myricetin in *an1* mutants (Gerats et al. 1982). Thus, the “early biosynthetic genes” are bracketed by *CHS* and *HT*, whereas *HF1* and *HF2* are “late biosynthetic genes”.

These steps of anthocyanin biosynthesis have been the basis of numerous biotechnological applications aimed at the manipulation of flower color in economically interesting species (Mol, Cornish, Mason, and Koes 1999; Shimada, Nakano-Shimada, Okinaka, Kiyokawa, and Kikuchi 2001; Tanaka, Katsumoto, Brugliera, and Mason 2005). For example, in order to redirect the metabolic pathway in flowers of *P. hybrida* (Surfinia Purple and Surfinia Purple Mini) from delphinidin-type anthocyanins to cyanidin-type anthocyanins, *F3′5′H* gene expression was down-regulated by sense suppression of the endogenous *F3′5′H* encoded by the *HF1* locus (Tsuda et al. 2004). Cyanidin-type anthocyanins dominated in these transgenic plants, which showed flower colors varying from pink/magenta to red, depending on concomitant suppression of *FLS*, *ART*, and *AAT* (see below).

13.3.6 The Role of Cytochrome b_5

It has been demonstrated that an additional Petunia gene, encoding a cytochrome b_5 ($CYTb_5$), is required for 3′5′ substitution of anthocyanins and blue/purple flower color. This gene was initially named *DIF-F* (*DIFFERENTIAL-F*), as it was identified in a collection of cDNAs whose corresponding mRNAs are downregulated in flowers with a mutation in the regulatory *AN1* gene. *DIF-F* transcripts accumulate in the limbs and tubes of the flower corollas and in the ovaries, but not in vegetative

organs such as leaves, roots, and stems. During petal development, the temporal *DIF-F* expression pattern closely matches that of other late anthocyanin biosynthetic genes (e.g., *DFR*), reaching a maximum just prior to unfolding of the petals. Transposon inactivation of *DIF-F* resulted in a decreased level of F3′5′H activity and reduced accumulation of 3′5′-hydroxylated anthocyanins in the petal (de Vetten et al. 1999).

Cytochrome b_5 participates in several systems where it functions as an electron transfer component. Among the proteins able to accept electrons from $CYTb_5$ are CYT P450s (like F3′5′H) that can use this electron source in addition to proteins such as NADPH:CYTP450 reductase. The first direct in vivo demonstration of the requirement for $CYTb_5$ for the full activity of a CYT P450 came from the *DIF-F* insertion mutant in Petunia. The analysis of this mutant has shown that the DIF-F-encoded $CYTb_5$ enhances the activity of both F3′5′H CYT P450 isoenzymes encoded by *HF1* and *HF2*.

The $CYTb_5$ encoded by *DIF-F* seems rather specific, as a *dif-f* mutation does not visibly affect the activity of other P450s involved in anthocyanin biosynthesis (e.g., HT1 and HT2), general phenylpropanoid metabolism (C4H), or other pathways (e.g., fatty acid biosynthesis, de Vetten et al. 1999).

It has been shown that F3′5′H activity, which is considered the critical step toward the generation of blue flowers in ornamental species, can be significantly increased by co-expression of the Petunia *DIF-F* gene (Brugliera et al. 2000). Expression of a Petunia *F3′5′H* in a carnation line that accumulated cyanidin-based pigments resulted in very low levels of delphinidin production and little effect on flower color, probably due to the failure of the introduced Petunia F3′5′H to compete with the endogenous carnation F3′H and DFR enzymes. However, when the Petunia *DIF-F* was co-expressed with *F3′5′H*, a dramatic improvement in the level of delphinidin production and a shift in the flower color from a variegated pink and red to a variegated mauve and purple was observed (Brugliera et al. 2000).

13.3.7 Synthesis of Flavonols

Dihydroflavonols are a common substrate for the synthesis of both anthocyanins and flavonols. Flavonols are colorless (or pale yellow) flavonoids that contribute to a bathochromic shift of anthocyanins (a phenomenon called co-pigmentation) and thus can have major effects on flower color. Co-pigmented anthocyanins are usually bluer than the corresponding anthocyanins alone. Flavonols of Petunia are mainly represented by quercetin and kaempferol glycosides; myricetin is of rare occurrence.

Synthesis of flavonols from dihydroflavonol substrates is controlled by the enzyme flavonol synthase (FLS), which is a 2-oxoglutarate-dependent dioxygenase. In Petunia flowers, the locus *FL* controls the formation of flavonols. In *fl* mutants only traces of quercetin and/or kaempferol occur, while in *FL* individuals, synthesis of kaempferol and quercetin proceeds at the expense of the formation of cyanidin-based pigments, and, to a lesser extent, of delphinidin derivatives. This is apparently

due to competition between the enzymes of flavonol and anthocyanin biosynthesis for dihydroquercetin, a common substrate. FLS could also compete with dihydroflavonol reductase (DFR) for dihydromyricetin as a common substrate. However, myricetin rarely accumulates to high levels in Petunia, presumably due to a low affinity of FLS for dihydromyricetin (Forkmann, de Vlaming, Spribille, Wiering, and Schram 1986). Only when accumulation of dihydromyricetin is stimulated, for example, in the case of a *dfr* mutation in *HF1* genotypes, is a considerable amount of myricetin synthesized (Gerats et al. 1982). The presence of small amounts of flavonols in *fl* mutants suggests that *FLS* in Petunia might be encoded by more genes (although genomic DNA hybridizations detected only one gene copy) or that the *fl* mutation is leaky.

A full-length cDNA clone coding for a putative FLS has been isolated and the gene shown to be closely linked to the *FL* locus (Holton et al. 1993a). The highest levels of transcripts for FLS were detected at the very early stages of flower development, decreasing thereafter. A similar pattern was described for FLS enzyme activity in Petunia petals (Forkmann et al. 1986). FLS activity was demonstrated in yeast expressing the Petunia cDNA clone. Sense and antisense suppression of *FLS* resulted in decreased amounts of flavonols and reddening of the flowers in both transgenic Petunia and tobacco (Holton et al. 1993a; Tsuda et al. 2004). Overexpression of a rose *FLS* homolog in Petunia (Surfinia Violet) produced paler flowers (due to the reduction in anthocyanins resulting from competition with DFR for the same dihydroflavonol substrate), and presence of myricetin (which the FLS from rose is able to synthesize, Tsuda et al. 2004). Taken together, these data strongly suggest that *FL* encodes the FLS enzyme, but they do not exclude the possibility that *FL* controls *FLS* expression.

13.3.8 From Dihydroflavonols to Anthocyanidins

The reduction of dihydroflavonols to leucoanthocyanidins is catalyzed by dihydroflavonol 4-reductase (DFR). Petunia contains three different *DFR* genes (*DFR-A*, *DFR-B*, *DFR-C*), but only the *DFR-A* gene is transcribed in floral tissues (Beld, Martin, Huits, Stuitje, and Gerats 1989). *DFR-A* gene expression is modulated during flower development, reaching maximum levels just before petal unfolding, similar to the pattern of all the "late" anthocyanin biosynthetic genes.

The *DFR-A* gene was shown to correspond to the *AN6* locus (Huits, Gerats, Kreike, Mol, and Koes 1994). In *an6* mutants, the synthesis of anthocyanins (or proanthocyanidins) is blocked in the flower limb, the flower tube, the pistil, the anther, and the seedcoat; it can be restored by the introduction of a wild-type *DFR-A* gene. Mutants containing the original *an6* reference allele, as well as *an6* mutants isolated in random transposon mutagenesis experiments, all display genetic instability. They normally bear white- or pale-colored flowers with pigmented spots or sectors. Although the phenotype resembles that of transposon-induced mutants, unstable *an6* alleles behave very differently from true transposon insertion alleles

in the way that (putative) germinal reversions are inherited. Moreover, no insertions could be found in the genomic fragment containing the *DFR-A* gene of *an6* mutants. The involvement of epigenetic phenomena is now being investigated (J. Kooter, personal communication).

The Petunia DFR enzyme preferentially converts dihydromyricetin to leucodelphinidin, whereas dihydroquercetin is a poor substrate and dihydrokaempferol is not accepted at all. Genetic evidence suggests that this substrate specificity of DFR is the reason that Petunia mutants with orange flowers (which accumulate pelargonidin) are missing. Expression of DFR from maize, gerbera, or rose, which can convert dihydrokaempferol into leucopelargonidin, in *hf1 hf2 ht1 ht2 fl* Petunia mutants resulted in formation of pelargonidin and a new brick-red flower color (Meyer, Heidmann, Forkmann, and Saedler 1987; Oud, Schneiders, Kool, and van Grinsven 1995; Tanaka et al. 2005),

The conversion of leucocyanidin and leucodelphinidin to the corresponding colored anthocyanidins is catalyzed by anthocyanidin synthase (ANS), belonging to the 2-oxoglutarate-dependent dioxygenase enzyme family. A Petunia gene coding for *ANS* has been isolated and functionally characterized (Weiss, Van der Luit, Kroon, Mol, and Kooter 1993; Nakajima, Tanaka, Yamazaki, and Saito 2001). So far, *ans* Petunia mutants have not been reported.

13.3.9 Modification of Anthocyanidins

Modification of anthocyanidins includes glycosylation, acylation, and methylation steps. All anthocyanins found in flowers of Petunia are glucosylated at the 3-position. Depending on the genetic background, anthocyanidin 3-glucosides can be modified by sequential rhamnosylation, acylation (generally with *p*-coumaric acid), glucosylation at the 5-position, and methylation at the 3′- and 3′,5′-positions. The reactions take place in this order, as the enzymes catalyzing these steps have been reported to exhibit strict substrate specificity toward the corresponding proper precursor. Consequently reactions such as glycosylation or acylation, which in themselves barely change the color of anthocyanins (generally speaking, each of these reactions leads to a slight blueing of the color), are important for subsequent modifications of anthocyanin molecules, that is, methylation, which can have major effects on flower color.

Glucosylations at the 3- and 5-positions are carried out by anthocyanidin 3-*O*-glucosyltransferase (3GT) and anthocyanidin 5-*O*-glucosyltransferase (5GT), respectively. No mutant lines specifically lacking 3GT or 5GT activity have been identified in *P. hybrida*. However, the genetic control and the biochemical properties of these enzymes have been characterized (Gerats, Wallroth, Donker-Koopmann, Groot, and Schram 1983; Jonsson, Aarsman, Van Diepen, Smit, and Schram 1984b); Yamazaky, Yamagishi et al. 2002). It appears that while 3GT has a wide specificity and is active on different anthocyanidins and flavonols, 5GT exhibits strict substrate specificity toward delphinidin 3-(p-coumaroyl)-rutinoside. Two cDNAs encoding

3GT and 5GT were isolated and the developmentally regulated expression of the corresponding genes in the Petunia corolla was reported (Yamazaky et al. 2002). Hybridization analysis of genomic DNA indicates that both *3GT* and *5GT* genes exist in two copies in *P. hybrida*.

Rhamnosylation of anthocyanidin-3-glucosides is catalyzed by a rhamnosyltransferase (ART) encoded by the *RT* locus (Brugliera et al. 1994; Kroon et al. 1994). The *rt* mutants accumulate red cyanidin-3-glucoside and/or dull gray delphinidin-3-glucoside in petals instead of the magenta or blue/purple derivatives found in petals of *RT* individuals. This phenotype indicates absence of functional redundancy of the *ART* gene. Downregulation of *ART* has been successfully obtained both by sense and antisense gene suppression (Kroon et al. 1994; Tsuda et al. 2004).

Most red-flowering commercial cultivars harbor *rt* (and *hf1 hf2*) mutations, resulting in the accumulation of cyanidin-glucosides. Most of these lines contain in the coding sequence of *ART* a *dTPH3* transposon insertion that does not transpose (Kroon et al. 1994), apparently because an active transposase source is missing (van Houwelingen et al. 1998). Because most lines contain the same *rt* allele, this presumably arose very early in the history of Petunia breeding (Nakajima et al. 2005).

In an *HF1* background, mutations in *RT* result in the accumulation of delphinidin-glucosides. Curiously, this is associated with a puckered or crumpled shape of the corolla. Because RNAi of *RT* also resulted in crumpling, this phenotype seems due to *rt* (or delphinidin accumulation), rather than a linked mutation (Tsuda et al. 2004). Crumpled corollas are apparently considered an inferior trait, explaining why delphinidin-accumulating mutants could not be found among 200 commercial varieties (Ando et al. 2004).

Acylation with *p*-coumaric acid is controlled by the locus *GF* and is a prerequisite for subsequent glucosylation at the 5-position. Because of this, *GF* was initially thought to encode a 5GT. A gene coding for anthocyanidin 3-rutinoside acyltransferase (AAT) has been isolated from a collection of cDNAs downregulated in *an1* mutant flowers (*DIF-C*, Koes et al. unpublished) and mapped close to the *GF* locus. The acylation activity of the *GF* locus had been previously proposed (Jonsson et al. 1984b) by comparison of extracts from wild type and *gf* mutant flowers. Because no difference in 5-*O*-glycosyltransferase activity was detectable, it was suggested that the *GF* locus controls a step preceding (and required for) glucosylation. The acyltransferase activity of *DIF-C* (later renamed *AAT*) was subsequently demonstrated (F. Brugliera, R. Koes – International Patent Publication Number WO01/72984, 2001) and this gene has been successfully used in transgenic approaches aimed at producing, by gene suppression, reddish flowers. In the transgenic plants, the ratio of malvidin-petunidin/delphinidin was reduced (Tsuda et al. 2004).

Anthocyanin methylation in Petunia depends on two pairs of genes (i) *MT1* and *MT2*, which control methylation at the 3′-position, and (ii) *MF1* and *MF2*, which control methylation at the 3′- and 5′-positions. When a dominant allele of *MT1* or *MT2* is present in an *mf1 mf2* background, mainly petunidin (purple) or peonidin (magenta) accumulates, but malvidin can also be present, accounting for up to 20%

of the total anthocyanin content. A dominant allele of one or both *MF1* and *MF2* is required for malvidin (blue/purple) to be the major pigment. *MF1* and *MF2* action is restricted to the flower limb. Genetic control and biochemical properties of the four flavonoid-*O*-methyltransferase activities, which are directly controlled by the corresponding methylation genes in Petunia flowers, have been well established (Jonsson, de Vlaming, Wiering, Aarsman, and Schram 1983; Jonsson, Aarsman, Poulton, and Schram 1984a).

A cDNA fragment (*DIF-E*) encoding an anthocyanin methyltransferase (AMT) has been isolated and shown to be co-expressed with the other late structural genes of the pathway, under the control of the same regulators (Quattrocchio et al. 1993). Northern blot analysis of the transcripts detected by *DIF-E* showed complete lack of mRNA only in *mt1 mt2 mf1 mf2* quadruple mutants; if just one of the four AMT coding genes was functional, transcripts hybridizing to *DIF-E* could be detected (Koes et al., unpublished data). This suggests that the *MT* and *MF* genes constitute a gene family encoding methyltransferases with slightly different activities, although other possibilities (such as *MT* and *MF* genes controlling expression of methyltransferases) cannot be ruled out in the absence of other genetic data.

13.3.10 Transport to the Vacuole

Anthocyanins are believed to be synthesized in the cytosol but they do not show their brilliant colors until they accumulate in the acidic vacuoles. How anthocyanins arrive at their destination is still a matter of debate. Different observations suggest that at least a gluthatione *S*-transferase (GST) and a vacuolar membrane-bound permease and/or transporter are necessary for vacuolar uptake of anthocyanins.

In Petunia, the gene *AN9* encodes a functional Phi-type GST involved in a genetically defined late step of the pathway controlled by the anthocyanin regulatory genes (Alfenito et al. 1998). Stable *an9* mutants display very pale flowers with reduced anthocyanin accumulation, whereas flavonol accumulation is not affected (Gerats et al. 1982). Unstable *an9* mutants, caused by insertion of a transposable element into the coding sequence, bear flowers with fully colored revertant somatic spots on a pale background. These spots have diffuse boundaries, implying that *AN9* controls pigmentation in a non-cell-autonomous manner (Alfenito et al. 1998). The presence of this zone of diffusion (a halo of lighter colored cells around a full red revertant sector) is a feature of all mutable alleles of structural genes in the anthocyanin pathway. The “fuzzy” appearance of revertant spots is ascribed to the diffusion of pathway intermediates from a revertant cell to the surrounding mutant cells. If the diffusing compound originates from a reaction occurring after the mutational block, it can be converted to a colored anthocyanin in the mutant cell. In contrast, unstable mutants of anthocyanin regulatory loci produce sharp revertant spots, in which pigmented revertant cells adjoin completely uncolored mutant cells. These observations strongly suggest that *AN9* is not the final gene in the anthocyanin pathway that is controlled by the regulators *AN1*, *AN2*, and *AN11*, but that another gene acting after *AN9* confers cell autonomy. One possibility is that an additional step involving

the expression of a vacuolar transporter could be under the control of the same regulatory genes. Recent reports suggest that these transporters could belong to the ABC family or MATE family (reviewed by Kitamura 2006).

The step of the pathway controlled in Petunia by *AN9* is controlled in maize by the locus *BZ2*. Both gene products are a GST, but the proteins belong to different classes (Alfenito et al. 1998). Although AN9 and BZ2 are functionally homologous (as demonstrated by cross-complementation experiments) they clearly have separate evolutionary origins. The Arabidopsis *TT19* locus controls the accumulation of both anthocyanins and proanthocyanidins and encodes a Phi-type GST. However, overexpression of the Petunia *AN9* gene can complement the *tt19* mutant for anthocyanin accumulation, but not for proanthocyanidin pigmentation in the seedcoat (Kitamura, Shikazono, and Tanaka 2004). The mechanism by which these GSTs contribute to the transport of anthocyanins to the vacuole is not yet clear. GS-anthocyanin conjugates have never been detected in plant extracts. Therefore roles of GST alternative to glutathionation of the anthocyanin molecules are being investigated (Mueller, Goodman, Silady, and Walbot 2000; Kitamura 2006).

13.4 Regulators of Anthocyanin Biosynthesis in Petunia

The production of anthocyanins in Petunia (and also in other plant species) takes place in specialized cells of some organs, at specific moments in development. This requires that the structural genes involved are expressed in a coordinate manner at the right time and the right place.

Early and late biosynthetic genes have remarkably similar expression patterns in developing flowers. During early stages of development they are already active in anthers. The expression in petal tube and limbs peaks at a later developmental stage, just before the corolla unfolds. *CHS*, *CHI*, and *DFR* are also expressed in the epidermis of ovules, but this does not result in anthocyanin accumulation. After fertilization, the ovule epidermis develops into a seedcoat and *DFR* expression is strongly induced, enabling the synthesis of proanthocyanidins. Reporter genes driven by *CHS*, *CHI*, and *DFR* promoters exhibit expression patterns similar to those of the endogenous genes, indicating that expression of structural genes is regulated primarily via transcription.

Mutations affecting anthocyanin biosynthesis in Petunia were already known in the 1980s to fall into two categories: those affecting only one step of the pathway (one enzymatic activity) and those affecting a set of steps (a whole group of enzymatic activities) (Gerats et al. 1984). Subsequent experiments revealed that *AN1*, *AN2*, *AN4*, and *AN11* control the mRNA expression of *CHS-J*, *DFR-A*, *ART*, *AMT*, and *AN9* (Beld et al. 1989; Quattrocchio et al. 1993) by regulating the activity of their promoters (Huits 1993). The same experiments revealed that the early biosynthetic genes *CHS-A*, *CHI-A*, and *F3H* are expressed independently from these regulators. The production of flavonols (and possibly other products of side branches of the same pathway) is therefore independent from the accumulation of anthocyanin,

and allows flavonol synthesis in tissues (leaves, stems, etc.) in which the anthocyanin pathway is inactive.

13.4.1 Regulators of Pigment Accumulation

The isolation and molecular characterization of the regulatory anthocyanin loci were facilitated by the availability of transposon-tagged mutants (see Chapter 17).

AN1 is expressed in pigmented tissues only (petals, anthers, seedcoat, etc.) and encodes a protein with a basic-helix-loop-helix (BHLH) domain, suggesting that it is a transcription factor (Spelt, Quattrocchio, Mol, and Koes 2000). As *an1* mutants lose pigmentation in all tissues, *AN1* function is indispensable for pigmentation and not redundant with other genes. As shown by the phenotype of *an1* petal and seedcoat cells, this locus is also involved in the control of cell shape and cell size (Spelt, Quattrocchio, Mol, and Koes 2002).

AN2 encodes a protein belonging to the R2R3-MYB class of transcription factors. *AN2* expression is essentially limited to the petal limb and nearly absent from other pigmented tissues, explaining why *an2* mutations affect pigmentation only of the petal limbs (Quattrocchio et al. 1999). Recent results indicate that pigmentation in other tissues is activated by very similar MYB proteins that are encoded by distinct genes with different expression patterns (see below).

AN11 encodes a WD40-repeat (WDR) protein that does not seem to be involved in determination of tissue specificity of pigmentation, as it is expressed in all parts of the plant, including those that never show pigment accumulation (de Vetten, Quattrocchio, Mol, and Koes 1997). Like *an1*, *an11* mutants lose pigmentation in all tissues and show similar defects in development of the seedcoat, but no defects are seen in other, uncolored, tissues that express *AN11*. The Arabidopsis *AN11* homolog *Transparent Testa Glabra 1* (*TTG1*) was shown to be involved in the development of trichomes on stems and leaves. Petunia *an11* mutants do not show any evident aberration in the development of trichomes, but, as already mentioned, other aspects of epidermal cell development are affected in seeds and flowers. Because AN11 has no similarity to known transcription factors and because most AN11 protein is in the cytoplasm, its precise role in the activation of the promoters of structural anthocyanin genes remains poorly understood (see below).

JAF13 encodes a BHLH protein. It was cloned by homology to genes from the maize *R* family (*R, Lc, B*), and, together with DELILA from snapdragon, GL3/EGL3 from Arabidopsis, and several other proteins from different species, it defines a class of BHLH proteins that is clearly distinct from that including AN1 and Transparent Testa 8 (TT8) from Arabidopsis. The similarity among the genes of the *JAF13* clade is not limited to homology of the proteins, as the gene architecture is also conserved, indicating that they probably share a common ancestor, while the genes of the *AN1* clade display a different intron/exon structure (Spelt et al. 2000).

Although of different evolutionary origins, AN1 and JAF13 display very similar activities. In yeast cells both can interact with AN2 and related MYBs, as well as with AN11. Moreover, in transient expression assays co-expression of AN2 with

AN1 or JAF13 is sufficient to induce the *DFR* promoter in cells where it is normally not expressed. Co-production of both AN1 and JAF13 with AN2 does not enhance *DFR* promoter activity (Quattrocchio, Wing, van der Woude, Mol, and Koes 1998; Spelt et al. 2000). However, JAF13 and AN1 cannot be fully interchangeable, as *an1* mutants are white even though they express JAF13 normally. Whether both proteins are required together for pigment accumulation is not yet clear. In mutants homozygous for the transposon insertion allele *jaf13*^{B2128}, anthocyanin accumulation is reduced by about 50% and *DFR* expression is reduced, but not abolished (Quattrocchio, Urbanus, and Koes, unpublished). This finding can be explained in three ways: (i) the JAF13 protein increases the strength of the transcription-activating complex by binding to AN1 (and to its MYB partner) and results in higher transcription of the target genes; (ii) JAF13 is necessary for the function of the transcription-activating complex, but *jaf13*^{B2128} is not a null allele; or (iii) *JAF13* is redundant with other genes, and therefore the mutation results in partial loss of activity. Further analysis of the existing *jaf13* mutant and/or the isolation of new mutant alleles are required to distinguish among these possibilities.

In contrast to AN1 and AN11, AN2 and AN4 are required for anthocyanin synthesis in a subset of colored tissues. Molecular analysis revealed that *AN2*, *AN4*, and *MYBB* are partially redundant genes that encode functionally similar MYB domain transcription factors, but have divergent expression patterns. *AN2* is expressed primarily in the petal limb. In *an2* mutants pigmentation of the petal is reduced but not abolished, resulting in a pale-colored petal limb and, in some backgrounds, a darker venation pattern. The residual pigmentation in *an2* may be due to *MYBB*, *AN4*, or both. Anthers express only *AN4*, while *AN2* and *MYBB* are not expressed in this tissue, explaining why pigmentation is fully blocked in *an4* anthers. The *an4* phenotype can be rescued by expression of *AN2* from a viral promoter, underscoring the functional similarity of the encoded proteins.

It is possible that pigmentation of the tube and the seedcoat of *an2 an4* double mutants is due to MYBB. As a mutant is not yet available, this cannot be proved. One other locus, *AN8*, is required for pigmentation of the tube of the corolla. Possibly *AN8* encodes another member of this MYB family, but the gene has not yet been cloned, Interestingly, proteins of the AN2/AN4/MYBB family are required for the transcription of *AN1*, as indicated by the absence of *AN1* transcripts in *an4* anthers and the restoration of *AN1* expression in the same tissue induced by a *35S:AN2* transgene (Quattrocchio et al. 1999; Spelt et al. 2000). Remarkably, wild-type amounts of *AN1* transcripts are expressed in the corolla of *an2* Petunias, suggesting that AN2 function might be redundant in these cells, possibly due to overlapping expression of *AN4* and/or *MYBB*.

13.4.2 Regulation of Vacuolar pH in Pigmented Cells

The regulation of vacuolar pH is an important factor for flower color. Recent results showed that the regulation of vacuolar acidification in petal epidermal cells is tightly linked to that of anthocyanin biosynthesis.

In plants and fungi, pumping systems powered by ATP or pyrophosphate are known to translocate protons across cellular membranes to create electrochemical gradients that drive transmembrane transport of a variety of solutes. A single-peptide ATPase (of the P-ATPase family) localized in the plasma membrane transports protons from the cytoplasm to the intracellular space, while a large multiprotein complex (V-ATPase) is responsible for proton transport across the tonoplast and several other endocellular membranes. Finally a PP-ase (a mono-peptide pump) also serves in proton transport across the vacuolar membrane in several cell types.

Mutants affecting this process are scarce, as they result in lethality in fungi and animals. One fortunate exception is represented by seven non-allelic Petunia mutants (*ph1* to *ph7*) that display bluer flower color than isogenic wild-type plants. These mutations do not affect anthocyanin composition; instead, the pH of the crude petal homogenate is increased up to one unit compared to that of the wild type. Because the absorption spectra of anthocyanins are shifted as consequence of a change in solvent pH, anthocyanins behave as pH indicators. By this criterion these mutants have been thought to affect the pH of the vacuolar compartment. The change in color is most spectacular in Petunia lines that synthesize malvidin or petunidin derivatives, in which the mutations result in a shift from purple to blue (Fig. 13.2). In a cyanidin background, the same shift results in a change from red to a dull gray.

Closer examination of *an1*, *an2*, and *an11* flowers revealed that they exhibit a shift in pH of the petal extract similar to that seen in the *ph1* to *ph7* mutants. Characterization of *ph6* mutants revealed that they carry peculiar alleles of the *AN1* gene. In the allele *ph6*$^{\mathrm{G621}}$ a transposon in the coding region, just before the BHLH domain, produces a very stable truncated AN1 protein. This truncated AN1 accumulates in cells at levels up to 25 times that of the full-length protein. It is able to induce nearly normal transcription of the structural genes for anthocyanin biosynthesis, but it cannot drive acidification of the vacuole. Given that the *ph6* mutant displays a flower color shift compared to wild-type flowers, it follows that the pH shift in *ph6* petal extracts also results from a change in vacuolar pH. In flowers of mutants carrying null alleles of *AN1*, loss of pigmentation is accompanied by increased vacuolar pH, that is, in these cells both *AN1* functions are lost. This finding, together with the study of allelic series of *AN1*, suggests that AN1 activates pigment synthesis and vacuolar acidification by distinct regulatory mechanisms that have been functionally separated in the *ph6* mutant (Spelt et al. 2002).

In addition to *AN1*, *AN2* and *AN11* are also required for vacuolar pH regulation. *AN11* mutants do not undergo the strong acidification of the vacuolar lumen in the epidermis of the petals that occurs in wild-type buds at the moment when flowers open. The *an2* mutants show only a partial block of this acidification process, possibly due to redundancy of *AN2* function, and the above-described *JAF13*$^{\mathrm{B2128}}$ mutant allele produces a pH increase of about one-half pH unit.

To understand how vacuolar pH is controlled, we set out to isolate *PH* genes by transposon-tagging and transcript-profiling strategies. Although the nature of the vacuolar acidification pathways in corolla is unclear, a preliminary analysis reveals that it is different from the classical “housekeeping” pH pathways described above.

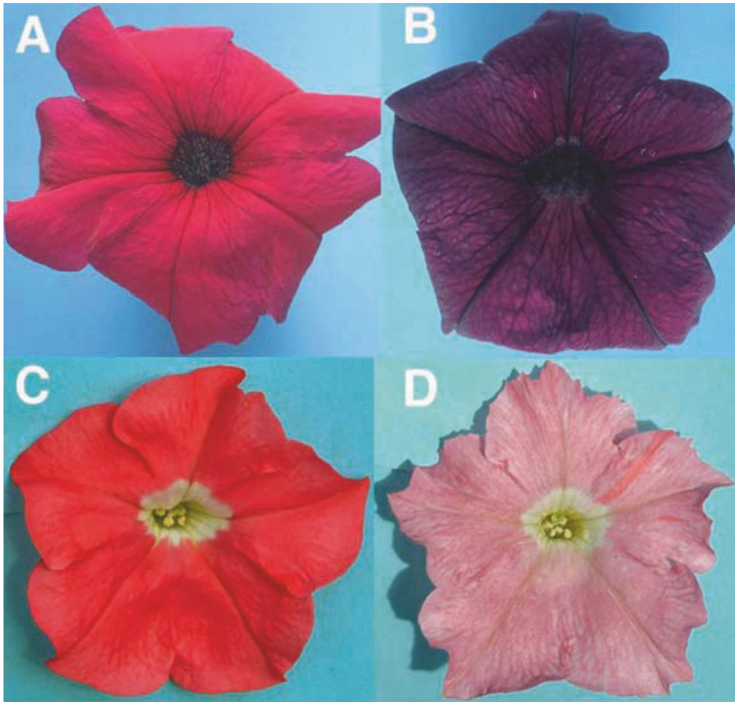

Fig. 13.2 Color shift due to vacuolar pH changes in the cells of the petal epidermis in Petunia lines that accumulate different anthocyanins. (**A**) WT Petunia flower (hybrid M1 × V30) accumulating malvidin; (**B**) Effect of knocking out the PH4 gene in the same background; (**C**) Flower from a line accumulating cyanidin 3-glucosides (line R27; hf1 hf2 rt fl); (**D**) Unstable ph5 mutant in the R27 background. Note the revertant red spots and small sectors

Flowers from *ph4* mutants are bluish, and show neither changes in pigment content nor any other aberration when compared to isogenic wild types. The *PH4* gene encodes an R2R3MYB protein that can interact with AN1. The AN1/PH4 complex acquires specificity for the activation of a set of target genes responsible for acidification of the vacuolar lumen (Quattrocchio et al. 2006).

This regulatory mechanism based on one BHLH factor (AN1) and multiple possible MYB partners (e.g., AN2 or PH4) gives flexibility to the system, integrates the regulation of the two (or more) phenomena in one network, and partially explains the double role of AN1 and the possibility of separating these two functions in alleles in which only one of the two is lost (see *an1* versus *ph6* mutants).

PH3 is a target gene of the AN1/PH4 complex, but rather than encoding an enzymatic function, it represents a new layer of regulation. *PH3* encodes a transcription factor which appears to work downstream of *AN1* and *PH4*, as *PH3* mRNA is reduced, but not abolished, in *an1* and *ph4* petals (Verweij 2007). Transcript profiling that aimed to identify the target genes of the AN1, PH3, and PH4 pathway yielded 10–20 genes that are downregulated in *an1*, *ph3*, and *ph4* petals (Verweij

2007). One of these genes was found to be identical to the genetically defined *PH5* gene and to encode a novel type of vacuolar ATPase which is expressed only in pigmented cells. Analysis of the other *AN1*/*PH3*/*PH4* target genes is underway.

Petunia epidermal petal cells have developed a novel vacuolar acidification mechanism that is independent of the already described vacuolar pumping systems, as shown by two lines of evidence: (i) the expression of V-ATPases and PPases is not affected by mutations in *AN1* or *PH4* and (ii) among the collection of *AN1*/*PH3*/*PH4* target genes no genes have been found that encode v-ATPase subunits. Instead, a novel type of proton pump, encoded by the *PH5* locus, has been identified in this collection, and we have shown that it is required for vacuolar acidification and red coloration of the corolla (Verweij, Spelt, Di Sansebastiano, Vermeer, Koes, and Quattrocchio, In press).

13.5 Stability of Pigment Molecules in Epidermal Cell Vacuoles

One more factor that contributes to the color of a Petunia flower is worth mentioning: stability of pigmentation (Fig. 13.3). Once the molecules of pigment are produced and accumulate in the vacuolar lumen of the petal epidermal cells, a phenomenon known as fading can still result in the disappearance of color from the corolla within a couple of days after the opening of the bud (de Vlaming et al. 1984).

The mechanism that results in fading (or prevents it in wild-type flowers) is unknown, but several genetic factors that are required for fading to occur are known:

Fig. 13.3 The fading phenomenon in Petunia corollas. All flowers in this picture are from the same plant of an F1 hybrid with an unstable *ph4* phenotype (which accumulates malvidin). The dark fully pigmented corollas are those of freshly opened flowers, while those of flowers a couple of days older are lighter and, again later, even white. The red spots and sectors that do not fade contain cells in which the *PH4* gene reverted to wild type, and therefore the vacuolar pH is back to a normal acidic value

- A dominant allele of the locus *Fading* (*FA*). Because *FA* is not yet cloned, its role is unclear.
- Highly substituted anthocyanins. Malvidin, petunidin, and peonidin derivatives can be subjected to fading, while cyanidin derivatives fade much less.
- A mutation in *PH4*, *PH3*, or AN1 (*ph6* allele). Interestingly *ph2* and *ph5* do not trigger fading, suggesting that fading is not due to high vacuolar pH alone, but requires some other defect present in *ph4*, *ph3*, and *an1* mutants.

These mutants open the fascinating possibility of learning how the stability of molecules that are stored in a subcellular compartment is dependent on a number of genetic factors possibly involved in the genesis of vacuolar compartments (Quattrocchio et al. 2006).

13.6 A Map of Interactions of Flower Color Regulatory Proteins

The multiplicity of factors involved in the activation of transcription of anthocyanin genes in Petunia and the phenotypes of single and double mutants suggest that the different proteins participate together in a complex with high specificity for the promoters of the target genes.

The N-terminal domains of AN1 and JAF13 have been shown to interact in yeast with several MYB proteins. A yeast two-hybrid screening in which this domain of AN1 was used as bait yielded, indeed, a small collection of Petunia MYBs that included the members of the AN2 family, PH4 and a short R3-MYB protein that we call MYBX due to its cryptic function (Kroon 2004; Quattrocchio et al. 2006).

Overexpression of *MYBX* in wild-type Petunia results in loss of pigmentation and high pH value of the crude corolla extract, as it prevents transcription of all sets of genes under the control of *AN1* (Spelt, Quattrocchio, Koes, in preparation). These results suggest the MYBX protein forms an inactive transcription complex with AN1. Although transgenic plants in which *MYBX* is silenced do not show defects in pigmentation or pH regulation, this inhibitor might be required to modulate other, yet unknown, functions of AN1.

The C-terminal region of AN1 can interact with the C-terminus of another AN1, producing a homodimer, and with the corresponding region of JAF13 (Kroon 2004). Curiously, the BHLH domain, a known dimerization interface, seems not to be involved in the AN1-AN1 or AN1-JAF13 interaction. Analysis of an allelic series shows that insertions of 1–3 amino acids in the BHLH domain of AN1 strongly reduce its activity, underscoring the importance of this domain (Spelt et al. 2002). However, the precise function of the BHLH regions of AN1 and JAF13 remains unknown.

As shown with yeast two-hybrid experiments, the AN11 protein contacts different proteins of this complex. The WDR motifs of AN11 can interact with the central domains of JAF13 and AN1, as well as with AN4 and MYBB. The specificity of interaction of AN11 with the MYB proteins is a good monitor of the diversification in this family, as AN2 is the only member of this group that cannot interact with AN11 (de Vries, Koes, and Quattrocchio, unpublished results).

The interaction of AN11 with these transcription factors, which are expected to be active in the nucleus, seems difficult to fit with the observation that most of the AN11 protein is in the cytoplasm (de Vetten et al. 1997). However, it is possible that a small amount of AN11 reaches the nucleus to contribute to the transcription-activating complex. Alternatively, AN11 could transiently bind to AN1, JAF13, AN4, and MYBB to activate them or to facilitate their transport into the nucleus, as proposed for the AN11 homolog from *Perilla frutescens* (Sompornpailin, Makita, Yamazaki, and Saito 2002). This is supported by the observation that a JAF13-GFP fusion protein localizes to the cytoplasm in transiently transformed *an11* petal cells, but to the nucleus when a construct overexpressing AN11 is co-transformed (Quattrocchio, Eppens, and Koes, in preparation).

This complex network of interactions, which is summarized in Fig. 13.4, was derived from experiments in yeast (and in the case of PH4 and AN1 also in vitro) by testing interactions between proteins one by one. It is difficult to reconstruct from such data the composition of the protein complexes as they occur in vivo, because we do not know how many of these interactions take place at the same time in the same cell, which are more stable, and which are mutually exclusive

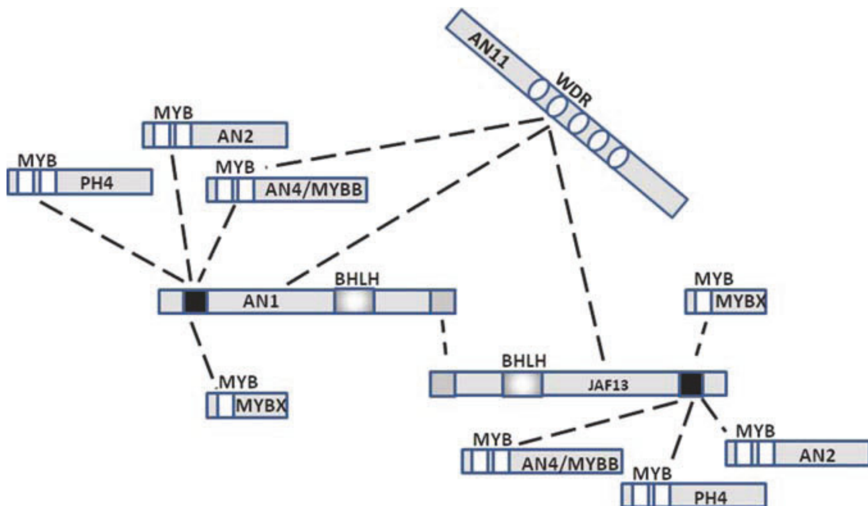

Fig. 13.4 Map of the interactions among factors of the regulatory network controlling flower color in Petunia. The interactions depicted in this schema have been shown by yeast two-hybrid assays and, in the case of AN1 and PH4, co-immunoprecipitation (Quattrocchio et al. 2006). The BHLH factors AN1 and JAF13 can interact with each other via their conserved C-terminal domains. Both can interact with the MYB-domain of different MYB proteins (AN2, AN4, MYBB, PH4, and MYBX) through their N-terminal conserved regions. The WDR factor AN11 can form complexes with the central part of the BHLH proteins AN1 and JAF13, as well as with the MYB proteins AN4 and MYBB, but not with the MYBs AN2 and MYBX (and not with the other MYB factors that are recognized by AN1 and JAF13). Because each of these interactions has been studied independently, it is impossible to define which of these factors are present at the same time in one single protein complex

(i.e., competing for the same binding site). Nevertheless, some educated guesses can be made from additional genetic and molecular evidence. Probably the complex contains only some of the MYB proteins (maybe even only one type), because (i) the proteins of the *AN2* family have only partially overlapping expression domains; (ii) the *an4* mutation is recovered by ectopic expression of *AN2*; and (iii) the *ph4* mutant is not affected in anthocyanin accumulation, but only in pH regulation. Because the same domain of AN1 can interact with different MYB proteins, it is possible that the MYB participating in the complex determines the specificity for the set of target genes (anthocyanin structural genes for AN2/AN4/MYBB, or pH structural genes for PH4). Similarly, when MYBX is in the complex any affinity for target promoters is lost, and no gene is activated, or, alternatively, the complex can still interact with target genes, but it is inactive and no transcription is induced.

