Genetics of Floral Development in *Petunia*

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ABSTRACT

In the last two decades the genetic and molecular research on floral development has advanced tremendously. Initially the research focused mostly on the two species of which the homeotic floral development mutants formed the basis for the ABC-model: *Arabidopsis* and *Antirrhinum*. In recent years the importance of studying a wider range of species, especially in an "evo-devo" context, has become more and more evident. This review summarizes advances in the understanding of the genetic control of floral induction, inflorescence formation, and floral organ formation in *Petunia*. Moreover, we put the knowledge on *Petunia* floral development in the broader perspective of what is known on floral development in other species, thus pointing out differences and resemblances in the regulatory systems that ultimately form the basis for the enormous variation in flower shapes.

I. INTRODUCTION

A. SOLANACEAE

Petunia belongs to the Solanaceae, which is a plant family of great economic importance. Solanaceaous species are used for food (e.g., potato, tomato, pepper, eggplant), as drugs (e.g., tobacco, deadly nightshade, mandrake), and as ornamentals (e.g., petunia, velvet tongue, *Datura* spp., *Schizanthus* spp.) (Knapp *et al.*, 2004). Solanaceae can be found worldwide, from the driest deserts to tropical rainforests. The highest species diversity is found in the Neotropics. Estimates of species diversity in the family range from 3000 to 4000 species, almost half of which belonging to the large cosmopolitan genus *Solanum* (see Knapp, 2002b for a review of the genera in the family). The family is diverse, both in terms of life form, with species ranging from ephemeral herbs (*Leptoglossis* and *Schizanthus* of the Chilean deserts) to large forest trees (*Duckeodendron* of the Amazon), and in flower and fruit morphology (Knapp, 2002a,b). A literature and illustrations database on Solanaceae can be found at (http://www.bgard.science.ru.nl/solanaceae). Moreover, a huge international effort, SOL, aims at sequencing the tomato genome (Mueller *et al.*, 2005). Members of the Solanaceae family show a wide range of floral morphologies. Some species have zygomorphic or monosymmetric flowers, while others have actinomorphic or radially symmetric flowers (Knapp, 2002b).

B. THE GENUS *PETUNIA*

In 1803, Jussieu established the genus *Petunia* (Solanaceae), later referred to as *Petunia sensu* Jussieu. In the 1980–1990s *P. sensu* Jussieu was divided into two genera: *Petunia* and *Calibrachoa*. Ando *et al.* (2005) performed a detailed phylogenetic analysis of *P. sensu* Jussieu and demonstrated that the

separation of *Petunia* and *Calibrachoa* into different genera is supported by chloroplast DNA RFLP data. Several clades in the *Petunia* phylogenetic trees were found to correspond with geographic distribution, suggesting that recent speciation occurred independently in different regions. To date, around 30 *Petunia* (sub)species have been described. The geographic origin of *Petunia* is the southern/central part of South America, and various species have been documented from collections made in Argentina, Brazil, Paraguay, and Uruguay (Ando *et al.*, 2005).

C. *PETUNIA* FLOWER CHARACTERISTICS

Petunia plants, as other Solanaceae species, exhibit determinate inflorescences composed of scorpioid or cincinnus cymes (Souer *et al.*, 1998; Weberling, 1989). At the base of each flower, two bracts are formed, each with a dormant (vegetative) meristem in its axil. A wild type flower contains 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 *Petunia* flower is zygomorphic in all floral whorls, which is partly due to the whorled or irregular arrangement of sepals and petals in the floral bud before it opens (Knapp, 2002b). Ovules have a single integument (Angenent *et al.*, 1995). The fruit is a capsule, containing variable amounts of seeds for different *Petunia* species (Gunn and Gaffney, 1974; Sink and Power, 1978). Seed capsules are conic, widest at the base and tapering to the apex. 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) (Fig. 1).

D. FLORAL DIVERSITY IN THE *PETUNIA* CLADE: POLLINATION SYNDROMES

Hawkmoth (in *Petunia axillaris*) and bee (in *P. integrifolia*) pollination form typical examples of pollination syndromes in the genus *Petunia*. These two representatives of two groups of *Petunia* species have a complex set of morphological and physiological traits that are adapted to their respective pollinators. *P. axillaris* has white flowers, with long petal tubes that exactly fit the length of the tongue of the hawkmoths that pollinate them (*Manduca contracta* and *M. diffissa* ssp. *Petuniae*). Moreover, for the nocturnally active hawkmoths a colored flower is not as important as a strong scent, and in accordance with that *P. axillaris* has white, nocturnally scented flowers (Ando *et al.*, 2001; Stuurman *et al.*, 2004). *P. integrifolia* has unscented purple colored flowers, with a wide and short petal tube. Flowers of

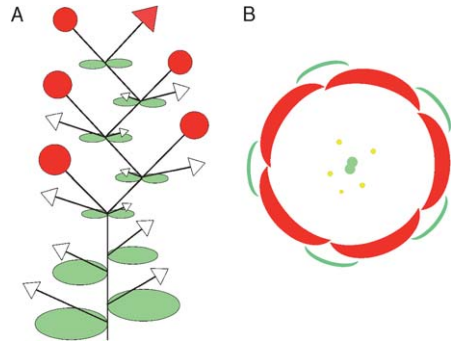

Fig. 1. (A) Schematic representation of *Petunia* branching in the reproductive phase (after Souer *et al.*, 1998). The position of the flowers is indicated by colored circles and the apical inflorescence meristem by a colored triangle. Leaves and bracts are shown by large and small green ovals, respectively. Vegetative axillary meristems are shown by open triangles. A smaller size of these triangles indicates a stronger dormancy. (B) Floral diagram of *Petunia* (after Knapp, 2002b). The floral organs, sepals (whorl 1), petals (whorl 2), stamens (whorl 3), and carpels (whorl 4) are depicted in green, red, yellow, and light green, respectively. The smaller stamen indicates the stamen in this position is reduced in some *Petunia* varieties.

P. integrifolia exhibit diurnal opening and closing movements synchronous with the activity period of the bee that pollinates them (*Hexantheda* sp.). Amounts of floral nectar in *P. axillaris* and *P. integrifolia* are within the range of hawkmoth- and bee-pollinated flowers, respectively (Ando *et al.*, 2001; Stuurman *et al.*, 2004). A thorough study in which the *Petunia* pollination syndromes have been dissected into their most important phenotypic and genetic components has been conducted by Stuurman *et al.* (2004) (Fig. 2).

Natural hybrids of *P. axillaris* and *P. integrifolia* have never been reported, even though artificial crosses can produce fertile hybrid offspring (garden petunias are known to be descendants of such a hybrid) and the two species grow together. Therefore, Ando *et al.* (2001) studied the reproductive isolation between the two species. Differential insect visitation of *P. integrifolia* and *P. axillaris* in sympatric populations was observed, suggesting an important biological meaning of the floral differences in color, scent, and amount of nectar. Insect visitation is not the only reproductive barrier among *Petunia* species, as genetic incompatibilities between ecotypes can also be considerable (Ando *et al.*, 2001). Still, the extensive divergence in the *Petunia* floral pollination syndromes indicates that insect visitation has certainly had a huge impact on the evolutionary history of the *Petunia* species (Stuurman *et al.*, 2004).

Fig. 2. (A) *P. axillaris* flower (left) and a *P. integrifolia* flower (right). Picture courtesy of Mary Hoballah and Jeroen Stuurman. (B) *Manduca sexta* hawkmoth on a *P. axillaris* flower. Picture courtesy of Mary Hoballah and Jeroen Stuurman.

Two important characteristics in pollination syndromes are scent production and floral color. In *Petunia hybrida*, the floral aroma is predominantly determined by volatile benzoids (Verdonk *et al.*, 2003). Verdonk *et al.* (2005) identified *ODORANT1* (*ODO1*), a member of the R2R3-type MYB family, as a candidate for the regulation of volatile benzoid production in *P. hybrida* "Mitchell" (W115) flowers. Underwood *et al.* (2005) demonstrated, using transgenic ethylene insensitive "Mitchell" lines, that the production of volatile organic compounds is regulated by ethylene. Once the flower has been pollinated and attraction of pollinators is no longer necessary, ethylene acts as a signal to downregulate the expression of scent biosynthetic genes (Negre *et al.*, 2003). Flower color is mainly determined by flavonoid components. The genetics, biochemistry, and molecular biology of flavonoid synthesis are fairly well understood in *Petunia* (Koes *et al.*, 2005; Martin and Gerats, 1993; Spelt *et al.*, 2002).

E. RESEARCH ON *PETUNIA* FLOWER DEVELOPMENT

Petunia has been studied since around 1830, and there are some early papers that mention specific flower developmental mutants. To quote Bailey (1896), who was referring to work by Harrison from around 1838: "Various curiously marked types of petunias have appeared and are lost. One of the early forms had a red body color with grass-green borders." Further, Bailey quotes Carman (Proc. Sixth Conv. Soc. Am. Flor., 1890) as obtaining plants with "rosettes of green leaves without the rudiments of calyx, corolla, stamens, or pistils." The last description is reminiscent of a full *sep* phenotype

(Ditta *et al.*, 2004). Levan (1937) describes a mutant in which ovules have been replaced by leaf-like structures, comparable to the phenotype of a double mutant for two MADS-box genes, *fbp2/fbp5* (Vandenbussche *et al.*, 2003b). More information on various aspects of the use of *Petunia* in research can be found in Gerats and Vandenbussche (2005). On the Petunia platform website (http://www.petuniaplatform.net) most groups working with *Petunia* as a main system are presented. A valuable resource for background information on culture and various research aspects of *Petunia* still is Sink's 1984 monograph "Petunia."

F. *PETUNIA* IN MOLECULAR STUDIES

A range of materials, techniques, and strategic approaches make *Petunia* a feasible system to work with. Besides easy culture conditions, an endogenous transposable element system is available, which can be used efficiently in both forward and reverse approaches. Two extensively used *Petunia* varieties in molecular research are the easy-to-transform "Mitchell diploid" and the high copy-number *dTph1* transposon line "W138" (for details see Gerats and Vandenbussche, 2005). Forward approaches are primarily performed by Transposon Display methods (De Keukeleire *et al.*, 2001; Van den Broeck *et al.*, 1998). Reverse approaches have been optimized over the years (Koes *et al.*, 1995; Vandenbussche *et al.*, 2003b). Many of the genes involved in floral development (floral transition, floral patterning) are MADS-box genes (see Irish, Chapter 3; Kramer and Zimmer, Chapter 9; and Soltis *et al.*, Chapter 12). Over time, a number of these have been studied by transgenic methods (e.g., Angenent *et al.*, 1994, 1995; Immink *et al.*, 1999) or by insertional mutagenesis as mentioned in an earlier section. For all major clades of MADS-box genes *Petunia* members are known (for an overview see Vandenbussche *et al.*, 2003b). In this review we only focus on the genes for which functional data are present.