Experiments in which an *an1* mutant was transformed with a construct expressing a fusion of AN1 and the ligand-binding domain of the rat glucocorticoid receptor (AN1-GR) have shown that AN1 can activate the promoter of target genes of pathways for both anthocyanin and pH (Spelt et al. 2000; Verweij 2007), even when the flowers are treated with a combination of dexamethasone and cycloheximide. This is a proof that AN1 directly activates transcription of these genes, without requiring the induction of intermediate factors. Although no direct evidence is available, it also suggests that AN1 partners, like AN2/AN11 and possibly JAF13, are direct activators of structural anthocyanin genes and are required either as components of a transcription complex or for its formation and/or activation.

Already in the 1970s Stafford proposed another level of regulation in the phenylpropanoid pathway, based on the organization of the metabolic enzymes into complexes that could control the flux of intermediates (Stafford 1974). Later, several lines of experimental evidence provided clues that the phenylpropanoid enzymes are organized into "metabolons," large multi-enzyme complexes representing the different branches of the pathway (Winkel 2004). In Petunia, however, the petals of unstable mutants for pigmentation enzymes have "fuzzy" (non-cell-autonomous) reversion spots, suggesting that the intermediates of the flavonoid pathway can move to neighboring cells. This observation is difficult to reconcile with the channeling of intermediates through enzyme complexes in Petunia epidermal cells.

With their large petals, the flowers of Petunia represent good biological material for biochemical experiments. The possibility is therefore open for using them in studies of the organization of the enzymes acting in the phenylpropanoid pathway.

13.7 Evolution of the Anthocyanin Pathway and Its Regulators

The appearance of the flavonoid pathway dates from very early in the history of the plant kingdom. This implies that the genes for the synthesis of products of this pathway are also ancient and that their regulation evolved with the appearance of different plant structures (e.g., seeds, flowers, etc.). The characterization of pigmentation regulatory mutants in Petunia (Quattrocchio et al. 1993) and a comparison with similar maize mutants has revealed that the same pathway is regulated differ-

ently in distinct species. This is probably related to the need for plants to accumulate different end products that allow them to fulfill a variety of functions in relation to different environmental settings and physiological requirements.

In maize the whole pathway, at least in the kernel aleurone, the plant body and leaves, is regulated as one unit, allowing for the accumulation of only colored anthocyanins as final products. In *an1*, *an2*, and *an11* regulatory mutants of Petunia messenger RNAs for only the genes controlling the late enzymatic steps (all steps from *DFR* down) and some early genes (*CHS-J*, *F3′5′H*) are affected. This implies that the other early genes of the pathway (e.g., *CHS-A*, *CHI*, *F3H*) are independently regulated.

According to a model proposed to explain the differences observed in the regulation of the pathway in different species, the early biosynthetic genes were duplicated, making it possible to combine one set of early genes with the newly arising late biosynthetic genes (those required to produce anthocyanins), and to regulate this whole new pathway independently from that involving the other copies of early genes, which are regulated by ancient as yet unknown regulators. This was achieved by linking the new part of the pathway to the ancestors of *AN1*, *AN2*, and *AN11*. At this point the ancestral angiosperm had two sets of specialized early biosynthetic genes devoted to the production of either anthocyanins or flavanons/flavonols, each coupled to different regulators. In this way newly evolved organs could produce colored pigments, while the green parts could still accumulate other flavonoids. Later in evolution, several species of flowering plants lost one of the two specialized copies of the early genes, depending on the need to produce specific side products of the pathway (Koes et al. 1994). According to this model, Petunia kept both copies of *CHS* (*A* being the original gene coupled to the unknown ancient regulators, and *J* the new gene connected to the *AN1/AN2/AN11* network) while it lost the newer copy of *F3H*. It can thus produce anthocyanins and flavonols independently. As a consequence, Petunia can accumulate flavonols even in cells where the anthocyanin pathway is inactive, green tissues, for example. Similarly colorless anthers that fail to accumulate anthocyanins due to the *an4* mutation can still be fertile, thanks to flavonoids produced by the early genes, which are not affected by this mutation.

A function of the ancestor of the *AN1/AN2/AN11* regulators in the interaction with pollinators showed up initially in plants that displayed complicated and attractive flower structure. In these "newer species" (among them Petunia), the regulators also controlled another factor: vacuolar pH in corolla cells. As no blue Petunias have ever been found in wild populations (while in greenhouse collections new *ph* mutants appear regularly and are completely viable), it is suggested that a blue corolla is disadvantageous in nature because it does not effectively attract pollinators. Furthermore, *ph* mutants often involve other defects of the petal cells that influence stability of the pigmentation phenotype (i.e., fading), which would also affect the interaction with pollinators.

The separated regulation of different subsets of structural genes of the flavonoid pathway has allowed for the appearance of new species: white flowers of wild Petunia accessions that carry mutations in *AN2*, do not produce anthocyanins, but do accumulate fluorescent flavonols, which attract different pollinators (see Chapter 2).

The color of the corolla in the Petunia wild accession presents a minimal palette of colors, indicating that the adaptation to specific pollinators does not allow for a lot of flexibility.

During evolution, the regulators of the anthocyanin pathway appear to have been reused several times through their coupling to different sets of target genes. As shown in Fig. 13.5, the same selection of regulatory factors, in slightly different combinations, is used in the petal epidermal cells to activate different sets of target genes, resulting in the activation of different developmental processes. The two examples presented in Fig. 13.5 are the two best understood, but the phenotypes of the *an1*, *an11*, and of some *ph* mutants suggest that more of these combinations are possible and that the target genes are probably many more and involved in more

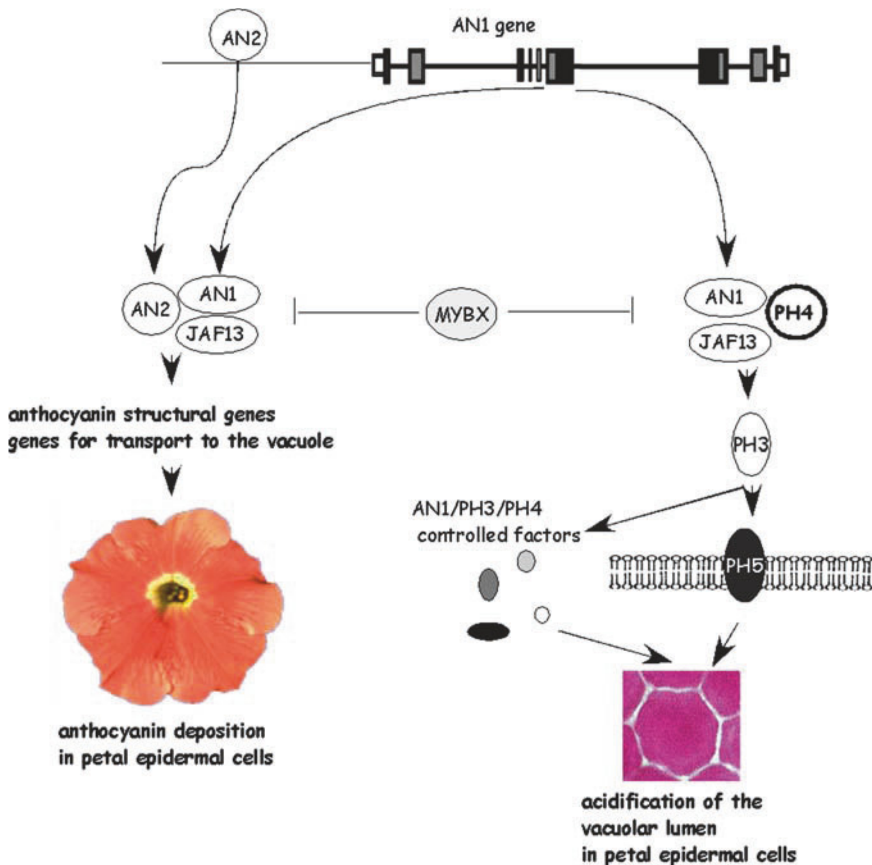

Fig. 13.5 Integration of the regulatory network controlling different aspects of epidermal cell differentiation in Petunia. Sets of regulators that can form slightly different protein complexes, mainly by recruiting different MYB factors, allow for specificity with different groups of target genes, and thereby coordinate induction of different pathways in the pigment-accumulating cells of the flower

processes (e.g., cell division and cell morphology, proanthocyanin accumulation, etc., Spelt et al. 2002). The biochemical pathway for flavonoid biosynthesis must be the oldest controlled by this regulatory network, as it is the most widespread in the plant kingdom, while other aspects of epidermal cells differentiation, such as trichome and root hair development, which are observed in only some dicot species (e.g., Arabidopsis, cotton), were probably linked to this regulation only much later in evolution.

The gene encoding the oldest regulator of this whole network is probably *AN11*. The protein encoded by this gene is a WDR factor, highly conserved, not only among plants, but also in fungi, worms, fish, and humans (de Vetten et al. 1997). Next to the homology among the proteins encoded by the AN11 orthologs in very different organisms, the functional conservation represents a strong indication of the common origin of these genes. In transient expression experiments, even the human AN11 homolog was able to restore activation of the *DFR* promoter in *an11* petals. This high degree of conservation indicates that the regulatory mechanism in which AN11 is involved is much older than the flavonoid pathway. It is easy to imagine that the molecular mechanism by which orthologous AN11 proteins function in very different organisms might be the same, although evolution has coupled its function with different target genes (and therefore totally different pathways) downstream of the regulatory step controlled by these WDR proteins.

13.8 Concluding Remarks

The history of Petunia flower color genetics is full of landmarks for the development of knowledge about the synthesis of secondary metabolites, gene regulation, gene expression, gene silencing technology, transposon biology, and evolutionary biology. Next to a long series of scientific achievements, the color of these small and very common flowers has been the protagonist of many applications in the flower industry. The unraveling of the genetic control of flower pigmentation has enabled the manipulation of flower color in ornamental species and the introduction of new, previously non-existing, purple colors in rose and carnation (Forkmann and Martens 2001; Tanaka et al. 2005).

The *PH* genes from Petunia are so far the only available source of characterized DNA sequences useful to produce constructs for the manipulation of the petal vacuolar pH in transgenic plants. The recently obtained insights into the genetic control of vacuolar pH open new avenues, and may ultimately lead to the production of the long-awaited blue rose. H^+ homeostasis is an essential parameter for cell viability. Thus far the genetic analysis of this process has been hampered because mutations in proton pumps in animals, fungi, and plants lead to severe developmental defects or even lethality. Petunia mutants (both *ph* and *an*) offer the unique opportunity to unravel the regulation of pH and the mechanism of accumulation and storage of solutes in intracellular compartments.

The hybrid Petunia, grown under greenhouse conditions and often hand pollinated, shows a very large variety of colors due to the blending of different products of the pathway in different proportions. The huge scale of colors reflects the mixing of different genomes, which allows for the bringing together of different alleles of both structural and regulatory genes. This results in a great "tool box" for the genetist who chooses Petunia as a model.

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Chapter 14
Petunia Flower Senescence

Michelle L. Jones, Anthony D. Stead, and David G. Clark

Abstract Senescence represents the last stage of floral development and is an active process that requires gene transcription and protein translation. A genetically controlled senescence program allows for the ordered degradation of organelles and macromolecules and the remobilization of essential nutrients from the petals. Petunia provides an excellent model system for studies of flower senescence because the plants flower profusely and have large floral organs amenable to molecular and biochemical analysis. While Petunia flowers have a finite lifespan that is under tight developmental control, petal senescence can be accelerated and synchronized by means of exogenous ethylene or by pollination. Petal senescence in Petunia is accompanied by decreased nucleic acid and protein content, DNA and nuclear fragmentation, and structural and compositional changes in the plasma membrane. These changes are correlated with increased mRNA abundance and enzyme activity of proteases, nucleases, and phospholipases. Major macronutrient levels in Petunia petals (collectively called the corolla) also decrease during senescence. These studies support cellular degradation and remobilization as the central functions of petal senescence. Ethylene is clearly involved in modulating the process, but the transcription factors and other components of the senescence signal transduction pathway(s) remain to be elucidated. Further studies focusing on early transcriptome changes during petal senescence will help to identify these early regulators, and subsequent studies of protein changes and the post-translational modification of senescence-related proteins will further our understanding of the pathways executing the senescence program.

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14.1 Petunia as a Model System for Studies of Flower Senescence

The postharvest working group of the American Society for Horticultural Science defines senescence as "those processes that follow physiological maturity or horticultural maturity and lead to death of tissue" (Watada, Herner, Kader, Romani, and Staby 1984). Flower senescence is therefore the last stage of flower development. Senescence is a programmed developmental event requiring the transcription of new genes, but it is also subject to post-transcriptional and post-translational regulation (Borochov and Woodson 1989; Thomas, Ougham, Wagstaff, and Stead 2003). Flower senescence culminates in cell death. In a recent review of plant cell death, it was suggested that the morphological characteristics of programmed cell death (PCD) in petals are most similar to those of autophagy in animal cells (van Doorn and Woltering 2005).

The flower is a complex plant organ made up of many different tissues with different functions in sexual reproduction. Senescence occurs in these tissues at different stages of floral development, and it can be characterized by distinct morphological changes and triggered by independent hormonal or other endogenous signals (Rogers 2006). This chapter will briefly discuss the coordination of senescence within the flower in the context of the pollination response, but it will focus on the changes occurring in the petals (collectively called the corolla). Research on flower senescence has focused on petals because in most ornamentals it is the longevity of the corolla that determines postharvest quality and garden performance.

Petunias are an ideal model system for studies of flower senescence because they flower profusely and the large flowers facilitate collection of floral tissue for biochemical and molecular analysis (Gerats and Vandenbussche 2005). While the longevity of the flowers can be decreased by environmental stresses such as high temperatures (Shvarts, Weiss, and Borochov 1997), flower senescence is largely irreversible and the timing can be precisely predicted. Significant research on flower senescence has been conducted in carnation, daylily, *Alstroemeria*, and *Sandersonia*, but functional analysis is hampered by the lack of quick and reliable transformation protocols for these species. *Petunia hybrida* can be easily transformed using *Agrobacterium tumefaciens* (Jorgensen, Cluster, English, Que, and Napoli, 1996; (see also Chapter 19)). Antisense, RNAi, and sense technologies have been used to further characterize senescence-related genes using transgenic Petunias. Virus-induced gene silencing (VIGS) based on Tobacco Rattle Virus has also been used to investigate the functional role of senescence-related genes in Petunia corollas (Chen et al. 2004; see Chapter 18).

Much of the recent research on flower senescence has been conducted in Petunia Mitchell (also known as Mitchell Diploid or MD), a doubled haploid derived from a *P. axillaris*/*P. hybrida* cv. "Rose of Heaven" hybrid (Gerats and Vandenbussche 2005). Petunia flowers have five petals that are fused to form a tube and limb. The petals are collectively referred to as the corolla. Flower longevity in Mitchell is terminated by corolla wilting, the first symptoms of which are usually observed at 6–8 d after flower opening. Wilting begins at the corolla margins and progresses until the entire corolla limb wilts and collapses. The corolla then desiccates, turns

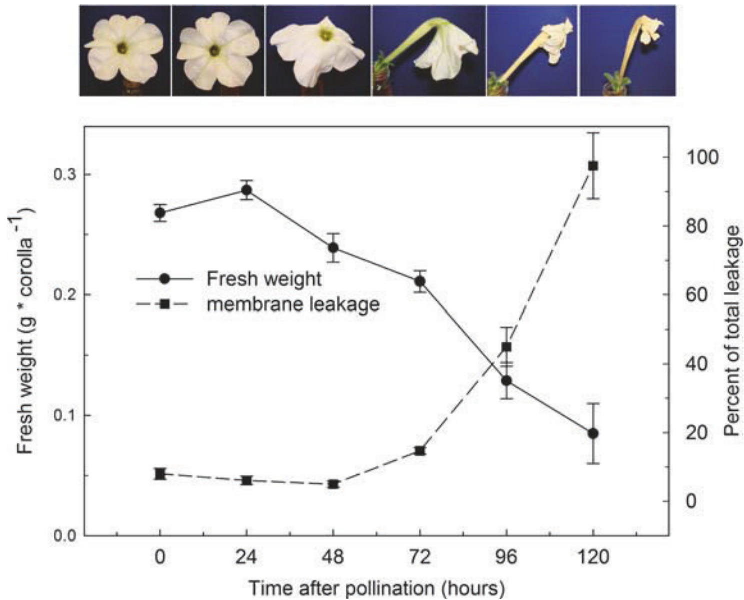

Fig. 14.1 Pollination-induced corolla senescence in Petunia Mitchell. Representative flowers at 0, 24, 48, 72, 96, and 120 h after pollination. Fresh weight changes in corollas and membrane leakage measured as a percentage of the total electrolyte leakage from the corollas. Data represent mean±SD (*n*=12)

brown, and is eventually shed. Petunia Mitchell flowers are self-compatible and pollination accelerates flower senescence. Corolla wilting can usually be observed within 48 h of pollination (Fig. 14.1).

14.2 Hormonal Regulation of Petunia Corolla Senescence

Flower senescence is a complex genetic program that is mediated in part by changes in ethylene, cytokinin, and abscisic acid (ABA) content and sensitivity. In many species petal senescence is controlled by ethylene, while in others it is not affected by ethylene and the endogenous senescence signals remain to be elucidated (van Doorn 2001).

The role of plant hormones in flower senescence has been studied largely by correlating endogenous hormone levels with symptoms of senescence and by evaluating the effects of exogenous treatments on flower longevity. The recent creation of transgenic Petunias that are insensitive to ethylene and those that overproduce cytokinins has provided researchers with useful tools for studying the roles of these individual hormones as well as the hormone interactions important in controlling flower senescence in Petunia (Wilkinson et al. 1997; Chang, Jones, Banowetz, and Clark 2003; Clark, Dervinis, Barrett, Klee, and Jones 2004; Shibuya et al. 2004).

14.2.1 Ethylene Controls Corolla Senescence in Petunia

The senescence of many flowers, including Petunia, is controlled by the gaseous plant hormone ethylene. Treating Petunia flowers with ethylene accelerates corolla senescence and the expression of senescence-related genes. Senescence can be significantly delayed, but not prevented, by treating flowers with inhibitors of ethylene action, including silver thiosulfate (STS) and 1-methylcyclopropene (1-MCP) (Lovell, Lovell, and Nichols 1987b; Porat, Reuveny, Borochov, and Halevy 1993; Serek, Tamari, Sisler, and Borochov 1995). The control of flower senescence by ethylene requires specific receptors to perceive the signal and a signal transduction pathway to coordinate the downstream responses that lead to petal senescence and cell death.

The mutated ethylene receptor gene *etr1-1* confers dominant ethylene insensitivity in Arabidopsis (Chang, Kwok, Bleecker, and Meyerowitz 1993), and ectopic expression of the *etr1-1* gene in heterologous species, including Petunia, tomato, and carnation results in plants with greatly reduced sensitivity to ethylene (Wilkinson et al. 1997; Bovy, Angenent, Dons, and van Altvorst 1999). Transgenic *etr1-1* (P_{35S}::*etr1-1*) Petunias have delayed corolla senescence and flower longevity twice that of nontransformed Mitchell flowers. Corolla wilting can be observed in Mitchell flowers at 6–8 d after flower opening, while symptoms of corolla wilting are not observed in unpollinated *etr1-1* flowers until approximately 14–16 d after flower opening (Gubrium, Clevenger, Clark, Barrett, and Nell 2000; Jones, Chaffin, Eason, and Clark 2005; Langston, Bai, and Jones 2005). While corolla longevity in wild-type flowers can be reduced by high temperatures, decreases in longevity are much more apparent in *etr1-1* flowers. Petunia Mitchell plants grown at 26/21°C day/night temperatures have an average flower life of 6.7 d, and this is decreased only to 6.0 d when plants are grown at 30/24°C. In contrast, flower longevity of unpollinated *etr1-1* flowers is reduced from 16.6 d at 26/21°C to 7.9 d at 30/24°C (Gubrium et al. 2000).

In most flowers, the role of the petals is to attract pollinators. The maintenance of petals is costly in terms of respiratory energy, nutrients, and water loss (Stead...); therefore the senescence of the corolla is often accelerated once a flower has been successfully pollinated. This accelerated senescence is largely dependent on ethylene signaling, as pollination-induced or accelerated senescence is not observed in ethylene-insensitive transgenic Petunias (Wilkinson et al. 1997; Gubrium et al. 2000; Shibuya et al. 2004). In most instances the flower longevity of unpollinated and pollinated *etr1-1* corollas is similar, but the longevity of pollinated *etr1-1* flowers depends on the growth rate of the ovary, which is faster at higher temperatures. At warmer temperatures the growing ovary actually pushes the corolla off the receptacle while it is still fully turgid (Fig. 14.2), while at lower temperatures the corolla may be wilted before it is pushed off by the growing ovary. When Mitchell flowers are pollinated at 26/21°C they last for approximately 2.1 d before showing symptoms of corolla wilting; this does not change if they are grown at 30/24°C. Pollinated *etr1-1* flowers last 16.9 d at 26/21°C, while their longevity is reduced to only 5.7 d when grown at 30/24°C (Gubrium et al. 2000).

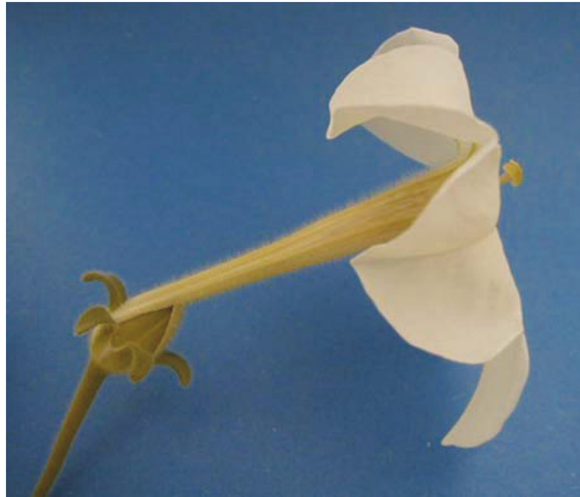

Fig. 14.2 Pollinated ethylene-insensitive transgenic Petunia flower (P_{35S}::*etr1-1*). The rapid growth of the pollinated ovary can result in the abscission of a fully turgid corolla

It is not clear if *etr1-1* corollas that abscise from the flower before wilting undergo the full senescence program. Messenger RNAs of the senescence-specific cysteine protease gene *PhCP10* are detected during the senescence of unpollinated *etr1-1* corollas at 16 d after flower opening, when the corollas are wilted (Jones et al. 2005). While *PhCP10* expression is induced in senescing pollinated Mitchell corollas, expression is not detected in pollinated *etr1-1* corollas that are partially detached and wilting at 8 d after pollination (Fig. 14.3B; Jones, unpublished). Transgenic Petunias that are insensitive to ethylene due to downregulation of the Petunia *EIN2* gene, a downstream gene in the ethylene signal transduction pathway, also have increased flower longevity, and pollination does not accelerate their corolla senescence (Shibuya et al. 2004).

While modifying ethylene sensitivity in Petunia has a positive impact on flower longevity, the plants themselves display other negative characteristics that reduce their usefulness to the horticultural industry. Both seed germination and adventitious rooting of vegetative cuttings, two characteristics with an important impact on production efficiency, are significantly reduced (Gubrium et al. 2000; Clevenger, Barrett, Klee, and Clark 2004). Despite this negative effect on horticultural performance, the plants provide a very useful tool for comparative analyses and investigations of the role of ethylene signaling in specific senescence responses. These plants have been used in such comparative analyses to determine the role of ethylene in protein and nucleic acid degradation and nutrient remobilization during corolla senescence (Jones et al. 2005; Langston et al. 2005; Jones, unpublished). Details of these experiments are provided in Sects. 14.3.2 and 14.3.3.

Flower senescence in Petunia is accompanied by increased endogenous ethylene production. Ethylene production from the corolla slightly precedes visual symptoms of corolla wilting, peaking when the corolla is wilted and decreasing at the

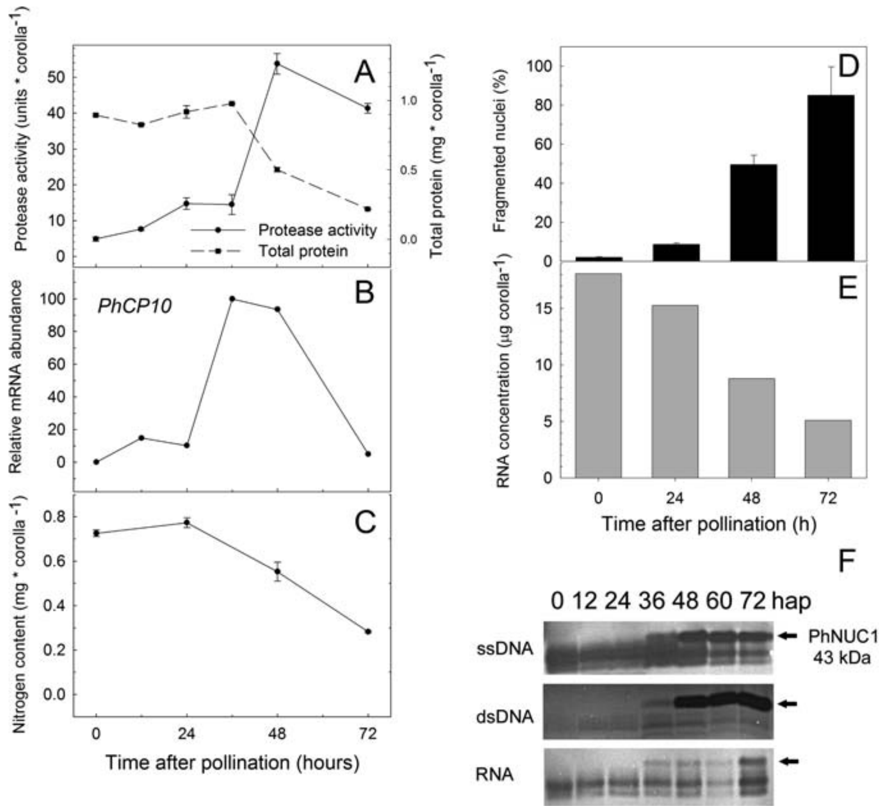

Fig. 14.3 Protein and nucleic acid degradation are central components of corolla senescence in Petunia Mitchell following pollination. (**A**) Changes in protease activity and total protein content of corollas at various times after pollination; (**B**) relative mRNA abundance of the senescence-specific cysteine protease gene *PhCP10* in pollinated corollas; (**C**) total nitrogen content of pollinated corollas; (**D**) percentage of fragmented nuclei in corollas as determined by the TUNEL assay; (**E**) RNA content of pollinated corollas; (**F**) In-gel activity assays showing senescence-specific activity of the endonuclease PhNUC1 against RNA, single-stranded DNA (ssDNA) and double-stranded DNA (dsDNA). Graphed values indicate means±SD ($n \geq 6$). Data in panels D, and F are from Langston, Bai, and Jones (2005) by permission of the Society for Experimental Biology

later stages of senescence when corollas are desiccating (Langston et al. 2005). This pulse of ethylene is referred to as the ethylene climacteric and is believed to coordinate the senescence program in petals. Making Petunias insensitive to ethylene via transformation with the *etr1-1* gene does not prevent ethylene production from the corolla or from other parts of the flower (Wilkinson et al. 1997; Langston et al. 2005). The climacteric peak of ethylene production is delayed in *etr1-1* flowers and correlates with corolla wilting at 12–16 d after flower opening (Langston et al. 2005).

Following pollination, ethylene production is also induced or accelerated (Fig. 14.4) and the ethylene climacteric is detected from the corolla at 36–60 h after pollination. Maximum ethylene production by the corolla at 48 h after pollination corresponds with the first visual symptoms of corolla wilting. As early as 3 h after pollination, increased ethylene production can be detected from the pollinated style. Stylar ethylene production decreases at 12 h and peaks again at 24 h after pollination. Increased ethylene production is detected from ovaries at 24 h after pollination and this ethylene peaks at 36 h, at which time the pollen tubes have just reached the bottom of the style (Clevenger et al. 2004; Jones, unpublished). The early ethylene

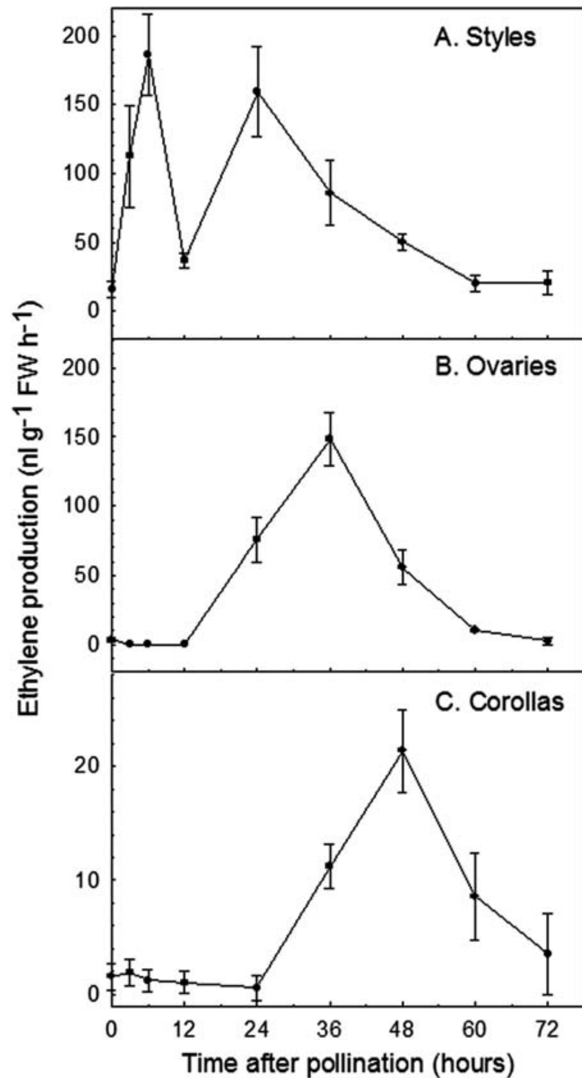

Fig. 14.4 Ethylene production in (**A**) stigma/styles; (**B**) ovaries; and (**C**) corollas from pollinated Petunia Mitchell flowers. Values are means±SD (n=12)

at 3 h after pollination is also detected in *etr1-1* flowers, and it is consistently higher than the levels detected from Mitchell flowers, suggesting a mechanism for feedback control of ethylene biosynthesis that is disrupted in the absence of ethylene perception (Wilkinson et al. 1997). The ethylene climacteric measured from *etr1-1* corollas is also higher than that measured from wild-type Mitchell corollas (Langston et al. 2005). This sequential production of ethylene from stigma, style, ovary and petals following pollination has been observed in other flowers and is thought to be the signal that coordinates the acceleration of corolla senescence in response to pollination (Jones and Woodson 1999a). This wave of ethylene production by the floral organs is controlled by the differential regulation of individual members of the gene families encoding the enzymes 1-aminocyclopropane-1-carboxylic acid (ACC) synthase and ACC oxidase (Tang, Gomes, Bhatia, and Woodson 1994; Jones and Woodson 1999b).

ACC oxidase (ACO) activity and *ACO* mRNA levels are high in unpollinated stigmas; this led to the hypothesis that ACC produced in the pollen provides a substrate for ACO and results in the increased ethylene production from the stigma/style that immediately follows pollination (Tang et al. 1994). To resolve this question in Petunia, the Woodson lab (Purdue University) created transgenic Petunias that overexpressed ACC deaminase (an enzyme that degrades ACC) under the control of the pollen-specific Lat52 promoter from tomato (Lei, Lindstrom, and Woodson 1996). Transgenic P_{Lat52}::*ACC deaminase* Petunias have reduced ACC levels in the pollen. Pollinating wild-type Petunia Mitchell stigmas with transgenic pollen reduced in ACC levels results in the same amount of ethylene production from the stigma as is detected after pollination by wild-type Mitchell pollen with normal ACC levels. Both types of pollen also accelerate corolla senescence, discounting the role of pollen-borne ACC in early senescence signaling. Pretreating pollinated styles with the ACC synthase inhibitor 2-amino-ethoxyvinyl-glycine (AVG) also results in decreased ethylene production from the style, indicating that de novo ACC synthesis in the style, not pollen-borne ACC, is required for early stylar ethylene production (Hoekstra and Weges 1986).

The nature of the signaling between the pollen and the stigma in Petunia is not yet clear, but there is substantial evidence that the Petunia style has a central role in signaling the corolla to senesce (Lovell, Lovell, and Nichols 1987a). Wounding or removing the stigma and style also results in accelerated senescence similar to the post-pollination response (Lovell et al. 1987b). Style dissection experiments in Petunia indicate that a transmissible signal has left the pollinated style and signaled the corolla to senesce as early as 4 h after pollination (Gilissen and Hoekstra 1984). In carnation flowers, inhibiting ethylene action in only the pollinated style prevents pollination-induced corolla senescence, supporting the importance of ethylene perception within the gynoecium in post-pollination signaling (Jones and Woodson 1997).

14.2.2 Cytokinins Delay Senescence by Decreasing Ethylene Sensitivity

The exogenous application of cytokinins to detached Petunia flowers delays corolla senescence (Taverner, Letham, Wang, Cornish, and Willcocks 1999).

This senescence delay is dependent on the type of cytokinin that is applied: 6-benzylaminopurine and zeatin riboside applications effectively delay senescence, while O-glucosyldihydrozeatin riboside is less effective (Taverner et al. 1999). It has been hypothesized that high levels of cytokinins in nonsenescing corollas have a senescence-delaying effect, and an inverse relationship between petal cytokinin content and flower senescence has been observed in some flowers. In carnations, roses, and *Cosmos*, cytokinin levels are highest in young flowers, decreasing during corolla development and senescence (Mayak, Halevy, and Katz 1972; Van Staden and Dimalla 1980; Saha, Nagar, and Sircar 1985). In Petunia flowers, endogenous cytokinin levels in the corolla (Taverner et al. 1999; Chang et al. 2003) do not support this theory. Total cytokinin levels in Petunia corollas increase during flower development and do not decrease until the very advanced stages of senescence (Taverner et al. 1999; Chang et al. 2003).

Cytokinins have many forms and not all of these have biological activity in all tissues and during all developmental events. It is therefore important to determine which specific cytokinins increase during petal senescence. Petunia corolla senescence is preceded by an increase in the level of O-glucosides, primarily O-glucosyldihydrozeatin (Taverner et al. 1999). Ethylene treatments that accelerate senescence promote the conversion of dihydrozeatin to inactive O-glucosides and result in the degradation of cytokinin ribosides to adenosine and AMP. Taverner and colleagues (1999) have therefore hypothesized that ethylene production during Petunia corolla senescence promotes the inactivation of cytokinins via O-glucosylation and degradation and that this may in some way facilitate senescence.

Expression of isopentenyltransferase (*IPT*), a cytokinin biosynthetic gene from *Agrobacterium*, under the control of the senescence-specific promoter SAG12 has been used effectively to delay leaf senescence in Arabidopsis and other species (Gan and Amasino 1995). Transformation of *P. hybrida* V26 with the P_{SAG12}::*IPT* construct results in plants with delayed leaf and flower senescence (Chang et al. 2003; Clark et al. 2004). Similar to what is observed in *etr1-1* Petunias, pollination does not accelerate the senescence of *IPT* Petunia corollas and wilting is delayed 6–10 d compared to that in nontransformed, pollinated V26 Petunias. This delay is accompanied by an increase in corolla cytokinin content, primarily zeatin and zeatin riboside. Cytokinin levels are higher in *IPT* corollas than WT corollas at all time points, but significant increases are detected in *IPT* corollas at 18–24 h after pollination, preceding the observation of visual symptoms of senescence in WT flowers (Chang et al. 2003). Cytokinin levels remain elevated in *IPT* corollas through corolla senescence at 12 d after pollination (Jones and Banowetz, unpublished). The senescence-delaying effects of cytokinins have been suggested to be the result of decreased ethylene production or sensitivity. While ethylene production was delayed in *IPT* Petunias, the wilting of the flowers was accompanied by an ethylene climacteric equivalent to that detected in V26 flowers (Jones, unpublished). In contrast, longer ethylene treatments were required to induce petal senescence and expression of senescence-related genes in *IPT* Petunias. These experiments support the idea that enhanced cytokinin levels in the *IPT* corollas result in decreased sensitivity to ethylene, which subsequently delays senescence (Chang et al. 2003).

14.2.3 *ABA Accelerates But Does Not Directly Regulate Senescence*

ABA is thought to be the primary hormonal regulator of petal senescence in ethylene-insensitive daylily flowers, but its role in other ethylene-insensitive flowers and in ethylene-sensitive flowers, like those of Petunia, is not straightforward (Panavas, Walker, and Rubinstein 1998). While the application of ABA to carnation flowers accelerates petal senescence, these effects seem to be secondary and are the result of increased ethylene production or sensitivity (Ronen and Mayak 1981). ABA levels in Petunia petals increase during the late stages of senescence when corollas are wilted (Chang et al. 2003; Ferrante, Vernieri, Tognoni, and Serra 2006). While ABA levels are higher in blue-flowering Petunias with high anthocyanin content, there is no correlation with flower longevity (Ferrante et al. 2006). Exogenous ABA application to Petunia flowers does not accelerate flower senescence, suggesting that the increase in the ABA content of the corollas is a by-product of senescence rather than a regulator (Ferrante et al. 2006).

The delayed senescence of *IPT* transgenic Petunias is accompanied by an increase in cytokinin content that reduces the sensitivity of the petals to ethylene. Wilted *IPT* Petunia corollas at the late stage of senescence do not have increased ABA levels (Chang et al. 2003; Jones and Banowetz, unpublished). Similarly, the senescence of ethylene-insensitive *etr1-1* transgenic Petunias is not accompanied by an increase in corolla ABA content (Jones and Banowetz, unpublished). These studies support the idea that increased ABA is the result of ethylene production by the corolla and that this response is dependent on ethylene sensitivity.

14.3 Ordered Cellular Disassembly and Nutrient Recycling

Flowers have a genetically determined lifespan because their role in plant development is that of sexual reproduction, and the maintenance of petals is costly in terms of respiratory energy, nutrients, and water loss. Once a flower has been pollinated or the stigma is no longer receptive to pollination, the petals are therefore programmed to senesce. A genetically controlled senescence program allows the plant to remobilize nutrients from dying petals to developing tissues in the flower (i.e., the pollinated ovary) or elsewhere in the plant before cell death occurs (Jones 2004; Stead et al. 2006). In support of this nutrient-recycling function, most of the genes and proteins that have been reported to be upregulated during petal senescence are those with putative roles in macromolecule or organelle degradation. The late stage of senescence in many flowers, including Petunia, is characterized by corolla wilting. In some species the functional lifespan of the flower is terminated by corolla abscission. It is in those species where petal abscission occurs before a decline in fresh weight or protein content that senescence does not result in a significant recycling of cellular constituents (Stead et al. 2006).

14.3.1 Plasma Membrane Changes Occur Without Destroying Integrity

The senescence of Petunia corollas is accompanied by compositional, structural, physical, and functional changes in the cellular membranes (Borochov, Cho, and Boss 1994; Borochov, Spiegelstein, and PhilosophHadas 1997). Most of the research on membrane changes during Petunia corolla development and senescence has been conducted on detached *P. hybrida* flowers. In these experiments, the fresh weight of detached Petunia flowers increases until 5 d after harvest, when the corollas first show signs of wilting (Borochov et al. 1994). A decline in membrane protein content is detected as early as 2 d after harvest, well before visible symptoms of corolla senescence. In general, the activities of enzymes that are involved in phospholipid synthesis decrease during flower development and senescence, but the ability to synthesize the phospholipids phosphatidic acid (PA) and phosphatidyl inositol 4,5-bisphosphate (PIP_2) is maintained from 1 to 4 d after harvest until just before the onset of corolla wilting (Borochov et al. 1994). This decline in phospholipid content is the main cause of decreased membrane fluidity together with increases in membrane permeability and leakage. The result is an eventual loss of membrane integrity late in the senescence process when corollas are fully wilted (Borochov, Drori, Tirosh, Borochovneori, and Mayak 1990).

When flowers are pollinated and remain on the plant, corolla wilting at 48 h after pollination is accompanied by a decrease in fresh weight (Fig. 14.1). The largest decreases in fresh weight are detected between 72 and 96 h. The ordered degradation and remobilization of cellular constituents require that cellular integrity be maintained until the senescence program is complete. Membrane permeability, as measured by the percentage of total ion leakage from the corollas, begins to increase at 72 h after pollination. Maximum membrane leakage is not detected until 120 h after pollination, when the corollas are brown and visibly dry. Similar changes in membrane permeability and membrane protein and phospholipid content have been reported in other flowers (Itzhaki, Borochov, and Mayak 1990).

The catabolism of membrane phospholipids is catalyzed by the activity of various phospholipases. Activities of both phospholipase A and C increase during the postharvest life of Petunia corollas, peaking when corollas are visibly wilted (Borochov et al. 1994, 1997). The activity of plasma membrane phospholipase A increases in detached Petunia flowers and peaks at 5 d after harvest when the petals are wilted (Borochov et al. 1994). Phospholipase A is associated with the degradation of membrane lipids and may function in membrane degradation occurring during the latest stages of senescence. In addition, the products of phospholipase A activity, free fatty acids, and lysolipids, can act as secondary messengers. Borochov et al. (1997) have suggested that the early increases in phospholipase activity (from 1 to 4 d after harvest) may function in the creation of products associated with senescence signaling. Phospholipase C activity results in the production of lipophilic diacylglycerol (DAG). The application of lipid metabolites like DAG (as determined by exogenous application of its analog PMA) accelerates ethylene production and senescence in cut Petunia flowers, suggesting that these by-products of lipid catabolism may be functioning as regulators of the senescence process (Borochov et al. 1997). It

would be interesting to determine if reducing endogenous Phospholipase C activity in transgenic Petunias would result in a significant delay in petal senescence.

14.3.2 Protein Levels Decrease and Protease Activity Increases

The degradation of proteins is a prominent process during senescence (Solomon, Belenghi, Delledonne, Menachem, and Levine 1999). Endopeptidases, or proteases, enzymes that degrade proteins by hydrolyzing internal peptide bonds, are the most commonly identified and characterized senescence-related genes from flowers (Jones 2004; Stead et al. 2006). Proteases that are upregulated during petal senescence have been cloned from *P. hybrida* (Jones et al. 2005) as well as *Dianthus caryophyllus* (carnation, Jones, Larsen, and Woodson 1995), *Hemerocallis* spp. (daylily, Valpuesta, Lange, Guerrero, and Reid 1995; Guerrero, de la Calle, Reid, and Valpuesta 1998), *Alstroemeria peruviana* (Wagstaff et al. 2002), *Sandersonia aurantiaca* (Eason, Ryan, Pinkney, and O'Donoghue 2002), *Narcissus pseudonarcissus* (daffodil, Hunter, Steele, and Reid 2002), and *Gladiolus grandiflora* (Arora and Singh 2004). Most of these are from the cysteine protease family of endopeptidases.

The senescence of unpollinated and pollinated Petunia flowers is accompanied by a decrease in the total protein content of the corolla (Fig. 14.3A; Jones et al. 2005). The protein content of naturally aging (unpollinated) Petunia corollas increases slightly from the day of flower opening (0 d) to 4 d. At 6 d there is a slight decline, but the largest decrease occurs between 6 and 8 d (Jones et al. 2005). Similar protein declines are measured in pollinated corollas, and by 72 h after pollination, when the corollas are completely wilted, protein levels have decreased by 75% compared to those on the day of flower opening. These decreases in protein content correspond with increased protease activity in the corolla. Maximal protease activity is detected after 8 d and 72 h in unpollinated and pollinated Mitchell corollas, respectively (Fig. 14.3A; Jones et al. 2005). The protease activity in senescing Petunia corollas can be attributed mainly to cysteine proteases (CP; ∼90%) and to a lesser extent metalloproteases (∼10%) (Jones et al. 2005; Jones, unpublished). Similar declines in protein content have been correlated with increased activity of CP during the senescence of ethylene-insensitive *Sandersonia* and *Iris* flowers (Eason et al. 2002; Pak and van Doorn 2005).

To determine the role of ethylene in regulating the age-related changes in total protein levels and protease activity, comparative analyses between Petunia Mitchell and *etr1-1* transgenic Petunias with reduced sensitivity to ethylene were conducted (Jones et al. 2005). The delayed senescence of *etr1-1* corollas is accompanied by a decrease in total protein content and an increase in protease activity. Protease activity peaks at 16 d after flower opening, when *etr1-1* corollas are fully wilted. The protease activity in *etr1-1* corollas is also due mainly to CP, and units of activity per corolla are similar in senescent Mitchell and *etr1-1* corollas (Jones et al. 2005). These studies support a role for ethylene in regulating the timing of flower

senescence and show that protein degradation is a major component of the senescence program even in the absence of the ethylene signal.

Nine Petunia cysteine protease genes that are expressed during corolla development and senescence have been identified (Jones et al. 2005). Six of these genes increase in abundance during corolla senescence. Of the six, only one (*PhCP10*) has senescence-specific expression. *PhCP10* is a homolog of the Arabidopsis senescence-associated cysteine protease *SAG12* (Weaver, Gan, Quirino, and Amasino 1998). The senescence-upregulated CP in Petunia have different temporal patterns of expression in the corolla, suggesting that they may play different roles in the execution of the senescence program. One group (*PhCP8* and *PhCP10*) has maximum transcript abundance at 6 d after flower opening, before visible symptoms of senescence. The second group (*PhCP2*, *PhCP3*, *PhCP5*, and *PhCP9*) has maximum transcript abundance in unpollinated flowers at 8 d after flower opening, when corollas are wilted. All six senescence-upregulated CP are also induced by pollination and maximal transcript abundance corresponds with corolla wilting (Fig. 14.3B; Jones, unpublished). Maximal expression of *PhCP2*, *PhCP5*, *PhCP8*, *PhCP9*, and *PhCP10* is delayed, and corresponds with corolla wilting and senescence, in *etr1-1* corollas (14–16 d after flower opening). These gene expression patterns support a role for ethylene in modulating the timing of corolla senescence. Experiments in the Clark laboratory (Shibuya et al. 2004) have demonstrated that transformation of Petunias with P_{35S}::*etr1-1* results in moderate to strong reductions in ethylene sensitivity. While the transgenic *etr1-1* Petunias are considered to be insensitive to ethylene, it is also possible that enough signal is able to accumulate over time to induce a similar, but delayed, senescence program in *etr1-1* Petunia corollas. Only *PhCP3* has a temporal gene expression pattern in *etr1-1* that is similar to that of Petunia Mitchell, with maximal transcript abundance at 8 d after flower opening, when Mitchell flowers are senescing. Regulation of *PhCP3* appears to be independent of ethylene signaling and may be the result of other age-dependent developmental cues.

While the treatment of cut *Sandersonia* and *Iris* flowers with cysteine protease inhibitors decreases endogenous protease activity and delays visible symptoms of senescence (Eason et al. 2002; Pak and van Doorn 2005), the number of CP identified to date in senescing Petunia corollas suggests that there is functional redundancy among the individual CP genes. In support of this idea, RNAi and antisense silencing of selected individual cysteine protease genes in Petunia does not result in a delay in corolla senescence (Jones, unpublished).

Senescence-enhanced proteolytic activity is generally thought to function in the large-scale protein degradation that allows for the remobilization of nutrients from dying tissues. On the other hand, those proteases that are upregulated before the visible symptoms of senescence and the large decreases in corolla protein content may be functioning in the specific cleavage and activation of enzymes (including other proteases) that have a role in the execution of the final stages of senescence. The activation of cysteinyl aspartate-specific proteases (caspases) plays a critical role in signaling and executing PCD in animals (Sanmartin, Jaroszewski, Raikhel, and Rojo 2005). PCD is regulated by a proteolytic cascade involving two groups of

caspases, the initiator (or signaling) caspases and the effector (or executioner) caspases. Initiator caspases activate downstream effector caspases which then cleave a variety of specific target proteins, resulting in the ordered disassembly of the cell. Plant orthologs of animal caspases have not yet been identified, but the metacaspases and the vacuolar processing enzymes, both of which are CP, have been suggested to have a caspase-like function during PCD in plants (Sanmartin et al. 2005). While there have been no reports of vacuolar processing enzymes expressed during Petunia flower senescence, we have recently identified a metacaspase that is upregulated during petal senescence (Jones, unpublished). Further characterization and functional analysis of this and other Petunia metacaspases will determine if they play a role in the initiation and/or execution of the senescence program in petals. Those proteases that are upregulated earlier in the senescence process may also have a defense-related role, protecting the flower from pathogen attack and allowing for the ordered disassembly of petal cells and remobilization of important nutrients.

14.3.3 DNA Fragmentation and Increased Endonuclease Activity

The purpose of large-scale nucleic acid catabolism during senescence is to degrade DNA and RNA and recycle the carbon, nitrogen, and phosphorus to developing tissues (Thomas et al. 2003). Both the DNA and RNA contents of Petunia corollas decrease during senescence and the largest decreases are detected in fully wilted flowers (Fig. 14.3E; Xu and Hanson 2000; Yamada, Takatsu, Kasumi, Ichimura, and van Doorn 2006). These observations suggest that nucleic acid degradation is a function of petal senescence in Petunia.

Hallmarks of PCD related to nucleic acid catabolism, including DNA degradation, chromatin condensation, and nuclear fragmentation, have been investigated during Petunia corolla senescence. The first phase of DNA degradation involves the generation of high molecular weight fragments of approximately 50 kbp. Subsequently the DNA is preferentially digested at internucleosomal sites, resulting in $\sim$160 bp fragments. DNA fragmentation can therefore be detected in situ using the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) method to fluorescently label the 3′-hydroxyl termini or by visualizing the 160 bp internucleosomal fragments (DNA ladders) on agarose gels. DNA laddering is detected in naturally senescing *P. hybrida* corollas and pollinated *Petunia inflata* corollas at the fully wilted stage (Xu and Hanson 2000; Yamada et al. 2006). TUNEL assays also indicate that the advanced stages of corolla wilting in *P. hybrida* during natural aging and following pollination are associated with increased DNA fragmentation (Fig. 14.3D; Langston et al. 2005). At 8 d after flower opening in unpollinated flowers and at 72 h after pollination, when corollas are completely wilted, DNA fragmentation can be detected in $>$85% of the nuclei. DNA fragmentation is also a component of the senescence program in ethylene-insensitive corollas, and similar percentages of TUNEL-stained nuclei can be detected in senescent *etr1-1* Petunia corollas at 16 d (Langston et al. 2005).

The number of distinct DNA masses increases and they decrease in size during petal senescence. This condensed chromatin is surrounded by individual nuclear membranes, suggesting that nuclear fragmentation accompanies DNA fragmentation during Petunia petal senescence (Yamada et al. 2006). DNA fragmentation also occurs during petal senescence in *Alstroemeria*, *Antirrhinum majus*, *Argyranthemum frutescens*, and daylily flowers (Panavas, LeVangie, Mistler, Reid, and Rubinstein 2000; Wagstaff et al. 2003; Yamada et al. 2006). While nuclear fragmentation similar to that observed in Petunia accompanies petal senescence in *Argyranthemum*, the condensation of chromatin masses inside the nuclei of *Antirrhinum* petals is not followed with nuclear fragmentation (Yamada et al. 2006). While this may represent a difference in the petal cell death morphology between different flower species, Antirrhinum corollas abscise and petal separation may merely occur before the senescence program is complete.

Nucleic acid catabolism during senescence requires the activity of RNases and DNases (Sugiyama, Ito, Aoyagi, and Fukuda 2000). The degradation of genomic DNA must be catalyzed by endonucleases capable of digesting both single- and double-stranded DNA. Multiple RNases and DNases have been identified in Petunia corollas using in-gel activity assays (Xu and Hanson 2000; Langston et al. 2005). Constitutive activities were found to increase during senescence and novel senescence-specific activities were also detected. DNases with activity against both single- and double-stranded DNA are induced during corolla senescence. The induction of a senescence-specific, 43 kDa endonuclease (PhNUC1) coincides with DNA fragmentation in both naturally senescing and pollinated Mitchell corollas (Fig. 14.3F; Langston et al. 2005). PhNUC1 also has RNase activity, which corresponds with a decline in the RNA content of the corollas (Fig. 14.3F; Xu and Hanson 2000; Langston et al. 2005). The bifunctional nuclease PhNUC1, a glycoprotein with a pH optimum of 7.0, is detected in nuclear but not cytoplasmic protein fractions. Its nuclear location and neutral pH optimum support a role for PhNUC1 in nucleic acid degradation during corolla senescence. The activities of most of the nucleases that are enhanced during Petunia corolla senescence require Ca^{2+} and Mg^{2+}, while PhNUC1 activity is dependent on Co^{2+}. Recently, other Co^{2+}-dependent nucleases have been reported to be involved in nucleic acid catabolism during leaf senescence (Lers, Lomaniec, Burd, and Khalchitski 2001; Canetti, Lomaniec, Elkind, and Lers 2002).

The activation of endonucleases and subsequent digestion of nuclear DNA and RNA appear to be modulated by plant hormones. The senescence of ethylene-sensitive Petunia flowers is accompanied by a burst of ethylene production from the corolla that occurs just prior to and during the advanced stages of senescence, when the corolla is wilting (Langston et al. 2005). The induction of PhNUC1 activity in the corolla corresponds with this ethylene production. Treating Mitchell flowers with 2.0 $\mu l\ l^{-1}$ ethylene for 12 h also results in the induction of PhNUC1 activity. As with what is observed for protease activity and gene expression, the induction of PhNUC1 activity is also delayed in *etr1-1* Petunias. PhNUC1 activity in *etr1-1* corollas is detected at 12–16 d after flower opening and corresponds with wilting and DNA fragmentation (Langston et al. 2005). These comparative analyses indicate that ethylene also modulates the timing of endonuclease activity during senescence.

14.3.4 Specific Nutrient Levels Decrease During Corolla Senescence

Ending the life of a flower by a genetically controlled senescence program allows the plant to salvage valuable nutrients from dying petals before the corolla is shed. Much of the research in Petunia flower senescence has focused on the characterization of protease, nuclease, and phospholipase genes and proteins that are upregulated during corolla senescence. These studies provide evidence that the degradation of macromolecules and cell membranes to allow for the remobilization and recycling of important nutrients is a central function of the senescence program in corollas. Since this nutrient remobilization requires an expenditure of energy, the specific nutrients and the levels that can be remobilized from senescing corollas must result in a net advantage to the growth and development of the plant.

Changes in the mineral nutrient content of Mitchell corollas have been reported during the development and senescence of unpollinated flowers (Verlinden 2003). In this study, Verlinden found that the carbon, nitrogen, phosphorus, and potassium content of corollas declines during the later stages of flower development, suggesting that the remobilization of these nutrients occurs during petal senescence. Carbon levels decline at a rate of 1.1 mg d^{-1} from flower opening to senescence. While these changes correlate well with the observed changes in corolla dry weight, it is unclear whether this is the result of C recycling or tissue respiration. The K content of Mitchell corollas declines progressively throughout development and is reduced by 35% when comparing corollas on the day of flower opening to fully senescent corollas. N and P levels decline by 50% and 75%, respectively, from corolla opening through senescence (Verlinden 2003). Similar declines in N and P have been detected during the pollination-induced senescence of Mitchell corollas (Fig. 14.3C; Jones, unpublished). No significant changes occur in the corolla content of the macronutrients sulfur, calcium, and magnesium, or the micronutrients boron, copper, iron, manganese, or zinc (Verlinden 2003).

In Arabidopsis it was reported that, in addition to reductions in carbon, nitrogen, phosphorus, and potassium levels, chromium, copper, iron, molybdenum, sulfur, and zinc levels decrease by greater than 40% during leaf senescence (Himelblau and Amasino 2001). Nitrogen, phosphorus, potassium, sulfur, calcium, and magnesium levels in corollas on the day of flower opening are much lower than levels detected in newly expanded Petunia leaves (Verlinden 2003). These experiments suggest that corollas are not as important a source of nutrients for recycling as leaves, and may explain why the plant expends energy to remobilize only the most essential and often limiting macronutrients during petal senescence.

14.4 The Role of Mitochondria Remains Unclear

In animals the release of Cytochrome c (Cyt c) from the mitochondria represents the point of no return, at which time the cell is committed to the cell-death program. During PCD, the release of Cyt c from the mitochondrial inter-membrane space pre-

cedes the morphological changes in the cell and initiates the cell death program via activation of the caspase cascade (Sanmartin et al. 2005). In *Petunia inflata* corollas, Cyt c remains localized within the mitochondrial protein fractions late into the senescence program, and it is not detected within the cytosol (Xu and Hanson 2000). These experiments suggest that Cyt c is not involved in signaling the initiation of corolla senescence in Petunia flowers.

Recently, two prohibitin genes that are downregulated during corolla senescence were identified from Petunia flowers (Chen, Jiang, and Reid 2005). Prohibitins are mitochondrial proteins that have a role in cell cycling and death in animals and yeast, and are thought to form a complex that stabilizes components of the respiratory chain (McClung, Jupe, Liu, and Dellorco 1995). Downregulating the *P. hybrida* prohibitin1 (*PhPHB1*) gene by approximately 88% was achieved using VIGS (see Chapter 18). The resulting plants have developmental abnormalities including accelerated corolla senescence (Chen et al. 2005). This reduced flower longevity is accompanied by an earlier rise in the senescence-associated climacteric peak of respiration. It has been suggested that disrupting mitochondrial homeostasis by downregulating prohibitin leads to increased oxidative stress and the generation of reactive oxygen species (ROS), which accelerate corolla death. While downregulating prohibitin may disrupt mitochondrial function and lead to accelerated senescence via the generation of ROS, it is still not clear if signaling from the mitochondria is involved in the normal initiation of the senescence program. Senescence is an active process that requires energy, and anything that disrupts mitochondrial function and results in a depletion of ATP could affect the senescence program. Further investigation of the prohibitins will help establish the role of mitochondria in the onset of corolla senescence in Petunia.

14.5 The Need for Profiling the Proteome and Transcriptome

Most molecular studies of flower senescence have focused on identifying changes in gene expression during corolla senescence and have resulted in the characterization of a few genes identified as senescence upregulated. DNA microarrays containing 500 and 1,300 genes in *Alstroemeria* and *Iris*, respectively, have been used to identify genes that are upregulated and downregulated during petal senescence (van Doorn et al. 2003; Breeze et al. 2004). The further characterization of these genes is hampered by the inability to transform the host species. Large-scale transcript profiling during Petunia corolla senescence and functional analysis of identified genes using VIGS or RNAi techniques is now needed to increase our understanding of the genes involved in the initiation and execution of corolla senescence in ethylene-sensitive flowers.

The authors of this chapter have been involved in a collaborative Petunia genome project that has resulted in the sequencing of more than 10,000 Petunia ESTs encoding approximately 4,500 unique genes from flowers. These sequences were used to design 70-mer oligonucleotide probes and to construct a Petunia DNA microarray

(D.C. Kiplinger Petunia Microarray). The Jones laboratory recently used this Petunia microarray to compare transcript profiles in nonsenescing corollas at 24 h after flower opening to the transcript profiles in corollas at 24 h after pollination. Flowers at 24 h after pollination are not yet showing symptoms of corolla wilting but are committed to the senescence program (Fig. 14.1). This experiment identified 55 genes that are downregulated and 236 genes that are upregulated by 24 h after pollination. Eight percent of the upregulated genes fall into the molecular function categories of transcription regulator activity and signal transducer activity. Further functional analysis of these genes using VIGS or RNAi will help identify those genes that have a critical role in the initiation of the senescence program. Although comprehensive microarray datasets are publicly available for young and old Arabidopsis petals, the analysis of these data cannot contribute much to our understanding of petal senescence, as petals of Arabidopsis flowers abscise without wilting.

While most of the studies of corolla senescence in Petunia and other flowers have focused on changes in gene expression, there is significant evidence that flower senescence is also regulated post-transcriptionally and post-translationally (Thomas et al. 2003). As in other species such as daylily, *Iris*, and carnation (Wulster, Sacalis, and Janes 1982; Lay Yee, Stead, and Reid 1992; van Doorn, Harkema, and Song 1995), treating detached Petunia flowers with the protein synthesis inhibitor cycloheximide significantly delays corolla senescence and provides evidence for the role of newly translated proteins in the senescence program (Fig. 14.5A). In an attempt to identify proteins that are upregulated during flower senescence a proteomic analysis was conducted in Petunia corollas using two-dimensional electrophoresis followed with mass spectroscopy. Two-dimensional protein gels were used to visualize protein profiles in senescing and nonsenescing corollas. Nonsenescing corollas at 0, 24, 48, and 72 h after flower opening were compared with those from pollinated flowers at 24, 48, and 72 h after pollination. No protein differences were detected between unpollinated and pollinated corollas at 24 h, suggesting that 24 h after pollination is too early in the senescence program to identify proteins that are newly translated in response to the pollination signal or those that have been post-translationally modified.

A total of 74 and 113 proteins were determined to be upregulated at 48 and 72 h after pollination, respectively, when compared to the profile of unpollinated flowers of similar age (Bai and Jones, unpublished). Functional classification of the proteins indicates that the majority of the upregulated proteins at 48 h after pollination are those involved in protein, nucleic acid, carbohydrate, and lipid metabolism, supporting the central function of macromolecule degradation during senescence (Fig. 14.5B). Seventeen percent of the upregulated proteins are those involved in stress or defense responses. Defense-related proteins have commonly been found to be upregulated during senescence and are believed to protect the senescing organ from pathogen attack to allow for the progression of senescence and recycling of nutrients (Jones 2004). In contrast to the genes that were upregulated early in the senescence program (at 24 h after pollination), no proteins involved in signaling were identified at 48 h after pollination. Many proteins are represented by multiple spots on the two-dimensional gels, and the differences in their molecular weights

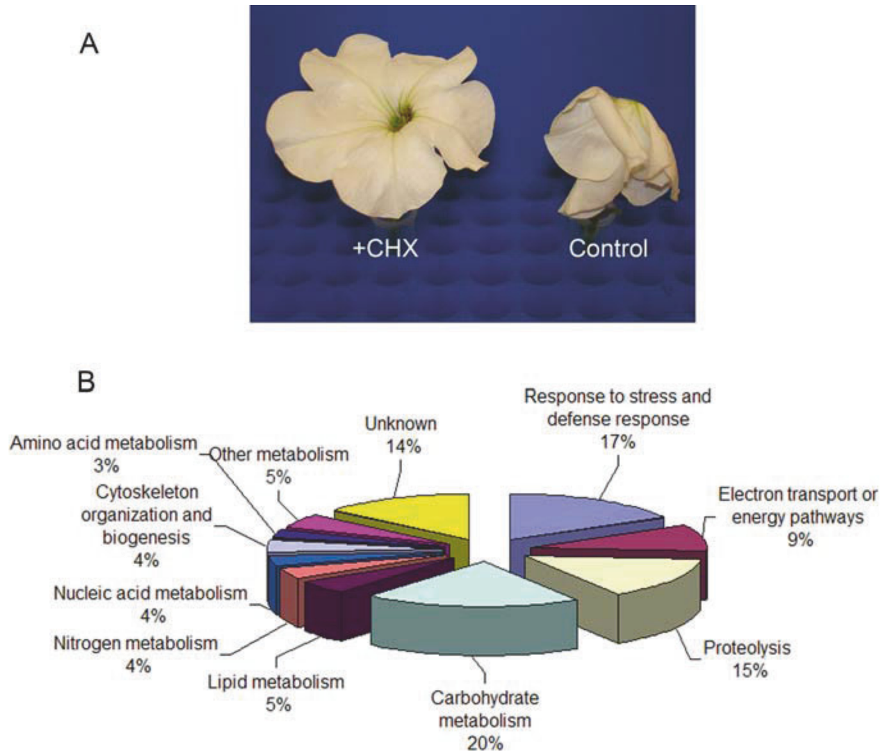

Fig. 14.5 Protein synthesis is required for Petunia corolla senescence, and proteins that are upregulated during pollination-induced corolla senescence can therefore be identified using a proteomics approach. (**A**) Detached Petunia Mitchell flowers after 5 d of continuous treatment with 50 μM cycloheximide (CHX) or water (control). Treatments began on the day that flowers opened fully. (**B**) Proteins that appear or are upregulated in senescing Petunia Mitchell corollas at 48 h after pollination compared with those of unpollinated corollas of a similar age. Total proteins from corollas were separated by two-dimensional gel electrophoresis and analyzed using PDQuest v 7.40 software (BioRad). Differentially expressed proteins were sequenced by the Cleveland Clinic Proteomics Laboratory and putative identities were assigned by searching the nonredundant protein database at NCBI. Biological functions were assigned using the TAIR database

suggest that they represent various post-translationally modified forms of the same protein. Other proteins are represented by both full-length proteins and truncated forms that increase in abundance during senescence and may represent enzymes that are activated by proteolytic cleavage. One such example is a protein with homology to the tomato beta-xylosidase-1, a protein involved in cell wall degradation (Itai, Ishihara, and Bewley 2003). The full-length protein and both N- and C-terminally truncated forms of the protein increase in abundance during corolla senescence (Bai and Jones, unpublished). Functional and biochemical analysis of these proteins is underway to provide a more comprehensive understanding of the biochemical pathways functioning during petal senescence.

14.6 Conclusions

Changes in gene expression, enzyme activities, and nutrient content during Petunia corolla senescence support a central role for cellular degradation and recycling during flower senescence. Despite considerable research on flower senescence in Petunia, these studies have failed to identify master regulators of senescence. Although transgenic Petunias with modified hormone synthesis and perception have increased flower longevity, knocking down the expression of individual senescence-upregulated genes has failed to significantly delay corolla senescence. These experiments confirm that a complex network of pathways is executing the senescence program and that there are many redundancies in these pathways. To date, changes in the transcriptome have predominated in studies of flower senescence, but future proteomic and metabolomic investigations are needed to determine the post-translational regulation of senescence. Additional studies are also needed to focus on upstream regulatory networks and their regulation by ethylene and other plant hormones. Petunia will continue to be an excellent model system for these investigations because of the growing number of gene sequences and the availability of a DNA microarray. The ability to efficiently transform Petunias, as well as the availability of very rapid transient assays like VIGS and *Agrobacterium* infiltration, will allow for functional and biochemical analysis of those genes and proteins identified from current and future genomic and proteomic studies. These analyses should lead to great advances in our understanding of the regulatory networks controlling petal senescence.

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Chapter 15
Genetic Recombination and Mapping in Petunia

Judith Strommer, Janny L. Peters, and Tom Gerats

Abstract For nearly a century Petunia has served as a model species for genetic and cytological studies. The list of mapped loci has grown from one for each of the seven chromosomes to more than 150, to which can be added several hundred, mostly AFLP-based, molecular markers. Mapping efforts provided early evidence for a number of phenomena which now appear to apply to a great number of plant and animal species, including a tendency toward tightly clustered gene family members, suppression of recombination in wide crosses, frequent chromosomal rearrangements, and active transposable element systems.

15.1 Introduction

Genetics research on *Petunia hybrida* was built on a strong foundation of cytological investigations. Although in the mid-1930s Marthaler (1936) reported published estimates of 14–35 chromosomes, the presence of a haploid complement of 7 chromosomes in *Petunia hybrida* and its ancestors was already clear to most workers in the field. Investigations of that decade tended to focus on comparisons of diploid and polyploid lines, the latter of interest for their commercial potential, and a number of researchers analyzed the behavior of meiotic chromosomes in such lines. Subsequent painstaking work, particularly at INRA in Dijon, France, and the University of Amsterdam in the Netherlands, relied on analyses of aneuploid, deletion and translocation lines to fuse cytology and genetics. By 1979, these efforts permitted the assignment of anchor genes to each of the seven chromosomes.

Classical genetic research blossomed for the next 20 years, with traits added by means of visual, biochemical, electrophoretic, and eventually DNA-based analyses. More recently, analyses combining molecular and computer-based approaches have carried Petunia genetics into the genomics era. In the process, much has been

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learned about chromosome structure and behavior in Petunia; for the most part, this work of the last few decades confirmed, extended and, in some cases, made practical use of the astute observations of early researchers in the field.

15.2 Early Cytological Observations

Studies in the early decades of the twentieth century were frequently carried out on lines of ambiguous ancestry, described as "Petunia" or "Commercial Petunia." Not surprisingly, descriptions of chromosomes and their behavior in Meiosis I were variable, with conflicting reports regarding both the relative lengths of homologs and the degree of chromosome pairing, with reports of homolog association ranging from normal to non-existent. Steere (1932) suggested that generally these disparities could be ascribed to a few variables, particularly differences in fixation techniques (poor fixation resulting in daisy chains of chromosomes linked end-to-end, or even as one large ring); and – most relevant to subsequent analyses – the use of different varieties derived from multiple species and their hybrids.

Steere wrote of the difficulty in defining species and ancestry in commercial varieties: "The commercial *P. 'violacea'*, which includes the common garden types, has been derived from crosses among *P. violacea, P. axillaris*, and *P. inflata*, so that it is a composite of two or three species, certain characteristics of each appearing in varying degrees in different strains" (1932). It was to this mixed ancestry that he ascribed the reports of loose pairing, and he therefore chose to base his studies on a pure culture of *P. axillaris*. This choice, together with his precision in regard to fixation methods and careful attention to meiotic progression, probably account for his reports, rare in the Petunia literature, of reasonable pairing of chromosomes and "regular" meioses.

Even the extent of pairing between genetically identical homologs, however, seems to have been modest compared to that observed in many other organisms. Using polyploids derived from inbred diploids, Levan (1938), for example, documented early terminalization of chiasmata and bivalents typically consisting of a single terminal chiasma joining the homologs end-to-end. Almost half a century later Cornu and Maizonnier (1983) reaffirmed Levan's descriptions of early terminalization and the prevalence of homologs joined by one chiasma. In the same paper they cited evidence provided by Sink (1975), again in agreement with their findings, that four potential parental species (*P. axillaris*, *P. inflata*, *P. violacea*, and *P. parodii*) all share genes with *P. hybrida* and were likely genetic contributors to the formation of the hybrid. The significance of these two attributes, limited chiasma formation and broad hybridity, acting singly or in combination, has been reinforced in subsequent mapping efforts which, for the most part, have provided evidence for limited genetic recombination during meiosis.

15.3 Putting Genes on Petunia Chromosomes

While a few genes associated with floral morphology and pigmentation had been identified by the end of the 1950s using classical genetic approaches (Paris and

Haney 1958; Bianchi 1959), the discipline of Petunia genetics grew more directly from a merging of cytology with segregation analysis. This approach was facilitated by the creation of a complete series of trisomics and other mutagenesis-derived chromosomal variants. Aberrant segregation data in these lines allowed the assignment of a range of genes to the appropriate trisomic chromosomes. Daniel Maizonnier, whose group had initially chosen Petunia as their model system specifically for mutagenesis-based research, created in 1971 a workable karyogram that distinguished five of the seven chromosomes (Maizonnier 1971), and in the same year Maizonnier and Cornu (1971) reported on the use of a spontaneous translocation to assign the flavonoid gene *Hf1* to Chromosome I. Shortly thereafter, Smith and Oud (1973) developed a fluorescence-based method for distinguishing the problematic Chromosomes, V and VI, and were able to place the flower-shaped gene *Un* on Chromosome V (Smith and Oud 1973; Smith, de Jong, and Oud 1975). Meanwhile, Wiering and de Vlaming had begun to build linkage groups, based largely on genes associated with pigmentation (Wiering and de Vlaming 1973, 1977).

As described a few years later by Cornu (1984), there emerged an agreed-upon set of independently segregating anchors or "linkage group heads" (*Hf1*, *Fl*, *Ht1*, *Bl*, *Hf2*, *Rt*, and *An4*), one for each of the seven chromosomes. (All of these genes have now been characterized at the molecular level; see, e.g., Chapter 13.) Maizonnier and Moessner (1979) presented a karyogram with each linkage group head assigned to a chromosome, in most cases with linked markers (Fig. 15.1A). In the same year, the Amsterdam and Dijon groups together published a comprehensive list of 74 genes of Petunia, 51 of them assigned to the linkage groups defined by the anchoring markers (Wiering, de Vlaming, Cornu, and Maizonnier 1979).

In the 1980s, the cytological and genetic tools developed in the previous decade were reinforced in several ways, through the development of new genetically characterized lines; broad application of novel isozyme analytical methods, particularly by van den Berg and Wijsman (1980); and the addition of a number of new researchers to the discipline. The confluence led to rapid development of an extended genetic

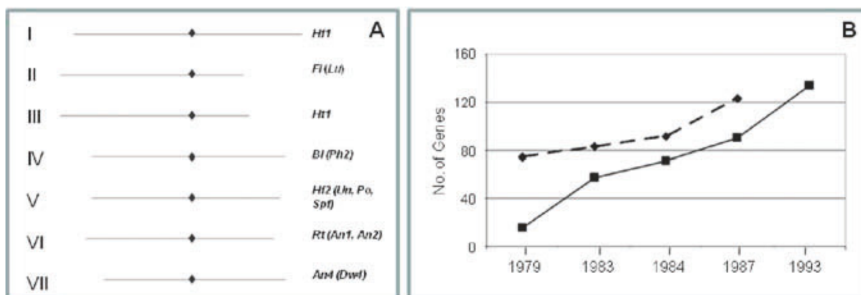

Fig. 15.1 Development of the Petunia genetic map. (**A**) Initial assignments of genes to cytologically distinguishable chromosomes, published by Maizonnier and Moessner (1979). Full names of genes are provided in Table 15.1. (**B**) Increases in the numbers of genes identified (upper curve) and mapped to chromosomes (lower curve) between 1979 and 1993. Fourteen years later, the number of mapped genes has increased slightly, to just over 150

Table 15. 1 Petunia genes assigned to chromosomes, from Gerats et al. (1993), except as indicated in footnotes

Ch I		*an11*	Anthocyanin 11	*gf*	Glycosylation at 5
		ch2	Choripetalous-2	*hf2*	Hydroxylation at 3'5'-2
an9	Anthocyanin-9	*ch3*	Choripetalous-3	*la*	Luteus activator
bi	Bifunctional	*dg5*	Dark green-5	*lh1*	Long hypocotyl-1
ch1	Choripetalous-1	*f6*	[c]	*mf2*	Methylation at 5'-2
dg6	Dark green-6	*fu1*	Folded up-1	*mt2*	Methylation at 3'-2
ea	Ears	*hf6*	[c]	*pAc1*	Actin-1
hf1	Hydroxylation at 3'5'-1	*ht1*	Hydroxylation at 3'-1	*pFLO6*	Floral-specific cDNA
ht2	Hydroxylation at 3'-2	*lg6*	Light green-6	*pFLO14*	"
le1	Lethal-1	*mf1*	Methylation at 5'-1	*pFLO21*	"
le3	Lethal-3	*mt1*	Methylation at 3'-1	pJMC13.8	Cyto P450-related
lg1	Light green-1	*pAc4*	Actin-4	*po*	Pollen colour
nl1	Narrow leaves-1	*ph4*	*pH-4*	*sdh*	Shikimic acid dehydrogenase
pAc9	Actin-9	*prxA*	Peroxidase-A	*sp1*	Spotted-1
ph1	pH-1	*prxD*	Peroxidase-D	*ua*	Ustulata
ph5	pH-5 (= *vt3*)	*rDNA1*	Ribosomal DNA-1	*ve1*	Veination-1
prxB	Peroxidase-B	*rDNA2*	Ribosomal DNA-2	*vs4*	Virescent seedling-4
vr1	Viridis-1	*rl1*	Rolled up leaves-1	*yg1*	Yellow-green-1
vs1	Virescent seedling-1	*S*	Self incompatibility[d]		
vs2	Virescent seedling-2	*sp3*	Spotted-3	Ch VI	
wi	Withered	*vs7*	Virescent-7		
		yg3	Yellow-green-3	*acs4*	ACC synthase-4
Ch II				*an1*	Anthocyanin-1
		Ch IV		*an2*	Anthocyanin-2
aco3	ACC oxidase-3			*ap2c*	Apetala-2-like[b]
aco4	ACC oxidase-4	*adh1*	Alcohol	*dfrC*	Dihydroflavonol reductase-C
chsC	Chalcone synthase-C		dehydrogenase-1	*dg1*	Dark green-1
chsE	Chalcone synthase-E	*adh3*	Alcohol dehydrogenase-3[e]	*dw2*	Dwarf-2
chsG	Chalcone synthase-G	*an3*	Anthocyanin-3	*e*	Element (= *ms4*)
chsL	Chalcone synthase-L	*an6*	Anthocyanin-6	*le2*	Lethal-2
cr	Crumpled	*apt*	Apetalous (= *gp*)	*ph6*	pH-6

dfrB	Dihydroflavonol reductase-B	*bl*	Blind[a]	*pFLO11*	Floral-specific cDNA
		dfrA	Dihydroflavonol reductase-A	*rf2*	Restorer of fertility-2
do2	Double-2	*dg2*	Dark green-2	*rt*	Rhamnose at 3
dw7	Dwarf-7	*dw1*	Dwarf-1 (= *dg4*)	*tu*	Trumpet
f3gt	Flavonol 3-galactosyl transferase[a]	*dw4*	Dwarf-4		
fl	Flavonol synthase	*f3bh*	Flavanone-3-b-hydroxylase	Ch VII	
ftr	Fluoro-tryptophan resistance	*pAc3*	Actin-3		
		pAc7	Actin-7	*acs2*	ACC synthase-2
gad	Glutamate decarboxylase[b]	*ph2*	pH-2	*acs3*	ACC synthase-3
		pJMC13.8	cyto P450-related	*an4*	Anthocyanin-4
grp	Glycine-rich protein	*prxC*	Peroxidase-C	*an12*	Anthocyanin-12
lg2	Light green-2	*rf1*	Restorer of fertility-1	*do1*	Double-1
lu1	Luteus-1	*st1*	Short tube-1	*fbp1*	MADS-box transcription factor
pAc1	Actin-1	*sx*	S-protein[b]	*gpiB*	Glucose phosphate isomerase-B
px	Phoenix	vs3	Virescent seedling-3	*lapB*	leucine amino peptidase-B
rm1	Recombination modulator-1	*yg4*	Yellow-green-4	*lg5*	Light green-5
				ls	Long style
rnx2	RNAase-X2[b]	Ch V		*ms3*	Male sterile-3
si	Short internodes			*pAc2*	Actin-2
ws	White stigma	*an8*	Anthocyanin-8 (= *fn*)	*pFLO1*	Floral-specific cDNA
		chiA	Chalcone isomerase-A	*pFLO3*	”
Ch III		*chsA*	Chalcone synthase-A	*pFLO9*	”
		chsD	Chalcone synthase-D	*ph3*	pH-3
ab5	[c]	*chsF*	Chalcone synthase-F	*prxF*	Peroxidase-F
adh2	Alcohol dehydrogenase-2	*chsH*	Chalcone synthase-H	*prxG*	Peroxidase-G
alf	Aberrant leaf/flower	*chsI*	Chalcone synthase-I	*prxH*	Peroxidase-H
an10	Anthocyanin 10	*fu2*	Folded up-2	*vs5*	Virescent-5
		G	Grandiflora (= *un*)		

[a]Miller et al. 2002; [b]Strommer et al. 2000; [c]Strommer et al. 2002; [d]ten Hoopen et al. 1998; [e]Garabagi and Strommer 2005.

map. This progress, illustrated by the maps published in 1983 and 1984 (Cornu and Maizonnier 1983; Cornu 1984; de Vlaming et al. 1984), owed a great deal to the ongoing collaborative spirit of the groups in Dijon and Amsterdam. Figure 15.1B, illustrating the increase in the number of reported chromosomal assignments of genes between 1979 and 1993, captures a sense of the spirit of this period.