G. FLORAL DEVELOPMENT

Flower development can be divided into several distinct phases: (1) transition to flowering, (2) inflorescence/flower meristem formation, and (3) floral organ patterning. It appears that, while in general molecular aspects of flower development are quite comparable for *Arabidopsis* and *Antirrhinum*, this can not always be generalized to fully encompass other species like *Petunia*. Thus, to discern ornamental differences from fundamental ones, it is important to develop insights in a range of systems.

II. THE TRANSITION TO FLOWERING

The transition from the vegetative to the reproductive phase is an important developmental shift in the plant life cycle, and its timing is critical for reproductive success. This shift is characterized by the induction and development of an inflorescence meristem that generates floral meristems. This morphogenetic change is controlled by endogenous factors, where the program to flowering is turned on after a certain time of vegetative growth or when a defined number of leaves or biomass is produced, and by environmental conditions. In *Arabidopsis*, a number of genetic pathways controlling flowering time (see Engelmann and Purugganan, Chapter 13) have been identified, and a lot of genes involved in these pathways have been studied extensively. Models now extend beyond "primary" controlling factors and show an ever-increasing number of cross-talks between pathways triggered or influenced by various environmental factors and hormones (mainly gibberellins) (reviewed in Bernier and Perilleux, 2005; Boss *et al*., 2004).

For *Petunia* there is less extensive knowledge on the regulatory mechanisms and genes involved in floral transition. We do know flowering in *Petunia* is photoperiodically controlled, and long day conditions or a night interruption with artificial light promote early flowering (Adams *et al.*, 1999). Moreover, quite some work on the participation of gibberellins and gibberellin-induced proteins in diverse developmental processes in *Petunia*, including flower induction, development, and pigmentation, has been done by the group of David Weiss (e.g. Ben-Nissan *et al*., 2004; Izhaki *et al*., 2001; Weiss, 2000).

When studying floral transition in *Petunia* and genes involved in the genetic pathways controlling flowering time, obvious candidates are genes homologous to *Arabidopsis* genes with a known function in floral transition. The key genes integrating multiple floral transition promoting pathways in *Arabidopsis* are *FLOWERING LOCUS T* (*FT*), *LEAFY* (*LFY*), and *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1* (*SOC1*; also known as *AGAMOUS-LIKE20*) (Blazquez and Weigel, 2000; Kardailsky *et al*., 1999; Kobayashi *et al.*, 1999; Lee *et al*., 2000; Nilsson *et al.*, 1998; Samach *et al*., 2000). These three floral pathway integrators have both overlapping and independent functions in the determination of flowering time and floral initiation (Moon *et al.*, 2005).

SOC1 is a MADS-box gene that has a promotive effect on flowering. *SOC1* is activated during the transition to flowering; transgenic plants overexpressing *SOC1* flower early while *soc1* mutants are delayed in flowering (Borner *et al.*, 2000; Samach *et al.*, 2000). The *SOC1* gene integrates signals from the photoperiod, vernalization, and gibberellin pathways (Blazquez, 2000;

Borner *et al.*, 2000; Moon *et al.*, 2003; Samach *et al.*, 2000). As can be seen in the phylogenetic tree in Fig. 3 four genes have been identified in *Petunia* belonging to the *SOC1*/*TM3* clade (Immink *et al.*, 2003). *Arabidopsis SOC1*/*TM3* clade members besides *SOC1* itself (*AGAMOUS LIKE14, AGL19, AGL42, AGL71, AGL72*). Therefore, without thorough functional analyses it is impossible to find out which *Petunia* gene, or which combination of genes, is functionally orthologous to the *Arabidopsis SOC1* gene. The *Arabidopsis SOC1* gene is expressed in most organs at variable levels, but upon floral induction its expression is rapidly upregulated in the apical meristems, whereas in vegetative plants only very little *SOC1* transcript can be detected in these meristems. Later, during floral development, *SOC1* is expressed in apical meristems and in procambial strands of developing inflorescences. Although *SOC1* is not expressed in emerging floral meristems, it was detectable in the center of floral meristems at a later stage (Borner *et al.*, 2000).

The *Petunia* members of the *SOC1*/*TM3* clade (Fig. 3), *FLORAL BINDING PROTEIN21* (*FBP21*), *FBP22, FBP28*, and *UNSHAVEN* (*UNS*; formerly called *FBP20*), have related expression patterns. All are mainly expressed in the vegetative tissues of the plant, however some differences in expression patterns have been observed (Immink *et al.*, 2003). It is not yet clear if an upregulation of expression of either of these *Petunia SOC1*/*TM3* clade genes upon floral transition takes place (as for *SOC1* in *Arabidopsis*).

Transposon insertion knockout mutants have so far only been identified for *UNS* and *FBP28*. The *uns* and *fbp28* single mutants are similar to

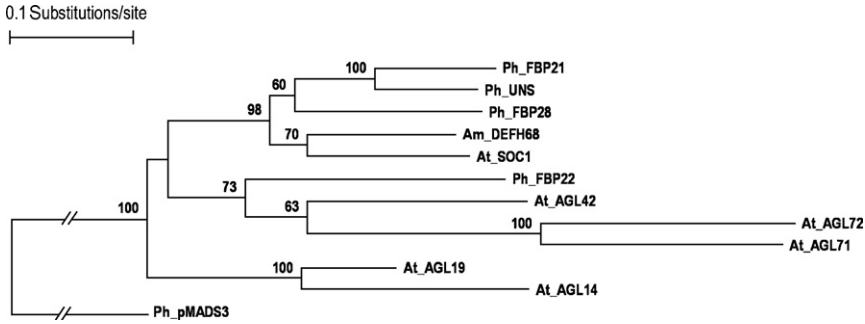

Fig. 3. Neighbor-joining tree of *SOC*/*TM3* clade MADS-box genes from *P. hybrida, A. thaliana*, and *A. majus*. The tree was rooted with *pMADS3*, a *P. hybrida* member of the *AG* clade. Altogether, 1000 bootstrap samples were generated to assess support for the inferred relationships. Local bootstrap probabilities (in percentages) are indicated near the branching points for branches with >50% support. This neighbor-joining tree, and the ones shown in Figs. 4–7, were obtained according to the methodology described previously (Vandenbussche *et al.*, 2003a).

wild-type plants, indicating that if the *Petunia SOC1*/*TM3* genes have a function in floral transition or development, they probably act redundantly.

Constitutive expression of *UNS* under control of the *Cauliflower mosaic virus* 35S (CaMV 35S) promoter indicated that *UNS* might nevertheless have a function in floral transition similar to that of *SOC1*. *UNS* overexpression leads to an acceleration of flowering, as also found in *SOC1* overexpressing plants. In addition, these *UNS* overexpressing transgenic plants exhibit ectopic trichome formation on floral organs and a conversion of petals into organs with leaf-like features, the so-called unshaven floral phenotype (Ferrario *et al.*, 2004). Ferrario *et al*. set up an experiment to find out if part of, or the whole phenotype could be due to a dominant-negative action of the protein, rather than showing the native protein function. A truncated version of *UNS*, lacking the MADS-box domain, was introduced. This truncated protein was shown not to be translocated to the nucleus, and any phenotype resulting from its introduction in the plant could, therefore, only be due to a dominant negative action of the protein. With overexpression of a truncated version of *UNS* the same floral phenotype, accompanied by a delay in flowering, was obtained. Thus, the conclusion was that the ''unshaven'' phenotype had nothing to do with the protein's function. However, the early flowering of the plants constitutively expressing *UNS* under control of the CaMV 35S promoter did represent the native function of the UNS protein (Ferrario *et al.*, 2004). As for its *Arabidopsis* homolog *SOC1*, overexpression of *UNS* has a promotive effect on flowering, which indicates that *UNS* is most likely also involved in the floral transition. The absence of a phenotype for the *uns* mutant leads to the conclusion that, contrary to *SOC1, UNS* must act in a redundant manner, probably with other *SOC1*/*TM3* genes.

The *Arabidopsis FRUITFULL* (*FUL*) gene belongs to the *APETALA1*/*SQUAMOSA* (AP1/SQUA) clade as do *AP1* and *CAULIFLOWER* (*CAL*). *FUL* plays a redundant role with *AP1* and *CAL* in *LFY* upregulation, thus promoting floral meristem specification. Moreover, *FUL* was found to have a floral meristem identity promoting activity independent of *LFY* (Ferrandiz *et al.*, 2000).

In *Petunia* four genes have so far been identified that belong to the *AP1*/*SQUA* clade: *PETUNIA FLOWERING GENE* (*PFG*), *FLORAL BINDING PROTEIN26* (*FBP26*), *FBP29* and *P. hybrida FRUITFULL*-like (*PhFL*) (Fig. 4). All of these harbor a paleo*AP1*/eu*FUL*-motif just like the *Arabidopsis FUL* gene (Ferrandiz *et al.*, 2000; Immink *et al.*, 1999, 2003; Litt and Irish, 2003; Vandenbussche *et al.*, 2003a). *FBP26, FBP29*, and *PFG* are expressed in most plant tissues, except stamens. Highest expression levels for *PFG* are found in vegetative and inflorescence meristems (Immink

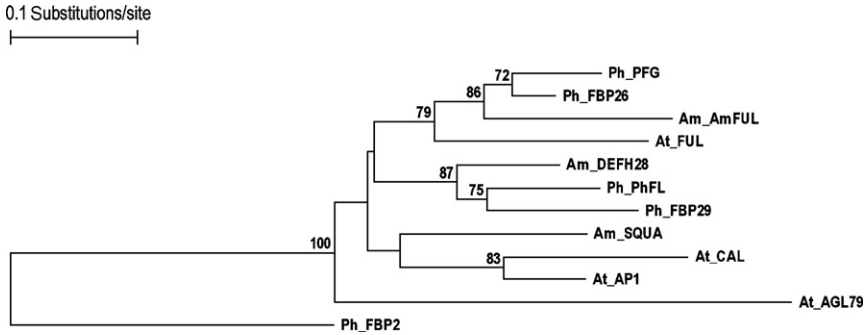

Fig. 4. Neighbor-joining tree of *SQUA/AP1* clade MADS-box genes from *P. hybrida, Arabidopsis, A. majus*, and a tomato *TM6* lineage gene. The tree was rooted with *FBP2*, a *P. hybrida* member of the *SEP* clade. See legend of Fig. 3 for technical details.

et al., 1999, 2003). No expression studies have been performed for *PhFL*. Vandenbussche *et al.* (2003b) isolated knockout alleles for both *FBP26* and *PFG* and demonstrated that single insertion mutants do not exhibit a phenotype when homozygous. No transposon insertion mutants have yet been found for *FBP29* or *PhFL*.