Between 1979 and 1984, the number of identified genes increased from 74 to 91 and the number of chromosomal assignments from 51 to 74. More than one-third of the genes listed and mapped in 1983 were associated with flower color; others covered traits affecting floral morphology, plant morphology/physiology, chlorophyll deficiencies, and fertility/lethality (Cornu and Maizonnier 1983). The authors noted that each linkage group contained at least one "block" in which meiotic recombination appeared to occur at a low frequency: Chromosomes I and V, each with 12 mapped genes, carried 7 and 6 loci, respectively, in such blocks. Cornu and Maizonnier (1983) referred to unpublished evidence suggesting that loci in a linkage block were not necessarily closely linked physically, and suggested evidence for the existence of "genetic modulators of recombination."

The first Petunia monograph, published in 1984, carried an illustration of the seven chromosomes with a total of 60 assigned genes (Cornu 1984). The 1984 report increased the number of roughly mapped loci to 74, with the introduction of results from classic two-point test crosses, including a number that relied on analysis of isozyme variants (de Vlaming et al. 1984).

The list of 122 genes published just a few years later by the Dutch–French collaborators (Gerats et al. 1987), with 88 markers assigned to chromosomes, was the final version lacking assignments based on molecular data (included, however, was a chloroplast DNA restriction map containing a number of genes). The updated CSH map released 6 years later (Gerats et al. 1993), in which 134 of 165 listed nuclear genes had chromosomal assignments, included for the first time loci mapped by restriction fragment length polymorphisms (RFLPs). Table 15.1 provides an updated, but undoubtedly incomplete, list of Petunia genes assigned to chromosomes, including those mapped by molecular methods. With the advent of molecular techniques, especially reverse genetics approaches, Mendelian mapping of genes fell out of fashion and, to the extent that maps are yet produced, they are either local (for map-based cloning, e.g., Bentolila, Zethof, Gerats, and Hanson 1998; Bentolila, Alfonso, and Hanson 2002) or based on anonymous molecular markers (for studies of recombination).

The blossoming of Petunia as a genetic system in the 1980s coincided with the debate over the choice of a plant model system to serve in parallel with *Drosophila melanogaster* and *E. coli*. The development, in Amsterdam, of lines such as V23 and R51 (Fig. 15.2), with readily identifiable polymorphic markers on each chromosome (first reported in Wallroth, Gerats, Rogers, Fraley, and Horsch 1986) along with high levels of DNA polymorphisms (McLean, Baird, Gerats, and Meagher 1987), offered a resource of great potential for molecular biologists. There were a number of supporters of Petunia, who recognized that it offered, in addition, a small number of well-characterized chromosomes, ease of maintenance and genetic manipulation, and easy collection of materials for biochemical analysis. Although the early Cold

Fig. 15.2 Four of the major players in genetic analyses of Petunia. Clockwise from upper left: *P. hybrida* V26, *blind* mutant, *P. hybrid* W 138 and Petunia Mitchell

Spring Harbor courses in plant molecular biology used Petunia as a model system, the number of converts to Arabidopsis grew in the early 1980s, and by the end of the decade the unremarkable weed, whose small genome and short generation time offered indisputable advantages, had clearly become the model of choice.

15.4 Molecular Maps

Given the great reservoir of genetic diversity, together with the availability of diverse inbred lines created and housed initially at the University of Amsterdam and later at the Free University of Amsterdam, Petunia was an attractive organism for molecular analyses based on DNA sequence variation. The analyses of gene families encoding chalcone synthase (Koes, Spelt, Mol, and Gerats 1987) and actins (McLean et al. 1987; McLean, Gerats, Baird, and Meagher 1990) made good use of the available resources and polymorphisms. The findings from this early "genomics" work revealed several similarities between the two gene families and foreshadowed a number of subsequent discoveries related to the structure and evolution not only of Petunia but of eukaryotic genomes in general.

Chalcone synthase genes (*CHS*) encode the enzyme associated with the first committed step in flavonoid biosynthesis. In the velvet-petaled deep purple V30 variety,

Koes and coworkers (1987) identified at least seven *CHS* genes (*CHS-A* to *G*), and recovered in addition five genomic clones carrying just one of the two exons (*CHS-H* to *L*). Surprisingly, they found evidence for expression of only two of the genes, *CHS-A* and *CHS-J*, during normal plant development in V30, with low expression of two others inducible by UV light treatment (Koes et al. 1986). Among the genes and gene fragments, they identified two subfamilies whose members share >95% identity in protein-coding and untranslated DNA regions (*CHS-C,E,L,G* and *CHS-H,I*), compared with the 75–80% identity shared among other members of the family. The genes designated *CHS-D,F, H*, and *I*, covering a genetic distance of about 5 cM, were shown to be closely linked to *Hf2* on Chromosome V. *CHS-A* mapped about 30 cM away from the cluster, and a sixth gene, *CHS-B*, was tentatively mapped to the same chromosome. *CHS-C, E, G*, and *L* were mapped to within 3 cM of one another, weakly linked to the Chromosome II markers. The genetic distances reflected measurements from one of the sets of crosses, as map distances were consistently greater in the V30×R135 than in V30×M7 crosses. A comparison of *P. hybrida* with a panel of potential ancestral species, which contained gene families of varied sizes and locations, suggested that the pattern observed in *P. hybrida* is the result of gene duplications, which apparently took place before the separation of the species groups bearing white versus colored flowers (Koes et al. 1987).

In the same year, Baird and Meagher (1987) reported the recovery of a number of different actin-like gene sequences from a genomic library prepared from Petunia Mitchell, a doubled-haploid derived from an F1 hybrid between *P. axillaris* and the *P. hybrida* cv. "Rose of Heaven" (Mitchell, Hanson, Skvirsky, and Ausubel 1980). Actin, required for normal cell structure and function, had been shown to be encoded by gene families in all organisms in which it had been studied, so it was no surprise to find a gene family in Petunia. Nonetheless, the number of genes estimated by Baird and Meagher (1987), at close to 200 copies, set an as yet unbeaten record. Although expression of individual genes was not analyzed, DNA sequence similarity to mRNA of leaf tissue was demonstrated for a number of the clones.

From sequence analysis of 20 members of this "gene superfamily" nine distinct subfamilies were identified, and representative genes for six of these were subsequently subjected to genetic characterization by McLean et al. (1987, 1990). The aforementioned V23-R51 lines, carrying 11 phenotypic polymorphisms, were used for mapping. The subfamily representatives mapped to five chromosomes: *PAc9* on Ch I; *PAc1* on Ch II; *PAc4* on Ch III; *PAc* 2 on Ch VII; and *PAc3*/*PAc7* tightly linked on Ch IV. Members within at least one subfamily, *PAc4*, were shown to be tightly clustered. The number of genes within each subfamily appeared to be variety specific, with lower numbers in V23 and R51 compared to the much higher numbers reported for Petunia Mitchell.

Examined together, these two studies portrayed what seemed at the time to be an unusual genome. It contained gene families (of sometimes surprisingly large size), dispersed overall but organized into genetically linked subfamilies. The size of the family and subfamilies in a given variety or lineage differed from those seen in progenitors and could be expected to vary among different varieties or lineages. It now appears that Petunia was not exceptional: a huge body of research on various

plant and animal genomes accumulated since the late 1980s generally supports this portrayal of eukaryotic genome structure and evolution. Petunia, as it turned out, had provided an excellent model to study the evolution, structure, and function of eukaryotic genomes.

DNA from the V23-R51 backcross lines used to map the actin gene subfamily members nearly 10 years earlier was pulled from storage in the mid-1990s to serve in the construction of an expanded RFLP map (Strommer, Gerats, Sanago, and Molnar 2000, 2001). This effort contributed additional evidence for the emerging pattern of genome organization. The work added 21 markers to the genetic map, including multiple representatives of 5 more gene families, in addition to the 6 actin genes and the 11 visual and electrophoretic markers segregating among progeny of V23-R51 hybrids. Included in the original map were four of the *CHS* genes (*CHS-A,C,F,G*) previously assigned to Chromosomes II and V by Koes and coworkers (1987). Two genes encoding aminocyclopropane carboxylic acid oxidase (*aco*) were shown to be tightly linked; of three genes for aminocyclopropane carboxylic acid synthase (*acs*), two were tightly linked and the third was on a different chromosome. Two alcohol dehydrogenase genes (*adh*) were unlinked (subsequent work identified a third *adh* gene linked to one of them (Garabagi and Strommer 2004; see Chapter 4)). Two *rDNA* clusters mapped to distinct regions of Ch III, but subsequent mapping placed the two ribosomal markers close together. The limited overall length of the genetic map, at less than 370 cM, and the evidence for clustering of loci, particularly on Chromosomes II and IV, reaffirmed the existence of "linkage blocks" and an apparent paucity of chiasma-associated recombination events.

The potential of the newly developed AFLP technology (Vos et al. 1995) to contribute to genetic and molecular studies of Petunia was recognized immediately (Gerats, de Keukeleire, Deblaere, van Montagu, and Zethof 1995). It provided a potential means for addressing a number of objectives: expanding the Petunia genetic map, facilitating map-based cloning, and shedding light on the unusual nature of recombination in the genus. A report on the construction and comparison of two sets of AFLP maps, based on segregation data from hybrids derived from four disparate varieties, appeared in 2002 (Strommer, Peters, Zethof, de Keukeleire, and Gerats). One set was based on the same V23-R51 hybrids used for RFLP mapping in the 1980s and 1990s. With the addition of 9 new RFLP and 127 AFLP markers, the map not only failed to grow but shrank from 368 to 332 cM. A second map, created from backcross progeny of a V26-W137 hybrid, carried 228 markers on an even shorter linkage map of just 261 cM, approximately one-fifth the length of the previously published RFLP map of tomato (Tanksley et al. 1992). In the V26-W137-derived map, more than two-thirds of the markers were congregated in high-density clusters (Fig. 15.3).

The parallel maps derived from two very different hybrids offered additional information. They demonstrated, for example, that the degree of marker clustering, which is inversely correlated with recombination frequencies, was strongly chromosome specific, and that the chromosomes specifically exhibiting the lowest levels of recombination depended on the parental lines (Fig. 15.3). Thus, from the V23-R51 hybrids, 28 of 36 markers on Ch II, and 26 of 31 markers on Ch V, lay within 17 cM

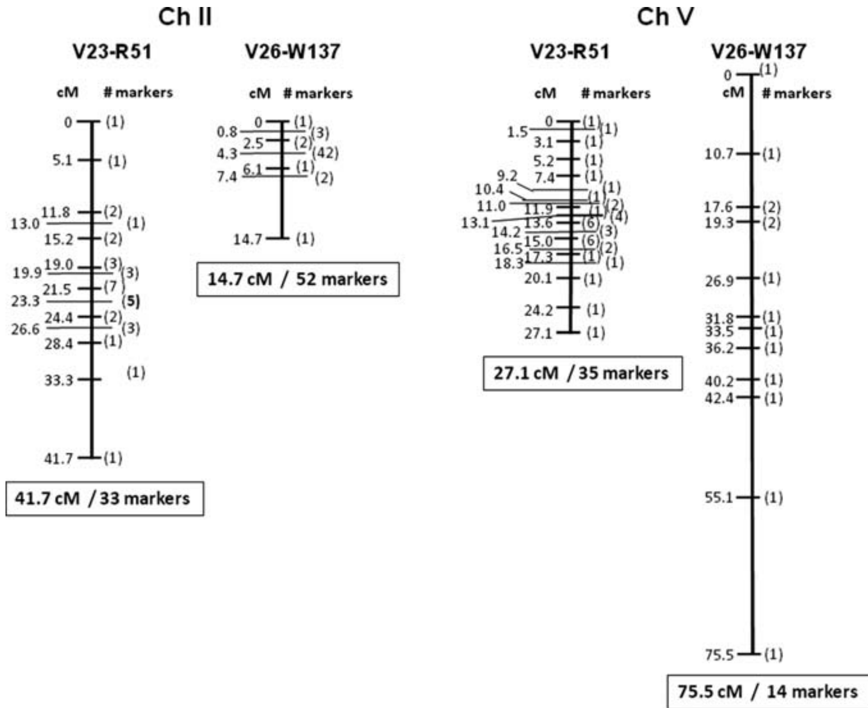

Fig. 15.3 Schematic maps illustrating differences in relative lengths of Chromosomes II and V derived from AFLP maps based on two different sets of hybrids (Strommer et al. 2002). Indicated at the right of each chromosome are the numbers of different markers that mapped to the same site, i.e., failed to segregate in any of the progeny. While recombination frequencies for both sets of crosses were low, regions of significant "recombination blocks" were cross specific

of one another on their respective chromosomes; there was much less evidence of marker clustering on other chromosomes. From the V26-W137 hybrids, on the other hand, there was no evidence for a linkage block on Ch V, but clustering was severe on Ch I, Ch II, and Ch VII: on Ch II, for example, there was no recombination seen among 42 of 52 markers. Artifacts related to the potential reliance on AFLP fragments generated from repetitive DNA regions were ruled out by demonstration that products obtained with a given set of AFLP primers from a tight linkage group showed no cross-hybridization. This mapping work provided confirmation of the presence of variety-specific differences in recombination frequencies, and thus measured map distances, and generally limited recombination in Petunia.

15.5 Physical and Genetic Maps

An enlightening study reported in a set of papers in the mid-1990s allowed a comparison of genetic and physical locations for transgenes in Petunia (Robbins, Gerats,

Fiske, and Jorgensen 1995; Fransz et al. 1996; ten Hoopen et al. 1996). The experimental plan was to map 135 independent transgene insertion sites using the antibiotic resistance marker provided by T-DNA, thereby defining a large set of randomly placed markers of potential use for mapping or gene cloning (Robbins et al. 1995). Transgenic V26 lines were crossed to two genetically distant lines, M1 and M59, to create the hybrids needed for mapping. Focusing on inserts linked to *Hf1* on Ch I and *Fl* on Ch II, the researchers described a set of transgenics with a low level of recombination between the T-DNAs and the phenotypic markers. From the crosses using M1 (M59), 4/6 (3/17) transgenes linked to *Hf1* lay within 1 cM of that marker on Ch I, and 5/9 (7/13) transgenes linked to *Fl* lay within 1 cM of that marker on Ch II. To test their hypothesis that recombination was suppressed as a consequence of the wide genetic distance between the two parental lines, they crossed pairs of transgenic V26 lines which carried different, but, according to the results from the first set of crosses, tightly linked insertions. Progeny of the outcross to the wild-type were scored for the frequency of crossovers between the two resistance markers. As predicted, recombination frequencies between insertion sites increased dramatically in the inbred hybrid compared to those in the wide hybrids. Insertion sites mapping approximately 16 cM apart on Ch I in the wide cross, for example, segregated as though unlinked in the V26-V26 cross, and the genetic distance between a set of insertions on Ch II increased from 0.9 to 11 cM.

Making use of six of the lines carrying T-DNAs linked to *Fl* on Ch II, ten Hoopen et al. (1996) then used fluorescence *in situ* hybridization (FISH) to compare genetic and physical distances between transgenes. Their results demonstrated that loci closely linked by genetic analysis spanned almost the full length of the chromosome. Two major conclusions came from this work: recombination over most of the chromosome in the wide-cross hybrid was extremely low; and recombination in general was suppressed around the centromere. The known relationships between centromeric regions and heterochromatin on the one hand, and heterochromatin and suppressed recombination on the other (Roberts 1965), provided the simplest explanation for strong centromeric suppression of recombination. Similar clustering of markers around centromeric regions of tomato (Tanksley et al. 1992) and rye (Bert, Charment, Sourdille, Hayward, and Balfourier 1999) have been reported, and in the latter case led to direct FISH-based visualization of tetrads (Kagawa, Nagaki, and Tsujimoto 2002), which documented heterochromatic suppression of recombination. The demonstration that recombination rates based on measurements from a given chromosome increased with inbreeding provided a direct explanation for the changeable but recurring linkage blocks documented in Petunia over many decades. Peltier and coworkers showed that blocks of RAPD markers in true Petunia species have been maintained as such in many cultivars (Peltier, Farcy, Dulieu, and Berville 1994).

In the past few years, evidence has been accumulating for suppression of recombination in hybrids derived from wide crosses involving other crops. On the one hand, there is no evidence for the phenomenon in the closely related tomato, chromosomes of which exchange information readily, even in interspecific hybrids (Chetelat, Meglic, and Cisneros 2000). For potato, on the other hand, Tanksley and coworkers (Bonierbale, Plaisted, and Tanksley 1988; Tanksley et al. 1992) estimated

a frequency of recombination approximately half that found in tomato, with some chromosomes more strongly affected than others. Caldwell, Russell, Langride, and Powell (2006) provided evidence for much higher levels of linkage disequilibrium among elite barley lines compared to those seen in landraces and other inbreeding populations. The extent of recombination suppression in Petunia, however, like the number of copies of actin genes, appears exceptionally high.

Petunia also appears to carry an unusually plastic genome, which, to the extent that sequence divergence restricts crossing over, can help explain the enigmatically short genetic maps. Most of these maps were created by crossing genetically distant plants, for example, different cultivars or a cultivar and a true species. Galliot, Hoballah, Kuhlemeier, and Stuurman (2006) presented a genetic map based upon interspecific crosses and ranging in length from typically short (for Petunia) to 400 cM. More enigmatically, they presented data to show that a map based on crosses between presumed subspecies (*P. axillaris axillaris* x *P. axillaris parodii*) was much smaller than the average. Despite the limited number of markers analyzed in that cross, the implication is that meiotic recombination was suppressed to an extreme extent, presumably due to enhanced differences in genome structure between the subspecies. Alternatively the phylogenetic position of the two subspecies may have to be revised (Galliot et al. 2006).

15.6 Modulators of Recombination

Maizonnier et al. (1984) observed that recombination between markers on Chromosome VI was extremely low, despite their positions at opposite ends of the chromosome, *An2* on the short arm and *Rt-An1* on the long arm. Using γ-irradiation-induced deletions, Gerats et al. (1984) found a surprising correlation between the length of the deletion on the short arm and the frequency of recombination between *Rt* and *An1*, closely linked on the long arm. In other words, the normally low frequency of recombination between *An1* and *Rt* increased as more of the short arm was lost due to chromosome breakage. Recombination events that restored the short arm reduced recombination on the long arm to the original levels.

Hypothesizing that the mechanism of inhibition of recombination on Ch VI might be related to the presence of heterochromatin, Maizonnier et al. (1984) shared the observation that patterns of heterochromatin differed between lines. Thus, it seemed plausible that heterochromatin generally, and heterochromatic variations specifically, might be key to explaining the low but variable levels of recombination characteristic particularly of wide crosses in Petunia.

The second potential explanation offered by Maizonnier, Cornu, and Farcy (1984) was the involvement of "a genic system". A line cultured in Amsterdam had been observed to be associated with generally higher rates of recombination; its ability to modulate recombination (*Rm*) became the subject of a series of papers published in *Genome* between 1989 and 1992 (Cornu, Farcy, and Mousset 1989; Robert, Farcy, and Cornu 1991; Abirached-Darmency, Tarenghi, and de Jong 1992). In this

work, recombination rates between two markers on each of the seven chromosomes were determined using the St 43 "*Rm*" line and appropriately marked control or "*rm*" lines. For the marker pairs on Ch I, II, V, VI, and VII, recombination rates were higher in the *Rm*-derived hybrids than in the controls (e.g., Ch I: 4.9 *cf.* 1.0%; Ch VII: 11.9 *cf.* 2.2%). For the Ch IV and Ch III pairs, however, recombination was lower in the *Rm*-associated lines (Ch IV: 25.9 *cf* 29.4% and Ch III: 2.9 *cf.* 6.8%). The authors concluded that the effect of *Rm* was greatest in crosses where recombination rates were the lowest (Cornu et al. 1989), although the conclusion, derived from such a small number of marker pairs, was not compelling. Because there was no maternal effect and the modulation appeared to segregate roughly 1:1 in testcross progeny, the *Rm* effect was assigned to the action of a nuclear gene that they designated as *Rm1*. Clearer results came from a subsequent effort to map *Rm1*. The experiment could detect linkage of the recombination-modulation effect with either *Hf1* on Ch I, or *Lu1* on Ch II, by co-segregation of the Ch I or Ch II marker with increased recombination between *An2* and *Rt* on Ch VI. Predicting equivalent rates of *An2-Rt* recombination among the four classes of testcross progeny (phenotypically *Hf1 Lu1*, *Hf1 lu1*, *hf1 Lu1*, and *hf1 lu1*), the authors found much higher rates of recombination in lines carrying *Lu1*. On the basis of this result they positioned *Rm1* closely linked to *Lu1* on Ch II (Cornu et al. 1989).

Subsequent papers in the series suffered from the complexity of the system. Robert et al. (1991) reported on a set of *Rm1* gene-dosage experiments. Data from three populations in which recombination rates in *Rm1* homozygotes, *Rm1-rm1* heterozygotes, and *rm1* homozygotes could be compared simultaneously – that is, in the same genetic background – for two unlinked sets of markers, were consistent with a semi-dominant mode of action for *Rm1*. Recombination increased with inbreeding, as reported later by Robbins et al. (1995), but the increase was sufficient to explain only about one-third of the variation in mean recombination rates. In work reported a year later, histological examination of pollen mother cells of *Rm* and *rm* plants revealed no clear differences in synaptonemal complexes reconstructed from three-dimensional serial sections, but pachytene chromosome spreads suggested that particularly around telomeres and pericentric regions, "synaptonemal complex formation is more regular and more efficient in the presence of *Rm1*" (Abirached-Darmency et al. 1992).

The *Rm1* story remains unfinished, but again it appears that Petunia is not unique. Background-dependent and localized variations in recombination rates have more recently been reported in a number of plants and animals (Koehler, Cherry, Lynn, Hunt, and Hassold 2002; Yandeau-Nelson, Nikolau, and Schnable 2006; Yang et al. 2006). The demonstrated region-specific dependence of recombination rates on a combination of *cis*- and *trans*-acting factors in maize (Yandeau-Nelson et al. 2006) seems to differ from that in Petunia only by degree; and the local sequence homology required for heteroduplex maintenance in mouse (Yang et al. 2006) may help explain the link between wide crosses and suppressed recombination in Petunia. Work to date is thus consistent with a genetic system in which a combination of one or more genes, local chromosome structure, and the degree of homology between paired chromosomes determines the level of recombination. The tendency for early

terminalization in Petunia meiosis may be a secondary consequence of these factors or, alternatively, independently depress the frequency of those crossovers that can be easily detected by classical genetic means.

15.7 Genome Rearrangements

As described above, there was early evidence for genic instability in Petunia. Rearrangements and phenotypic reversions have been ascribed to a number of potentially interrelated sources: breakage–fusion–bridge cycles, translocations and reversible genic systems. The breakage–fusion–bridge cycle appears to have received little attention since Daniel Maizonnier's doctoral research (Maizonnier 1976). The evidence for unstable phenotypes reminiscent of those associated with transposable elements in snapdragon and maize, however, was of particular interest to members of the Amsterdam group. Bianchi, Cornelissen, Gerats, and Hogervorst (1978) described a revertable mutant allele of *An1* associated with a system, which bore similarities to Barbara McClintock's controlling elements of maize, although, as they reported, their work did not address the potential of the mutable element for transposition. The subsequently identified *dTph1* transposable element system is described in detail in Chapter 17, and its applications for molecular genetic studies is demonstrated by the large number of references to its use throughout this book.

A perplexing finding from efforts to merge genetic maps was that the location assigned to a gene or the structure of a gene family defined in one variety of Petunia did not necessarily reflect chromosomal position or gene copy number in other varieties. In the cases of both chalcone synthase (Koes et al. 1987) and actin genes (McLean et al. 1990), the numbers of genes in subfamilies were shown to be variety specific. The *CHS-A* gene, which mapped to Ch V in V30, V23 and R51 varieties (Koes et al. 1987; Strommer et al. 2000), was found near the tip of Ch III in V26 and Mitchell (Fransz et al. 1996). In generating the Petunia RFLP map, one progeny set had evidence of a newly arising rearrangement affecting alleles for three genes near the tip of Ch IV (Strommer et al. 2000). In the absence of direct comparisons, it is not clear whether Petunia is typical or exceptional in regard to either the generation of, or tolerance for, such rearrangements.

15.8 Conclusions

While the era of classical genetic mapping has passed, Petunia researchers of the last century bequeathed to us not only more than 150 mapped genetic loci but also molecular maps useful for current applications, such as map-based cloning and comparative genomics. Scientifically, they raised issues of broad relevance in relation to phenomena such as variable rates of recombination, genome instability, and genome evolution. Petunia researchers, now developing DNA sequence and transcriptome

libraries, continue the tradition that built over the last century, the century of genetics, working collaboratively across wide geographic and disciplinary distances.

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Chapter 16
Impact of Retroelements in Shaping the Petunia Genome

Katja R. Richert-Pöggeler and Trude Schwarzacher

Abstract Retroelements, defined by their dependence on reverse transcription for replication, are found in the genomes of bacteria, fungi, animals and plants. This chapter summarizes current knowledge about the structure, function and evolution of representatives from two retroelement groups identified in Petunia. The presence of both a viral retroelement – an inducible endogenous plant pararetrovirus, EPRV- and non-viral retroelements in the form of LTR-retrotransposons makes Petunia an ideal model system to study possible retroelement interactions. Phylogenetic relationships have been determined and chromosomal co-localization of EPRV and *Metaviridae*, one group of LTR-retrotransposons, has been demonstrated. The impact of partly overlapping replication pathways on element interference is discussed. While studies in Petunia and related species have led to tremendous progress in our understanding of these elements we are just beginning to comprehend the consequences of their presence and activities in their hosts.

16.1 Introduction

Retroelements comprise a class of diverse elements that have in common a dependence on reverse transcription for their replication (Hull 2002). DNA is synthesized from the RNA template employing an RNA-dependent DNA polymerase, the reverse transcriptase that is encoded by the retroelement. All steps of reverse transcription occur in the cytoplasm, thus circumventing competition with host DNA synthesis in the nucleus. Retroelements are not only ubiquitous components of plant genomes but also abundant in other major taxonomic groups of bacteria, fungi and animals.

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In this chapter we review two retroelement groups, the endogenous pararetroviruses (EPRVs) and retrotransposons, of which distinct members have been identified in genomes of solanaceous plants including Petunia. The endogenous Petunia vein-clearing (pararetro)virus (ePVCV), representative of the first group, is of special interest, as it combines features of both viral and non-viral retroelements and much is known about its function (Richert-Pöggeler and Shepherd 1997; Richert-Pöggeler, Noreen, Schwarzacher, Harper, and Hohn 2003). Long terminal repeat (LTR)-retrotransposons illustrate the second group. They are non-viral retroelements that play a major role in plant gene and genome evolution and, indeed, overall plant genome size (Wright and Voytas 1998; Kumar and Bennetzen 1999; Hansen and Heslop-Harrison 2004; Vitte and Bennetzen 2006). In the following we summarize what is known about genomic occurrence and distribution, as well as replication, of these retroelements in Petunia. Furthermore we highlight potential modes of element interaction as well as their possible impact on genome plasticity in relation to both evolutionarily significant and epigenetic modifications.

16.2 Classification of Retroelements

Ever-increasing DNA sequence information is revealing a level of dynamic diversity of retroelements that makes classification a challenge. More and more types of elements have emerged showing a mixture or rearrangements of features that had previously been assigned to a specific group. Classification is therefore constantly evolving to accommodate new and transitional elements. Furthermore, novel features have been revealed that beg for reorganization of taxonomy and the creation of subgroups (Fauquet, Mayo, Maniloff, Desselberger, and Ball 2005). In Fig. 16.1 we give an overview of the retroelements discussed in this chapter and highlight the common DNA sequence and gene features.

Historically the gene order and phylogeny of encoded reverse transcriptase, the core enzyme of retroelements, has been used to distinguish two major groups of LTR-retrotransposons (Xiong and Eickbush 1990; Hansen and Heslop-Harrison 2004; Havecker, Gao, and Voytas 2004). The Ty3/*gypsy* group features a gene order analogous to that of retroviruses, in which the integrase (IN)-encoding domain is located downstream of the *pol* domain that encodes reverse transcriptase (RT) and RNAse H (RH), while in the Ty1/*copia* group the gene coding for IN is found upstream of the *pol* region (Fig. 16.1A).

The presence of a retroviral-like envelope gene (*env*), which is essential for infectivity in animal cells, in some members of LTR-retrotransposons (Laten, Majumdar, and Gaucher 1998; Wright and Voytas 1998) and the ability to form virus-like particles (VLPs) (Jääskeläinen et al. 1999; Takeda, Sugimoto, Kakutani, and Hirochika 2001) led to the classification of Ty3/*gypsy* elements as *Metaviridae* and the Ty1/*copia* elements as *Pseudoviridae* (Fig. 16.1), with three genera each (Peterson-Burch and Voytas 2002; Havecker, Gao, and Voytas 2005). *Pseudoviridae* carrying an *env* gene are assigned to the genus *Sirevirus,* ToRTL1 of tomato

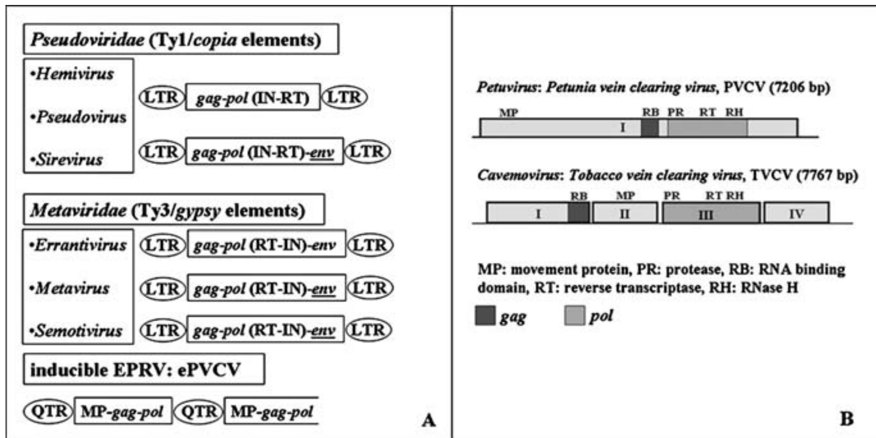

Fig. 16.1 Sequence elements, genes and structures of retroelements discussed in this chapter. (**A**) Current classification of LTR-retrotransposons (Fauquet et al. 2005) and genomic organization of family members. A simplified genomic organization of ePVCV as a tandem array that allows for direct transcription is depicted. Note that the second ePVCV genome in the tandem array is incomplete (*open box*). Preserved domains of retroelement genes necessary for encapsidation (*gag*) and replication (*pol*) are indicated, and an EPRV-specific gene domain for virion transport within plants (movement protein, MP) and a LTR-retrotransposon-specific domain for integrase (IN), promoting active integration, are illustrated. LTR: long terminal repeats, QTR: quasi terminal repeats due to tandem array of integrated viral genomes; *env*: *env* gene, not present in all members; RT, reverse transcriptase. Boxes indicate open reading frames. (**B**) Genomic organization and consensus domains of pararetroviruses for which homologous sequences have been found in genomes of the Solanaceae

(Peterson-Burch and Voytas 2002) and *Endovir* of *Arabidopsis thaliana* (Peterson-Burch, Wright, Laten, and Voytas 2000), for example. The remaining *Pseudoviridae* were further subdivided into *Hemi-* and *Pseudovirus* due to distinct strategies used in priming minus-sense DNA synthesis (Peterson-Burch and Voytas 2002); however, members of the *Hemivirus* genus have not yet been found in plants.

Although the genome organization among *Metaviridae* is conserved, analysis based on the reverse transcriptase domains has enabled definition of three genera: *Metavirus*, including the elements identified in plants; *Errantivirus*; and *Semotivirus*, the latter two abundant in animal genomes (Eickbush, Boeke, Sandmeyer, and Voytas 2005). *Metaviridae* containing an *env* gene are found in all members of the genus *Errantiviruses* and in some members of the genera *Metavirus* and *Semotivirus*.

The phylogeny of LTR-retrotransposons and related viruses based on amino acid sequences of RT and RH domains reveals a close relationship of *Metaviridae* elements and *Caulimoviridae*, which comprise pararetroviruses of plants (Eickbush and Malik 2002; Hansen and Heslop-Harrison 2004). These are called “para” retroviruses (PRVs, Hohn and Richert-Pöggeler 2006) because they are similar to retroviruses with regard to genome organization and replication, but they are distinct

in two major features: (i) their virions harbor double-stranded DNA in contrast to retroviruses, which encapsidate two RNA molecules; and (ii) they lack an integrase gene that promotes active integration into the host genome in retroviruses. Differences in particle morphology, genome organization and RT sequence phylogeny led to the definition of six different genera among plant pararetroviruses: *Caulimovirus*, *Soymovirus, Cavemovirus*, *Petuvirus*, *Badnavirus* and *Tungrovirus* (Fauquet et al. 2005). It has been shown that among this virus family the *Petuvirus* and *Cavemovirus* clades branch closest to the *Metaviridae* clade (Richert-Pöggeler and Shepherd 1997; Hohn et al. 2008). Interestingly, the nearest relatives to plant pararetroviruses are found among *Metaviridae* derived not only from plants, but also from insects and fungi.

16.3 Genomic Diversity and Distribution of Retroelements

Our current knowledge of retroelement presence and structure in the Solanaceae taxon (summarized in Table 16.1) is fragmental and relates mostly to species that are economically important for agriculture or horticulture, such as potato, tobacco, tomato and to some extent Petunia. Nevertheless, recurrent patterns of distribution within and between genomes, as well as certain conclusions about the origins of these mobile elements, can be recognized (see below). "Solanaceous" genome sequencing projects started with tomato (Mueller et al. 2005), which contains the smallest genome (see Table 16.1) and will, if sequenced totally, provide the first complete picture of retroelement diversity and distribution within a solanaceous plant genome. The first sequenced genome of a dicotyledon was obtained from a member of the Brassicaceae, *Arabidopsis thaliana*, with a genome about six-fold smaller than that of tomato and one-tenth that of Petunia (Arabidopsis Genome Initiative 2000). In *A. thaliana* the contribution of retroelements to genome shape has been studied comprehensively; it has therefore been included in Table 16.1 for comparison.

Information about *LTR-retroelements* in the Solanaceae has been obtained from experiments with defined primers or probes using PCR (Rogers and Pauls 2000; Hansen and Heslop-Harrison 2004) and from the screening of genomic DNA and BAC libraries (Guyot et al. 2005; Wang et al. 2006). In addition, retroelements have been discovered by virtue of their insertions into genes (Grandbastien, Spielmann, and Caboche 1989; Camirand and Brisson 1990). *Tnt1*-like elements of the family *Pseudoviridae* are widespread among solanaceous plant species and are showing diversity among regulatory sequences in their LTR regions that are essential for transcription (Grandbastien et al. 1991, 2005; Casacuberta, Vernhettes, Audeon, and Grandbastien 1997; Manetti, Rossi, Costa, Clausen, and Van Sluys 2007). The observed sequence plasticity may account for the adaptation and concomitant preservation of these elements within distinct host genomes after invasion by an ancestral *Tnt1*-like element early in the evolution of the Solanaceae. Chromosomal mapping of the *Tnt1* superfamily in tomato located the majority

Table 16.1. Identified EPRV and LTR-retrotransposons in Solanaceae compared with those of Arabidopsis

Species	Mean genome size[1]	EPRV[2]	Homologous PRVs	*Metaviridae*[2]	*Pseudoviridae*[2]	References
Petunia spp.	1362	ePVCV (50-100)	PVCV	3-24, 4-18	Petunia *copia*, rTph 1, pToml.1	Voytas et al. 1992, Rogers and Pauls 2000, Richert-Pöggeler et al. 2003, Matsubara et al. 2005
Solanum lycopersicon	985	*Lyc*EPRV	TVCV	Jinling elements (≥2000)	ACO1PRT (high), Lerel, Retrolyc (30-40), ToRTL1 (11-57, T135 (22-58), TnT1 (29-51), pTom1.1 TCI-4	Blume et al. 1997, Grandbastien 1998, Rogers and Pauls 2000, Mao et al. 2001, Wang et al. 2006, Staginnus et al. 2007, Tam et al. 2007
Solanum tuberosum	1376	SoTuI, SotuIII	TVCV	Prt group	Tst1	Camirand and Brisson 1990, Grandbastien 1998, Hansen et al. 2005
Nicotiana spp.	3699	eTVCV, *Ns*EPRV (>1000), *Nto*EPRV (>1000)	TVCV		Tnt (383-673), Tto (30)	Grandbastien et al. 1989, 2005, Pouteau et al. 1991, Lockhart et al. 2000, Gregor et al. 2004

Table 16.1. (continued)

Species	Mean genome size[1]	EPRV[2]	Homologous PRVs	*Metaviridae*[2]	*Pseudoviridae*[2]	References
Arabidopsis thaliana	157	none		Athila group (30), Tat1 (2-10), Gimli, Gloin, Legolas, Tft, Tma	Endovir, Artl AtC group, AtR group (few), Evelknievel (few), Metal-1, Ta1-3	Martin and Llorens 2000, Peterson-Burch 2000, Peterson-Burch and Voytas 2002, Vitte and Panaud 2005 and references therein

[1]Royal Botanic Grandens Kew average DNA C-values for 1C in Mbp. [2]Copy number in brackets, where available. Underlined elements are transcriptionally active

of insertions in centromeric or neighboring regions and identified a few elements residing at more distal sites along the chromosomal arms (Tam et al. 2007). The most abundant LTR-retroelement family of tomato is represented by the *Jinling* elements, belonging to the *Metaviridae*, and specific to the pericentromeric regions (Wang et al. 2006), which are rich in retroelements in general (Yang et al. 2005).

Retroelements with pararetroviral counterparts, the *EPRVs*, are ubiquitous in economically important solanaceous crop plants like tobacco, tomato, potato and Petunia, as well as their respective wild relatives and progenitors. The majority of identified elements show sequence homology to *Cavemoviruses* (Fig. 16.1B). It appears that host-genome invasion by pararetroviruses occurred several times during the evolution of the Solanaceae, as integrated sequences show different degrees of decay (Staginnus and Richert-Pöggeler 2006). Highly degenerated non-functional sequences, as well as complete and inducible integrants homologous to episomal viruses, have been isolated (Jakowitsch, Mette, Van der Winden, Matzke, and Matzke 1999; Lockhart, Menke, Dahal, and Olszewski 2000; Richert-Pöggeler et al. 2003; Gregor, Mette, Staginnus, Matzke, and Matzke 2004; Hansen, Harper, and Heslop-Harrison 2005; Staginnus et al. 2007). The copy numbers of EPRVs are quite variable and can reach into the thousands in large host genomes like *Nicotiana* spp. (Gregor et al. 2004). Most copies are concentrated in pericentromeric heterochromatin and they are often found associated with *Metaviridae* sequences (Richert-Pöggeler et al. 2003; Gregor et al. 2004; Hansen et al. 2005; Staginnus et al. 2007); see Fig. 16.3A.

16.3.1 Diversity of LTR-Retrotransposons Among Petunia Species

As retroelements insert themselves into genomes they act as mutagenic agents, thereby providing a putative source of biodiversity (Hirochika, Sugimoto, Otsuki, Tsugawa, and Kanda 1996; Heslop-Harrison et al. 1997; Ellis, Poyser, Knox, Vershinin, and Ambrose 1998; Flavell, Knox, Pearce, and Ellis 1998) and serving as markers of diversity. Petunia contains both viral and non-viral retroelements, as revealed in several reports by others and by our own studies (Voytas, Cummings, Konieczny, Ausubel, and Rodermel 1992; Rogers and Pauls 2000; Richert-Pöggeler et al. 2003; Matsubara, Kodama, Kokubun, Watanabe, and Ando 2005). The amino acid sequences of consensus domains of *gag* and *pol* regions for LTR-retrotransposons identified in Petunia (Richert-Pöggeler et al. 2003) reveal homology to elements found in other plant families.

In order to study the diversity of LTR-retrotransposons in Petunia more closely, we isolated 35 new RT/RNAse H sequences from *P. hybrida* variety V26 and its ancestral and wild relatives *P. axillaris, P. parodii*, *P. inflata* and *P. parviflora*, using the *Metaviridae* and *Pseudoviridae* degenerate primers described by Flavell, Smith, and Kumar (1992) and Friesen, Brandes, and Heslop-Harrison (2001). Phylogenetic analysis based on maximum parsimony of the DNA sequences (Fig. 16.2), together

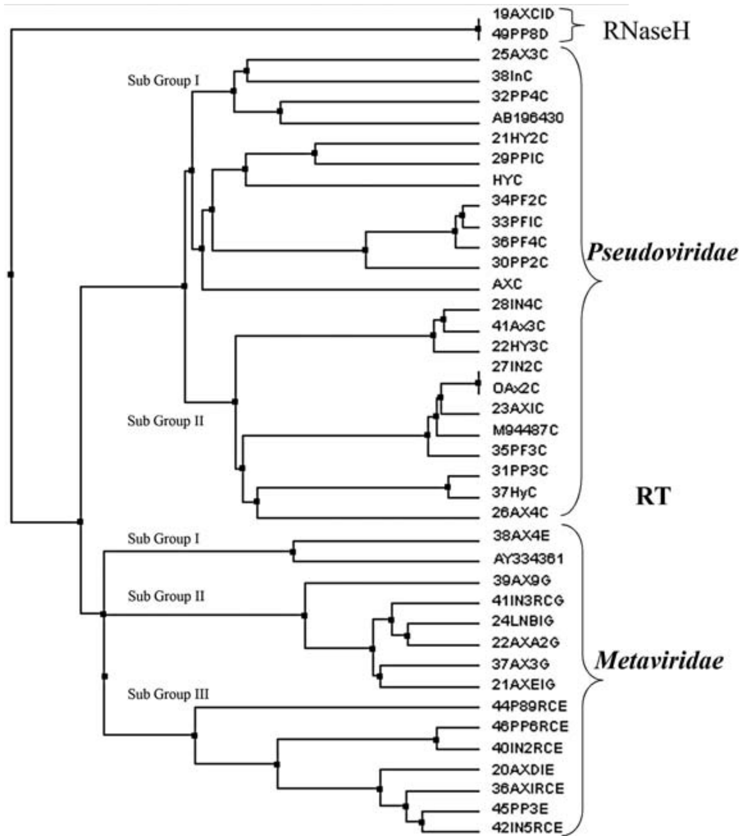

Fig. 16.2 Phylogenetic tree of RT sequences of *Metaviridae* and *Pseudoviridae* of Petunia. Maximum Parsimony tree of 35 RT and 2 RNAse H retroelement DNA sequences isolated with universal primers (Flavell et al. 1992; Heslop-Harrison et al. 1997; Kubis et al. 1998; Friesen et al. 2001) from *Petunia hybrida* V26 (HY), *P. axillaris* (AX), *P. inflata* (IN), *P. parodii* (PP) and *P. parviflora* (PF). Corresponding sequence fragments were taken from the retrotransposon-like clone AB196430 in Petunia (Matsubara et al. 2005; *rTph1*, see Table 16.1), M94487 (Voytas et al. 1992; Petunia *copia,* see Table 16.1) and lambda clone 3, which also contains the *metaviridae*-related sequence 3–24 (see Table 16.1), at position 4509 (AY334361; Richert-Pöggeler et al. 2003). *Pseudoviridae* sequences (names ending with C, EMBL accessions AM941495–AM941498 and AM944800–AM944816) are split into two subgroups. *Metaviridae* sequences (ending with E or G, EMBL accessions AM941494 and AM991944–AM991958) are split into three subgroups

with three published sequences, shows that *Pseudoviridae* and *Metaviridae* form distinct groups. Additionally, the *Pseudoviridae* sequences are subdivided into two groups and the *Metaviridae* sequences into three. Interestingly, neither of these subgroups contains sequences restricted to a single Petunia species, or even closely related species, white- or purple-flowering. This is in contrast to many species

groups, in which retroelements have been found to be species specific and to reflect species phylogeny (Katsiotis, Schmidt, and Heslop-Harrison 1996; Tatout, Lavie, and Deragon 1998; Friesen et al. 2001). Indeed they are often argued to be older components of the genome (Heslop-Harrison 2000), their turnover and loss likely to have occurred in a directed manner (Tatout et al. 1998), leading to characteristically different retroelement compositions between species. The heterogeneity of RT sequences maintained among the Petunia species studied support the notions of ongoing speciation (Kulcheski et al. 2006) and recent isolation of taxonomic units, allowing for continued frequent crossing and back-crossing among the species and hybrids, with consequent exchange of retroelements.

16.3.2 Chromosomal Distribution of Retroelements in Petunia

LTR-retroelements are generally dispersed throughout the genome (Heslop-Harrison 2000). They have been identified within or adjacent to genes and may associate with, or avoid, particular genomic regions such as rDNA and heterochromatin, which consist of tandemly repeated DNA elements (White, Habera, and Wessler 1994; SanMiguel et al. 1996; Kamm, Doudrick, Heslop-Harrison, and Schmidt 1996). In Petunia, *Metaviridae*-like sequences are found in the pericentromeric regions of all chromosomes (Fig. 16.3A) but show slight variations in signal strength between chromosomes. This pattern is indicative of nested insertions that are chromosome specific. The chromosomal localization of a sequence homologous to an RNAse H domain showed a dispersed distribution including all chromosomes (Fig. 16.3B). This part of the retrotransposon is less conserved and any given sequence will pick up only the closest related family members under the hybridization conditions used. The lack of strong signal sites would argue that clusters of retroelements are made up of different family members. Alternatively, the two sequences used for FISH come from very distinct retroelement families with distinct distribution patterns, as has been described for barley, where different *Metaviridae* families are either centromeric or dispersed (Vershinin, Druka, Alkhimova, Kleinhofs, and Heslop-Harrison 2002).

Endogenous PVCV sequences have also been found mainly in pericentromeric regions (Richert-Pöggeler et al. 2003), but only in selected chromosomes, with some variation between accessions (Hohn et al. 2007; also see Fig. 16.3C and 16.3D). Whereas ePVCV sequences produce one coherent signal, the signal for *Metaviridae*-like sequences seems to be more dispersed (compare Fig. 16.3A with 16.3C and 16.3D). LTR-retrotransposon distribution similar to that observed in Petunia has also been found in tomato (Staginnus et al. 2007), where EPRV sequences are found in the same chromosomal regions. The close chromosomal proximity of *Metaviridae* and EPRVs is not surprising, as we have even found them residing on the same lambda clone (Richert-Pöggeler et al. 2003), and points to a possible functional association.

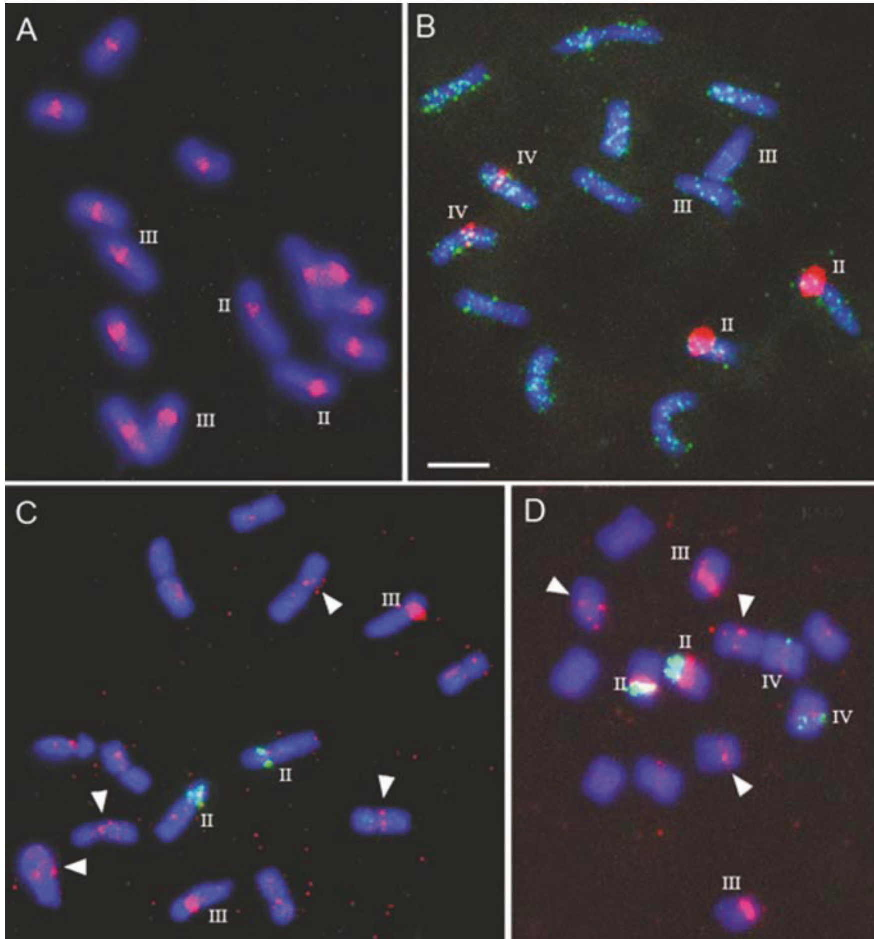

Fig. 16.3 Fluorescent in situ hybridization (FISH) of retrotransposons and ePVCV in Petunia. Chromosomal distribution of *Metaviridae* retroelements (A, B) and ePVCV sequences (C, D) in Petunia demonstrated by double-target FISH. Chromosomes II, III and IV were identified by morphology and 5S rDNA hybridization signal. (**A**) *Metaviridae* clone 3–24, isolated from lambda clone 3 (nt 2174-3349 of AY334361) obtained from a genomic library of *P. hybrida* (W138), shows hybridization to all centromeric regions in *P. hybrida* "Himmelsröschen"; weak fluorescence was detected along all arms. (**B**) A clone containing the RNAse H domain of *Metaviridae* (*green*) hybridized to metaphase chromosomes of *P. axillaris* that were also probed with 5S rDNA (*red*). The signal is dispersed along most of the chromosome arms with no amplification in any certain region. C, D) PVCV (*red*) and 5S rDNA (*green*) sequences were hybridized to *Petunia hybrida* "Himmelsröschen" (**C**) and "W138" (**D**) metaphase chromosomes stained blue with DAPI. In both varieties strong signal is visible near the centromeres of chromosome III and several other chromosomes (*arrow heads*). On chromosome II, near the 5S rDNA site, a second strong PVCV site is detected in "W138", but not in "Himmelsröschen"

16.4 Replication of Retroelements

The replication cycles of both viral and non-viral retroelements involve several common steps that take place in different cellular compartments (Fig. 16.4). Transcription of the element occurs in the nucleus, while translation followed with DNA synthesis from this RNA template by RNA-dependent DNA polymerase, the reverse transcriptase, takes place in the cytoplasm. Formation of VLPs in the case of LTR-retrotransposons and EPRVs probably allows for assembly of replicative complexes that remain intracellular (Fig. 16.4). Intercellular transport of mature

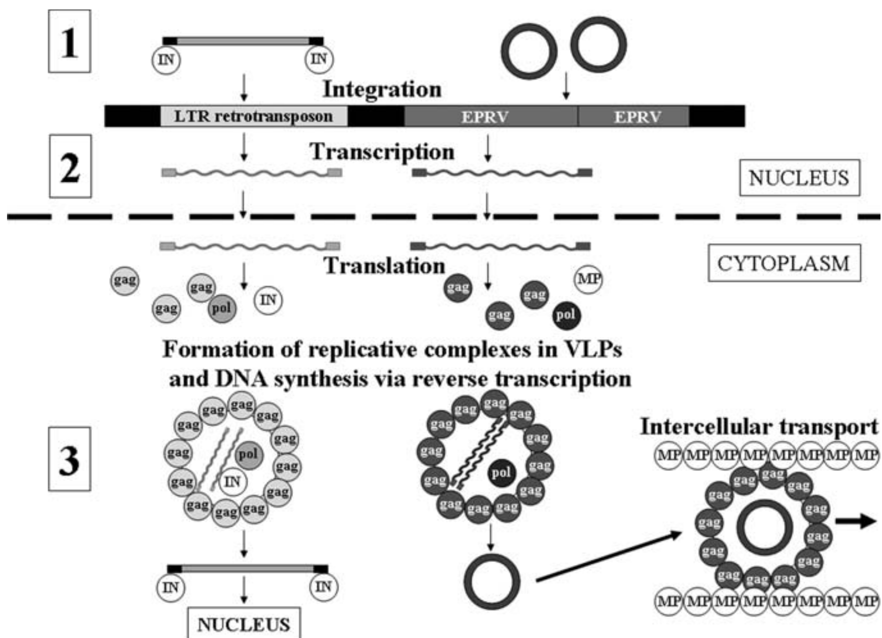

Fig. 16.4 Proposed scheme for replication of ePVCV and associated *Metaviridae* in Petunia, awaiting experimental verification. For detailed explanation see text sections on replication and integration. Putative interactions within different cellular compartments are indicated by numbers. (**1**) Illegitimate and homologous DNA recombination during double-strand DNA break repair. (**2**) Read-through transcription producing transcripts comprising EPRV and *Metaviridae* sequences. (**3**) RNA-mediated recombination events during replication in VLPs. Encapsidation of heterologous RNA molecules in VLPs followed by template switches of reverse transcriptase between heterologous RNA molecules would produce chimeric dsDNA molecules containing both *Metaviridae* and EPRV sequences. Active integration *via Metaviridae* integrase would be possible due to the presence of LTRs on chimeric DNA. Note: for pararetroviruses the composition of the replication complex containing two copies of RNA has not been experimentally demonstrated. At step 3 the circular dsDNA molecules have single-stranded gaps at respective primer binding sites for minus-strand and plus-strand DNA synthesis (not indicated). *Black boxes* depict plant genomic DNA; *light gray boxes*, integrated LTR-retrotransposon DNA; and *dark gray boxes*, integrated EPRV in the neighborhood of LTR-retrotransposons interspersed with plant DNA. Abbreviations are the same as those used in Fig. 16.1

virions happens only for ePVCV, an activatable EPRV, and is promoted by the virus-encoded MP. We postulate that putative interactions between elements, including recombination, template switches and read-through transcription, can take place during multiple steps of the replication cycle (Fig. 16.4) and may explain the often integrated DNA structures containing adjacent *Metaviridae* and EPRV sequences.

16.4.1 Mechanisms for Retroelement Integration into the Host Genome

Active integration of episomal DNA molecules into host chromosomes is promoted in the case of LTR-retrotransposons by the element-encoded integrase (IN) protein (Fig. 16.4, Step 1). IN is most likely the primary determinant for nuclear localization of LTR-retrotransposon DNA and, in addition, is involved in cleavage of target DNA and joining of target and episomal DNA ends. The resulting gaps of single-stranded DNA are repaired by the host recombination/repair machinery (Voytas and Boeke 2002).

Evidence accumulated so far indicates that integration of EPRV into the plant genome does not occur actively but as a by-product of the DNA repair machinery of the host. The generally accepted mechanism of such integration events is double-strand break repair (Puchta 2005), which can lead to either homologous recombination or random integration, the latter being the predominant mode in plants. The passive mode of integration by homologous recombination also plays a role for the actively inserting retrotransposons (Voytas and Boeke 2002). The association of pararetroviruses and retrotransposons found in almost all cases of described EPRVs, including Petunia (Richert-Pöggeler et al. 2003; Fig. 16.2), other solanaceous species (Staginnus et al. 2007) and banana (Hohn et al. 2008), may be functional. Intact retrotransposons may supply the missing viral integrase function in *trans* or through related structural sites of the retrotransposons (Hull, Harper, and Lockhart 2000; Fig. 16.4).

Besides the interactions between chromosomal and episomal DNA that will lead to invasion of the host genome, homologous and/or heterologous molecules of episomal DNA can recombine before integration. In step 1 of Fig. 16.4, only the repaired circular dsDNA forms of EPRVs in the nucleus are shown. The precursors of those repaired DNA molecules with single-strand breaks can be used as templates for the dsDNA break-repair machinery (Hohn et al. 2008). Encapsidated concatamers of viral genomes that could have been generated during replication have been found for one pararetrovirus infecting sugarcane (Geijskes, Braithwaite, Smith, Dale, and Harding 2004). Additionally, once these chimeric DNA molecules are integrated into the host genome, they could form higher-order repetitive DNA structures that are amplified by mechanisms of repetitive sequence amplification (see, e.g., Schwarzacher 2003), such as unequal and illegitimate crossing over or replication slippage of conserved short repeats as are found within L*yc*EPRVs and related sequences (Staginnus et al. 2007).

Furthermore, extrachromosomal recombination of foreign DNA after uptake into the cell has been shown to occur in plants (reviewed by Hanin and Paszkowski 2003). Their observations indicate that tandem arrays or clusters of viral as well as non-viral sequences that have been identified in Petunia (Richert-Pöggeler et al. 2003) could have been formed prior to genome invasion. Although random integration of PVCV sequences into the Petunia genome may occur quite frequently in somatic cells during virus infection, only integrants in reproductive cells will be inherited and therefore traceable (Staginnus and Richert-Pöggeler 2006; Hohn et al. 2008), but any sequence insertions that have caused infertility or lethal mutations in the genome will not be preserved.

16.4.2 Common and Distinct Features in Replication

EPRVs have, by definition, abandoned their episomal stage and become components of their host genomes. Accordingly, horizontal virus transmission from plant to plant has been replaced with vertical transfer of virus sequences *via* reproductive cells. The EPRVs identified in the Solanaceae show homology to episomal virus forms of *Petuvirus* and *Cavemovirus* (Fig. 16.1B). These plant pararetroviruses (PRVs) together with *Caulimovirus* and *Soymovirus* represent genera among the family *Caulimoviridae* that form isometric virions. Generation of icosahedral VLPs in plants has been reported for the active *Pseudoviridae* BARE-1 in barley (Jääskeläinen et al. 1999). However, these virions remain intracellular and do not move between cells, as is possible for inducible EPRVs that encode an additional MP enabling cell-to-cell transport in plants (see Figs. 16.1A, 16.B and 16.4). Thus it is not surprising that infectious LTR-retrotransposons have been identified in animals, in which the *env* gene is sufficient to mediate intercellular spread, but not yet in plants, because LTR-transposons lack a movement or analogous protein essential for intercellular transport in plants across the cell wall. Common to both EPRVs and LTR-retrotransposons is not only a reproducing, but also an amplifying, replication cycle (Fig. 16.4). The replication cycle of activatable EPRVs has not been investigated specifically but it is assumed to be analogous to that of CaMV, the best-studied member of the *Caulimoviridae* (for details see recent review of Hohn and Richert-Pöggeler 2006). For the episomal form of activatable EPRVs like ePVCV in Petunia, a so-called "minichromosome" – a circular double-stranded (ds) DNA molecule associated with histones (histones are not displayed in Fig. 16.4) – serves as template for transcription in the nucleus, mediated by host enzymes. Thus the polyA signal in close proximity to the promoter is over-read at first encounter and recognized only on the second round, resulting in a full-length, terminally redundant RNA transcript (Fig. 16.4).

This is also the transcription product of LTR-transposons; however, as templates they use linearized DNA molecules with LTR that are integrated in the host chromosome (Grandbastien 1998; Harper, Hull, Lockhart, and Olszewski 2002). EPRVs in tandem array, which have been found for some copies of ePVCV in the genome, resemble LTR-retrotransposons in structure and allow for direct transcription of

integrants (Richert-Pöggeler et al. 2003). The transcribed RNA serves as a template for translation and DNA synthesis (Fig. 16.4). The terminal redundancy of the generated RNA molecule is essential for reverse transcription, as it enables the polymerase template switches that produce the specific DNA molecules mentioned above. Common to both pararetroviruses and LTR-retrotransposons in plants is their reliance on a particular host t-RNA to prime reverse transcription for producing the minus-sense DNA strand (Peterson-Burch and Voytas 2002; Hohn and Richert-Pöggeler 2006) and polypurine-rich tracts to prime for synthesis of the plus-sense DNA strand. The resulting double-stranded DNA molecule is linear, with terminal redundancy in the case of LTR-retrotransposons and circular in the case of pararetroviruses and inducible EPRVs, due to different termination points of the reverse transcription (Fig. 16.4).

Compartment formation in VLPs (Fig. 16.4) may be essential for the replication reactions as well as for separation from host metabolism (Voytas and Boeke 2002). Heterologous encapsidation of *Metaviridae* and EPRV transcripts may provide templates for the production of chimeric sequences that undergo integration (Fig. 16.4, Step 3; Matzke et al. 2004). Studies of retroviral and *Pseudoviridae* recombination indicate that RNA structure leading to a pause in reverse transcription and action of RNAse H are involved in template switches between RNA molecules (Voytas and Boeke 2002; Roda et al. 2003).

16.5 Viral Plant Cell Invasion

Every EPRV group is presumed to have originated from its cognate exogenous pararetrovirus (PRV, Staginnus and Richert-Pöggeler 2006). Accordingly, one prerequisite for being invaded by PRVs is that the plant represents a suitable host and allows for viral replication. In the case of ePVCV it could be shown that integrated sequences remain infectious and can trigger PVCV infection and expression of vein-clearing symptoms in provirus (ePVCV)-free *P. parodii* when introduced by biolistic inoculation (Richert-Pöggeler et al. 2003). The only known disease caused naturally by PVCV is in members of the Petunia taxon and commercially available hybrid Petunia (Harper et al. 2002; Staginnus and Richert-Pöggeler 2006). Even though the exogenous counterpart of ePVCV has been identified as a member of the genus *Petuvirus* (Richert-Pöggeler et al. 2003; Fauquet et al. 2005), the question of how virions were initially delivered into plant cells is unresolved. No natural transmission has yet been found under normal growth conditions in the greenhouse, and PVCV transmission from infected plants is possible only by grafting (Richert-Pöggeler et al. 2003).

Petunia species seem to be ideal PVCV hosts, as all members of this taxon tested so far could be infected with PVCV using grafts, whereas this was not true for other members of the Solanaceae, including *N. tabacum*, *Datura metel*, *Datura stramonium*, *Solanum lycopersicum*, *Physalis peruviana, Nicandra physaloides,* and *Solanum melongena* (Richert 1992; Richert-Pöggeler et al. 2003). Detailed analysis

of potential vectors for horizontal transmission, such as insects or fungi that "visit" Petunia for pollination or as food resource in the original habitat of Petunia species in South America, will help to resolve the mode of horizontal transmission. On the other hand it is possible that ePVCV and its derived episomal form have lost the gene responsible for vector transmission and therefore lack the capacity for horizontal spread. Within the PVCV genome no homology to an aphid transmission factor of insect-transmissible *Cauliflower mosaic virus*, the type member of *Caulimovirus*, has been found (Richert-Pöggeler and Shepherd 1997). The scenario is even more mysterious in the case of eTVCV, which seems to be an inducible EPRV in *Nicotiana edwardsonii*, where only vertical, but no horizontal, transmission has been reported so far (Lockhart et al. 2000); even artificial transmission using grafting was unsuccessful. Despite the isolation of distinct EPRVs with homology to TVCV from several *Nicotiana* spp., Datura and tomato (Jakowitsch et al. 1999; Harper et al. 2002; Staginnus and Richert-Pöggeler 2006), no such integrants have been found in the Petunia genome.

16.6 Control and Activation of Retroelements

For Petunia, with a relatively small plant genome (Leitch, Soltis, Soltis, and Bennett 2005), it is likely to be essential that self-amplifying genomic elements such as LTR-retrotransposons and ePVCV are controlled in order to prevent genomic overload. Indirectly these mobile elements become inactivated over time due to mutations accumulated during replication that relies on error-prone reverse transcriptase. Such generated sequence diversity, reminiscent of the quasi-species nature of viruses (Casacuberta et al. 1997), can, on the other hand, offer high adaptive capacity, as demonstrated for regulatory sequences of *Tnt1* elements in the Solanaceae (Beguiristain, Grandbastien, Puigdomenech, and Casacuberta 2001; Grandbastien et al. 2005; Manetti et al. 2007): it has been shown that the host directly takes part in controlling the copy number of these elements by DNA removal *via* homologous and illegitimate recombination (Kalendar, Tanskanen, Immonen, Nevo, and Schulman 2000; Vitte and Bennetzen 2006).

Additionally, the plant uses epigenetic gene silencing as an active defense against retroelements as a means of maintaining its genome size and organization (Feschotte, Jiang, and Wessler 2002; Staginnus and Richert-Pöggeler 2006). For EPRVs in Petunia and tomato it could be shown that epigenetic control includes both transcriptional gene silencing (TGS) and posttranscriptional gene silencing (PTGS) pathways, and is therefore active in the nuclear and cytoplasmic compartments, respectively (Noreen, Akbergenov, Hohn, and Richert-Pöggeler 2007; Staginnus et al. 2007). Nevertheless, active forms of LTR-retrotransposons and EPRVs exist in solanaceous genomes and have been studied intensively in *Nicotiana* spp. (*Pseudoviridae*) and Petunia (ePVCV). Stress activation has been shown for both elements (Hirochika 1993; Grandbastien et al. 1997; Takeda et al. 2001; Richert-Pöggeler et al. 2003; Noreen et al. 2007). Expression of the two active

LTR-retrotransposons in tobacco, *Tnt1 A* and *Tto1*, is induced by biotic and abiotic factors that can elicit plant defense responses (Grandbastien 1998). This activation is transcriptionally regulated and responsive promoter elements have been isolated (Grandbastien 1998). For ePVCV it has been proposed that a resetting or loosening of epigenetic control occurring in dedifferentiated cells of callus tissue leads to its release (Richert-Pöggeler et al. 2003; Noreen et al. 2007).

16.7 Outlook

Petunia represents a young taxon thought to have diverged about 25 million years ago from other clades and showing ongoing speciation (Kulcheski et al. 2006). Despite high morphological diversification, low genetic differentiation exists and retroelement genome sizes are quite uniform (Mishiba et al. 2000; Kulcheski et al. 2006). LTR-retrotransposons in Petunia taxa are not species specific; we also know that PVCV, as well as EPRVs of the progenitor species, are integrated into the *Petunia hybrida* genome, providing evidence that the current taxa split after retroelement integration, but were probably differentially amplified thereafter. Genome sequencing of Petunia will help us to understand the complete composition of retroelements in the genome and allow us to determine in more detail their interactions, times of invasion and bursts of amplification.

Furthermore, the diversity of retroelements in larger genomes is expected to be even greater, and in order to understand plant/host specific impacts of these diverse retroelements we need several relevant genomes to be sequenced. It also has yet to be determined if sequences other than that of Petuvirus-like EPRV are present in the Petunia genome, indicating that other genera of *Caulimoviridae* have been able to replicate and integrate in Petunia. In this context it will also be resolved whether there is a competition among retroelements for "genomic niches", resulting in the presence of only a few distinct elements, or coexistence, leading to a higher diversity of elements.

The described stress-related activity of some retroelements in tobacco and Petunia will be of special interest in relation to plant breeding programs and plant regeneration systems using tissue culture. Both retroelement groups discussed here contain regulatory regions in their sequences and can influence gene expression at their sites of insertion. RNA-mediated gene silencing has a major impact on retroelement control. For viruses several suppressors of gene silencing have been described. Studies on ePVCV also indicate the existence of an EPRV-encoded suppressor (Noreen et al. 2007). However, for retrotransposons the involvement of such an antagonistic player in gene silencing has not been investigated yet. In *Nicotiana* spp. it has been pointed out that high copy numbers of EPRVs (>500) may contribute to virus resistance (Mette et al. 2002). Petunia on the other hand, harboring only 100–200 ePVCV copies, can still be infected by a homologous virus.

The actions of the described retroelements can differ among cells and cell types and therefore may be multiple and complex in multicellular organisms such as

plants. The field for research on the interactions of these mobile elements with their hosts is wide open, for we are only beginning to imagine the extent of interference. The impact of retroelements may be far beyond structural effects, influencing physiological and metabolic processes as well.

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Chapter 17
Identification and Exploitation of Petunia Transposable Elements: A Brief History

Tom Gerats

Abstract Although it is generally accepted that transposable genetic elements are ubiquitous, their full mutagenic capacity has been exploited in only a few species. Among plants these are, most notably, maize, Antirrhinum and Petunia. Representatives of all three major groups of class II elements, viz., HAT-, CACTA- and Mutator-like elements, have been identified in Petunia. Here we describe the Petunia two-element *Act1-dTph1* system and the development of its application in forward and reverse genetics studies.

17.1 History

The first specific descriptions of variegated flower-color phenotypes in Petunia, indicative of transposable element-induced mutations, appeared in reports from Malinowski and Sachs (1916) and Dale (1941), although one can find occasional remarks on unstable phenotypes throughout the older literature. There is some evidence that transposons were activated as the result of interspecific hybridization, which was historically the main source of varieties and cultivars in Petunia. If so, the transposable elements of Petunia can be cited as an example of Barbara McClintock's postulated "genomic shock" (McClintock 1983) as the trigger for transposon activation. This in turn suggests a natural role for transposons as a kind of ultimate barrier against unwanted interspecific hybridizations: transposon activity can be expected to produce mainly negative mutations and thus enable selection against hybrids.

Among the classic mutable lines in Petunia are an unstable dwarf line, in which reversions in the different tunica layers of the flower lead to changes in flower size, most notably in petals (Bianchi, de Boer, and Pompe 1974), and a set of lines bearing

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radiation-induced genic instabilities for the flower-color genes *An2* and *Rt* (Cornu 1977). In the mutant collection at the University of Amsterdam unstable alleles were also maintained for the color genes *An3*, *An6* and *Ph4*.

In the late 1960s, it was claimed that white-flowering Petunia plants could be transformed to give rise to sectored red-flowering plants by administering DNA from red-flowering plants to their white-flowering counterparts (Hess 1973 and references therein; see also Chapter 19). When Bianchi tried to repeat these experiments, he obtained sectored red-flowering plants from the acceptor line even in the absence of any exogenous DNA. This discovery intrigued him so much that he started to analyze a comparable case, in which the red-colored line "Roter Vogel" ("Red Bird") spontaneously gave rise to some progeny that were white-flowering but with revertant red spots and sectors at a low frequency (less than one per flower). The subsequent basic description of the reversion behavior of *an1*-unstable alleles in derivative lines like W17 and W28 can be regarded as the true start of Petunia "transposonology" (Bianchi, Cornelissen, Gerats, and Hogervorst 1978), the report appearing just ahead of the Big Bang of plant molecular biology.

17.1.1 Genetic Definition of the **Act-dTph1** *System*

In this pioneering period classical genetic experiments were performed to elucidate the basic features of the a*n1* unstable system. Many sub-lines that exhibited genetic changes in reversion frequencies could be recovered, as proved by the faithful transmission of the novel reversion frequency to subsequent generations. Indeed lines with more than 10,000 revertant spots per flower were recovered (Bianchi et al. 1978; Doodeman, Boersma, Koomen, and Bianchi 1984a). When the influence of environmental conditions on reversion frequency was analyzed, it was found that when plants were shifted from 18 to 25°C the original unstable *an1* allele reverted less frequently. Surprisingly, a newly selected unstable allele derived from a revertant of the original unstable allele exhibited the opposite response: a clear increase in reversion frequency under the higher temperature. In both cases, sporogenic reversion frequencies remained unchanged, at a level roughly one hundredfold higher than the somatic reversion frequency of the original allele (Doodeman, Bino, Uytewaal, and Bianchi 1984b). This last result contrasted with the otherwise surprisingly parallel *Antirrhinum* work as described, for example, by Harrison and Fincham (1964).

Based on the analysis of the positions and sizes of revertant sectors, ranging from a single cell to complete flowers, it was concluded that reversion can occur at any time during the lifecycle of the plant and that it is linked to cell division, specifically to DNA replication: temperature-shift experiments showed that in the final 5– 6 days before maturity, reversion patterns could not be modulated. A corollary of this work was the demonstration that no more cell divisions take place during this period in flower development, and flower-bud opening is thus primarily, if not exclusively, a matter of differential cell elongation. Moreover, based upon exactly

coinciding abaxial and adaxial revertant sectors at the corolla's distal end, it was concluded that two rings of meristematic cells independently give rise to the lower and upper epidermis of the corolla and that these rings frequently flip to exchange their positions (Martin and Gerats 1993).

While it was remarked that the occurrence of somatic and sporogenic reversions were reminiscent of McClintock's transposable elements, it was also stated that "...the postulation of transposition of the controlling element is unnecessary", with the addition that "...[this] does not permit the conclusion that in Petunia no transpositions take place" (Bianchi et al. 1978). At that time, genomes were regarded to be extremely stable, and transposition was generally considered a deviant process, no more than an exceptional peculiarity, occurring in just a few systems.

The stability of fully colored sporogenic revertants was tested by crossing a number of them to a stable white *an1* mutant. Among the 40,000 *An1/an1* heterozygous red-flowering progeny plants analyzed, nine white-flowering mutant plants were recovered, two of which exhibited a new reversion pattern. One of the latter has been maintained as W138, a line upon which most subsequent work has been based (Bianchi et al. 1978).

In the 1980s two major points were established: in 15–20% of the progeny derived from self-fertilization of derivative lines, notably W138, new mutant phenotypes were encountered at high frequencies (Doodeman et al. 1984c; Gerats, Beld, Huits, and Prescott 1989), and the unstable system behaved like a two-element system, similar to McClintock's *Ac-Ds* and McClintock's/Petersons' *En/Spm* systems (Gerats, Wallroth, de Vlaming, and Bianchi 1985; Wijsman 1986). Some of the earliest transposon-induced mutants recovered were *yg3* (*yellow green3*), *px* (*phoenix*, giving rise to new flowers that emerge from senescing flowers) and, depicted in Fig. 17.1, *alf* (*aberrant leaf and flower*, the Petunia counterpart of *leafy/floricaula*, see Souer et al. 1998). The high incidence of new mutations finally led to the recognition that transposons were on the move.

Crosses between line W138 and a divergent set of varieties and species revealed that the ability of *an1* unstable alleles to revert (leading mostly to red spots on a white background) depended on the presence of a single Mendelian factor, named *Activator* (*Act1*), which appeared to occupy a fixed position in the genome. Mapping experiments invariably located the *Act1* element on chromosome I (Huits, Wijsman, Koes, and Gerats 1995). Although an active *Act1* element was never identified in any of the accessions of pure species tested, it is, with one exception, ubiquitously present in modern cultivars. This finding argues for the presence of the system in an inactive state in at least one of the species that gave rise to the wide array of current cultivars, an argument that may be interpreted as evidence for McClintock's genomic shock theory. Indeed, it was eventually shown that a collection of *P. axillaris* accessions from Uruguay harbor a variable number of transposon-related sequences (Stuurman, personal communication, and unpublished results). Hence by 1985 it was clear that endogenous transposable elements, like in other species, were capable of inducing mutations at a high frequency and in a broad range of genes in Petunia, at least in *Petunia hybrida*.

Fig. 17.1 Growth habit of the *alf* (*aberrant leaf and flower*) mutant, one of the first transposon mutants derived from line W138. Note the revertant flower at *upper left*

17.1.2 Isolation of the dTph1 *Element and Its Basic Characteristics*

Subsequently, it took some years to obtain a molecular entry point into the system. The molecular identification of *dTph1* (*defective Transposable element P. hybrida 1*), which was isolated from the *dihydroflavonol reductase C* (*DFR-C*) gene, was published in 1990. It was argued that *DFR-C* should logically coincide with *An1*, as the presence/absence of the element was reported to correlate with the phenotypes underlying the different *An1* variants (Gerats et al. 1990).

This claim could not be substantiated and has caused confusion, as *An1* later was identified as a transcription factor of the basic helix-loop-helix (bHLH) type (Spelt, Quattrocchio, Mol, and Koes 2000). Whatever the source of the confusion, the *dTph1* element appeared unmistakably responsible for most of the phenotypes that have been defined and analyzed subsequently.

The various elements that have been described for Petunia are listed in Table 17.1. The list includes representatives of five diverse families of elements, including *copia*, Mutator, *Ps*1, *Ac-Ds*, and *En/Spm*. Without question, it is the *Ac-Ds*-like family of elements that has been exploited most successfully for both forward and reverse genetics investigations in Petunia. For that reason the *dTph* family and its applications are the focus of the rest of this chapter.