An indication for a role for *PFG* in the floral transition came from transgenic plants in which *PFG* expression was inhibited by cosuppression. In these plants the formation of inflorescences was completely blocked and vegetative growth was maintained, with the morphological characteristics typical of vegetative growth (Immink *et al.*, 1999). This nonflowering phenotype of *PFG* cosuppression plants is much more drastic than the slightly late flowering phenotype of *ful* single mutants. The flower-promoting activity of the *Arabidopsis FUL* gene is thought to be largely obscured by other highly redundant activities (Ferrandiz *et al.*, 2000). Not surprisingly, when the mutants were analyzed, not only the expression of *PFG* but also that of *FBP26* turned out to be downregulated (Immink *et al.*, 1999). This could well be expected as the putative protein sequences of *FBP26* and *PFG* are very similar. Vandenbussche *et al.* (2003b) showed that, in contrast to the *PFG* cosuppression line which gave a drastic nonflowering phenotype, homozygous *fbp26/pfg* double mutants only exhibit a subtle phenotype. Thus, to obtain the drastic nonflowering phenotype of the *PFG* cosuppression line, besides downregulation of *PFG* and *FBP26* at least a third gene needs to be knocked out. Looking at the sequences of *PFG* and *FBP26*, candidates to be knocked out by the *PFG* cosuppression construct (CaMV 35S promoter with the full-length *PFG* gene) are likely found in the *AP1/SQUA* clade of MADS-box genes.

III. MERISTEM IDENTITY GENES: INFLORESCENCE AND FLOWER ARCHITECTURE

Inflorescence architecture is highly variable in *Petunia*. In some species, the inflorescence consists of a single flower, whereas other species generate more complex inflorescences with multiple flowers arranged in various patterns. The diversity in inflorescence architecture is the result of a difference in action of meristematic cells, also called stem cells, in the inflorescence meristem of the different species. Development and maintenance of stem cells in general, both in inflorescence meristems as well as in the vegetative shoot apical meristem, is governed by regulatory circuits that integrate cues from different cellular origins, like the meristem itself or the young lateral organ primordia. Several genes have been identified that play an important role in these processes. *WUSCHEL* (*WUS*) expression is required for stem cell maintenance, while the *CLAVATA* (*CLV*) genes act antagonistically by inhibiting the proliferation of stem cells in a feedback loop with *WUS* (Brand *et al*., 2000; Laux *et al*., 1996; Schoof *et al*., 2000). Sharing labor with *WUS* is *SHOOTMERISTEMLESS* (*STM*), which is required to suppress differentiation throughout the meristem dome, thus allowing stem cell division to occur, while the daughter cells differentiate into organs (Lenhard *et al*., 2002).

Stuurman *et al.* (2002) identified the *Petunia WUS* homolog *TERMINATOR* (*TER*, also called *PhWUS*) and the *Petunia STM* homolog *PhSTM*, in a study on the *Petunia HAIRY MERISTEM* (*HAM*) gene. The *HAM* gene is essential for shoot apical meristem maintenance. *HAM* is a GRAS protein family member, like the *Arabidopsis SCARECROW* protein, which is required to prevent stem cells in the root meristem from adopting the fate of their differentiated neighbors (Bolle, 2004; Sabatini *et al*., 2003). The *Petunia HAM* protein was shown to act in parallel with *TER/PhWUS*, and is required for the cellular response to *TER/PhWUS* and *PhSTM*. *HAM* mRNA is expressed in L3-derived cells of lateral organ primordia and stem provasculature. This expression pattern suggests that the *HAM* gene acts non-cell-autonomously in a signaling system through which the differentiating tissues play a role in maintaining the undifferentiated state of the shoot apical meristem (Stuurman *et al*., 2002). Like *Arabidopsis wus* mutants, *Petunia ter* mutants stop shoot development after the first true leaves, continuously reiterating ectopic leaves and defective meristems from flat apices. This leads to very bushy plants that flower only occasionally. When flowers appear on these plants they have fewer organs per whorl, strongly resembling *wus* mutants (Laux *et al*., 1996; Stuurman *et al*., 2002).

A. *PETUNIA* INFLORESCENCE ARCHITECTURE

Members of the Solanaceae, such as *Petunia*, are considered to possess a cymose inflorescence that terminates in a flower. Growth continues from a sympodial meristem in the axis of this flower (Child, 1979). The formation of floral meristems in wild-type *Petunia* plants starts with the simultaneous generation of two bracts by the inflorescence meristem, before a bifurcation of the central dome yields two diversifying meristems (Souer *et al.*, 1998). One develops as a determinate floral meristem, that soon after the bifurcation starts to generate sepals, the first floral organs. The other remains meristematic and will continue with a new division, perpendicular to the last division, to form two new bracts and a new floral meristem. The same floral meristem initiation pattern is also found in tomato and pea, where flower formation also involves bifurcation of the inflorescence meristem (Souer *et al.*, 1998). *Petunia* inflorescence development thus is mainly directed by two processes: bifurcation at a predetermined position and induction of floral meristem identity. Once these processes have taken place and the floral meristem is established, meristem identity genes are necessary to determine the position of floral organ primordia. The last step than is the determination of organ identity for the primordia, which is regulated according to the ABCDE-model of flower development (see in a later section).

The *Petunia EXTRA PETALS* (*EXP*) gene is required for the split of the inflorescence apex into a floral and an inflorescence meristem (Souer *et al.*, 1998). *exp* mutant inflorescences consist of a single terminal flower that almost completely lacks the pedicel. Several lines of evidence indicate that the formation of a terminal flower in *exp* mutants is due to the complete transformation of the apical meristem into a floral meristem. First, no remains of the inflorescence meristem are detectable after this transformation. Moreover, the flower is located apically and once a terminal flower is generated the *exp* mutant loses its apical dominance (just like wild-type plants from which the inflorescence is manually removed). Consequently, the dormancy of the vegetative meristems in the axils of existing leaves is broken and a new stem with leaves will be generated from these axils, which will terminate again with the formation of a single flower (Souer *et al.*, 1998). The *exp* mutant is not the only *Petunia* mutant in which sympodial branching is lost and a single solitary flower is formed per infloresence; *hermit* and *sympodial* mutants also show this phenotype. These three mutants represent at least two different loci. *EXP* and *HERMIT* have been transposon tagged and cloned and are now being studied in more detail (Angenent *et al.*, 2005).

In *Arabidopsis*, meristem identity genes, such as *LFY*, *AP2*, and the *AP1/SQUA* clade genes *AP1, CAL*, and *FUL*, which are expressed in the newly

formed floral primordia, are responsible for the fate of floral meristems, and thereby their determinacy (Bowman *et al.*, 1993; Ferrandiz *et al.*, 2000; Weigel *et al.*, 1992). *FUL* plays a redundant role with *AP1* and *CAL* in *LFY* upregulation, thus promoting floral meristem specification (Ferrandiz *et al.*, 2000). Ditta *et al.* (2004) demonstrated by mutant and overexpression analysis that the *SEPALLATA* clade gene *SEP4* also plays a role in promoting flower meristem identity. In the complete absence of meristem identity gene activity, the floral meristems remain fully or partially inflorescence meristems, the apparent default pathway.

ABERRANT LEAF AND FLOWER (*ALF*) is the *Petunia* ortholog of *LFY* from *Arabidopsis* (Gerats *et al.*, 1988; Souer *et al.*, 1998). A study showed that *LFY* and *ALF* are very similar both in structure and in function in specifically inducing floral fate during the reproductive phase, whereas *LFY* homologs from mosses have a truly different biochemical function (Maizel *et al.*, 2005). No differences between *alf* and wild-type plants can be detected during their vegetative phase. Only after transition of the vegetative shoot meristem to an inflorescence meristem the differences become evident. The *alf* mutant inflorescence is a continuously bifurcating structure bearing only bracts, but no flowers because floral meristems fail to adopt their identity and develop as inflorescence meristems instead. The expression of *ALF* marks the formation of the floral meristem in the inflorescence (as *LFY* expression does in *Arabidopsis*), before the bifurcation of the apex becomes visible (Souer *et al.*, 1998). As is clear from scanning electron microscope studies, the bifurcation of the inflorescence meristem takes place as normal in *alf* mutants, and it is only the subsequent transition from inflorescence meristem identity to floral meristem identity that is affected. *exp*/*alf* double-mutant plants, like *alf* mutants, have an indeterminate inflorescence that contains bracts and completely lacks flowers. In addition, sympodial branching is lost in the *exp*/*alf* double mutants due to the *exp* mutation. *EXP* and *ALF* thus function in two distinct processes (Souer *et al.*, 1998).

DOUBLE TOP (*DOT*) is the *Petunia* ortholog of *UNUSUAL FLORAL ORGANS* (*UFO*) of *Arabidopsis*, and is, together with *ALF*, required to specify floral meristem identity (Tobena-Santamaria *et al.*, 2002).

All four *AP1/SQUA* clade genes identified in *Petunia* so far harbor a paleo*AP1*/eu*FUL*-motif and therefore are most likely *FUL*-homologs (as noted), but there are undoubtedly more clade members (Litt and Irish, 2003; Vandenbussche *et al.*, 2003a). The available single mutants (*fbp26* and *pfg*) exhibit a wild-type phenotype. At this point, therefore, no conclusions can be drawn on the role of *FUL* or *AP1* homologs in *Petunia* meristem identity. To ascertain these roles, first the entire set of *Petunia AP1/SQUA*

clade genes needs to be known and mutants need to be isolated for all of them.

Several *Petunia* genes are known to be required for marking the boundaries between different floral organ primordia, and thereby determining the position of the different primordia. The *Petunia NO APICAL MERISTEM* (*NAM*) gene and its identified putative orthologs from *Arabidopsis CUC1–CUC3* are involved in the formation of the shoot apical meristem during embryogenesis and are required for establishing the boundary between the cotyledons (Aida *et al.*, 1997; Souer *et al.*, 1996; Vroemen *et al.*, 2003). The *CUC* genes are thought to act upstream of *SHOOTMERISTEMLESS* (*STM*), as they are redundantly required for the expression of *STM* in the initiation pathway of the shoot apical meristem (Aida *et al.*, 1999). The expression pattern of the *Petunia NAM* gene in the inflorescence suggests that the *NAM* gene product acts very early in floral development, as the gene expression already marks the boundaries between different primordia before their separation becomes visible. The phenotype of occasional flowers on *nam* mutants (which in most cases even fail to produce the first leaves, let alone flowers) indicates that *NAM* is required in the cells around the stamen primordia in whorl three to prevent this region from developing into a primordium (Souer *et al.*, 1996). Even though *NAM* is also expressed at other sites in the inflorescence apex (e.g., at the boundaries of the site at which bract primordia will appear, and between developing carpel primordia), no corresponding phenotypic changes are observed in *nam* inflorescences. This is most likely due to redundancy in *NAM* function at these sites, as *NAM* is a member of a gene family; likewise, a high degree of redundancy was found for its *Arabidopsis* homologs, the *CUC*-genes (Souer *et al.*, 1996; Vroemen *et al.*, 2003). It will be interesting to analyze the effect of the *nam* mutation on the expression of *PhSTM*, to see if the relation between *CUC* and *STM* as found in *Arabidopsis* is conserved in *Petunia*.