The initially isolated *dTph1* element was 284 bp in length. Based on sequence composition, specifically the terminal inverted repeats, internal conserved motifs and the typical eight bp target-site duplication (TSD), the *Act1-dTph1* system is assigned to the *Ac-Ds*, or, more broadly, the HAT, family of elements. The *Act1*

Table 17.1 Chronological overview of the identification and application of Petunia transposable elements

Aspect	Gene	Element	Ref
Transposon trapping	*DfrC*	***dTph1*** (*Ds*-like)	a
Gene cloning; functional analysis	*Rt*	*dTph1* and ***dTph3***	b
Classical 3D screening	10 genes	*dTph1*	c
Inverse PCR screening	*An3*	*dTph1*	d
Gene cloning; functional analysis	*NAM*	*dTph1*	e
Transposon trapping	*Nia*	*dTph1*-3 ; ***dTph4***	f
Gene cloning; functional analysis	*An11*	*dTph1*	g
Gene cloning; functional analysis	*Alf*	*dTph1*	h
Transposon trapping	*Hf1*	***Ps1*** (*En*/*Spm*-like)	i
Gene cloning; functional analysis	*An9*	*dTph1* and *dTph4*	j
Transposon Display (TD)	*Fbp1*	*dTph1*	k
Massive gene cloning	12 genes	*dTph1*, ***dTph2***, *dTph3*	l
Gene cloning; functional analysis	*An2*	*dTph1*, ***dTph5***, ***dTph6*** (*Ps1*-like)	m
Gene cloning; functional analysis	*Cytb5*	*dTph1*	n
Epigenetic interactions	*An3*	*dTph1*	o
Gene cloning; functional analysis	*An1*	*dTph1*	p
Gene cloning; functional analysis	*FAD2*	*dTph1*	q
Gene cloning; functional analysis	*Ap2*	*dTph1*	r
Gene cloning; functional analysis	*NEC1*	*dTph1*	s
Gene cloning; functional analysis	*FLZ*	*dTph1*	t
Gene cloning ; new elements; functional analysis	*An1*	*dTph1*, *dTph5* *dTph6* (*Ps1*-like)	u
Gene cloning; functional analysis	*ham*, *ter*	*dTph1*	v
Advanced 3D screening; new element; gene cloning	20 MADS-box genes	*dTph1*, ***dTph8***	w
Gene cloning; functional analysis	*UNS*	*dTph1*	x
Gene cloning; functional analysis	*PhSUP1*	*dTph1*	y
New elements	*Hf1*	***rTph1*** (*copia*-like) ***dTph9*** (*Mu*-like)	z
New element	*Rt*	***dTph3**-C* (active)	aa
Unusual transposon	*DAD1*	*dot*	bb
Additional Activator	*An1*	***Act2***	cc
Gene cloning; functional analysis	*Stig1*	*dTph1*	dd
Gene cloning; new elements; functional analysis	*Ph2*, *Ph3*, *ph4*	*dTph1, dTph6*, ***dTph7***	ee
Gene cloning; functional analysis	*Tm6*	*dTph1*	ff
Gene cloning; functional analysis	*PMT1*	*dTph1*	gg
Proviral activation by *dTph1*	PVCV		hh
Gene cloning; functional analysis	*Blind*	*dTph1*	ii
454 pyrosequencing/TD	8 *NAM/NAC*	*dTph1*	jj

*Boldface indicates first report of element; [a]Gerats et al. 1990; [b]Kroon et al. 1994; [c]Koes et al. 1995; [d]Souer et al. 1995; [e]Souer et al. 1996; [f]Renckens et al. 1996; [g]de Vetten et al. 1997; [h]Souer et al. 1998; [i]Snowden and Napoli 1998; [j]Alfenito et al. 1998; [k]Van den Broek et al. 1998; [l]Van Houwelingen et al. 1998; [m]Quattrocchio et al. 1999; [n]de Vetten et al. 1999; [o]van Houwelingen et al. 1999; [p]Spelt et al. 2000; [q]Verwoert et al. 2000; [r]Maes et al. 2001; [s]Ge et al. 2001; [t]Tobeña Santa-Maria et al. 2002; [u]Spelt et al. 2002; [v]Stuurman et al. 2002; [w]Vandenbussche 2003; [x]Ferrario et al. 2004; [y]Nakagawa et al. 2004; [z]Matsubara et al. 2005; [aa]Nakajima et al. 2005; [bb]Snowden et al. 2005; [cc]Stuurman and Kuhlemeier 2005; [dd]Verhoeven et al. 2005; [ee]Quatrocchio et al. 2006; [ff]Rijpkema et al. 2006; [gg]Garrido et al. 2006; [hh]Noreen et al. 2007; [ii]Cartolano et al. 2007; [jj]Vandenbussche et al. 2008.

"controlling" element remains to be identified at the molecular level. While in cultivar crosses *Act1* appears to be a unique activator, Stuurman and Kuhlemeier (2005) described the action of a second activator-like element, identified from a *P. inflata* introgression in W138. De Keukeleire, De Schepper, Gielis, and Gerats (2004) have proposed a PCR approach to identify autonomous HAT elements in a range of species to help clarify the origin and diversity of *Act* elements.

In all examples identified to date, *dTph* integration sites contain the eight bp TSD that is standard for HAT elements. No sequence preference for integration has been reported. A broad variation in TSD-derived footprints left behind upon excision, however, has been reported in the studies that looked into this aspect (Wessler 1988). Not only is there variation within the footprint, excision can remove the whole TSD or add additional nucleotides, and even part of the genomic environment can be excised, or left-over bits of an element may be retained. This can lead to the selection of large allelic series of mutants, especially in the case of an easy-to-score visible phenotype like that associated with the *An1* gene (Spelt el al. 2002).

17.2 Reverse and Forward Insertional Mutagenesis

A reverse genetics method for efficient detection of *dTph1* and related elements in specific genes was published in 1995 (Koes et al. 1995), based on analogous work in *Drosophila* (Ballinger and Benzer 1989; Kaiser and Goodwin 1990). Initially the method employed PCR reactions, combining gene-specific and *dTph1*-specific primers with sets of DNA samples which had been pooled in a 3-D matrix, such that each plant was represented in three pools, one from each dimension. These experiments for identifying insertions in genes of interest typically covered 1000 individuals in an array of $10 \times 10 \times 10$ samples harboring 100 plants each. Originally, the method involved separation of PCR products on agarose gels, which were then blotted and the filters hybridized with gene-specific probes.

If positive signals were detected, a second gel was run, part of which was Southern blotted to identify the location of the desired product, and the fragment was then isolated from the non-blotted part of the gel, re-amplified and subcloned for further analysis (Koes et al. 1995). This laborious method was applied, for example, to identify and characterize insertions in *Apetala2* genes (Maes et al. 2001). A comparable method involving inverse-PCR and differential screening was proposed by Souer and coworkers (Souer, Quattrocchio, de Vetten, Mol, and Koes 1995); like its predecessor, it was abandoned with the development of more sensitive methods.

In a bid for higher efficiency, several technical improvements were proposed, tested and eventually adopted. These included the use of labeled gene-specific primers to circumvent tedious blotting and hybridization procedures. A second critical improvement was the adoption of polyacrylamide gel electrophoresis (PAGE). PAGE provided much higher resolution of fragment sizes, while allowing for isolation of fragments of interest directly from the gel. A third significant step forward was the replacement of gene-specific with family-specific primers. Such primers

are based on a specific sequence motif conserved throughout the family of interest. This approach allows one to catch several independent insertions simultaneously and, moreover, permits identification of hitherto unknown family members (Vandenbussche et al. 2003).

17.2.1 Transposon Display

An alternative approach, which in principle allowed for the recovery of unknown genomic sequences flanking *dTph1* elements, was presented by Van den Broeck et al. (1998). This method, called transposon display (TD; see Fig. 17.2), is a variation of the AFLP technique, a highly successful source of molecular markers (Vos et al. 1995). The TD approach is based on the use of two restriction endonucleases, one a "six-cutter" that cleaves within the *dTph1* element and the other a "four-cutter" that cuts in the neighboring genomic DNA. Adaptors are ligated as in standard AFLP protocols; in contrast with normal AFLP procedure, however, the sequence of the six-cutter adaptor is not random but defined by the four *dTph1* nucleotides next to the restriction site: ACAC to amplify in one direction, AACC if one needs (or prefers) to go the other direction. The adaptor at the four-cutter end carries no selective nucleotides at this stage. In this way PCR products are enriched for sequences

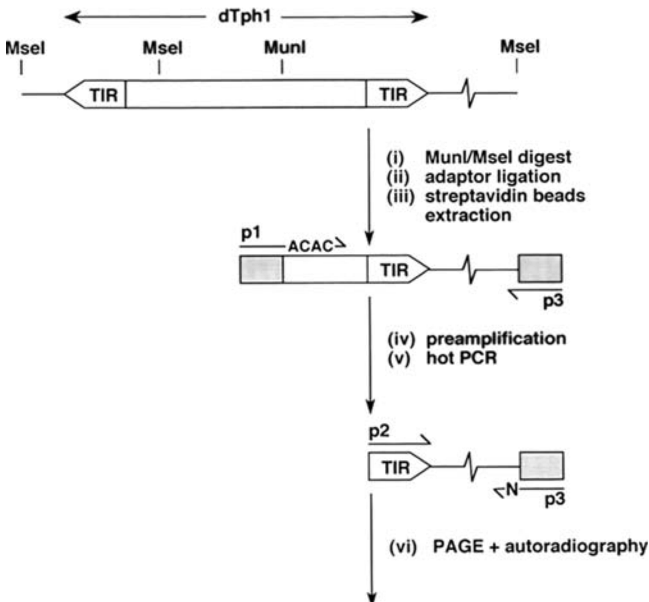

Fig. 17.2 Strategy for recovery of *dTph1* insertion-flanking sequences by means of Transposon Display (TD). Genomic fragments lying either 5′ or 3′ to the site of insertion can be recovered; the method allows for the detection of insertion events during plant development, as well as the correlation of TD fragments with phenotypes in segregating families

neighboring *dTph1*-insertion sites. In a subsequent nested PCR, a labeled primer based on the terminal inverted repeat of *dTph1* is used, in combination with the four-cutter-based primer. The latter primer can be extended by an extra nucleotide, such that if all four possible extensions are used, the amplified fragments are partitioned into four subsets.

The nested PCR approach basically ensures that all amplified fragments are derived from true *dTph1* insertion sites. On the other hand, elements that lack the necessary criteria (presence of the internal restriction site, sequence conservation at sites critical for PCR), will not be recognized or, if an outside restriction site is too close by or too far away, will not be amplified sufficiently for recovery. The technique thus is restricted to those elements that can respond to its mandatory conditions. Based on comparisons with Southern blot analyses we guesstimate that more than 90% of *dTph1* elements are responsive to this approach. While on the one hand deviant *dTph1* elements might be missed, on the other hand distinct but related elements responsive to the TD procedure can be recovered. It should be noted that for most of the cloned genes mentioned in Table 17.1 the insertion element has not been fully analyzed, and thus variants of *dTph1* might have escaped attention.

TD can also be used for forward genetics analyses: correlating a phenotypic mutant of interest with a particular TD fragment allows for isolation of the gene underlying the mutant phenotype, although the causal relationship between insertion and mutant phenotype must then be confirmed unequivocally. There are four standard approaches for such confirmation. A second, independent insertion event can be demonstrated to result in a similar mutant phenotype; silencing of the candidate gene by RNAi can be shown to lead to the expected phenotype; or, in the case of an unstable mutant, partial or total phenotypic reversion can be shown to accompany excision of the element. Homozygous revertants will specifically lack the original TD fragment. Finally, one can try to complement the mutant with a construct harboring the WT gene.

A complication is that roughly two out of three insertion mutants appear to be phenotypically stable; that is, the mutant phenotype is maintained even upon excision of the element. As the typical reversion footprint for members of the HAT family of elements is eight bp, such a stable mutant phenotype is the norm following excision of an insertion in the reading frame of the mutated gene. This can pose a serious problem in efforts to correlate a mutant phenotype with a specific TD fragment, because the mutant may or may not still carry the causative insertion, and thus a correlation between phenotype and presence of the element can no longer be made. One way to minimize this problem is to analyze all members of the family in which a new phenotype segregates, in the hope of catching the causative insertion before it excises from the mutant allele. Another strategy is to immediately stabilize the insertion by crossing out the *Act1* element (see below).

17.2.2 Transpositional Behavior of **dTph1**

In an in-depth study of the behavior of the *dTph1* family as a whole, De Keukeleire et al. (2001) followed the frequency and timing of insertion events throughout

the lifecycles of several generations of plants. A puzzling outcome was the finding that the number of *dTph1* elements increased regularly over five unselected generations; simple extrapolation argues clearly that such an increase cannot be maintained. Thus, transposition frequencies almost certainly cycle over time. Alternatively, selection for maintenance of overall plant vigor, as has been practiced over time by both nature and humans, may have been a factor minimizing the mutational damage caused by enhanced numbers of transpositional events.

One of the outcomes of De Keukeleire's work was confirmation that reversion can take place throughout the lifetime of a plant (Fig. 17.3). The work also revealed that insertions occur with a frequency of 10–20 sporogenic events per generation. About 60 TD fragments remained stable in the material over five generations. An analysis of plants of the ancestor line Roter Vogel, by then separated for approximately 50 generations, revealed the presence of most of these fragments in Roter Vogel, indicating that they represent stably positioned elements (defined as

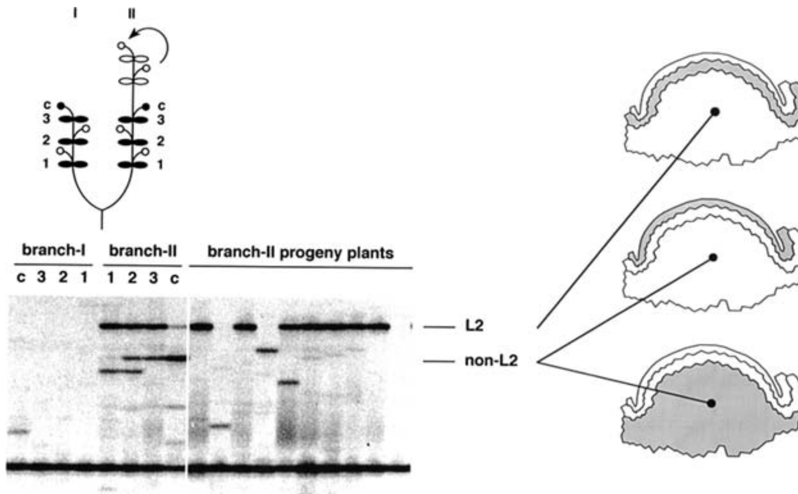

Fig. 17.3 TD reveals the apical lineage of somatic insertion events. Somatic insertion events are transmitted or not, depending on the tunica layer in which the event occurred. A schematic longitudinal section through the shoot apical meristem is presented on the right. L1 and L2 are independent cell layers, each a single cell thick; L3 forms the corpus. Sporogenic cells are produced exclusively from the L2 layer. On the left, part of a gel image representing a TD analysis is presented. The *left panel* shows products from two different branches, each represented by three leaves and a corolla. The progeny panel exhibits fragments from single-leaf samples taken from ten progeny plants obtained by self-fertilization of a flower from branch II of the parental plant. "Ancestral" fragments exhibit equal intensity in all samples and represent an element present in all cells and tissues; "transient" fragments appear and may subsequently disappear, due either to excision or to failure of the cell lineage to contribute to the formation of subsequent leaves. L2 events are characterized by a low signal ratio in corolla/leaf and by transmission to the next generation, where they appear as "ancestral"; non-L2 events are presumably L1 events (L3 does not multiply by cell lineages). They are characterized by a high signal intensity ratio in corolla/leaf comparisons and are never transmitted to the progeny (De Keukeleire et al. 2001)

fragments capable of amplification by the two-stage TD procedure). These fragments might represent degenerate elements or elements that are trapped by some other means in their sequence environments. Overall it can be concluded that individuals of line W138 harbor a minimum of 100–150 active *dTph1* elements, of which 10–20 occupy new genomic positions in the next plant generation. This indicates that a library of 1000 plants can be expected to carry 10,000–20,000 first-generation insertion events. Unpublished results indicate these to be minimum numbers.

So far about 200 insertions, mostly *dTph1* or closely related elements, have been characterized in approximately 100 genes, as summarized in Table 17.1. In most cases, the system was used in a reverse way, whereby sequence information was available and mutants were recovered by selection as a means of providing an entry to their functional analysis. Although a literature search argues that forward genetic screens are employed less often than reverse screens, they have been successfully applied as well, despite the high *dTph1* copy number. Examples are provided by the *nam* (Souer, van Houwelingen, Kloos, Mol, and Koes 1996) and *blind* (Cartolano et al. 2007) mutants.

17.3 Development of a Saturated Insertion Library

While insertion screens are thus already relatively efficient, we searched for possibilities to enhance the system further. This was realized when “454 pyrosequencing” technology came to the market (Margulies et al. 2005). Core to this method is the current capability of amplifying and sequencing 400,000 DNA fragments of about 250 bp in a single experiment. We aimed to adapt this technology, replacing “DNA fragments” with “insertion-site sequence tags obtained by Transposon Display”. As developed, the procedure applies the TD approach to pooled DNA samples. In a 1000-plant library, organized in a $10 \times 10 \times 10$ matrix, each plant is addressed individually by three coordinates. The 30 samples each consist of a pool of 100 individuals and each sample is separately amplified by TD. The identity of individual samples is safeguarded by the use of sample-specific primers that harbor a sequence identification tag of four nucleotides. This sequence tag is included in the sequence analysis of each fragment and thus leads to the identification of the sample of origin, ideally defining one of the three dimensions of an individual plant in the 3D pool. Identification of sequence tags for the remaining two dimensions then leads to identification of the plant of origin for this insertion tag; hence this plant can be checked and progeny analyzed for phenotypes (Vandenbussche et al. 2008). In the first experiment, more than 10.000 *dTph1*-flanking sequences were identified. The majority of these plant-of-origin-linked sequences have been put into a database, which can be screened upon request.

To evaluate whether this database represents a reliable mutant identification source, in silico screening for insertions into members of the *NAM/NAC* transcription factor family (Souer et al. 1996) was performed. *NAM/NAC* genes have been identified as important regulators involved in a range of aspects of plant

development (reviewed by Olsen, Ernst, Leggio, and Skriver 2005). DNA sequences of the 22 known *Petunia NAC* family members were extracted from Genbank and blasted against the Insertion-Flanking Sequence database. The search resulted in identification of nine different putative insertions into the coding sequences of eight Petunia *NAC* members. Five of these correspond to known Petunia *NAC* members, while the remaining four represent insertions into new *NAC* family members, illustrating the additional benefits of this methodology as a gene discovery source. In addition, three putative insertion events were identified with one of their coordinates missing (2D hits), while 15 putative insertions were identified as 1D sets.

Such incomplete coordinate sets may represent somatic insertion events, present in only one or two of the three samples taken from each individual; alternatively, they might represent true germinal insertion events for which amplified fragments from one or more of the coordinates may be missing for technical reasons. To check the automatic assignment of insertions to individuals and small families, the 30 DNA pools of the library were screened by the conventional gene-specific screening method (Vandenbussche et al. 2003). All in silico assigned insertion events were confirmed by PCR screening. Furthermore, presumably due to the higher sensitivity of the gene-specific screening method, the missing coordinate for all three 2D sets, and for two of the three 1D hits, could be determined. These results confirmed both the occurrence of somatic insertion events (ID hits remaining 1D), and the incompleteness of the sequence analysis (identification of missing coordinates). The latter result clearly indicated that either some templates were not amplified or, alternatively, that not all templates were represented in the sequence collection. Finally, the presence of the respective insertion events in progeny of positive individuals could be confirmed in all cases (Vandenbussche et al. 2008).

17.4 Conclusion and Outlook

As demonstrated in a number of chapters of this book as well as Table 17.1 of this chapter, methods developed for exploiting transposable elements of Petunia have proved highly useful for gene identification and isolation. Further improvements to this technology will make it feasible to construct an essentially saturated insertion library. This will be a great addition to the already wealthy set of materials and procedures presently available for functional gene analysis.

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Chapter 18
Virus-Induced Gene Silencing for Functional Characterization of Genes in Petunia

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Abstract Although functional analysis of genes can be readily carried out in Petunia using standard transformation/regeneration techniques, this process is time- and labor-consuming. High throughput analysis of gene knockouts has been made possible by the use of virus-induced gene silencing (VIGS): fragments of target plant genes are included in the genome of a viral vector, the plant silences them as part of its viral defense mechanism, and the consequences of gene inactivation can be readily analyzed. In Petunia, we use a modified tobacco rattle virus (TRV) vector for VIGS. Infection typically results in chimeric plants, and it is therefore desirable to have a reporter that can show where target genes have been silenced. Inserting a fragment of the gene encoding *PHYTOENE DESATURASE (PDS)* results in silencing-induced photobleaching of leaves; inserting a fragment of the gene encoding *CHALCONE SYNTHASE (CHS)* allows us to visualize silencing in floral tissues of purple-flowered Petunia cultivars as white patches, sectors or even entire corollas. We have shown that the VIGS system can silence as many as five independent genes at one time. We describe here the methods that we have found to be efficient and effective for VIGS in Petunia, and describe some results obtained by silencing a range of genes, including some transcription factors.

18.1 Introduction

Reverse genetics, particularly the analysis of effects of knocking out expression of a target gene, is a powerful tool for studying gene function. In Arabidopsis, T-DNA insertional mutant analysis has proved to be a useful means for genome-wide analysis of the genes identified in the Arabidopsis genome project. This approach has some limitations, such as requirements for stable transformation, difficulty in

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producing knockouts to cover every gene in the genome (genome saturation), lack of visible phenotype when the T-DNA insertion is in a gene of a multigene family or a duplicated gene, and embryo lethality associated with inserts in genes that are essential for survival. Furthermore, multiple T-DNA insertions may lead to simultaneous disruption of several genes in a single plant, which complicates downstream analysis. Alternative silencing methods such as dsRNA-mediated suppression (hairpin RNAs, antisense RNAs and co-suppression) have been developed, but this post-transcriptional gene silencing (PTGS) approach requires generation of inverted-repeat constructs and also depends on stable transformation and regeneration. Virus-induced gene silencing (VIGS) offers an attractively quick method for a loss-of-function assay, including that for proteins encoded by multigene families. VIGS can reveal the mature phenotypes of embryo-lethal disruptions of gene function and avoids the need for the time-consuming (and sometimes problematic) processes of transformation and regeneration.

18.2 Virus-Induced Gene Silencing

PTGS is one of the most adaptable and specific mechanisms for protection of the genome and elimination of foreign DNA or RNA, and culminates in the sequence-specific degradation of so-called aberrant RNA. This highly conserved process was first identified in plants by the apparently bizarre silencing of *CHS* in Petunia plants that had been engineered to overexpress this gene (Napoli, Lemieux, and Jorgensen 1990). PTGS has now been shown to function as an endogenous defense mechanism against viruses by directly targeting the replicative form of the virus (Voinnet 2001). During replication of the virus, double-stranded chimeric intermediates are produced. The plant cell recognizes these intermediates as foreign, and a specialized enzyme (Dicer) degrades the double-stranded RNA into small oligonucleotides (siRNA). The single strands of siRNA molecules, bound to an RNA-induced silencing complex (RISC) that degrades any transcripts with identical or highly similar sequences, serve as specific templates. In VIGS, this mechanism is co-opted to target endogenous host mRNAs by the simple expedient of including fragments of target host genes in the viral genome. The viral silencing mechanism does not differentiate these fragments from viral sequences, and this provides a simple means of downregulating host gene expression (Kumagai et al. 1995).

The technique has been extended to model plants such as Arabidopsis (*Arabidopsis thaliana*, Turnage, Muangsan, Peele, and Robertson 2002) and crops such as tomato (*Lycopersicon esculentum*, Fu, Zhu, Zhu, Jiang, and Luo 2005; Liu, Schiff, and Dinesh-Kumar 2002), potato (*Solanum tuberosum*, Faivre-Rampant et al. 2004), and barley (*Hordeum*, Holzberg, Brosio, Gross, and Pogue 2002; Lacomme, Hrubikova, and Hein 2003). Our laboratory has exploited VIGS for testing gene function in Petunia (Chen et al. 2004; Chen, Jiang, and Reid 2005).

There are several advantages to VIGS over gene-silencing methods involving expression of inverted-repeat constructs in transgenic plants:

1. The constructs can be easily created by directly cloning gene fragments into the viral vector without the need for generating inverted repeats.
2. The VIGS-induced phenotype can be observed in a relatively short time (in Petunia as short as 10 days after inoculation).
3. VIGS can reveal the phenotype of embryo-lethal knockouts.

18.2.1 VIGS Vectors

Many plant viruses have been used to develop VIGS vectors. Early vectors included tobacco mosaic virus (TMV, Kumagai et al. 1995; Lacomme et al. 2003), potato virus X (PVX, Angell and Baulcombe 1997; English, Mueller, and Baulcombe 1996; Lu et al. 2003a) and tomato golden mosaic virus (TGMV, Kjemtrup et al. 1998). All these viruses may cause strong infection symptoms including chlorosis, leaf distortion and necrosis. In addition, they are incapable of infecting the apical meristem and are therefore unlikely to provide information about genes involved in the identity and development of plant tissues and organs. Tobacco rattle virus (TRV)-based vectors have been suggested to have potential for use with a wide range of plant species due to the wide host range of TRV (Ratcliff, Martin-Hernendez, and Baulcombe 2001; Liu et al. 2002). These TRV vectors overcome many of the difficulties associated with PVX, TMV and TGMV. For example, the TRV vector induces mild symptoms, infects large areas of adjacent cells and silences expression of genes in the meristem. The TRV-based VIGS system has been shown to function effectively in Arabidopsis, *Nicotiana benthamiana*, tomato (Liu et al. 2002; Fu et al. 2005), lettuce (Wagstaff, Jiang, and Reid, unpublished), *Aquilegia* (Gould and Kramer 2007) and California poppy (Wege, Scholz, Gleissberg, and Becker 2007).

TRV is a two-particle positive-sense RNA rod-type virus. RNA1 encodes two replicase proteins, a movement protein, and a cysteine-rich protein. RNA2 encodes the coat protein and two non-structural proteins. Because TRV RNA1 can replicate and move systemically in the plant in the absence of RNA2, researchers reasoned that RNA2 could be modified for insertion of fragments of genes targeted for silencing. They constructed binary transformation vectors using T-DNA from *Agrobacterium* engineered to carry 35S promoters and the RNA1 and RNA2 of TRV (Ratcliff et al. 2001; Liu et al. 2002). The 35S promoters stimulate initial viral transcription, thus ensuring rapid infection once plant cells have been infected by the *Agrobacterium*. The RNA2 genes encoding non-structural proteins were replaced with a multiple cloning site (MCS) into which fragments of target genes can readily be inserted. In our studies we have used the vector described by Liu et al. (2002; Fig. 18.1).

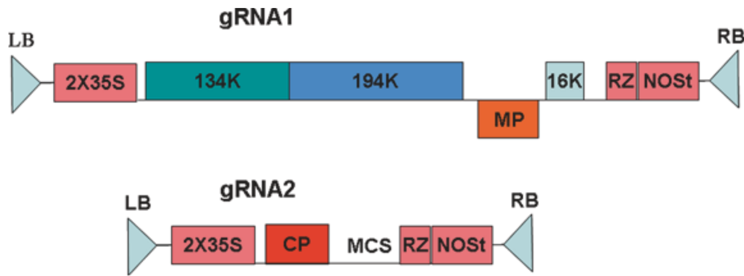

Fig. 18.1 The tobacco rattle virus VIGS vector comprises two plasmids encoding viral RNA1 and RNA2. Constitutive promoters (2X35S) and terminators (NOSt) flank coding regions, and a multiple cloning site (MCS) allows for insertion of target gene fragments into RNA2. Gene designations: 134 K and 194 K, replicases; MP, movement protein; RZ, self-cleaving ribozyme; CP, coat protein

18.3 Use of TRV with Petunia

The TRV VIGS system offers a number of particular advantages for use with Petunia. The virus infects floral tissues and produces only mild symptoms. Infection with TRV containing a fragment of the *PDS* cDNA to provide a visible reporter of gene silencing results in systemic infection and silencing of the host *PDS* as indicated by photobleaching of the leaves (Fig. 18.2). The silencing phenotype is non-uniform and varies among cultivars.

Fig. 18.2 Infection of Petunia with TRV containing a fragment of the tobacco *PDS* gene, resulting in characteristic photobleaching of leaves

18.3.1 Plant Material and Growth Conditions

In our initial studies we found that growth conditions were critical for efficient VIGS silencing in Petunia. Plants grown under the relatively changeable conditions of a standard greenhouse showed variable degrees of silencing; we found it essential to provide a controlled day/night temperature variation. Accordingly, we grow Petunia in growth chambers with a 16 h light/8 h dark cycle and a day/night temperature regime of 25°C/20°C. The need for a day/night temperature variation for successful systemic infection has also been reported for topovirus infection of *Capsicum* and *N. benthamiana* (Roggero, Dellavalle, Ciuffo, and Pennazio 1999). These authors suggested that long-distance transport of the virus may be inhibited at high temperature. Fu et al. (2006) reported that gene silencing with TRV in tomato was enhanced by low temperature and low humidity, whereas Szittya et al. (2003) found that low temperatures inhibited silencing by inhibiting siRNA generation. These apparently conflicting findings may reflect a requirement for low temperature to allow for initial viral replication and systemic movement, and higher temperatures to activate the silencing mechanism sufficiently to silence endogenous gene expression.

18.3.2 Plasmid Construction

Gene fragments for VIGS in Petunia were amplified from Petunia cDNA sources using primers designed to generate a 120–250 bp fragment. The RNA2 vector can accept inserts up to 1.5 kb, but silencing is highly efficient with fragments in the 150 bp range. Amplified products were cloned into the pGEM-T Easy vector (Promega, Madison, WI) to confirm the DNA sequence and facilitate subsequent cloning into TRV RNA2. In initial studies, we used ESTs obtained from the Petunia Floral EST database from the University of Florida, excising inserts from the plasmids and cloning them directly into the MCS of the TRV RNA2 vector. Puzzled by the low level of silencing obtained with these constructs, we eventually deduced that the polyA tail on the EST clones interfered with the silencing mechanism (Chen, Jiang and Reid, unpublished). The problem was overcome by interposing an amplification step, using primers specific to each EST.

18.3.3 Reporter Genes

Because VIGS typically results in chimeric plants with some portions uninfected and therefore unsilenced, it is essential to incorporate the silencing of a reporter gene as a tool to indicate sites of silencing. Early studies used the silencing of *PDS* to show the effect of VIGS; infected plants show characteristic photobleaching symptoms resulting from the inhibition of biosynthesis of protective carotene (Fig. 18.2). While silencing of *PDS* serves as a clear reporter, the phenotype has obvious disadvantages, as the photobleaching is concomitant with destruction of the photosynthetic apparatus.

Less detrimental reporters for silencing in green tissues would be useful. One candidate is green fluorescent protein (GFP) in plants that are constitutively expressing this transgene (Burch-Smith, Schiff, Liu, and Dinesh-Kumar 2006). Because we are primarily interested in flower senescence, we have been able to use a fragment of an endogenous petunia chalcone synthase gene (*CHSJ*; genbank accession number X14599) in the silencing construct. Silencing is seen as white spots, sectors, and flowers on infected plants (Chen et al. 2004).

18.3.4 **Agrobacterium-*Mediated Infection***

Viral infection is achieved by *Agrobacterium*-mediated infection of Petunia seedlings. The constructs, RNA1 (pTRV RNA1 construct) and RNA2 (pTRV RNA2 construct) or its derivatives, are transformed independently into *Agrobacterium tumefaciens* strain GV3101 by electroporation. The bacteria are cultured overnight at 28°C in LB or YEP medium using gentamycin (20 μg/ml) and kanamycin (40 μg/ml) for selection (transformed cells are Gent and Kan resistant). The *Agrobacterium* cells are then harvested and resuspended in inoculation buffer (10 mM $MgCl_2$, 10 mM MES, 200 μM acetosyringone) to an absorbance of 2.0 at 550 nm and left at room temperature for 3 h. The bacteria containing RNA1 and those with the modified RNA2 are then mixed together in a 1:1 ratio (Fig. 18.3).

18.3.5 Inoculation Method

Optimal efficiency is obtained when the Petunia plants are young (1–2 true leaves) and growing rapidly. The primary leaves are inoculated by injection of the mixed

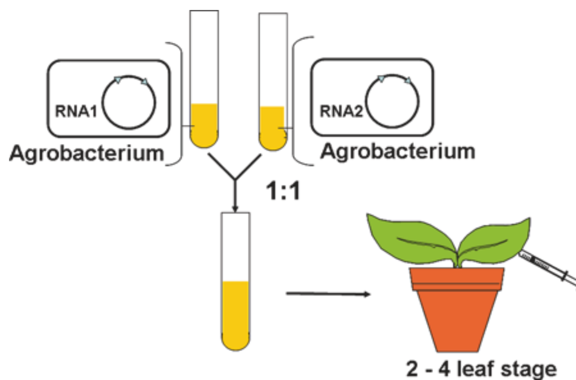

Fig. 18.3 The VIGS experimental system. Separate *Agrobacterium* cultures are transformed with the two viral vectors (RNA1 and RNA2); the RNA2 vector contains a fragment of the gene(s) to be silenced. Following growth of the transformed *Agrobacterium,* the combined cultures are used to infect primary leaves of young Petunia plants, by infiltration through the abaxial leaf surface, using a needle-less disposable syringe

bacterial culture using a 1 ml disposable syringe without a needle (Fig. 18.3). An alternative technique that we have tried with some success is vacuum infiltration of naked seedlings (ca. 1 week old). Other researchers have been successful, in other species, in inoculating older tissues, including developing fruits (Fu et al. 2005). Another technique that warrants testing in Petunia involves dripping the mixed inoculum onto the meristem then gently pinching, a technique reported to be effective with *Eschscholtzia* (Wege et al. 2007).

18.4 Evaluation of Silencing

Our primary evaluation tool is the visible symptom of *PDS*-silencing (photobleaching of the leaves) or *CHS*-silencing (white floral spots, sectors, or corollas). To confirm that silencing has been achieved we typically test transcript abundance using semi-quantitative or real-time RT-PCR (see Fig. 18.4; Chen et al. 2004). Total RNA is extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA) and treated with RNase-free DNase (Promega, Madison, WI) to remove any contaminating genomic DNA. The first strand cDNA is synthesized using the SuperScript III kit (Invitrogen, Carlsbad, CA). PCR primers for amplifying transcripts must be designed outside the region targeted for gene silencing to avoid amplification of the fragment included in the viral RNA2 construct.

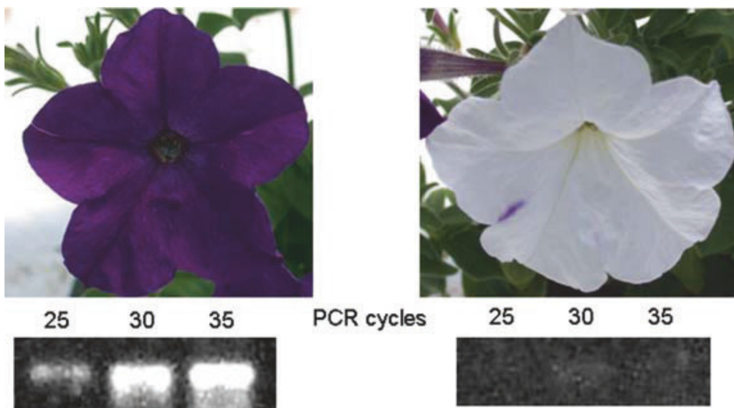

Fig. 18.4 Effect of silencing *CHS* on flower color and *CHS* transcript abundance in Petunia. Plants were infected with TRV containing a fragment of the Petunia *CHS* gene. Petals were harvested and transcript abundance was visualized by RT-PCR amplification of *CHS* in total RNA using primers binding to a region outside the silencing fragment

18.5 Cultivar Variations

We initially used the homozygous model cultivar V26 in our studies of silencing floral genes, but under our experimental conditions the silencing phenotype was

Fig. 18.5 Cultivar variation in the silencing response. Plants of the V26 and *Ultra Blue* cultivars of Petunia were infected with TRV containing a fragment of the Petunia *CHS* gene. Silencing in V26 was limited and diffuse. In *Ultra Blue*, silencing produced clear white sectors and even completely white corollas

limited to diffuse small white spots on the corollas (Fig. 18.5) and did not lend itself to biochemical or physiological characterization.

We therefore tested the effects of silencing *CHS* on a range of purple-flowered commercial cultivars (Goldsmith Seeds, USA), and found significant variations in the silencing phenotype. In some, clear silenced sectors and whole silenced corollas were common; others showed polka-dotted and/or diffuse silencing patterns similar to that seen in V26 (Fig. 18.5). The genetic basis for these differences is unknown; possible explanations include differences in movement of either the virus or the silencing signal between cells. This phenomenon is not restricted to Petunia. In studies with silencing *PDS* in tomato, we have also seen cultivar-dependent variation in the silencing phenotype. Whatever the cause, it is obviously important for investigators to choose a cultivar that responds optimally to VIGS.

18.6 Silencing Multiple Genes

Infection of purple-flowered cultivars with TRV containing fragments of both *PDS* and *CHS* resulted in leaf photobleaching and white patterns on the flowers. The abundance of both transcripts was reduced, confirming simultaneous silencing of both genes at the same location (Chen et al. 2004). These results demonstrated the feasibility of using *CHS* as a reporter for the silencing of genes of unknown function. Lu, Martin-Hernandez, Peart, Malcuit, and Baulcombe (2003b) suggested that homologous inserts as short as 23 nucleotides are sufficient to induce gene silencing, although the silencing is less extensive and more transient than with inserts carrying longer homologous sequences. This implies that having inserts with short regions of very high identity with the target gene is more important than

Fig. 18.6 Effects of including multiple gene fragments in the silencing vector. Petunia plants were infected with TRV bearing fragments of *CHS* and *PDS*. Both genes are silenced (as evidenced by the photobleached leaves and the white corolla), and the silencing is coordinated: branches with photobleached leaves have white flowers

having a larger fragment with moderate overall similarity. The TRV RNA2 vector can accept inserted gene sequences up to ca. 1.5 kb in length, which theoretically permits generation of multiple siRNAs from one vector. Using fragments from multiple members of a Petunia multigene family, we were able to show that as many as five genes (including *CHS*) could be silenced simultaneously (Fig. 18.6). The theoretical limit for the number of genes that could be silenced is ca. 10 (150 bp per fragment; 1.5 kb in total length).

An advantage of the VIGS system is that careful selection of the silencing fragment permits simultaneous silencing of homologous genes. For reducing levels of ACC oxidase (ACO, acting at the terminal step in ethylene biosynthesis), we used a fragment from *ACO4* (accession number L21979), and showed that the silenced (white) flowers produced much less ethylene and lasted longer. In Petunia, ACO is encoded by a multigene family, and we showed that the reduced ethylene production of the silenced sectors was correlated with a reduction not only in the abundance of *ACO4* transcripts (to 5% of the controls), but also in *ACO1* transcripts (to 23% of the controls).

18.7 Testing the Effects of Silencing Petunia Floral Genes

The efficacy of tandem constructs was initially tested by observing the effects of infection with TRV *CHS*/*ACO4*. In addition to the reduced abundance of transcripts encoding ACO, and reduced ethylene production in white sectors of infected flowers, these sectors senesced later than uninfected purple sectors (Fig. 18.7).

Fig. 18.7 Use of *CHS* as a reporter gene. Petunia plants were infected with TRV bearing fragments of *CHS* and ACC oxidase (*ACO4*) genes. Flowers showing silencing symptoms were excised, placed in deionized water, and then pollinated to induce rapid ethylene-mediated senescence. The photographs taken after 1 (*left*) and 5 (*right*) days demonstrate that senescence was earlier in the non-silenced (purple) sectors of the flower

18.7.1 Prohibitins

Prohibitin, a highly conserved mitochondrial protein, has been found in all eukaryotes. Its functions in yeast and animals have been well characterized (Nijtmans et al. 2000; Artal-Sanz et al. 2003), and there are indications that it is important for maintenance of cellular vitality. For example, downregulating prohibitin abundance in yeast reduces the replicative lifespan. Mitochondria are thought to act as biological clocks that define animal age, and declining mitochondrial function is a common feature in animal aging (Balaban, Nemoto, and Finkel 2005). Like their counterparts in other eukaryotes, plant prohibitins are mitochondrial (Snedden and Fromm 1997; Takahashi et al. 2003), but attempts to demonstrate their function using conventional reverse genetics have failed due to embryo lethality in transformed plants with either down- or upregulated prohibitin (Takahashi et al. 2003). Using VIGS with a fragment of a Petunia prohibitin gene (accession number CV294646) we were able to provide the first evidence for the function of plant prohibitins (Chen, Jiang, and Reid 2005).

Silencing of prohibitins in Petunia resulted in early petal senescence, and leaves and flowers of infected plants were smaller and distorted (Fig. 18.8). Cells in silenced sectors of corollas were larger (Fig. 18.9), implying a substantial reduction in cell number. Respiration of silenced flowers was higher than that of controls, and there was a marked increase in levels of transcripts for a catalase and a small heat-shock protein. In addition, prohibitin transcripts declined during natural petal senescence. These results indicate that prohibitins play a role in petal senescence and suggest that there is a conserved mechanism in animal and plant aging. Whether mitochondrial function declines during petal wilting is still unknown, but it may point to a new direction in studies of plant senescence.

Fig. 18.8 Effect of silencing prohibitin on vegetative and floral phenotypes. Petunia plants were infected with TRV containing *CHS* and *PROHIBITIN1* gene fragments. Infection resulted in distortion and reduction in size of leaves and corollas

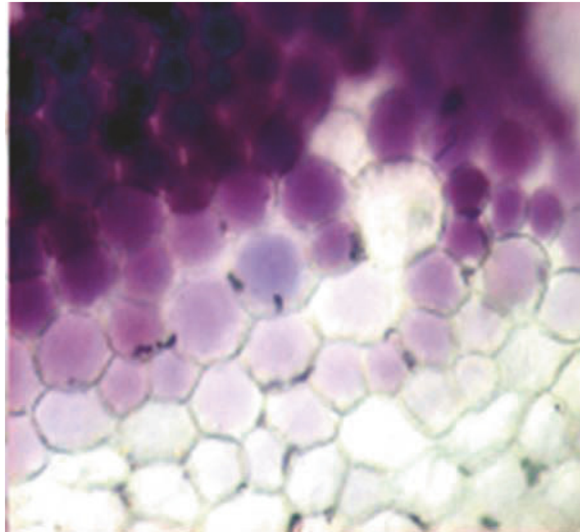

Fig. 18.9 Effect of silencing prohibitin on size of cells in the petal epidermis. An epidermal peel was removed from a of a mosaic flower on a plant infected with TRV containing *CHS* and *PROHIBITIN1* gene fragments. Mounted in water, the peel was photographed using a Zeiss compound microscope. Note the clear difference in cell size between unsilenced (*purple*) and silenced (*white*) sectors

18.7.2 MADS-Box Genes

Genes containing the highly conserved MADS-box DNA-binding motif are important determinants of floral organ identity (Coen and Meyerowitz 1991; see Chapters 10 and 11). A tomato ripening mutant with extended shelf-life has been demonstrated to be due to a mutation in a MADS-box gene, *rin* (Vrebalov et al. 2002).

The recovery of a MADS-box gene from a differential screen for senescence-associated genes in daylily (Lange, Valpuesta, Napoli, and Reid 1996) suggested

Fig. 18.10 Effect of silencing the Petunia *PhDEF*(*MADS1*) gene on floral morphology. Plants were infected with TRV-bearing fragments of *CHS* and *PhDEF*. Floral morphology was strongly affected, depending on the stage of the meristem when viral infection occurred

the possibility that MADS-box genes might play a role in floral senescence as well. Silencing of *MADS1*, the "green petal" MADS-box gene now designated *PhDEF*, had the anticipated effects on floral development: infection early in floral development led to a substantial modification of floral organs (Fig. 18.10; van der Krol, Brunelle, Tsuchimoto, and Chua 1993). Later infection resulted in more nearly normal flowers, some with unsilenced sectors. Comparison of the lifespans of pollinated purple and white flowers from plants infected with TRV *CHS/PhDEF* revealed a substantial difference in longevity, suggesting that, as in the ripening of tomatoes, MADS-box genes may play a role in the onset of floral senescence.

18.8 Future Prospects

Our experience, and the data that we have obtained, demonstrate the value of VIGS based on TRV vectors as a tool for reverse genetic analysis in Petunia. In our laboratory, infection routinely results in a very high frequency of silencing symptoms. We are using this tool to examine the effects of silencing a wide range of transcription factors on floral and other phenotypes. Among the technical issues that remain to be settled is the maximum number of independent transcripts that can be silenced using a single vector. With more transcript fragments per vector, the throughput of the VIGS technique increases, opening the possibility of genome-wide functional analysis.

Another interesting avenue worth exploring is the use of VIGS to silence all members of a multigene family, thereby generating information on the function of genes whose redundancy makes functional analysis challenging using other reverse genetic approaches. Petunia has proved to be an excellent model plant for the application of VIGS, and the technique is increasingly being used in a range of other ornamental, model, and crop species. The wide host range of TRV suggests that the technique will be applicable in many taxa, and we are presently exploring this potential.

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Chapter 19
Transformation and Regeneration of Petunia

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Abstract Petunia has played a central role in transformation research since the earliest reports of plant transformation. It was a key model system when the first definitive accounts of *Agrobacterium*-mediated transformation and direct DNA transfer unequivocally established the transfer and expression of foreign genes in plants. Petunia subsequently played an important role in elucidating many of the characteristics of plant transformation, including unique sites of insertion, variable expression levels, and modified T-DNA structures among independently derived transformants. It was central in the demonstration of transient expression immediately following co-cultivation and transgene-induced silencing of gene expression, two phenomena currently of great importance in studies of gene function. One of the key reasons for the importance of Petunia in plant transformation research has been the selection for and/or identification of genotypes well suited to growth and regeneration in culture, for example, Petunia Mitchell, for which a simple transformation protocol is described. Agro-infiltration for transient gene expression and the development of intragenic vectors to effect gene transfer without the integration of "foreign" DNA represent recent advancements in Petunia transformation. Ease of transformation, coupled with other favorable biological characteristics, ensure that Petunia will remain a valuable model system for studies of gene function in plants.

19.1 Introduction

Plant transformation provides a valuable experimental approach that is now almost routine in efforts to understand the functions of genes and their products in plants. Gene transfer provides a convenient tool to add alleles or genes, as well as to silence endogenous genes through antisense or RNAi strategies. In this manner it underpins

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investigations associated with functional genomics by allowing many hypotheses involving gene function to be tested.

Petunia has been at the forefront of many of the key developments in plant transformation. In addition to being an important model system for establishing approaches for plant transformation, Petunia made a substantial contribution to the understanding of the key outcomes of plant transformation. Research based on Petunia transformation has provided the foundation both for a number of highly significant contributions in plant biology and in efforts to develop new cultivars for the ornamental horticulture industry (see Chapter 20). This chapter summarizes the historical role played by Petunia in developing methods for plant transformation and elucidating characteristics of the process. In addition it discusses the importance of genetic selection for tissue culture performance, provides a protocol for Petunia transformation, and summarizes recent developments in Petunia transformation.

19.2 Petunia and the Development of Plant Transformation

19.2.1 Early Attempts at Plant Transformation

The first claims of genetic transformation in higher plants followed the treatment of Petunia plants with exogenous DNA (Hess 1969a, b, 1970, 1972, 1973). When seedlings of a white-flowered plant were treated with DNA (M.W. $\approx$2–88$\times 10^5$) from a red-flowered Petunia, the recovery of up to 2730 "transformed plants" was reported (Hess 1969a, b). Although genetic analysis of these putatively transformed plants established that most were heterozygous, some were, surprisingly, homozygous for anthocyanin-inducing alleles (Hess 1970, 1972). Criticisms of these experiments have been raised on the basis that somatic mutations occurring in the embryonic cell layers of Petunia could account for the appearance of pigmented flowers at the reported frequency (Bianchi and Walet-Foederer 1974). The appearance of faint red coloration in homozygous white-flowered Petunia treated with its own DNA (Hess 1969a, b) suggested environmental factors may also have played a role in the appearance of color in at least some of the putatively transformed plants. In the developmental stage of seedlings treated with exogenous DNA, the shoot apices have three embryonic cell layers, and it has been argued that transformed flowers produced on the plants treated with DNA would have been expected to appear as mosaics rather than being uniformly colored (Bianchi and Walet-Foederer 1974). In subsequent experiments the uptake of labeled bacterial DNA (Hess, Lörz, and Weisert 1974a), proteins and entire bacteriophage (Hess, Gresshoff, Fielitz, and Gleiss 1974b) by germinating Petunia pollen were reported.

The molecular tools commonly used to confirm transformation events, such as nucleic acid hybridization and PCR, were not available at the time of the early work. Analysis of putative transformants was therefore limited to phenotypic assessment, classical genetic analysis and in some cases biochemical assays, providing ready grounds for criticism of experimental results and their interpretation (Bianchi and

Walet-Foederer 1974). Nevertheless, Hess (1977) rightly maintained that there were certain features of his experiments for which transformation provided an explanation as plausible as any other. Indeed, more recent experiments involving the direct uptake of naked DNA by plant tissues, followed by the molecular verification of subsequent integration and expression in plant genomes (reviewed in Petolino 2002), provides some credibility to these early reports.

19.2.2 **Agrobacterium-*Mediated Transformation***

The plant transformation field rapidly gained momentum with the development of vectors for gene transfer to plants. A major breakthrough came with successful exploitation of the natural gene transfer ability of *Agrobacterium tumefaciens,* and in 1983 the transfer and expression of foreign genes in plant cells was definitively established (Bevan, Flavell, and Chilton 1983; Fraley et al. 1983; Herrera-Estrella et al. 1983). In all three studies *Agrobacterium*-based transformation was achieved by placing the coding region of the bacterial neomycin-phosphotransferase gene under regulatory control of the promoter of the nopaline synthase gene carried by the T-DNA of *A. tumefaciens*. Following co-cultivation with the genetically modified bacteria, successful transfer and expression of the *nptII* gene allowed for phosphorylation and detoxification of kanamycin, and transformed plant cells could thus be identified by their acquisition of kanamycin resistance. Collective evidence for plant transformation included the phenotypic display of kanamycin resistance, Southern-blot demonstration of integration of the foreign gene into the genome of plant cells, northern analysis demonstrating accumulation of RNA transcripts of the correct size, and enzyme assays that verified the activity of neomycin-phosphotransferase in the plant cells. Petunia was one of the key species in these three “break-though” studies (Fraley et al. 1983), based on transformation by co-cultivation of *A. tumefaciens* with leaf protoplasts.

In a subsequent landmark paper, Horsch et al. (1985) reported a simple approach for plant transformation based on the regeneration of transformed shoots obtained from leaf explants. Discs were punched from the leaves of Petunia, tobacco and tomato, then dipped into a suspension of a disarmed *A. tumefaciens* strain harboring a co-integrate vector with both a selectable marker gene (kanamycin resistance) and a reporter gene (nopaline synthase). Selection was applied 2 days after co-cultivation, and transformed plants were regenerated. In this study Petunia was the species on which most of the data were based, with detailed molecular analysis confirming the transgenic status of recovered plants. Variant forms of this simple approach for plant transformation, originally applied to Petunia, have since been adapted to develop transformation systems in a vast array of plant species.

Petunia also played an important role in establishing the general applicability of variant approaches to *Agrobacterium*-mediated transformation. Transgenic plants were obtained among the progeny of plants derived from Petunia shoot apices co-cultivated with *Agrobacterium* (Gould, Ulian, and Smith 1993; Ulian, Magill,

and Smith 1994). Pollination of Petunia flowers with pollen previously infiltrated with *Agrobacterium* was reported to result in a high frequency of transgenic seedlings among the progeny (Tjokrokusumo, Heinrich, Wylie, Potter, and McComb 2000). These authors also generated transgenic Petunia, although at much lower frequencies, by applying *Agrobacterium* to the stigma just prior to pollination (Tjokrokusumo et al. 2000).

19.2.3 Direct Gene Transfer

Direct gene transfer involves the integration of DNA into the genome following the uptake of naked DNA by plant cells. When this approach to plant transformation was established it was generally believed that protoplasts, with their "naked" plasma membrane, were necessary for direct DNA uptake. The first reported successes used Petunia as a model system for uptake, integration, and expression of isolated Ti plasmid DNA (Davey, Cocking, Freeman, Pearce, and Tudor 1980; Draper, Davey, Freeman, Cocking, and Cox 1982). Following definitive experiments in tobacco for the recovery of transgenic plants by direct gene transfer (Paszkowski et al. 1984), this approach to plant transformation was quickly adapted to a wide range of species, including Petunia (Potrykus, Shillito, Saul, and Paszkowski 1985).

Currently direct gene transfer can be effected by a wide range of approaches, including passive uptake, electroporation, treatment with polyethylene glycol, electrophoresis, cell fusion with liposomes or spheroplasts, microinjection, silicon carbide whiskers, and particle bombardment (see Petolino 2002). The target cells have been extended from isolated protoplasts to isolated cells, cultured tissues, organs, intact pollen, seeds, and whole plants (Petolino 2002). Although these variant approaches were first demonstrated with other species, Petunia played an important role in establishing the applicability of a number of them, including microinjection (Griesbach 1987), fusion with liposomes (Ballas, Zakai, Sela, and Loyter 1988) and particle bombardment (Buising and Benbow 1994; Clark and Sims 1994).

19.3 Role of Petunia in Elucidating Transformation Characteristics

19.3.1 Sites of Insertion into Plant Genomes

Southern analysis of plants derived from early transformation experiments revealed differences in restriction fragment banding patterns among independently derived transgenic plants. This variability suggested that each integration event occurred at a unique chromosomal site. Genetic mapping of inserted T-DNAs in Petunia definitively established that foreign DNA could be incorporated into any of the chromosomes, each transformation event characterized by a unique site of insertion (Wallroth, Gerats, Rogers, Fraley, and Horsch 1986). For these experiments, a

Petunia F1 hybrid (Violet 23 x Red 51), heterozygous for at least one phenotypic marker on each of the seven chromosomes, was subjected to *Agrobacterium*-mediated transformation with a kanamycin resistance gene plus a nopaline synthase gene. Backcrossing of recovered transgenic plants to the parental lines allowed for linkage of inserted T-DNAs to be assigned to the uniquely marked chromosome pairs. In this way, insertion events in nine independently derived transgenic lines were assigned to four chromosomes (I, III, IV and V). Foreign DNA was shown to be capable of insertion into chromosomes derived from either the ovule or pollen parent, with multiple insertions in the same transgenic line attributable either to multiple insertions in one chromosome or to insertions in multiple chromosomes (Wallroth et al. 1986).

19.3.2 Expression, Inheritance, and Structure of Insertion Events

Transgenic Petunia plants also played a lead role in the demonstration of variability in the expression levels, inheritance patterns and T-DNA structures of foreign DNA in independently derived transgenic plants (Jones, Gilbert, Grady, and Jorgensen 1987; Jorgenson, Snyder, and Jones 1987; Deroles and Gardner 1988a, b). Using a population of more than 100 independently derived transgenic Petunia lines Deroles and Gardner (1988a) demonstrated that expression and inheritance of kanamycin resistance varied markedly among transformation events.

Only about half of the lines exhibited normal Mendelian ratios of inheritance for one or two loci, while many others showed aberrant segregation patterns, usually with fewer than the expected number of transgenic progeny. Southern analysis of these transgenic lines established that only about half contained intact copies of the inserted T-DNAs (Deroles and Gardner 1988b). The most common rearrangements in the remaining lines involved simple deletions with one or both ends of the T-DNA missing, with some lines exhibiting gross T-DNA rearrangements. Anomalous segregation patterns among progeny of transgenic Petunia plants were often associated with high copy number and/or rearrangements of insertion events (Jones et al. 1987; Jorgenson et al. 1987; Deroles and Gardner 1988a, b), a feature later recognized as being common among transformants.

19.3.3 *Transient Expression Following* Agrobacterium *Transformation*

Transgene expression in plant tissue shortly after co-cultivation with *Agrobacterium* was first observed for the gene *nopaline synthase* in Petunia and was hypothesized to result from transient expression of non-integrated copies of T-DNA (Horsch and Klee 1986). This was subsequently established in an unequivocal manner, also using Petunia as an experimental system (Janssen and Gardner 1989). The construction of a *β-glucuronidase* (*GUS*) reporter gene capable of expression in plant but not

bacterial cells provided a straightforward means of monitoring transgene expression immediately following gene transfer by *Agrobacterium*. Localized regions of leaf explants exhibited transgene expression within 2 days of co-cultivation, a peak at 3–4 days, then a decline. Applying selection for transformation resulted in increased *GUS* expression again after 10–14 days, due to growth of the relatively rare cells with integrated transgenes. GUS activity assays of leaf tissue shortly after co-cultivation provided evidence that gene expression was taking place in all cells of the localized regions. This was demonstrated to arise from transient expression of non-integrated T-DNAs in the cytoplasm, presumably in a double-stranded conformation (Janssen and Gardner 1989). Transient expression assays have since become a valuable tool for rapid assessment of gene expression, underpinning identification of genes through functional genomics (see Sect. 19.6.1).

19.3.4 Transgene Silencing

Transgene-induced silencing, which can occur as a result of antisense expression, transcriptional silencing, or post-transcriptional silencing, has played a key role in the understanding and manipulation of gene expression in plants. Many of the early observations of transgene-induced silencing involved the use of Petunia as a model system. One of the first efforts to deliberately silence an endogenous gene in plants involved the expression of an antisense Petunia *CHALCONE SYNTHASE* gene in order to inhibit flower pigmentation in Petunia and tobacco (van der Krol et al. 1988).

Transcriptional gene silencing was first observed in Petunia, in plants transformed with a maize *DIHYDROFLAVONOL-4-REDUCTASE* (*DFR*) gene under control of the 35S promoter (Meyer, Heidmann, Forkmann, and Saedler 1987). DFR enzymatic activity resulted in the biosynthesis of pelargonidin, producing novel brick-red flower coloration. Occasionally, however, when the transgene was within or adjacent to a hypermethylated locus, the gene was silenced due to promoter methylation. The phenomenon occurred more frequently in plants subjected to environmental stress (Meyer et al. 1992; Meyer and Heidmann 1994). It was also demonstrated that an active copy of the transgene can be methylated and silenced when combined by crossing with an unlinked silenced homologous transgene (Meyer, Heidmann, and Niedenhof 1993).

Post-transcriptional gene silencing was seen in Petunia in experiments intended to overexpress *CHS* and *DFR* genes as a means of enhancing floral pigmentation (Napoli, Lemieux, and Jorgensen 1990; van der Krol, Mur, Beld, Mol, and Stuitje 1990). Unexpectedly, a block in anthocyanin biosynthesis was observed in a high proportion of the transgenic lines; the block was shown to be associated with a substantial reduction in expression of both the endogenous gene and the homologous transgene. This post-transcriptional gene silencing, also referred to as co-suppression, was subsequently demonstrated to result from the failure not of transcription but of RNA accumulation; the phenomenon has been associated with the production of aberrant RNAs (Vaucheret et al. 1998).

19.4 Genetics of Performance in Tissue Culture

Genetic variation for performance in cell and tissue culture is well known for most crop species. Petunia is no exception: some genotypes exhibit poor tissue culture performance while others demonstrate a high aptitude for growth and development in tissue culture (e.g., Izhar and Power 1977; Mitchell, Hanson, Skvirsky, and Ausubel 1980; Raquin 1982). Different genotypes may exhibit different requirements for optimal levels of growth regulators in culture (Skvirsky, Hanson, and Ausubel 1984). Following hybridization of Petunia genotypes differing in tissue culture performance, the analysis of progeny allowed the genetic variation to be attributed to dominance effects (Mitchell et al. 1980; Raquin 1982; Skvirsky et al. 1984). Screening of a backcross population of hybrids provided evidence that the capacity for callus growth is controlled by a few dominant genes (Izhar and Power 1977).

Consistent with this simple dominance model for inheritance of performance in tissue culture, attempts at breeding for improved regeneration from tissue culture have proved highly successful in Petunia, even after selection within one or two generations (Mitchell et al. 1980; Raquin 1982). Following a study on the inheritance of response to anther culture in Petunia, an individual haploid plant with high regeneration potential was derived from a plant (A $\times$ AC-1) originally selected among the progeny of a *P. axillaris* x (*P. axillaris* x *P. hybrida* "Rose du Ciel") backcross (Mitchell et al. 1980). This regenerated haploid line, presumably selected as a rare individual from among a large population of male gametophytes segregating for amenability to tissue culture regeneration, was designated the "Mitchell" line and, although initially clonally propagated in vitro as a haploid, doubling of the chromosomes restored fertility and allowed for seed propagation as a homozygous diploid line (Ausubel, Bahnsen, Hanson, Mitchell, and Smith 1980). Petunia Mitchell was subsequently used in many of the pioneering experiments in plant transformation (Fraley et al. 1983; Horsch et al. 1985; Deroles and Gardner 1988a, b; Janssen and Gardner 1989). Due to ease of transformation, as well as vigorous growth habit, it remains a popular choice as a model plant system, although accessions of pure *P. axillaris* are performing equally well in transformation.

19.5 Protocol for Transformation of Petunia Mitchell

Many cultivars of Petunia are routinely transformed and used extensively in plant genetic research. The most common method for producing transgenic Petunia is via *Agrobacterium*-mediated transformation, with selectable markers genes including a range of antibiotic and herbicide resistance genes (Deroles, Bradley, Davies, and Schwinn 1996; Deroles, Boase, Lee, and Peters 2002). Although exact procedures employed by various laboratories vary depending upon the Petunia genotype and research history of the lab, we routinely develop transgenic Petunia plants using *Agrobacterium*-mediated transformation based on the methods of Horsch et al. (1985).

Young leaves from small (3- to 6-week-old) glasshouse-grown plants are used as the explant source. These are surface-sterilized by immersion with gentle shaking for 10 min in 10% commercial bleach (1.5% sodium hypochlorite) containing a few drops of 1% Tween 20, followed by several washes with sterile water. Leaf discs of 6 mm diameter are cut with a paper punch and immediately immersed for about 2 min in an early log-phase culture of the disarmed *Agrobacterium* strain LBA4404 (Hoekema, Hirsh, Hooykaas, and Schilperoort 1983) carrying the appropriate binary vector. The *Agrobacterium* culture is grown at 28°C with vigorous shaking in YN broth (3 g/l Difco Bacto beef extract, 5 g/l Difco Bacto Peptone, 10 g/l Difco Bacto yeast extract, 8 g/l NaCl, pH 7.3) containing an appropriate bacterial selection agent for the binary vector and 20 μM acetosyringone. When the absorbance of the culture at 550 nm reaches about 0.6 (equivalent to early log-phase growth for most *Agrobacterium* strains) the culture is used for co-cultivation.

After co-cultivation the leaf discs are blotted with sterile filter paper and placed onto PS medium, consisting of: MS salts (Mursashige and Skoog 1962), B5 vitamins (Gamborg, Miller, and Ojima 1968), 3% sucrose, 3 mg/l BAP, 0.2 mg/l IAA and 0.7% agar at pH 5.8. After 2 days they are transferred to PS medium containing 500 mg/l cefotaxime, which prevents *Agrobacterium* overgrowth, and a selective agent to allow the preferential growth and regeneration of transformed cells carrying the binary vector T-DNA. The preferred selective agent is kanamycin, for which 300 mg/l is optimal for selection with Petunia Mitchell (Fig. 19.1).

After 4 weeks, the regenerating shoots are harvested, cut into nodal segments and placed onto fresh PS medium with cefotaxime and kanamycin, in order to generate up to ten clonal copies of each putatively transformed shoot. This step also provides an additional selection step to eliminate non-transformed "escapes". After 4 more weeks, the cloned shoots are dipped into a sterile solution of 100 mg/l IAA and transferred to PR medium (MS salts, B5 vitamins, 3% sucrose) containing 500 mg/L

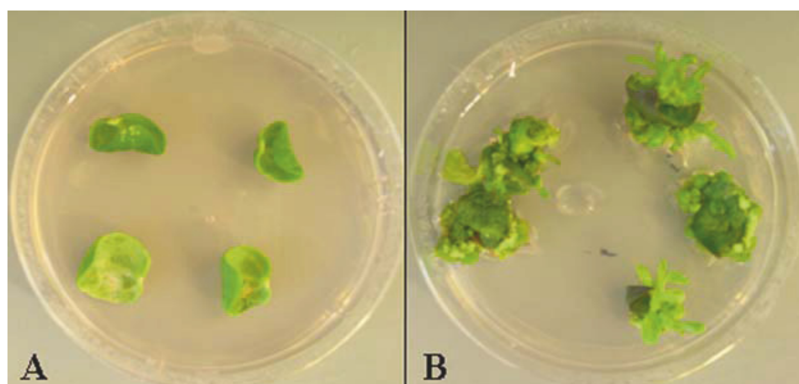

Fig. 19.1 Leaf discs from Petunia Mitchell under kanamycin selection (300 mg/l) (Albert 2006). (**A**) non-transformed leaf discs. (**B**) leaf discs transformed with *A. tumefaciens* carrying a binary vector with a kanamycin resistance gene

cefotaxime and 100 mg/l kanamycin). Shoots initiating roots in the presence of kanamycin are designated putative transgenics and transferred to the glasshouse.

19.6 Recent Developments in Petunia Transformation

19.6.1 Agro-infiltration for Transient Expression

Transient expression refers to the rapid transcription and translation of constructs upon introduction into a cell. The phenomenon as observed shortly after *Agrobacterium* co-cultivation is described in Sect. 19.3.3. Transient expression offers a valuable research tool for rapid evaluation of gene constructs, bypassing the time and effort needed to develop transgenic plant lines, yet allowing for evaluation of the consequences of targeted changes in gene expression *in planta*, within a limited region of either healthy or compromised plant tissue. These assays can be simply conducted on localized regions of intact plants, providing a convenient system to investigate functional activities of genes. The method is especially useful in analysis of genes that may result in semi-lethal or lethal responses in plant cells when overexpressed, silenced, or mutated.

Agro-infiltration is achieved by “injecting” an *Agrobacterium* suspension into plant tissue. Both petals and leaves are easy organs to infiltrate in Petunia. The method was well illustrated by infiltration of *Agrobacterium* containing binary vectors, using either an intron-containing *GUS* gene or a *GFP* gene under the control of a 35S promoter, into detached Petunia flowers (Shang et al. 2007). Strong transient expression was observed in petals from flowers of any size, ranging from 15 mm-long buds to fully opened flowers. Similar success is possible with infiltration of leaves. In this case, a syringe lacking a needle is placed against the underside of a leaf (abaxial surface) and, with fingers providing support from the adaxial side of the leaf, pressure is gently applied to the syringe. Infiltration of the liquid *Agrobacterium* suspension into the leaf tissue can be readily observed and, if necessary, the zone of *Agrobacterium* exposure can be recorded directly on the leaf surface with a universal pen in order to facilitate subsequent analysis of gene expression. The plants are then returned to normal growing conditions for 2–7 days, after which gene expression is analyzed (Fig. 19.2).

19.6.2 Intragenic Vectors

Petunia not only provides a valuable model system for transformation-based research, it also offers opportunities for the development of new cultivars for the ornamental horticulture industry (see Chapter 20). The release of transgenic crops for commercial use has raised many ethical, religious and other concerns, especially among the general public, politicians and bureaucrats (Conner, Glare, and Nap 2003; Nap, Metz, Escaler, and Conner 2003). A major underlying concern is the transfer

Fig. 19.2 Enhanced expression of anthocyanin biosynthesis 3 days following agro-infiltration of intact leaves. Petunia Mitchell plants were infiltrated with *A. tumefaciens* harboring a binary vector with an anthocyanin regulatory gene under control of the 35S promoter

of genes across very wide taxonomic boundaries. The potential exists, however, to address many of the concerns by achieving gene transfer without the presence of "foreign" DNA.

Recent progress in the design of vectors for plant transformation has resulted in the development of the intragenic vector system (Conner et al. 2007). This system involves identification of functional equivalents of vector components in plant genomes and the use of these DNA sequences to assemble transformation vectors that can then effect gene transfer without the introduction of foreign DNA. A binary vector for *Agrobacterium* transformation with the T-DNA region completely constructed from three fragments of Petunia DNA has been created (Fig. 19.3) and shown to be effective in Petunia transformation (Conner et al. 2007). Particularly for the transfer and manipulation of Petunia genes within the *Petunia* genus, such intragenic vectors should help to alleviate some of the public concern over the deployment of transgenic plants in the ornamental horticulture industry. Once again, while Petunia has allowed proof of principle, the approach is adaptable to a wide variety of ornamental and crop plants.

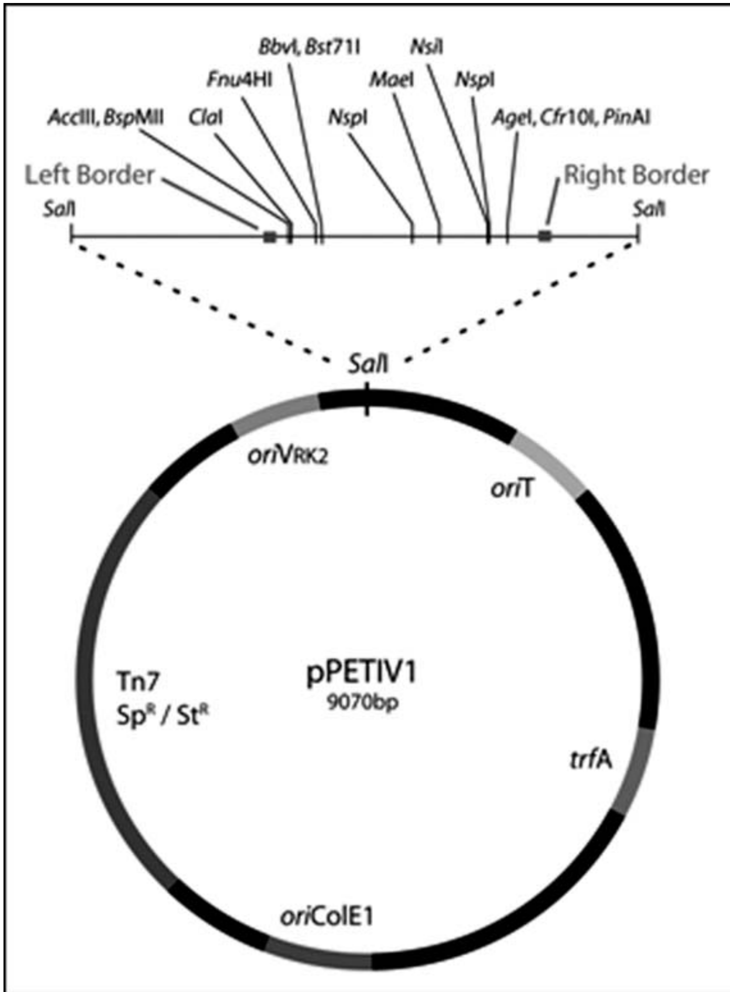

Fig. 19.3 An intragenic binary vector, pPETIV1, with a 1066 bp Petunia-derived T-DNA region inserted as a *Sal*I fragment into the 8004 bp backbone of pART27 (Gleave 1992). The Petunia-derived T-DNA region is composed of the complete sequence of the 394 nucleotide fragment from SGN-E521144, followed by the reverse complement of nucleotides 85–540 from SGN-E534315, and the reverse complement of nucleotides 121–336 from SGN-U207691

19.7 Conclusion

Ease of transformation, coupled with other favorable biological characteristics, has made Petunia a valuable model system for plant science research. Petunia transformation has provided an important model system for establishing the technologies associated with plant transformation and contributing to our understanding of

the outcomes of gene transfer, integration and expression. Consequently, transgenic Petunia plants have made numerous important contributions to our understanding of the functional genomics underpinning plant traits. Some key functional genomics studies have included those examining the regulation of the flavonoid biosynthetic pathway (Oud, Schneiders, Kool, and van Grinsven 1995; Bradley, Davies, Deroles, Bloor, and Lewis 1998; Tanaka, Tsuda, and Kusumi 1998), male sterility (Ylstra et al. 1994), flower development (Angenent, Franken, Busscher, Weiss, and van Tunen 1994), plant form (Winefield, Lewis, Arathoon, and Deroles 1999), alkaloid production (Thomas, Akroush, and Adamus 1999), ethylene perception (Gubrium, Clevenger, Clark, Barrett, and Nell 2000), self-incompatibility (Dowd et al. 2000), and fungal resistance (Esposito et al. 2002). With the current rapid rate of accumulation of "omics" data on Petunia and related Solanaceae, Petunia transformation remains an invaluable tool in efforts to understand fundamental biological processes.

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Chapter 20
Petunia Biotechnology

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Abstract Over the past two decades Petunia has served as an excellent model system for uncovering the molecular, biochemical and physiological bases of several plant processes. The experimental tools available in Petunia have allowed researchers to examine genetically controlled changes in plant morphological characteristics at both the cellular and whole-plant levels. Many of the basic experiments conducted with Petunia in recent years have led to the development of new biotechnologies that are being tested for potential commercial utility. Although most commercial advancements in Petunia in the near future will almost certainly come from the hands of conventional breeders, Petunia as a biotechnology model system has provided a proof-of-concept platform through which new technological advancements for horticultural crops have been well tested. While the worldwide commercial Petunia market is significant, the greatest benefits of Petunia biotechnology have most certainly been for the larger plant science community. As we proceed into the future it will undoubtedly continue to serve both roles.

20.1 Introduction

The biotechnology age was conceived on April 2, 1953, with the publication of Watson and Crick's model for the DNA double helix (Watson and Crick 1953a, b). In the field of plant science, modern biotechnology bore fruit 30 years later, shortly before publication of the first Petunia monograph, with reports of the first transgenic plants. Significant credit is due the groups of Mary Dell Chilton at Washington University (Framond, Bevan, Barton, Flavell, and Chilton 1983) and Jeff Schell at the University of Ghent (Schell et al. 1983), and also to the group at Monsanto, who stably transformed Petunia Mitchell ("Mitchell Diploid") in the very early days of

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Table 20.1 Why Petunia is a good model system for scientific research

Ease of genetic transformation
Hundreds of seeds from a single pollination
Relatively short life cycle
Complete flowers
Strong, well characterized floral aroma
Large floral organs which are easy to handle and cross pollinate
Progenitors of *Petunia hybrida* (*P. axillaris x P. integrifolia*) known and available
Existence of self-incompatibility systems
Large amounts of plant tissue
Available mutant collections
Efficient insertion mutagenesis
Existence of plants with a range of habits and genetic diversity
Economic importance as an ornamental crop
Abundant genomic resources for related solanaceous species
Continuous development of new scientific resources including microarrays, cDNA collections, and transposon mutants
Highly collaborative, growing multi-national group of researchers working on Petunia (www.petuniaplatform.net)

plant transformation (Fraley, Rogers, and Horsch 1983a; Fraley et al. 1983b). Using *Agrobacterium tumefaciens* Ti plasmids engineered with chimeric genes containing the neomycin phosphotransferase coding region driven by 5′ and 3′ regulatory regions from the nopaline synthase gene, the first dominant selectable markers were generated for transgenic plants. These transformation vectors were subsequently used as a backbone on which insect- and herbicide-resistance transgenic traits were built and commercialized in food and fiber crops in the USA over the last decade. Since the publications by Fraley et al. (1983a, b), many plant species have been genetically transformed and Petunia has remained important as a model system for proof-of-concept research and the development of new biotechnologies (Table 20.1). This review provides insight into the importance of Petunia as a biotechnology model system from a historical perspective. It also helps establish its future importance as scientists proceed in a post-genome world where analysis of gene function is garnering much attention.

20.1.1 Manipulation of Gene Expression in Transgenic Petunia

Petunia was the model system for early foundational gene-silencing work, based on co-suppression and antisense technology, that has proved key to the biotechnological modification of flower color, phytohormone synthesis and perception, and modification of floral scent. During the 1980s, antisense RNA was discovered to provide a successful means for silencing gene expression in many different organisms including plants (Ecker and Davis 1986). Following the discovery that antisense silencing occurs in the plant kingdom, van der Krol and colleagues stably transformed and regenerated Petunias expressing the *CHALCONE SYN-*

THASE (*CHS*) gene in antisense orientation (1988). *CHS* encodes an enzyme required for anthocyanin pigmentation in flower petals and is an easily scored, visual marker. Antisense-*CHS* plants had reduced *CHS* mRNA levels as well as reduced levels of CHS protein and anthocyanins, demonstrating that antisense technology could be used for genetic manipulation of plant traits.

Overexpression of chalcone synthase in transgenic Petunia led to the surprising discovery of co-suppression or sense suppression, a phenomenon wherein transcripts from sense-orientated transgenes and native endogenous genes are suppressed. Sense-*CHS* plants exhibited highly variable pigmentation patterns and lacked petal anthocyanins in discrete sectors of the petals (Fig. 20.1; Napoli,

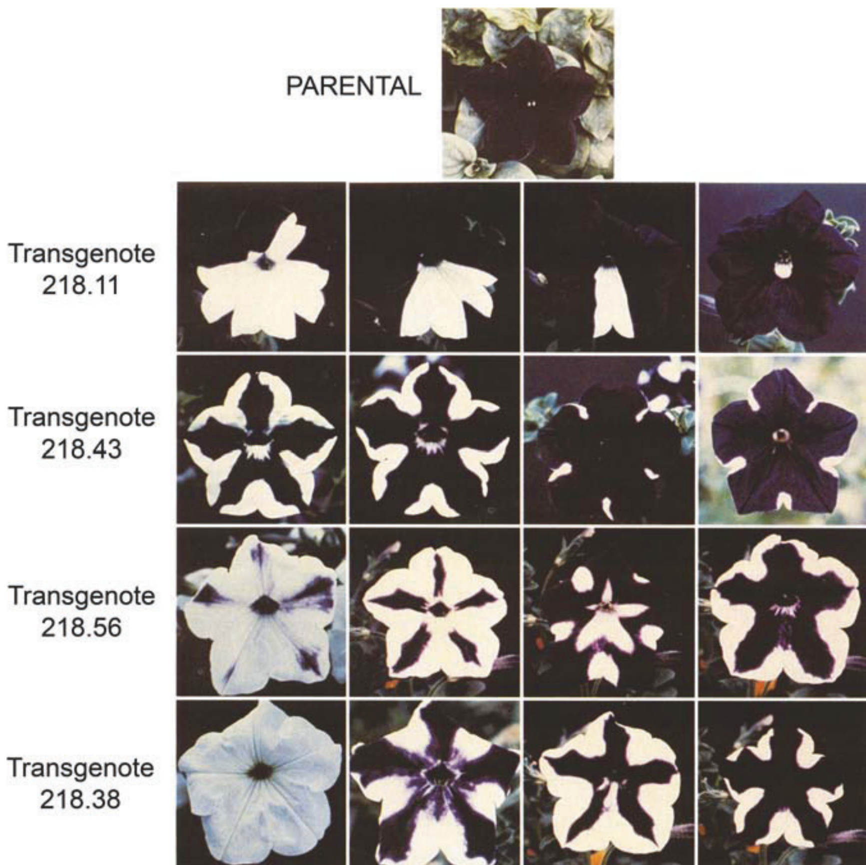

Fig. 20.1 Phenotypes of *CHS* co-suppressed Petunia flowers. Control parental flower ('V26') is shown in *top picture* and representative flowers from four transgenic lines are shown in the *rows below*. Modified from Napoli et al. 1990. Reprinted with permission, ©American Society of Plant Biologists

Lemieux, and Jorgensen 1990; van der Krol, Mur, Beld, Mol, and Stuitje 1990), similar to plants expressing *CHS* in the antisense orientation. While the co-suppression approach is clearly useful as a tool for reducing expression of specific genes, obtaining high levels of expression of Petunia genes can thus be problematic, as overexpression can also lead to silencing. Although there are documented examples of successful overexpression in transgenic plants (e.g., Kater et al. 1998; Nakagawa et al. 2005), we have observed repeated examples of co-suppression in attempts to overexpress Petunia genes using the figwort mosaic virus 34S and cauliflower mosaic virus 35S promoters (D. Clark, personal observation). One solution to this problem is to use genes from heterologous plants for expression in Petunia, as demonstrated by Wilkinson and colleagues (1997), who used the mutant Arabidopsis *ETHYLENE RECEPTOR* gene *ETR1-1* for constitutive expression in Petunia. However, this is not an absolute solution since novel genes introduced into the genome are also subject to silencing depending on insertion copy number and environmental factors (Linn et al. 1990; Meyer et al. 1992).