In *Arabidopsis* the *SUPERMAN* (*SUP*) gene is involved in establishing a boundary between whorls three and four and in ovule development (Sakai *et al.*, 2000). *PhSUP1* from *Petunia* can partly complement the *Arabidopsis sup* mutant, indicating *PhSUP1* is an ortholog of *SUP* (Nakagawa *et al.*, 2004). *PhSUP1* plays a role in ovule development in *Petunia* as *SUP* does in *Arabidopsis*. Moreover, the gene may contribute to flower morphogenesis by preventing over-progression of intercalary growth. Presumably, this particular role of *PhSUP1* has co-evolved with the flower structure of *Petunia*. *PhSUP1* also seems to play a role in placenta and anther morphogenesis. In summary, the early floral meristem function and late function in ovule development of the *SUP* gene, originally discovered in *Arabidopsis*, are conserved in *Petunia*. Furthermore, *PhSUP1* has some additional functions

in placenta and anther morphogenesis, which have not been described for the *Arabidopsis SUP* gene (Nakagawa *et al.*, 2004).

As in the process of floral transition, hormones are also involved in inflorescence and flower formation. Tobena-Santamaria *et al.* (2002) analyzed the *FLOOZY* (*FZY*) gene, which is involved in synthesizing a signalling compound, most likely auxin, required for floral organ initiation. In *fzy* mutants the formation of floral organ primordia in the outermost three floral whorls and one of the two bracts at the base of the flower is blocked at an early stage (Tobena-Santamaria *et al.*, 2002).

IV. FLORAL ORGAN IDENTITY DETERMINATION

During floral organogenesis, five different types of organ primordia emerge from the floral meristem and differentiate into the floral organs. These floral organs are organized in concentric whorls: sepals, petals, stamens, carpels, and in the center of the flower, the placenta bearing the ovules. What organ is formed where is specified by a combinatorial action of five functional classes of genes. This was first formulated in the famous ABC-model, which has formed the foundation for our understanding of floral development, and was later extended with two extra functional classes D and E (Angenent *et al.*, 1994, 1995; Coen and Meyerowitz, 1991; Honma and Goto, 2001; Pelaz *et al.*, 2001). Almost all of the identified players in this model belong to closely related paralogous lineages of the MADS-box gene family. The different lineages have arisen by duplication events, although the exact timing of the duplications and the exact relationship of the lineages to each other is not yet fully known (Becker *et al.*, 2000; Nam *et al.*, 2003; Purugganan *et al.*, 1995; Theissen *et al.*, 2000; see Irish, Chapter 3 and Kramer and Zimmer, Chapter 9). The B- and C-function lineages appear to be among the oldest lineages, as genes belonging to these clades are involved in the development of the female and male reproductive organs already in gymnosperms (Becker *et al.*, 2000; Tandre *et al.*, 1998). Younger lineages, which are angiosperm specific, are those of the *SQUA/AP1* clade and *SEP* clade (Litt and Irish, 2003). One of the most important characteristics of MADS-box proteins is that they can form (multimeric) protein complexes with each other and probably also with other partners (de Folter *et al.*, 2005; Gutierrez-Cortines and Davies, 2000; Immink *et al.*, 2003; Masiero *et al.*, 2002). This efficiently creates a large collection of different transcription activation complexes that can regulate different sets of target genes, thus resulting in the formation of specific organs at specific times and specific positions in the floral meristem (Theissen and Saedler, 2001).

A. THE A-FUNCTION GENES

In *Arabidopsis* two genes are generally considered to represent the A-function: the MADS-box gene *APETALA1* (*AP1*) and *AP2*, the only non MADS-box gene in the ABC-model so far. However, *AP1* may be interpreted as a meristem identity and flower induction pathway gene, as it does not truly belong to this functional class. *AP1* function is not essential to identify sepals and petals, as it is actually the overexpression of *AGL24* in *ap1* mutants that is responsible for many aspects of the *ap1* floral phenotype, including defects in the first and second whorl floral organ development. Some floral organ defects of *ap1–1* mutants, especially the absence of petals, can partly be rescued by the absence of *AGL24* in an *ap1/agl24* double mutant (Kramer and Hall, 2005; Yu *et al*., 2004).

The *Arabidopsis AP2* gene fulfils two roles in the process of floral organ identity determination: a cadastral function consisting of repressing the C-function gene *AGAMOUS* (*AG*) and promoting an organ specification function in the perianth (sepals and petals) (Jofuku *et al*., 1994). In *Antirrhinum* the *AP2*-like genes *LIPLESS1* (*LIP1*) and *LIP2* are together essential for sepal and petal specification, but unlike the *ap2* mutants in *Arabidopsis*, the *lip1*/*lip2* double mutants do not show any ectopic C-class gene expression (Keck *et al*., 2003). In *Petunia* three *AP2*-like genes have been identified: *P. hybrida APETALA2A* (*PhAP2A*), *PhAP2B*, and *PhAP2C*. *PhAP2A* has a high overall sequence similarity with the *Arabidopsis AP2* gene and a similar expression pattern during flower development, suggesting that they are orthologs. *PhAp2B* and *PhAp2C* encode for *AP2*-like proteins that belong to a different subgroup of the *AP2* family of transcription factors and exhibit divergent, nearly complementary expression patterns during flower development compared to *PhAp2A*. The only clear overlap in expression between the three *PhAp2* genes is in the endosperm where all three are strongly expressed (Maes *et al*., 2001). *PhAP2A* is the functional ortholog of the *Arabidopsis AP2* gene, as it can complement the *Arabidopsis ap2–1* mutant. Surprisingly, several *phap2a* transposon insertion mutants in which the *PhAP2A* gene was knocked out, did not exhibit a mutant phenotype in floral development. Thus, *PhAP2A* is not essential for normal perianth development (Maes *et al*., 2001). Because the sequences of *PhAP2B*/*PhAP2C*, and their expression patterns during flower development, are very different from those of *PhAP2A*, it is very unlikely that they are functionally equivalent, and would act in a redundant way in the *phap2a* mutant. *Petunia* thus might differ from both *Antirrhinum* and *Arabidopsis* in this respect. The A-function, as encoded by *AP2* of *Arabidopsis* and *LIP1* and *LIP2* from *Antirrhinum*, does not seem to exist as such in *Petunia*. It seems as if these

three species have each found a different way to encode the A-function. In *Arabidopsis* one gene both has a cadastral and an organ identity function. In *Antirrhinum* two homologous genes function in organ identity specification together, while other genes must be responsible for the cadastral function. In *Petunia* all the knowledge suggests that *AP2*-like genes are not involved in either organ identity specification, or setting boundaries for expression of C-function genes.

Nevertheless, a *Petunia* A-function mutant has been known for a long time, *blind* (*bl*) (Maes *et al.*, 2001; Vallade *et al.*, 1987). Unfortunately the identity of the *BLIND* (*BL*) gene is still unkown. *bl* mutant flowers display a homeotic conversion of the corolla limb into antheroid structures in the second whorl and, under certain conditions, homeotic conversion of the tips of the first whorl sepals into carpelloid tissue (Vallade *et al.*, 1987). The *bl* phenotype is quite variable, but the pistil tube is never affected and the mutant does not show the complete A-function conversion as observed in the *Arabidopsis ap2* mutant.

Tsuchimoto *et al.* (1993) and Kater *et al.* (1998) demonstrated that the *bl* phenotype is caused by ectopic expression of the C-function genes *pMADS3* and *FBP6* in the first two floral whorls of the *bl* mutant. In addition, ectopic expression of *pMADS3* and *FBP6* was also observed in leaves of the *bl* mutant, although the *FBP6* hybridization signal was only detectable after long exposure. These results indicate that the *BL* gene product is involved in the suppression of both petunia *AG* homologs in leaves and in the first two floral whorls (Kater *et al.*, 1998).

In search of the *BL* gene, Mayama *et al.* (2003) studied the *Petunia* orthologs of one of the *Arabidopsis* cadastral genes, *CURLY LEAF* (*CLF*), which is required to repress transcription of the class C gene *AG* in the first and second floral whorls and also in vegetative organs. *CLF* encodes for a protein with extensive similarity to the product of the *Drosophila* Polycomb-group gene *Enhancer of zeste* (*E*(*Z*)) (Goodrich *et al.*, 1997). *Petunia* harbors at least two *CLF* homologs (*PhCLF1* and *PhCLF2*). The two *PhCLF* proteins share two conserved domains with related proteins. Both *PhCLF1* and *PhCLF2* are expressed in all the floral organs, but the amounts of *PhCLF1* and *PhCLF2* transcripts differ. The *PhCLF1* transcript contains alternatively spliced RNA species encoding proteins truncated in the C-terminal region. Neither *PhCLF1* nor *PhCLF2* appears to coincide with the *BL* gene, but their expression is affected by homeotic transformations in the *bl* mutant flower (Mayama *et al.*, 2003).

An important step in understanding how the A-function is regulated in *Petunia* will be the discovery of the sequence underlying the mutation causing the *bl* phenotype. Currently, everything points in the direction that

at least some aspects of A-function regulation in *Petunia* will be organized differently compared to *Arabidopsis*.

B. THE B-FUNCTION GENES

The most extensively studied B-function genes are from *Arabidopsis* and *Antirrhinum, AP3* and *PISTILLATA* (*PI*), and *DEFICIENS* (*DEF*) and *GLOBOSA* (*GLO*), respectively. They are mainly expressed in the second and third whorl, consistent with their function in petal and stamen identity specification. The *DEF/AP3* and *GLO/PI* lineage genes are thought to represent paralogous genes that arose from a duplication event that occurred before the origin of the angiosperms (Kramer *et al.*, 1998; Purugganan, 1997; Theissen *et al.*, 2000; Kim *et al.*, 2004). The AP3 and DEF proteins form heterodimers with respectively PI and GLO (Riechmann *et al.*, 1996; Schwarz-Sommer *et al.*, 1992). These heterodimers are important in the autoregulation of the expression of *DEF/AP3* and *GLO/PI*, as the heterodimer formation enhances the initially low-expression levels of the genes and maintains their expression (Honma and Goto, 2000; Saedler and Huijser, 1993; Schwarz-Sommer *et al.*, 1992; Zachgo *et al.*, 1995). This was long believed to be the general system of B-function, but more and more deviating systems are being discovered in different species. Often, for one or both of the gene lineages *DEF*/*AP3* and *GLO*/*PI* more representatives are found which can have (partly) redundant but also diverged functions. Not uncommon is a shift in the expression pattern of one or more B-function genes, often resulting in different floral morphologies (Kanno *et al.*, 2003; Kramer *et al.*, 2003; Nakamura *et al.*, 2005).