The co-suppression phenomenon and the unpredictable and variable expression of transgenes underscore the importance of generating and analyzing multiple transgenic lines. Peer-reviewed publications that discuss the numbers of transgenic plants generated indicate the need for analysis of many transgenic lines to find only a few lines with the desired expression pattern (e.g., Nakagawa et al. 2005). Factors contributing to expression variability include differences in transgene copy number, position of transgene integration into the genome, and environmental factors (Finnegan and McElroy 1994).

20.1.2 Petunia as a Model for the Study of Gene Silencing

After the discovery of transgene-mediated co-suppression in Petunia and tobacco, gene silencing was found to be a widespread phenomenon in plants, protists, fungi, and animals. Elucidation of the silencing mechanisms was critical to understanding a near-universal genome regulatory phenomenon essential for genome stability, development, and adaptive responses. This knowledge of silencing mechanisms has also allowed for improved plant transformation approaches.

Petunia flavonoid biosynthesis continues to be a favored model for studying gene silencing for a number of reasons: the visual flower phenotype is easily scored, it is essentially cell-autonomous, and pigmentation is a dispensable process (Jorgensen et al. 1996). Experiments from the 1990s clearly showed that transgene structure and copy number affect patterns of sense-mediated co-suppression. Jorgensen and coworkers (1996) produced hundreds of transgenic sense and antisense-*CHS* plants and correlated their pigmentation patterns with organization of the transgene DNA (tDNA). Single-copy sense transgenic plants produced white sectored patterns known as the junction pattern. In contrast, sense transgenic plants with multiple direct and indirect tDNA repeats produced complex pigmentation patterns lacking discrete wedges of color, similar to the complex patterns seen in

antisense-*CHS* transgenic plants. Fully pigmented flowers were more commonly observed in transgenic lines with multiple tDNA copies, indicating inter-transgene paramutation-like interactions wherein the transgenes silenced themselves but not the endogenous *CHS*.

There are two forms of homology-dependent gene silencing (HDGS) observed in transgenic plants: transcriptional gene silencing (TGS) and post-transcriptional gene silencing (PTGS). TGS reduces transcription as a result of promoter methylation and chromatin modification; it is meiotically heritable, and is not graft transmissable (Meyer et al. 1993; Park et al. 1996; Mourrain et al. 2007). In contrast, genes undergoing PTGS are transcriptionally active, but steady-state levels of transcripts are reduced, siRNAs (small interfering RNAs) accumulate, and the silencing is graft transmissible (Van Blokland et al. 1994; Hamilton and Baulcombe 1999). These findings have led to the development of RNAi (RNA interference) constructs for reducing expression of endogenous genes. Waterhouse and colleagues (1998) made RNAi plant transformation constructs that simultaneously expressed sense and antisense RNA for targeted gene silencing, an approach that has since proved to silence endogenous genes at a higher frequency than sense transgenes alone (Beclin et al. 2002).

Due to HDGS, introduction of multiple transgenes through cross-breeding of transgenic plants or sequential transformations can lead to transgene inactivation (Matzke et al. 1989). Transgene methylation and loss of expression was observed when plants with related tDNAs were brought together, with reactivation occurring only after segregation of the two tDNAs. Interestingly, the tDNAs were not identical, with as little as 90 bp of promoter homology sufficient to cause trans-inactivation (reviewed in Finnegan and McElroy 1994). These concepts will be important to keep in mind particularly when introducing multi-gene traits into plants.

20.1.3 Proof-of-Concept Transgenic Plants

Petunia has been a successful test system for new technologies, both to demonstrate commercial utility for novel Petunias as floriculture crops and to serve as a model for technology application in other plant species. Traits such as novel flower color and patterning, longer-lasting flowers, stay-green leaves, larger flowers, and dwarf habit are some of the traits that have been realized through genetic engineering of Petunia.

20.2 Flower Color

One of the principal areas of ornamental plant research focuses on the modification of flower color. Flower color is of significance because it is a primary visual element of pollinator attraction and it provides aesthetic appeal for the commercial market. Three different types of compounds give rise to the colorful floral display

in the angiosperms: carotenoids, betalains, and anthocyanins. Carotenoids are of major significance in all plant species for their essential roles in photoprotection and in the light-harvesting complex. They are the major contributor to flower color in marigolds (Moehs, Tian, Osteryoung, and DellaPenna 2001; reviewed in Grotewold 2006), but their contribution to flower coloration has yet to be demonstrated in Petunia. Betalains are nitrogen-containing, water-soluble compounds synthesized from tyrosine, and are restricted to the order Caryophyllales (reviewed in Grotewold 2006). Anthocyanins are a major contributor to flower color in a broad distribution of flowering plants and the chief contributor to color in Petunia corollas. They are water-soluble compounds that give flowers red, orange, purple, and blue color; these anthocyanin pigments will be the focus of the remainder of this section.

Anthocyanins encompass a group of hundreds of distinct compounds derived from a branch of the flavonoid pathway. They are composed of three cyclic rings decorated with hydroxyl and methyl groups at different positions on the B-ring of the compound. The first step of flavonoid synthesis is catalyzed by the enzyme chalcone synthase (CHS) and involves the condensation of *p*-coumaroyl CoA with three molecules of malonyl-CoA. This reaction provides the substrate for many types of flavonoids, including the anthocyanins, which branch off into different pathways after chalcone is converted to a flavonone by chalcone isomerase (CHI). Dihydroflavonones are formed from hydroxylation of flavonones by flavonone-3′-hydroxylase (F3′H) and flavonone-3′5′-hydroxylase (F3′5′H). Dihydroflavonol 4-reductase (DFR) catalyzes the formation of the leucoanthocyanidins from dihydroflavonols (reviewed in Grotewold 2006). There are further steps for anthocyanin biosynthesis after formation of leucoanthocyanidins; however the steps presented here represent the main points of the biosynthetic pathway that have been targeted for introducing new flower colors into Petunia and other plant species such as carnations and roses (see Chapter 13 for an in-depth look at anthocyanin biosynthesis in Petunia).

Novel flower colors and patterns can be achieved by changing expression patterns of endogenous genes or by introducing new genes from other species. The first application of biotechnology to Petunia was the production of plants with reduced flower color through direct reduction of transcript levels for the endogenous *CHS* gene by antisense and co-suppression techniques (van der Krol et al. 1988; Napoli et al. 1990; van der Krol et al. 1990). Plants with reduced *CHS* expression had flowers with less pigmentation and with coloration that was restricted to specific regions of the flower (Fig. 20.1). These experiments demonstrated that by blocking the first step in the flavonoid pathway a white-flowering phenotype could be achieved from colored Petunias and novel color patterning in the corollas could be produced. van der Krol et al. (1990) also demonstrated this for another enzyme, DFR, which catalyzes the reduction of dihydroflavonols to leucoanthocyanidins.

Pale yellow and light purple flowers were obtained by introducing the *Medicago sativa* chalcone reductase gene, *CHR*, into Petunia (Davies, Sheikh, Ratcliffe, Coupland, and Furner 1998). CHR activity reduces 6′-hydroxychalcones to 6′-deoxychalcones. When *CHR* was introduced into the white-flowered Petunia Mitchell background, corollas accumulated novel 6′-deoxychalcones, had lower levels of flavonols, and were a pale yellow color. When *CHR* was introduced

into a purple background (V26 x [Petunia Mitchell x V26]), flowers had a pale purple color, accumulated 6′-deoxychalcones, and had reduced levels of flavonols and anthocyanins. As the Petunia chalcone isomerase does not accept 6′-deoxychalcones as substrates, novel chalcone accumulation was thereby introduced into Petunia.

A novel orange color was first introduced into Petunia by incorporating a maize *DFR* gene into the genome of a Petunia variety lacking F3′H and F3′5′H activity (Meyer, Heidmann, Forkmann, and Saedler 1987). Petunias lacking F3′H and F3′5′H activity accumulate the dihydroflavonol dihydrokaempferol because the Petunia DFR does not accept dihydrokaempferol as a substrate for leucoanthocyanidin synthesis. By introducing the maize DFR, which accepts dihydrokaempferol as a substrate, a pathway for the orange pelargonidin anthocyanins was created. However, flowers on these plants did not produce a commercially acceptable deep orange color and showed frequent reversion to the original color (Meyer, Linn, Heidmann, Meyer, Niedenhof, and Saedler 1992). Oud, Schneiders, Kool, and van Grinsven (1995) introgressed the maize *DFR* into elite breeding lines that accumulated cyanidin, peonidin, delphinidin, petunidin, and malvidin anthocyanins. After three generations of selfing the F1 crosses, color had stabilized and the flowers exhibited a range of novel, improved orange colors.

20.3 Phytohormones

Because hormones are involved in regulating a wide range of plant processes important to commercial crop production, modification of phytohormone synthesis and perception is a major area of research in plant biotechnology. Genetic engineering provides a means for more precise delivery of novel traits through the use of developmental or tissue-specific promoters, which can reduce grower input and reduce environmental costs. For example, the hormone cytokinin delays leaf senescence, a process involving the ordered breakdown of cellular macromolecules and reallocation of nutrients to other parts of the plant. Control of this process is of major importance to improving the quality of many crop plants, particularly ornamentals, as delaying senescence cosmetically enhances plants grown in less-than-ideal conditions by garden center retailers and consumers. Plants constitutively producing cytokinins do not exhibit normal plant architecture (Faiss et al. 1997) but when cytokinin synthesis is limited to specific stages of development, such as senescence, the value of the genetic change surfaces. The utility of promoters conferring expression at specific stages and leading to appropriate levels of gene activity is a theme that repeatedly arises in plant biotechnology, especially when manipulating phytohormone synthesis and perception, and is a point that cannot be overemphasized.

20.3.1 Cytokinins

Cytokinins regulate a number of developmental processes in plants. These include promoting cell division, transducing nutritional signals, promoting shoot initiation,

increasing crop productivity and delaying senescence (Mok and Mok 1994; reviewed in Sakakibara 2006). The first cytokinin biosynthetic gene identified and characterized was the *ISOPENTENYL TRANSFERASE* (*IPT*) gene from *A. tumefaciens* (Akiyoshi, Klee, Amasino, Nester, and Gordon 1984). The IPT protein catalyzes synthesis of the cytokinin Δ^2-isopentenyladenosine monophosphate through a transferase reaction between dimethylallylpyrophosphate (DMAPP) and 5′AMP. This enzyme is encoded on the Ti plasmid of *A. tumefaciens* and is integrated into the host genome after infection. The enzyme is active in plants, causing increased cytokinin production in plants transformed with *IPT* (Medford, Horgan, El-Sawi, and Klee 1989). Not until recent years have cytokinin biosynthetic genes been isolated from plants (Takei, Sakakibara, and Sugiyama 2001; Zubko et al. 2002); therefore work to date has focused on using the *A. tumefaciens IPT* gene to modify endogenous cytokinin levels.

There have been many attempts to engineer delayed leaf senescence via higher cytokinin levels in plants, most resulting in plants with abnormal growth (Faiss, Zalubilova, Strnad, and Schmulling 1997; McKenzie, Mett, Reynolds, and Jameson 1998; Smart, Scofield, Bevan, and Dyer 1991). Gan and Amasino (1995) designed constructs for auto-regulated, senescence-specific expression of the *IPT* gene using the *SAG12* promoter from Arabidopsis. This construct confers elevated cytokinin levels when the plant initiates senescence, which then attenuates senescence processes and expression of *IPT*, thereby preventing overproduction of cytokinins.

The SAG12::*IPT* construct was introduced into V26 Petunia (Dervinis 1999; Jandrew 2002; Chang, Jones, Banowetz, and Clark 2003; Clark, Dervinis, Barrett, Nell, and Klee 2004). More than 20 SAG12::*IPT* lines were generated, two of which displayed a normal growth phenotype and delayed leaf senescence. The remainder presumably had "leaky" expression of the *IPT* transgene, which caused an unacceptable growth phenotype (Dervinis 1999). Delayed leaf senescence was screened by means of drought stress, which consistently induces leaf senescence in Petunia, does not induce *SAG12* expression (Weaver, Gan, Quirino, and Amasino 1998), and mimics a situation commonly encountered in retail garden centers. Leaf senescence following drought stress was delayed in transgenic SAG12::*IPT* Petunia plants, and the number of chlorotic basal leaves on untransformed V26 plants was 14 times that seen on transgenic SAG12::*IPT* V26 Petunias (Table 20.2; Dervinis 1999; Clark et al. 2004). Continued drought stress caused yellowing and death of the lower leaves of "V26" Petunias, while SAG12::*IPT* lines recovered and leaf chlorophyll levels were actually higher than those seen in nonstressed plants (Dervinis 1999; Clark et al. 2004).

These two SAG12::*IPT* lines (I-1-7-22 [IPT22] and I-3-18-34 [IPT34]) were also characterized as having delayed flower senescence (Chang et al. 2003). Unpollinated SAG12::*IPT* flowers showed symptoms of corolla senescence, including wilting and color fading, at 12 days after flower opening compared to 7 days in wild-type "V26" flowers. Increased accumulation of *IPT* transcripts and increases in the total cytokinin content of the IPT22 and IPT34 corollas were detected after pollination. Elevated cytokinin levels led to delays in corolla senescence, similar to that observed in Petunia leaves. While pollinated V26 flowers senesce at 48 h after

Table 20.2 Numbers of senescing and chlorotic leaves in T_2 progeny from two transgenic SAG12::*ipt* Petunia lines after drought stress. Adapted from Clark et al. 2004

Family	Segregant	# Senescing leaves	# Chlorotic leaves
"V26" control	Negative	12.3±0.77	6.04±0.67
I-1-7-1	Negative	12.0±1.48	5.83±1.2
I-1-7-1	Positive	0.02±0.01	0.00±0.00
I-1-7-2	Positive	0.05±0.05	0.00±0.00
I-1-7-3	Positive	0.66±0.45	0.52±0.36
I-3-18-1	Positive	0.42±0.33	0.13±0.09
I-3-18-2	Positive	0.85±0.54	0.46±0.46

pollination, corolla wilting was not observed in pollinated IPT34 and IPT22 flowers until 11 and 12 days after pollination, respectively. These flowers were also less responsive to ethylene than V26 flowers (see Chapter 14), and provide a good system for studying the importance of hormone interactions during flower senescence.

The SAG 13 promoter and the maize *KNOTTED1* gene (*KN1)* have also been used in constructs designed to delay leaf senescence. KN1 is a homeobox protein intimately associated with meristem formation, cytokinin synthesis, and repression of senescence. Like SAG12::*IPT* transgenic plants, SAG13::*IPT* and SAG12::*KN1* petunias exhibit delayed leaf senescence after drought stress (Jandrew 2002). There were a number of effects of the SAG13::*IPT* transgene in these plants: transgenic plants were more branched, leaves were thicker and larger, root growth was reduced, and flowering was delayed. These phenotypes were ascribed to "leaky" expression of the *IPT* transgene (Jandrew 2002). New growth after nutrient stress was chlorotic and necrotic in both. Severe growth-arrest responses were observed under nutrient-limiting conditions, presumably due to altered source-sink relationships (Jandrew 2002). Following an outbreak of *Cercospora* in the greenhouse, it was observed that SAG13::*IPT* and SAG12::*KN1* lines were more tolerant to pathogens (Jandrew 2002). *Cercospora* causes loss of photosynthetic tissue and has been documented to result in yield losses up to 50% in peanut (Subrahmanyam, McDonald, and Gibbons 1982). In controlled greenhouse experiments in which *Cercospora* was spray-inoculated onto plants, control plants had six-fold more senescing leaves than SAG13::*IPT* or SAG12::*KN1* plants (Jandrew 2002). Inoculated transgenic plants exhibited a local hypersensitive response and did not become chlorotic, while leaves on inoculated control plants became chlorotic and senesced. This transgenic stay-green technology presents a novel means for enhancing pathogen tolerance, as leaves of the plants do not senesce in response to infection. These experiments also highlight the value of exhaustive horticultural evaluations of transgenic plants.

Khodakovskaya et al. (2005) utilized cor15a, a cold-inducible promoter from Arabidopsis, to drive *IPT* expression in Petunia plants as a means of delaying senescence under conditions of stress that readily occur during commercial horticultural production, including darkness (experienced during transport) and cold storage (associated with plug production). Under normal conditions transgenic cor15a::*IPT* plants exhibited a phenotype similar to that of controls. Analysis revealed that

cytokinin levels increased in cold-treated transgenic plants and leaves maintained normal chlorophyll levels and resisted senescence after cold and dark treatments.

IPT has also been shown to confer larger fruit and flower size in Petunia when produced under control of the Arabidopsis *AP3* promoter (US Patent #7253340). *AP3* is a class B floral organ identity gene specifying petal and stamen identity, with expression restricted to the petals and stamens (Tilly, Allen, and Jack 1998). Petunia plants producing β-glucuronidase (GUS) under control of the *AP3* promoter showed *GUS* expression in floral organs only (Verdonk et al. 2008). Evaluation of more than 60 AP3::*IPT* transgenic lines identified five with increased flower diameter (Fig. 20.2). Flowers from these lines had significant increases in the diameters of individual floral organs, including a 20–30% increase in limb diameter and >90% increase in whole flower fresh weight. Some lines had >60% increase in overall fruit weight while others had fruit weights similar to those of controls. These observations underscore the importance of generating several independent transgenic lines when trying to produce plants with commercially important characteristics.

20.3.2 Ethylene

Ethylene is a two-carbon unsaturated gas that is involved in many aspects of plant growth and development, including seedling growth, flowering, fruit ripening, floral senescence, root formation, and stress responses (Abeles, Morgan, and Saltveit 1992). Because ethylene is required for, and involved in, many aspects of plant growth and development, there has been considerable interest in understanding its biochemistry and molecular biology as a prelude to biotechnological manipulation. The biosynthetic pathway of ethylene has been elucidated (Yang and Hoffman 1984), and many components of the biosynthetic and signal transduction pathways have been cloned. Ethylene is perceived by plants through transmembrane receptor proteins encoded by the *ETR* (*ethylene resistant*) gene family, typically consisting of >5 genes.

Ethylene receptors have been identified in EST collections from more than 20 plant species, including monocots, dicots, and lower plants (Mount and Chang 2002). They are thought to have a plastid origin, having arisen from a cyanobacterial ancestor, thus predicting universality in the means by which plants perceive ethylene. Ethylene binding has been demonstrated in cyanobacteria, some species of fungi, the green alga *Chara*, lower vascular plants including ferns, fern allies and bryophytes, and angiosperms (see Chen, Etheridge, and Schaller 2005; Wang et al. 2006). While studies of receptor function at the molecular level have not been carried out in many plant species, the proteins are highly conserved in regions which are functionally important (Mount and Chang 2002; Wang et al. 2006). It is therefore likely that the receptors act in a similar fashion in plant species other than tomato and Arabidopsis, that is, as negative regulators of ethylene responses. In the absence of ethylene, ethylene responses are repressed and in the presence of ethylene repression of ethylene responses is lifted. Arabidopsis plants carrying the *etr1-1* receptor mutation are almost completely insensitive to ethylene (Chang, Kwok, Bleecker,

Fig. 20.2 AP3::*IPT* transgenic Petunias have increased flower and fruit size. (**A**) Petunia Mitchell control flower on *far left* and flowers from two AP3::*IPT* lines in center and *far right*; (**B**) longitudinal section of Petunia Mitchell fruit; (**C**) longitudinal section of AP3::*IPT* fruit; (**D**) cross section of Petunia Mitchell fruit; (**E** cross section of) AP3::*IPT* fruit. Photographs shown in panels B–E were taken 15 days after pollination. Adapted from Verdonk et al. (2008). Reprinted with permission, ©John Wiley and Sons. Ltd.

and Meyerowitz 1993). Another central component of the pathway, *EIN2*, is critical for ethylene signaling. Arabidopsis *EIN2* loss-of-function mutants are unable to respond to ethylene (Roman et al. 1995). Both *ETR* and *EIN2* are critical to plant ethylene responses, making them good candidates for manipulating ethylene responses at the molecular level.

Petunia has proved to be an excellent model system for engineering flower longevity. The flowers are ethylene sensitive, producing ethylene after pollination then senescing within 2–3 days. Longer-lasting flowers have been attained by overexpression of the mutant ethylene receptor gene, *etr1-1*, from *Arabidopsis thaliana* (Wilkinson et al. 1997) and by reduction of *EIN2* expression using RNAi or co-suppression (Shibuya et al. 2004). Corollas from Petunias expressing the *etr1-1* gene do not wilt after pollination or exposure to ethylene and can stay attached to the plant until the developing fruit severs petal connections to the receptacle, up to 17 days after pollination depending on the temperature (see Chapter 14). Unpollinated flowers of *etr1-1* plants senesce at the same rate as pollinated flowers, in approximately 17 days (Gubrium, Clevenger, Clark, Barrett, and Nell 2000; Jones, Chaffin, Eason, and Clark 2005; Langston, Bai, and Jones 2005). *EIN2* RNAi and co-suppression Petunia plants also display delayed corolla senescence after pollination or exposure to exogenous ethylene (Fig. 20.3A, 20.3B). These flowers do not exhibit visual signs of senescence until more than 9–12 days after pollination (Shibuya et al. 2004).

While longer-lasting flowers clearly add value to commercial Petunias, research with transgenic ethylene-insensitive lines has shown that ethylene sensitivity is critical for overall plant health and the ability to respond promptly to changes in the environment. Ethylene-insensitive plants are less able to adapt to rapid environmental changes, exhibit delayed fruit ripening, have a higher incidence of mortality, and fail to form adventitious roots and therefore cannot be propagated by cuttings (Clark, Gubrium, Barrett, Nell, and Klee 1999; Gubrium et al. 2000; Clevenger, Barrett, Klee, and Clark 2004; Shibuya et al. 2004; Barry 2004). Fruit ripening is also delayed by ethylene insensitivity in Petunia: the time from pollination to fruit ripening in Petunias with reduced *EIN2* expression was delayed up to 28%, while that of *etr1-1* plants was delayed by 19% (Shibuya et al. 2004). Classical experiments with tomato have shown that ethylene is a critical signal for fruit ripening. Unlike tomato, Petunia fruit dry and dehisce upon ripening; the experiments with Petunia thus show that ethylene is involved in the ripening of dry, dehiscent fruits as well. Both 35S::*etr1-1* and *EIN2* RNAi/co-suppression Petunias failed to produce adventitious roots from vegetative cuttings (Fig. 20.3C; Clark et al. 1999; Shibuya et al. 2004), and treatment with auxin, a phytohormone used to stimulate rooting, did not overcome this inhibition, indicating that ethylene perception is critical for this process (Clark et al. 1999). Ethylene insensitivity conferred by *etr1-1* and reduced *EIN2* expression also led to increased mortality. Side-by-side in the greenhouse, during both spring and fall growing seasons, up to 50% of *etr1-1* ethylene-insensitive plants died, while there was no death among control plants (Figs. 20.3D, 20.4; Barry 2004). The cause of death in the *etr1-1* ethylene-insensitive Petunias remains unknown.

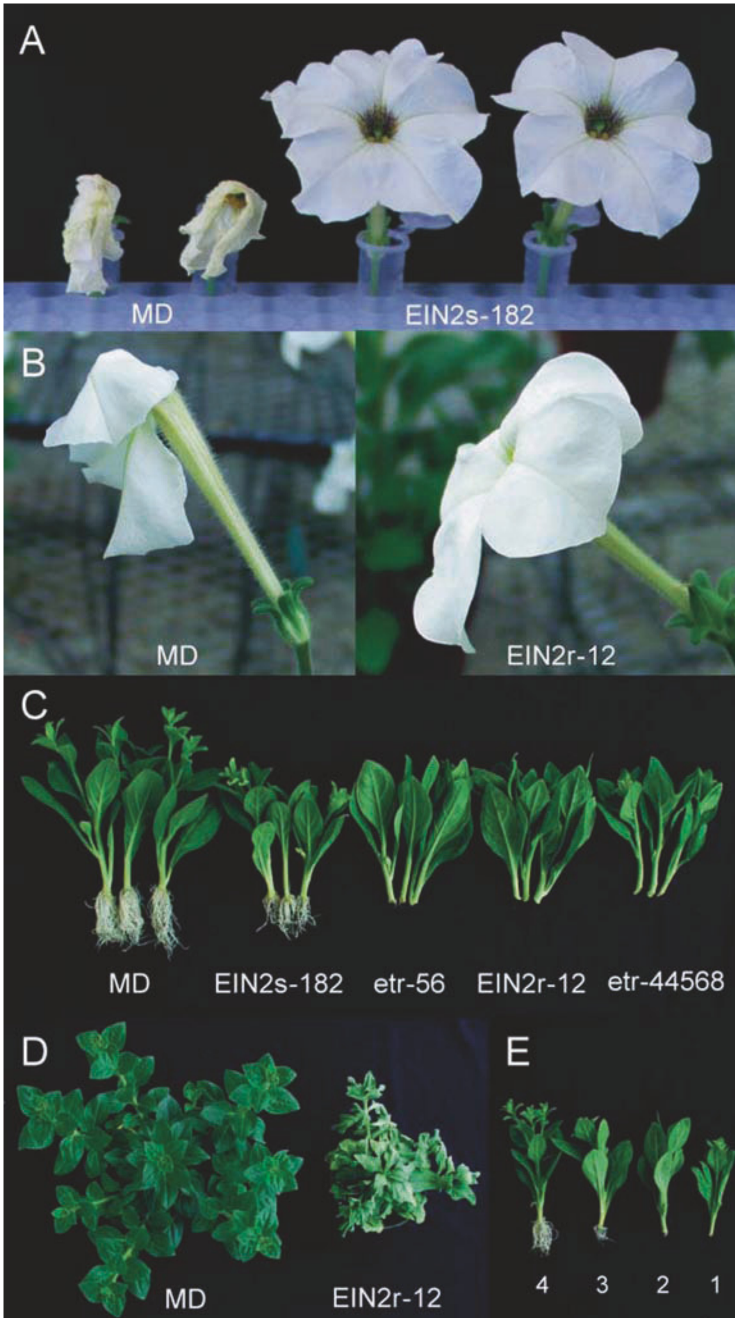

Fig. 20.3 Effects of ethylene insensitivity in P. Mitchell (MD). (**A**) Delayed corolla senescence in response to ethylene; (**B**) delayed pollination; (**C**) reduced ability to form adventitious roots; (**D**) increased mortality. (**E**) Adventitious root development scale in control Petunia Mitchell. "EIN" designates ethylene-insensitive plants. From Shibuya et al. 2004. ©American Society of Plant Biologists, reprinted with permission

Fig. 20.4 Transgenic 35S::*etr1-1* plants exhibit unexplained death. Petunia Mitchell is on the *left*; a 35S::*etr1-1* transgenic plant is on the *right*

While the 35S::*etr1-1* and *EIN2* RNAi/co-suppression lines are not commercially viable, the transgenic plants were critical for answering many basic questions about ethylene biology. All of the experiments pointed to the importance of ethylene in plant growth and development and showed that whole-plant ethylene insensitivity renders plants weaker and less amenable to commercial production and marketing. Shibuya et al. (2004) showed that the degree of ethylene insensitivity in different tissues varied among transgenic lines, again emphasizing the necessity for several independent transgenic lines in any efforts to develop a commercial idea. Promoters that drive specific expression of *etr1-1* in the petals or flowers may also provide a solution to the negative horticultural traits observed when entire plants are made insensitive to ethylene.

20.3.3 Gibberellic Acid

Since the days of the green revolution, management of plant height has been of great interest in relation to crop plants. In floricultural crops, plant height is regulated during production through the use of chemical growth regulators that control the synthesis of gibberellic acid (GA), a phytohormone that promotes stem elongation. Because labor and chemicals increase production and environmental costs, and compact plants are easier to ship to the consumer, the ideal crop plant would produce optimal patterns of growth regulators internally.

Fig. 20.5 Petunias expressing the CaMV35S::*AtGAox7* are dwarf and exhibit a range in height. In the far left is a Petunia Mitchell control followed by three independent transgenic lines carrying CaMV35S::*AtGAox7*. Adapted from Clark 2004. Reprinted with permission from John Wiley and Sons, Ltd.

Plant height can be controlled by lowering the endogenous levels of GA in plants either by inhibiting GA biosynthesis or by increasing GA degradation. The genetics of GA biosynthesis and degradation have been investigated in Arabidopsis. While more than 120 different GAs have been identified, many are precursors or breakdown products and only a handful are biologically active in plants. Several GA oxidase enzymes that hydroxylate GA precursors to form inactive GA products have been identified in Arabidopsis (Thomas et al. 1999; Schomburg, Bizzell, Lee, Zeevaart, and Amasino 2003). Two GA2-oxidases (*AtGAox7* and *AtGAox8*) have been shown to hydroxylate C_{20}-GA precursors, but not active C_{19}-GAs (Schomburg et al. 2003). Based on work by Schomburg et al. (2003), Petunias with reduced plant height were produced by overexpressing GA oxidase (*CaMV35S*::*AtGAox7* and *AtGAox8*), thereby reducing endogenous levels of GA. Transgenic GA oxidase Petunias have a range of dwarf phenotypes (Fig. 20.5 and Clark, unpublished). The dwarf phenotype can be rescued by application of exogenous C_{19}-GAs, a feature useful to commercial growers as a means of elongating cuttings for vegetative propagation.

20.4 Floral Scent

Modification of floral scent through biotechnology has not been a major focus of research until recent years. In many ornamental crops floral scent has been lost as a result of breeding programs that were focused on selection of other traits. Research on floral scent has importance for many reasons: (1) volatiles are a major cue for insect and animal pollinators, so an understanding of how pollinators perceive volatiles and how floral volatiles are regulated and produced could lead to improved fruit and seed set in an agricultural setting; (2) some of the volatiles emitted by

flowers play a role in repelling pathogens and pests (reviewed in Holopainen 2004); (3) since floral scent has not commonly been a trait of interest in commercial breeding programs, introducing and improving floral fragrance could increase the value of many ornamental varieties; and (4) many plant-produced volatile compounds are documented to have positive effects on human behavior and therefore could be used as tools for improving the health and welfare of humanity (Diego et al. 1998; Lehrner, Eckersberger, Walla, Postsch, and Deecke 2000; Komiyaa, Takeuchib, and Harada 2006).

The biochemistry and molecular biology of floral scent have been investigated in a handful of genera including *Petunia* (see Chapter 3; Negre et al. 2003; Verdonk et al. 2003; Verdonk, Haring, van Tunen, and Schuurink 2005; Underwood et al. 2005; Dexter et al. 2007), *Clarkia* (D'Auria, Chen, and Pichersky 2002), *Antirrhinum* (Kolosova, Gorenstein, Kish, and Dudareva 2001; Dudareva et al. 2003), *Stephanotis* (Pott et al. 2004), and *Rosa* (Guterman et al. 2002; Shalit et al. 2003; Scalliet et al. 2006). While some of the genes responsible for volatile synthesis have been cloned and characterized (Pichersky, Lewinsohn, and Croteau 1995; Murfitt, Kolosova, Mann, and Dudareva 2000; Negre et al. 2002, 2003; Simkin et al. 2004; Pott et al. 2004; Underwood et al. 2005; Orlova et al. 2006; Guterman et al. 2006; Kaminaga et al. 2006; Dexter et al. 2007; Tieman, Loucas, Kim, Clark, and Klee 2007; Dexter et al. 2008), few lines of transgenic plants with altered fragrance have been created and characterized by human sensory panels. While there are obvious benefits to studying floral scent in multiple plant species, (Knudsen, Tollsten, and Bergstrom 1993), Petunia has emerged as a key model system for studying floral scent. In recent years, the fragrance profile has become well characterized and many of the genes responsible for Petunia fragrance have been isolated. To date, the floral fragrance of Petunia has been modified both by changing the expression levels of endogenous genes, thereby altering ratios of volatiles naturally produced in Petunia, and by introducing novel biosynthetic pathways. Clearly the area of genetic engineering of floral scent is in its infancy and many basic questions about pollination biology (see Chapter 2) and improving floral fragrance in ornamental plants (see Chapter 3) remain.

20.4.1 Genetic Engineering of Regulatory and Structural Genes

One regulatory gene and several biosynthetic genes controlling synthesis of most of the major volatile compounds in Petunia have been identified, and transgenic plants with altered volatile profiles have been produced. Verdonk and coworkers (2005) identified a transcriptional regulator of floral scent biosynthesis, ODO1. Plants with a decreased level of *ODO1* expression emit lower levels of all the major volatile benzenoid compounds that comprise Petunia fragrance. The discovery of *ODO1* could thus be key for future genetic manipulation of floral fragrance. It will be interesting to learn if higher level expression of *ODO1* will lead to increased fragrance production. It will also be important to study *ODO1* expression and activity in other plant species that have lost floral scent through selective breeding for other

traits. In a recent set of experiments benzyl benzoate and phenethyl benzoate levels were decreased in benzylalcohol/phenylethanol benzoyltransferase (*PhBPBT*) RNAi Petunia plants (Orlova et al. 2006; Dexter et al. 2008). The volatile isoeugenol was significantly reduced from the volatile profile in plants with silenced coniferyl alcohol acyltransferase (*CFAT*) expression (Dexter et al. 2007). Silencing carotenoid cleavage dioxygenase 1 (*CCD1*) expression reduced β-ionone emission up to 76% (Simkin et al. 2004) compared to that measured in Petunia Mitchell flowers. While β-ionone is a minor peak in the volatile profile, the odor threshold is very low compared to that of other volatiles (Baldwin, Scott, Shewmaker, and Schuch 2000), and therefore it is likely an important component of the Petunia floral fragrance perceived by humans.

Recently the genes responsible for synthesis of the floral volatiles 2-phenylethanol and phenylacetaldehyde were cloned and characterized (Kaminaga et al. 2006; Tieman et al. 2007). Phenylethanol, a volatile normally produced in Petunia, is one of the more abundant floral volatiles in the plant kingdom, having been identified in more than 34 plant species (Knudsen et al. 1993). It is also an important flavor component and fragrance chemical for cosmetics. The first step of the pathway, converting phenylalanine to phenylacetaldehyde, is catalyzed by the enzyme phenylacetaldehyde synthase (PAAS) (Kaminaga et al. 2006). Transgenic Petunia plants with silenced *PAAS* expression emitted neither phenylacetaldehyde nor 2-phenylethanol (Kaminaga et al. 2006). The second step, catalyzed by phenylacetaldehyde reductases (PAR), converts phenylacetaldehyde to 2-phenylethanol (Tieman et al. 2007). Transgenic Petunias constitutively expressing PAR produced higher levels of 2-phenylethanol and accumulated less phenylacetaldehyde than control flowers (Tieman et al. 2007). It will be interesting to see how humans perceive the fragrance of the *PAR* overexpressing flowers, as 2-phenylethanol is thought to be one of the most important floral volatiles in fragrant plants. It will also be of interest to use this gene to drive higher 2-phenylethanol production in commercially important rose varieties, many of which suffer from low fragrance.

Introduction of a novel monoterpene synthesis pathway into Petunia has been attempted. Lücker and colleagues transformed Petunia Mitchell plants with the *Clarkia breweri S-LINALOOL SYNTHASE* (*LIS*) gene (Lücker et al. 2001). Linalool synthase catalyzes the formation of S-linalool from geranyl pyrophosphate (Pichersky et al. 1995). Linalool is an acyclic monoterpene that has pest repellant activity (Hori 1998) and is released from plants after herbivore attack (Pare and Tumlinson 1999). While it is synthesized in flowers of more than 60 plant species (Knudsen et al. 1993), it is not emitted from *Petunia hybrida* flowers. Transgenic plants constitutively expressing *LIS* did not emit linalool, but accumulated a conjugated non-volatile form, *S*-linalyl-β-D-glucopyranoside (Lücker et al. 2001).

Altering the synthesis of volatiles in Petunia by genetic engineering has been demonstrated to lead to changes in the human perception of the fragrance profile. This was shown with the major floral volatile in Petunia fragrance, methyl benzoate (Underwood et al. 2005). In Petunia, synthesis of methyl benzoate is catalyzed by the benzoic acid:salicylic acid carboxyl methyltransferases BSMT1 and BSMT2 (Negre et al. 2003); in plants engineered for reduced expression of both *BSMT1* and *BSMT2*, the emission of methyl benzoate was reduced by 95% (Underwood

et al. 2005). Flowers from transgenic *BSMT* RNAi plants were used in sensory-panel studies to determine if the change in fragrance was perceptible and to assess how the change was perceived. In this study, 80% of the panelists were able to detect a difference between control and *BSMT*-RNAi flowers, and most found the knockdown fragrance less appealing (Underwood et al. 2005). These studies are important for demonstrating both that the technology works and that human panels provide valuable feedback on the effects of changes in the fragrance profile.

20.5 Conclusions

Biotechnology has been useful for generating novel flower colors and color patterns, manipulating phytohormone synthesis and perception, and modifying floral scent in Petunia. Promoters conferring specific expression of cytokinin biosynthesis genes in certain developmental stages or plant tissues have been used successfully to engineer stay-green leaves and larger fruit and flowers and will be key to continued success in the genetic engineering of Petunia. Promoters that drive specific expression of transgenes such as *etr1-1* will be critical for making genetically engineered plants with long-lasting flowers a commercial reality. For these and all biotechnology-based strategies, the generation and characterization of multiple transgenic lines will be essential in efforts to develop concepts to commercial viability. Studies of the horticultural performance of genetically engineered crops are critical for evaluating the effects of transgenes on plant growth and development, determining their utility in a commercial setting, and answering basic questions about effects on overall plant physiology. While the Petunia genome remains to be sequenced, useful genetic and molecular resources are available, including EST (Solanaceous Genomics Network) and cDNA collections (University of Florida, University of Fribourg), oligo arrays (Ohio State University, Kiplinger array), and mutant transposon insertion lines. Bolstered by these tools and the general amenability of Petunia for transgenic research, biotechnology is becoming a significant partner to traditional breeding for both Petunia and the floriculture industry as a whole.

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