1. GLO/PI *lineage genes*

While *Arabidopsis* and *Antirrhinum* each have only one *GLO/PI* lineage gene, *Petunia* harbors two *GLO*/*PI* lineage genes: *P. hybrida GLOBOSA1* (*PhGLO1*; formerly called *FBP1*) and *PhGLO2* (formerly called *PMADS2* or *FBP3*) (Fig. 5). In wild-type *Petunia* flowers, the expression domain of *PhGLO1* and *PhGLO2* is mainly confined to the second and third whorl, and signals are slightly stronger in younger buds (Angenent *et al.*, 1992; van der Krol *et al.*, 1993; Vandenbussche *et al.*, 2004). The expression patterns of *PhGLO1* and *PhGLO2* are thus very similar to those of their *Arabidopsis* and *Antirrhinum* counterparts. *PhGLO1* and *PhGLO2* act largely redundant in petal and stamen formation. The differences between the function of the two genes become visible as unique phenotypical aspects of *phglo1* single mutants: petal midveins are greenish (sepaloid) and stamen filaments are not fused to the petal tube. This indicates that *PhGLO1*, and not *PhGLO2*,

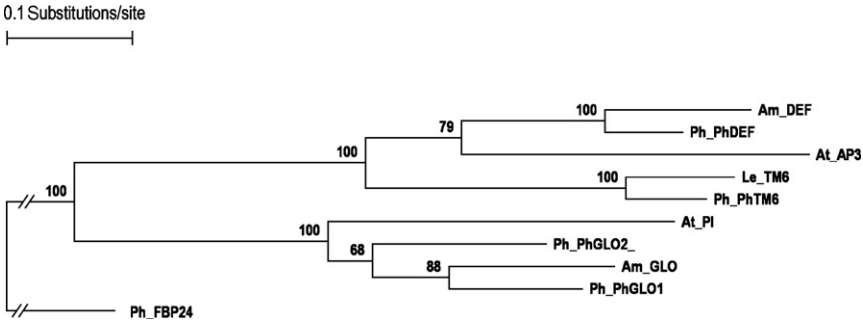

Fig. 5. Neighbor-joining tree of B-class MADS-box genes from *P. hybrida, Arabidopsis, A. majus*, and a tomato *TM6* lineage gene. The tree was rooted with *FBP24*, a *P. hybrida* member of the B_{sister} (B_s) MADS-box subfamily (Becker *et al.*, 2002). See legend of Fig. 3 for technical details.

controls the formation of the petal midvein and growth under the zone of petal and stamen initiation, which causes the corolla tube and stamen filaments to emerge as a congenitally fused structure (Vandenbussche *et al.*, 2004). Apart from these differences the two *Petunia GLO*/*PI* lineage genes act redundantly in petal and stamen formation and only the *phglo1*/*phglo2* double mutant shows a complete conversion from petals to sepals and stamens to carpels. The two *Petunia GLO*/*PI* genes together thus function in the same way as their *Arabidopsis* and *Antirrhinum* orthologs *PI* and *GLO*.

2. DEF/AP3 *lineage genes*

Within the *DEF*/*AP3* lineage, two clades can clearly be distinguished on the basis of their completely different C-terminal motifs (Kramer *et al.*, 1998). The first motif is referred to as the paleo*AP3* motif and is found in *DEF*/*AP3* proteins from basal eudicots, magnoliids monocots, and basal angiosperms, while a second type, named the eu*AP3* motif, is uniquely present in *DEF*/*AP3* proteins from core eudicots. A number of core eudicot species contain both the eu*AP3* and paleo*AP3* type of genes, termed eu*AP3* and *TOMATO MADS BOX GENE6* (*TM6*) lineages, respectively (Kramer and Irish, 2000).Vandenbussche *et al.* (2003a) have shown that the eu*AP3* motif most likely resulted from a simple frameshift mutation in one of the copies of the duplicated ancestral paleo*AP3*-type gene. Lamb and Irish (2003) published data indicating that paleo*AP3* and eu*AP3* motifs encode different functions: a chimeric construct made up of an *Arabidopsis AP3*-gene, containing a paleo*AP3*-motif from *Dicentra eximia* instead of its own eu*AP3*-motif, could not rescue petal formation in an *ap3* mutant. In contrast to

these findings, Whipple *et al.* (2004) demonstrated that the full-length maize paleo*AP3* encoding gene *Silky* is capable of identifying and properly regulating the genes necessary for normal petal and stamen development in the *Arabidopsis* eudicot flower. Therefore, at the moment it is not clear what the overall functional significance is of the acquirement of the new eu*AP3* motif during evolution.

Both the *Antirrhinum DEF* gene and the *Arabidopsis AP3* gene belong to the "modern" clade of the *DEF*/*AP3* lineage and harbor a eu*AP3*-motif. These species do not have an ancestral *DEF*/*AP3*, with a paleo*AP3*-motif. *Petunia* however harbors both types present within the *DEF*/*AP3* lineage: *PhDEF* (formerly known as *GREEN PETAL* (*GP*) or *PMADS1*), and *P. hybrida TM6* (*PhTM6*) (Angenent *et al.*, 1992; Kramer and Irish, 2000; van der Krol *et al.*, 1993; Vandenbussche *et al.*, 2004). The *PhDEF* gene contains a eu*AP3* motif, while the *PhTM6* gene contains a paleo*AP3* motif. Thus, while several core eudicots apparently have lost the gene copy containing the paleo*AP3* motif, *Petunia*, as well as at least two other Solanaceous species, tomato and potato, harbors a paleo*AP3* as well as a eu*AP3* gene (Fig. 5) (Kramer *et al.*, 1998; Vandenbussche *et al.*, 2003a). For a recent and more comprehensive overview of B-class MADS-box gene phylogeny, we refer to Kim *et al.* (2004).

3. PhDEF

In wild-type *Petunia* flowers, the expression domain of the eu*AP3*-type gene *PhDEF* is mainly confined to the second and third whorl, with slightly stronger expression in younger buds (Angenent *et al.*, 1992; van der Krol *et al.*, 1993; Vandenbussche *et al.*, 2004). Low levels of *PhDEF* are detectable in the first and fourth whorls (Tsuchimoto *et al.*, 2000; Vandenbussche *et al.*, 2004), which has also been reported for *DEF* in *A. majus* (Schwarz-Sommer *et al.*, 1992). Surprisingly, mutations in *PhDEF* cause homeotic transformations only in one whorl: petals are converted to sepals, whereas stamens remain unaffected (de Vlaming *et al.*, 1984; van der Krol *et al.*, 1993). This indicates that *PhDEF* is essential for petal formation, but might act redundantly with other factors in stamen development (Vandenbussche *et al.*, 2004).

4. PhTM6: *an atypical and interesting B-function gene*

While the expression patterns of *PhGLO1, PhGLO2*, and *PhDEF* are very similar to those of their *Arabidopsis* and *Antirrhinum* counterparts, the expression of the paleo*AP3*-type gene *PhTM6* differs drastically (Vandenbussche *et al.*, 2004). In small buds the strongest signals for *PhTM6* transcripts are detected in carpels and stamens, while the expression level in sepals and petals

is much lower. Later in development, the expression level for *PhTM6* remains high in the fourth whorl, while declining in the stamens at the time of maturation (Vandenbussche *et al*., 2004). Remarkably, the expression pattern of *PhTM6* thus is much more C-class-like. Moreover, in the A-function *blind* (*bl*) mutant, *PhTM6* expression is extended from the third and fourth whorl to all floral whorls, which is exactly what happens with the expression pattern of the *Petunia* C-class MADS-box genes *pMADS3* and *FBP6* (see later section).

The *PhTM6* expression pattern offers a logical explanation for the phenotype of both *phdef* flowers and *phdef*/*bl* double mutant flowers (see later section). Since in wild type plants, *PhTM6* is mainly expressed in whorls three and four, *PhDEF* is the only *DEF*/*AP3* lineage member expressed at high levels in petals, while expression of both *PhDEF* and *PhTM6* in anthers suggests that they might act redundantly in stamen formation. Likewise, *phdef* mutants only display a homeotic conversion of petals to sepals, while anthers remain virtually unaffected (Vandenbussche *et al.*, 2004). The question remains whether it is the lack of expression of *PhTM6* in the second whorl, or the inability of the paleo*AP3*-clade protein PhTM6 itself, that blocks a function in the petal developmental program.

Although the full homeotic conversion of petals to sepals in *phdef* single mutants suggests full absence of B-function activity in the second whorl of *phdef* flowers, *phdef*/*bl* double mutants develop antheroid structures in the second whorl, as in *bl* single mutants, although one would rather expect carpels in the second whorl as would be predicted for an A/B double mutant. This indicates ectopic B-function activity in the second whorl of *phdef*/*bl* flowers, which is not present in *phdef* single mutants, suggesting that the ectopic *PhTM6* expression in the *bl* mutant background might account for this (Vandenbussche *et al*., 2004).

5. *Interactions between the* Petunia *B-function proteins*

The *phdef*/*phglo2* double mutant shows a complete conversion of petals to sepals and stamens to carpels, which clearly demonstrates that the PhTM6-PhGLO1 heterodimer is either not formed or not sufficient to confer petal and stamen identity. Yeast two-hybrid studies suggest that this might be due to the specificity of the PhTM6 protein for PhGLO2, as PhTM6 only interacts with PhGLO2 and not with PhGLO1, while the PhDEF protein does interact with both PhGLO1 and PhGLO2 (Vandenbussche *et al.*, 2004). *PhTM6* together with *PhGLO2* expression on the other hand, is sufficient to induce stamen development. This is supported by the phenotype of the *phdef*/*phglo1* double mutant, which shows no additional phenotype compared with the *phdef* single mutant (Vandenbussche *et al*., 2004).

In conclusion, the *PhGLO1* and *PhGLO2* genes act largely redundantly in petal and stamen formation, with the only apparent differences between the two being the function of *PhGLO1* in the formation of the petal midveins and the fusing process of stamen filaments and tube. More divergence is observed in the *DEF/AP3*-lineage. *PhTM6* apparently does not have a function in petal development, where *PhDEF* has. In addition, while *PhDEF* expression is sufficient for stamen formation together with either *PhGLO1* or *PhGLO2, PhTM6* interacts specifically with *PhGLO2* and not with *PhGLO1* in the induction of stamen development (Vandenbussche *et al.*, 2004). Analysis of *phtm6* single mutants and double and triple mutants of *phtm6* with the other *Petunia* B-function gene mutants *phdef, phglo1*, and *phglo2*, will certainly provide more clarity on the B-function as encoded in *Petunia*.

The C-class expression pattern of the B-function gene *PhTM6* allows for speculation on the origin of *PhTM6* and B-function genes in general. At this point it is impossible to decide whether the C-class expression pattern of *PhTM6* reflects the original function of *PhTM6* (and thus of B-function genes in general), or that these characteristics are the result of a divergence in function that is specific for *Petunia* (or maybe Solanaceaous species). The *PhTM6* homologs from tobacco and potato or other Solanaceaous species, have not yet been studied in enough detail to allow a final interpretation.

C. THE C-FUNCTION GENES

In *Arabidopsis* the gene responsible for the C-function is *AGAMOUS* (*AG*). Loss of *AG* function results in the conversion of stamens into petals and in the absence of the fourth whorl carpels, which are replaced by indeterminate perianth whorls (Yanofsky *et al.*, 1990). *AG* thus has two functions: establishing stamen and carpel organ identity and maintaining meristem determinacy. In *Petunia*, and other species like *Antirrhinum* and maize (Davies *et al.*, 1999; Mena *et al.*, 1996) the C-function is encoded by two or more genes in a redundant manner.

Two *Petunia* genes are known with sequences highly homologous to that of *AG*: *Petunia MADS3* (*pMADS3*) and *floral-binding protein 6* (*FBP6*) (Angenent *et al.*, 1993; Tsuchimoto *et al.*, 1993) (Fig. 6). At an early stage, when the sepal primordia become apparent on the flanks of the floral meristem, *pMADS3* and *FBP6* transcripts start to accumulate in cells that later give rise to the stamen and carpel primordia. When the stamen primordia are clearly visible and carpel primordia start to develop, *pMADS3* and *FBP6* are expressed throughout the central part of the floral apex that develops into the pistil. No expression can be detected in sepal or petal primordia. At later stages during flower development, *pMADS3* and *FBP6*

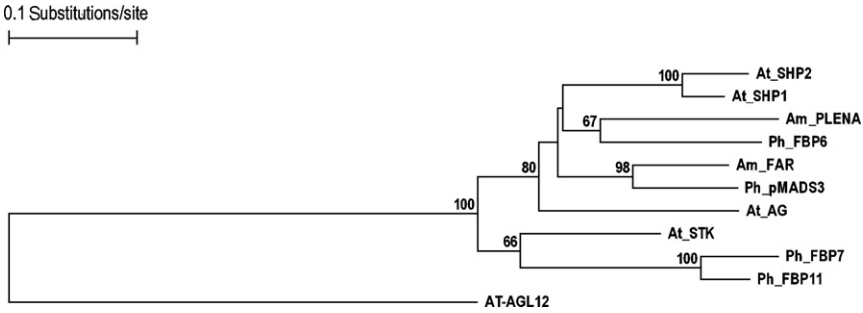

Fig. 6. Neighbor-joining tree of C- and D-class MADS-box genes from *P. hybrida, Arabidopsis*, and *A. majus*. The tree was rooted with *AGL12*, an *Arabidopsis* MADS-box gene. See legend of Fig. 3 for technical details.

become differentially expressed. *FBP6* then is highly expressed in the stigma and transmitting tissue of the style, while *pMADS3* is more abundant in the ovules, vascular tissue and the nectaries (Kater *et al*., 1998). Kapoor *et al*. (2002) found that the gene structure of *pMADS3* is consistent with that of the other dicot C-function genes. In *AG, cis*-elements including the binding sites for regulatory proteins have been identified in the second intron. The same kind of *cis*-elements (*LEAFY*, homeodomain protein, and MADS-box protein consensus binding sites), and a conserved stretch of 70 bp, were found in the second intron of *pMADS3* (Kapoor *et al*., 2002).

Due to a lack of transposon insertion mutants that knock out the expression of *pMADS3* or *FBP6*, the functional characterization of these two genes has so far only been carried out by the analysis of overexpression and cosuppression mutants (Kapoor *et al*., 2002; Kater *et al*., 1998; Tsuchimoto *et al*., 1993). The conclusion can be drawn that *pMADS3* is the *Petunia* ortholog of *AG* and is required for stamen and carpel development (Kapoor *et al*., 2002; Kater *et al*., 1998; Tsuchimoto *et al*., 1993). Several lines of evidence support this conclusion: first, the spatial and temporal expression pattern of *pMADS3*, and the overall sequence similarity with other C-function genes completely correspond with a C-function role. Second, in the *Petunia* A-function mutant *blind, pMADS3* is ectopically expressed in the first and second whorl, where the homeotic conversions take place: corolla limbs into antheroid tissue and small parts of sepals into carpelloid tissue (Tsuchimoto *et al*., 1993). Thirdly, transgenic plants overexpressing *pMADS3* under control of the constitutive CaMV 35S promoter phenocopy the A-function mutant *blind*. These transgenics show petal limbs that are largely reduced in size and have antheroid tissue at the fusion site of the petals.

Their sepals are curled up at the tip and stylar and stigmatic tissues are sometimes present on these sepal tips (Kater *et al.*, 1998; Tsuchimoto *et al.*, 1993). A fourth indication for the function of *pMADS3* came from a transposon insertion mutant, in which the transposon was inserted in one of the *pMADS3* introns in such a way that it induced *pMADS3* overexpression. This was the third type of plant in which *pMADS3* was overexpressed, and again it showed the same *blind*-like mutant phenotype (Kater *et al.*, 1998).

In contrast to *AG* and *PLE* (Mizukami and Ma, 1992; Saedler and Huijser, 1993), ectopic expression of *pMADS3* is not able to induce a complete homeotic conversion of the sepals and petals into reproductive organs. The sepals are typically largely unchanged, and especially the petal tube is always completely unaffected. This suggests that C-activity repression in the outer two whorls might be difficult to override by ectopic C-function gene expression (also implying that repression of C-activity in the *blind* mutant is not completely abolished), or alternatively, that *pMADS3* requires additional factors to give a full spectrum of C-function activity. On the other hand, ectopic expression of cucumber *AG* homolog *CUM1* in *Petunia* did result in a much more complete conversion of petals to anthers and sepals to carpels (Kater *et al.*, 1998). At first sight, this might also point in the direction that the C-function in *Petunia* is controlled by two or more genes whose functions are combined in this single cucumber gene, *CUM1* (Kater *et al.*, 1998). Equally possible, the cucumber CUM1 protein is less prone to C-activity repression in the outer whorls of the *Petunia* flower due to the heterologous nature of this experiment.

An obvious candidate for defining the C-function together with *pMADS3* is *FBP6*. Yet, despite the similarities between *pMADS3* and *FBP6* with respect to sequence and expression pattern, overexpression of *FBP6* did not result in a homeotic conversion of sepals into carpels and petals into stamens (Kater *et al.*, 1998). In line with this, in the *blind* mutant in which both *pMADS3* and *FBP6* are overexpressed, there was no additional phenotype when compared to the *pMADS3* overexpressor (in which *FBP6* was normally expressed in whorls three and four). Only the *fbp6*/*pmads3* double mutant will give solid proof if it is really *pMADS3* together with *FBP6* that defines the C-function in *Petunia*, or whether additional genes are involved.

All analyzed *pMADS3* overexpressors only gave indications for a role of *pMADS3* in stamen and carpel development. However, the phenotype of the transgenic plants in which the *pMADS3* gene was silenced (Kapoor *et al.*, 2002), suggests an additional function for *pMADS3* in controlling determinacy in the flower as has been found for *AG* (Yanofsky *et al.*, 1990). Silencing of *pMADS3* resulted in homeotic conversion of stamens into petaloid structures, whereas the carpels were only weakly affected. But most remarkable

were the emerging ectopic secondary inflorescences from the interstamenal region in the third whorl, while the fourth-whorl carpels were unaffected. Third-order inflorescences emerged at corresponding positions in the third whorl of inner flowers of secondary inflorescences, indicating reiterative conversion of parts of the floral meristem into an inflorescence meristem (Kapoor *et al*., 2002). Noteworthy is that, whereas *ag* mutant flowers develop indeterminate floral organs in the fourth floral whorl, the *pMADS3* knock-out plants demonstrate indeterminate organ formation in the third floral whorl. It is interesting to speculate on the question whether this is a fundamental difference between *Petunia* and *Arabidopsis*, or whether it is simply the absence of the carpels in *Arabidopsis* that makes the difference, while the location of formation of indeterminate organs is in fact the same. In *Arabidopsis* a negative feedback loop in the floral meristem, involving *WUS*, the floral meristem identity gene *LEAFY* (*LFY*) and the C-function gene *AG*, is thought to be responsible for *WUS* suppression in the floral meristem. Suppression of *WUS* then leads to termination of the floral meristem (Lenhard *et al.*, 2001; Lohmann *et al.*, 2001). In *Petunia, pMADS3* together with the E-function protein FBP2 could be responsible for terminating meristematic activity in the third whorl region of the floral meristem, by suppressing *TER* in the center of the flower (see later section and Kapoor *et al*., 2002).

D. THE D-FUNCTION GENES

1. Pistil and ovule development in Petunia

The *Petunia* pistil is composed of two completely fused carpels that arise separately from the floral apex in the center of the flower. Only immediately after the induction of sepal, petal, and stamen primordium formation are the two carpel primordia morphologically distinguishable. The two horseshoe-shaped primordia soon fuse to form a circular structure (Angenent *et al*., 1995). This primordial cylinder extends, and before the gynoecium closes at the top, the placenta starts to develop in the center of the flower. As the gynoecium closes, style formation starts. The style elongates and transmitting tissue differentiates to form a tract through which pollen tubes can grow (Angenent *et al*., 1995). At the same time ovule primordia arise from the placental tissue as a dense group of meristematic cells. Within each ovule primordium a single megasporocyte is formed from which eventually a seven-cell embryo sac develops. During this process, the ovule becomes stalked and an integument is initiated at the base of the nucellus. This integument elongates and grows over the nucellus and finally forms the micropyle. At the micropyle, a pollen tube penetrates the ovule to deliver

the sperm cell into the embryo sac for the double-fertilization process (Angenent *et al.*, 1995).

In 1995, a novel functional class of MADS-box genes, highly homologous to C-class MADS-box genes (Fig. 6), was discovered in *Petunia*, involved in ovule development (Angenent *et al.*, 1995; Colombo *et al.*, 1995). More recently, the MADS-box genes involved in the process of ovule development in *Arabidopsis* were described (Favaro *et al.*, 2003; Pinyopich *et al.*, 2003). The genes in *Petunia* are *FLORAL BINDING PROTEIN7* (*FBP7*) and *FLORAL BINDING PROTEIN11* (*FBP11*). The putative protein products of these genes share ~90% of their overall amino acid sequence (Angenent *et al.*, 1995). At the sequence level the putative proteins of *FBP7* and *FBP11* are most similar to the *Arabidopsis SEEDSTICK* (*STK*, formerly called *AGAMOUS LIKE11*), which was shown to play a role in ovule development in *Arabidopsis* (Pinyopich *et al.*, 2003). The *STK* gene is also required for normal development of the funiculus, a stalk-like structure that connects the developing seed to the fruit, and for dispersal of the seeds when the fruit matures. In promoting ovule identity, *STK* acts redundantly with the C-class genes *SHATTERPROOF1* (*SHP1*), *SHP2*, and *AG* (Pinyopich *et al.*, 2003).

FBP7 and *FP11* are expressed in the center of the gynoecium before ovule primordia become visible. At a later stage they are restricted to the ovules, predominantly the endothelium, which is the innermost cell layer of the integument. The expression levels of both *FBP11* and *FBP7* increase immediately after pollination and decline in developing seeds (Colombo *et al.*, 1995, 1997).

The conclusion that *FBP11* and *FBP7* encode a new floral function that specifies ovule identity was based on the analysis of *FBP11* cosuppression plants. In the ovary of these transformants, at many of the positions normally taken up by ovules carpelloid spaghetti-shaped structures developed. These carpelloid structures originate directly from the placenta and consist of tissues characteristic of style and stigma. Although at early developmental stages, irregular structures with a chimeric identity were observed, the identity of these structures in mature ovaries was more uniform. Either these structures elongated and developed into carpelloids, or morphologically normal ovules were formed (Angenent *et al.*, 1995). All seeds produced by these ovules had developmental defects (Colombo *et al.*, 1997). The frequency of ovule conversion seemed to be related to the residual *FBP11* expression in the mutants. Low-residual gene expression in the primary transformants was sufficient to overcome a certain threshold, required for normal ovule development. As the overall sequence similarity between *FBP11* and *FBP7* is very high, it was not surprising to find that in the *fbp11* cosuppression plants,

the expression of *FBP7* was also reduced to approximately the same extent as *FBP11* (Angenent *et al.*, 1995). The suppression of *FBP7* expression could also point to a regulatory role for *FBP11* determining *FBP7* expression levels. This is quite unlikely, however, since in the *FBP11* overexpression mutants, *FBP7* expression was not upregulated. Thus, although primordia are still formed from the placenta without *FBP7* and *FBP11*, the expression levels of *FBP11* and *FBP7* determine which type of development takes place after this primordium formation, that is, toward the formation of real ovules or carpelloid structures (Cheng *et al.*, 2000).

The phenotype of the *FBP11*/*FBP7* cosuppression mutant is reminiscent of that of the *stk*/*shp1*/*shp2* triple mutant. In the *stk*/*shp1*/*shp2* triple mutant, normal ovule and seed development is completely disrupted, with some ovules converted to leaf-like or carpel-like structures. In addition, the *Arabidopsis* C-class gene *AG* was also found to play a role in promoting ovule identity (Pinyopich *et al.*, 2003). If the redundancy between D- and C-class genes is conserved between *Arabidopsis* and *Petunia*, this would suggest that in *Petunia FBP11* and *FBP7* might act redundantly with C-function genes *pMADS3* and/or *FBP6*.

When ectopically expressed, *FBP11* can induce the formation of ovule-like structures on sepals, and, rarely, on petals (Colombo *et al.*, 1995). The presence of ovule-like structures on the adaxial side of the sepals is accompanied by a transformation of the sepal inner epidermis into placenta-like tissue. However, even though ovule-like structures are sometimes also found on the petals of these *FBP11* overexpressing plants, there their presence is not accompanied by the presence of placenta-like tissue (Colombo *et al.*, 1995). Ectopic expression of *FBP11* thus is sufficient to promote ovule development, as is ectopic expression of *STK* in *Arabidopsis* (Favaro *et al.*, 2003; Pinyopich *et al.*, 2003).

2. *Interacting proteins*

In yeast two-hybrid experiments FBP11 was shown to interact specifically with the three very closely related E-function (*SEPALLATA*) MADS-box proteins *FLORAL BINDING PROTEIN2* (*FBP2*), *FBP5*, and *FBP9* (Ferrario *et al.*, 2003; Immink *et al.*, 2002). Furthermore, Immink *et al.* (2002) demonstrated in a *FRET-FLIM* experiment that *FBP11* is only transported to the nucleus when a physical interaction takes place with the E-function protein *FBP2*. Expression analysis showed that *FBP2, FBP5*, and *FBP9* are expressed in ovules (Ferrario *et al.*, 2003). Further, *in situ* hybridization on sepals of the *FBP11* overexpression plants revealed the presence of *FBP2* mRNA in the ectopically formed ovules. This suggests that there might be a function for *FBP2-FBP11* and possibly *FBP5-FBP11* protein

complexes in ovule development. Definite proof that the *SEP*-genes are involved in ovule development comes from the *fbp2*/*fbp5* double mutants (see later section), in which leaf-like organs emerge from the positions normally occupied by ovules in the wild type. Remarkably, hardly any ectopic ovules were found on floral organs other than the sepals in the *FBP11* overexpression plants, even though *FBP2* is also expressed in petals and stamens (Colombo *et al.*, 1995; Immink *et al.*, 2002). This suggests the presence of (an) other interaction partner(s), indispensable for ovule formation. An indication that these other interaction partners of *FBP11, FBP7*, and *SEPALLATA* proteins might be C-funtion proteins came from experiments on *Arabidopsis* proteins. Favaro *et al.* (2003) showed that the *Arabidopsis* counterparts of these *Petunia* proteins *STK, AG, SHP1*, and *SHP2* can form multimeric complexes and that these interactions require *SEP* proteins.

E. THE E-FUNCTION GENES

Indications for the existence of an E-function were presented in 1994 based on the phenotypes of *FLORAL BINDING PROTEIN2* (*FBP2*) and (*TOMATO MADS5*) *TM5* cosuppression lines in *Petunia* and tomato, respectively (Angenent *et al.*, 1994; Pnueli *et al.*, 1994). However, the E-functional class was generally accepted and understood only in 2000, when Pelaz *et al.* published a triple mutant of the *Arabidopsis* homologs of *FBP2*/*TM5*, the *SEPALLATA* genes *SEP1* (formerly called *AGAMOUS-LIKE2*), *SEP2* (*AGL4*), and *SEP3* (*AGL9*). From this *sep1*/*sep2*/*sep3* mutant it was evident that B and C floral organ identity functions require SEP1, SEP2, and SEP3 for the formation of petals, stamens, and carpels because in the triple mutant all these organs are converted into sepals. In addition, these three genes are required to prevent the indeterminate growth of the flower meristem (Pelaz *et al.*, 2000). Ditta *et al.* (2004) characterized another *SEPALLATA* gene *SEP4* (formerly called *AGL3*), which turned out to be involved in the flower meristem identity and organ identity together with the other three *SEP*s. Although the *sep4* single mutant appears wild type, the floral organs are converted into leaf-like organs in *sep1*/*sep2*/*sep3*/*sep4* quadruple mutants, indicating the involvement of all four *SEP* genes in the development of sepals. Moreover, *sep4* also contributes to the development of petals, stamens, and carpels, and plays an important role in meristem identity (Ditta *et al.*, 2004).

In *Petunia*, six genes have so far been identified that belong to the *SEP* clade: *FLORAL BINDING PROTEIN2* (*FBP2*), *FBP4, FBP23, FBP5, FBP9*, and *PETUNIA MADS BOX GENE12* (*pMADS12*) (Angenent *et al.*, 1992; Ferrario *et al.*, 2003; Immink *et al.*, 2003; Vandenbussche *et al.*, 2003b).

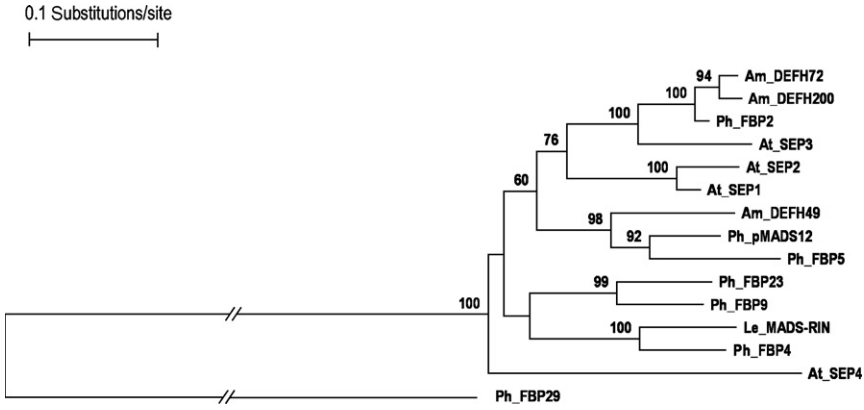

Fig. 7. Neighbor-joining tree of *SEP* clade MADS-box genes from *P. hybrida, Arabidopsis, A. majus*, and a tomato *SEP* clade gene. The tree was rooted with *FBP29*, a *P. hybrida* member of the *AP1*/*SQUA* clade. See legend of Fig. 3 for technical details.

See Fig. 7 for a simplified phylogeny of the *Petunia SEP* clade genes together with those of some other species. In an article by Zahn *et al.* (2005), a thorough phylogenetic analysis of the entire *SEP*-clade can be found. *FBP2, FBP5, FBP23*, and *pMADS12* are solely expressed in the floral domains, whereas *FBP4* and *FBP9* are also expressed outside of the floral organs (Ferrario *et al.*, 2003). *FBP5* and *pMADS12* transcripts can be detected already in the inflorescence meristem, while *FBP2* can only be detected later, in the central dome of the floral meristem, after it splits from the inflorescence meristem. In contrast to *FBP2*, the expression of both *FBP5* and *pMADS12* can be detected throughout the floral meristem, whereas at later stages when sepal primordia emerge, it becomes confined to the inner three floral whorls, like *FBP2* (Ferrario *et al.*, 2003). *FBP4, FBP9*, and *FBP23* are expressed in all floral whorls, except for the stamens. Furthermore, *FBP4* is also expressed in bracts, while *FBP9* accumulates in all green tissues of the plant; no transcript of *FBP23* can be detected in vegetative tissues. *FBP23* and *FBP4* are expressed in seed pods (Ferrario *et al.*, 2003). More details on expression patterns and protein–protein interactions for the *Petunia* E-function genes can be found in Ferrario *et al.* (2003).

In *Arabidopsis* three *SEP* genes need to be knocked out to obtain a full conversion of the second, third, and fourth whorl organs to sepals and meristem indeterminacy in the center of the flower (Pelaz *et al.*, 2000). The *Petunia FBP2* cosuppression mutant showed a quite similar phenotype.

An explanation for this might be that the E-function in *Petunia* is mainly encoded by a single gene, more particularly *FBP2*. But alternatively, cosuppression in these transgenics might have caused downregulation of multiple *SEP* genes simultaneously. Research suggests that the latter possibility most likely explains this seeming difference between *Petunia* and *Arabidopsis*. Ferrario *et al.* (2003) showed that another *SEP* homolog, *FBP5*, a gene unknown at the time of publication of the *FBP2* cosuppression experiments, was downregulated together with *FBP2*. Moreover, the phenotype of *fbp2*/*fbp5* double mutants in which the expression of *fbp2* and *fbp5* was specifically knocked out by transposon insertions (Vandenbussche *et al.*, 2003b, see later section) still was much less severe than that of the *FBP2* cosuppression mutants and the *Arabidopsis sep1*/*sep2*/*sep3* triple mutants. Therefore, in the *FBP2* cosuppression lines, at least three *SEP* genes must be downregulated. This indicates that the full E-function in *Petunia* is redundantly encoded by multiple *SEP* genes, as has been found in *Arabidopsis*. Nevertheless, the unique phenotype of *fbp2* insertion mutants and the *fbp2*/*fbp5* double mutant indicate differences in the degree of redundancy among the *SEP* genes between *Arabidopsis* and *Petunia*.

Two independent insertion alleles for *FBP2*, which both contained a *dTph1* insertion in the K-domain region, were identified. Plants homozygous for either insertion allele display an identical phenotype, in which the normal shaped petals exhibit an overall diffuse green hue, which is strongest in the areas surrounding the main veins and at the edges of the petals (Vandenbussche *et al.*, 2003b), indicating a partial conversion of petal to sepal identity in these regions. The most remarkable phenotype, however, is the presence of secondary inflorescences in the third whorl, positioned between the stamens near the nectaries at the base of the pistil. These secondary inflorescences are formed relatively late during development, when all organs of the primary flower have already been formed, and they rarely develop beyond a very young stage. The appearance of secondary inflorescences strongly suggests a loss of determinacy in the third whorl, and is exactly what Kapoor *et al.* found in flowers in which the C-function gene *pMADS3* is downregulated (see previous part and Kapoor *et al.*, 2002). In *Arabidopsis* loss of C-function is also associated with indeterminacy in the center of the flower, although the location of the formation of indeterminate floral organs seems different in *Arabidopsis* (as previously discussed and Yanofsky *et al.*, 1990). A yeast four-hybrid experiment revealed interactions between the FBP2 protein, a B-function heterodimer, and the C-function protein pMADS3 (Ferrario *et al.*, 2003). All together, these results strongly indicate that *FBP2*, together with *pMADS3*, is essential for meristem identity (Vandenbussche *et al.*, 2003b).

While loss of *FBP2* function by itself is sufficient to induce an E-function mutant phenotype, *fbp5* single mutants morphologically appear as wild-type (Vandenbussche *et al.*, 2003b) suggesting functional redundancy as observed in *Arabidopsis*. This was indeed confirmed by the phenotype of *fbp2/fbp5* double mutants. Flowers of *fbp2-2/fbp5-1* double mutants display an enhanced phenotype compared with *fbp2* mutant flowers. The petals of *fbp2-2/fbp5-1* plants show an increased petal-to-sepal conversion compared with *fbp2* petals, and sepal-like structures covered by trichomes develop on top of the anthers. In the fourth whorl a dramatic phenotypical change occurs in the *fbp2-2/fbp5-1* mutants: a huge pistil-like structure, without transmitting tissue, develops, covered with trichomes and often consisting of more than two carpels that never fuse at the top. Inside these pistils, leaf-like organs develop instead of ovules, supporting a function for *FBP2* and/or *FBP5* in directing ovule development, as discussed before. The development of secondary inflorescences in the third whorl of the double mutant is not enhanced significantly compared with the *fbp2* mutant (Vandenbussche *et al.*, 2003b).

The enhanced phenotype of *fbp2-2/fbp5-1* double mutants, compared with *fbp2* mutants, demonstrates that *FBP2* and *FBP5* act in a largely redundant manner, while *FBP2* has a unique function in the maintenance of deteminacy in the third whorl. Furthermore, the sepaloid characteristics of the petals, stamens, and pistil of the *fbp2-2/fbp5-1* double mutant indicate that *FBP2* and *FBP5* are required for B and C organ identity functions as the *Arabidopsis SEP* genes are. *FBP2* is essential for meristem determinacy, most likely together with the C-function gene *pMADS3*. Compared with the *Arabidopsis SEP* gene analyses (Ditta *et al.*, 2004; Goto *et al.*, 2001; Pelaz *et al.*, 2000), the research on these two *Petunia SEP* genes already shows that clear differences in redundancy, within the *SEP* clade, exist between the two species. These differences in redundancy between species can be very helpful in uncovering functions that would otherwise be missed. The role of the *Arabidopsis SEP* genes in ovule development could only be determined by indirect evidence (as discussed in an earlier section) because the phenotype of the *sep1/sep2/sep3* triple mutant is so strong that no ovary is formed at all in the fourth whorl. The *Petunia fbp2/fbp5* double mutant, however, does make ovaries, and clearly shows that these *SEP* genes are essential for ovule formation.

So far, for only two *Petunia SEP*-genes a detailed functional analysis has been performed. The expression patterns and different protein–protein interaction partners suggest different roles for the other *Petunia SEP*-genes. In order to fully analyze the functions and redundancy within this subfamily, transposon insertion mutants will have to be identified for all of the genes belonging to this subfamily.

V. CONCLUSIONS

So far, the research on the genetic regulation of floral transition in *Petunia* has focused mostly on genes from two MADS-box gene clades: representatives of the *TM3*/*SOC1* clade, and FUL-like genes from the *AP1*/*SQUA*-clade. Genes from these clades were shown to be important in floral transition in *Petunia* in a redundant manner, as is also the case for their *Arabidopsis* homologs. More extensive work has been done on meristem identity genes and their role in inflorescence architecture. With its cymose inflorescence *Petunia* clearly differs from racemose species like *Arabidopsis* and *Antirrhinum*, implying that the initiation of meristematic processes in inflorescence development will proceed differently.

Three main processes direct *Petunia* inflorescence and flower architecture: the bifurcation of the inflorescence meristem in two parts, the determination of floral meristem identity in one part, and the establishment of boundaries between the different floral organ primordia. The *EXP* gene is essential for the bifurcation process, while *ALF*, the *Petunia* ortholog of *LFY*, is indispensable in the establishment of floral meristem identity. After that, meristem identity genes like *PhSUP* and *NAM* are involved in determining the boundaries between different floral whorls and thus in positioning of the floral organ primordia. More genes important in determining inflorescence architecture in *Petunia* are known and are being studied, so considerable progress can be expected in this field in the years to come.

To date, the most intensively studied part of floral development in *Petunia* is the process of floral organ patterning. However, regarding the A-function numerous questions still remain (see Irish, Chapter 3; Kramer and Zimmer, Chapter 9; Soltis *et al.*, Chapter 12). The function of the *Petunia PhAP2* seems different from that of its *Arabidopsis* ortholog *AP2*; alternatively, the *PhAP2* gene may act in a redundant manner with other genes. And most important: which gene product is affected in the *blind* A-function mutant? It will be interesting to see if the A-function as encoded by the *BL* gene is conserved in other species.

Petunia harbors four B-function genes: two *GLO*/*PI* lineage representatives that are nearly completely complementary and two *DEF*/*AP3* lineage genes, of which *PhDEF* harbors a eu*AP3* motif, while *PhTM6* represents the ancestral gene with a paleo*AP3* motif. The *Petunia* B-function gene set clearly shows the result of divergence in function that has occurred after duplication of both the ancestral *AP3*/*DEF*-lineage gene (probably at the same time the core eudicots arose), and the *GLO*/*PI*-lineage gene (probably more recent). Divergence in function is most obvious in the *DEF*/*AP3* gene lineage. Whereas the eu*AP3* gene *PhDEF* contributes to petal and stamen

formation, the paleo*AP3* gene *PhTM6* is involved only in stamen formation. Moreover, the *PhTM6* protein has evolved a dimerization preference for *PhGLO2*. These two genes offer a great opportunity to study the significance of gaining a novel C-terminal motif and gene expression pattern shifts in evolution. In addition, research on the *Petunia* B-function genes revealed a novel function specifically controlled by one of the possible petunia B-function heterodimers. In wild-type *Petunia* flowers, as in many other species of Solanaceae, the stamen filaments are partially fused to the petal tube, probably out of the necessity to support the long thin filaments in an upward position. In both *phglo1* and *phdef* mutants, the stamens emerge as free-standing structures, indicating that the *PHDEF*/*PHGLO1* heterodimer specifically controls this process. In *Arabidopsis* and *Antirrhinum*, such a function does not exist, since anthers emerge as free-standing structures in these species. This might be an example of a subtle difference in function that accounts for species-specific differences in floral architecture.

Two *AG*-homologs have been identified in *Petunia*: *pMADS3* and *FBP6*. *pMADS3* has been shown to be required for stamen and carpel development, while the role of *FBP6* is less clear; there might even be other *AG*-homologs and/or different genes involved in the C-function process. *pMADS3* is also thought to be involved in meristem determinacy in the third floral whorl, together with the E-function gene *FBP2*. The *Petunia* D-function genes *FBP7* and *FBP11* are involved in ovule formation and thereby also important for seed formation. In the ovule formation process, D-function proteins act together with E-function proteins, while C-function proteins might very well be involved, too.

Like *Arabidopsis, Petunia* has several different *SEP*/*AGL2* clade genes. So far only two of the six *Petunia SEP*/*AGL2* clade genes have been analyzed in detail. *FBP2* and *FBP5* were found to act in a redundant manner in the development of petals, anthers, carpels, and ovule formation. The *Petunia SEP*/*AGL2* genes vary in sequence, expression pattern, and protein–protein interaction partners. This, together with the mutant phenotypes of the *fbp2* single and the *fbp2*/*fbp5* double mutant, leads to the conclusion that the *Petunia SEP*/*AGL2* clade genes have diverged in function. A detailed study on the other clade members will have to show what functions have been acquired by its other representatives. Though we have focused on MADS-box genes for which functional data are available, genes belonging to other major MADS-box gene family clades have also been identified in *Petunia*; these can thus also become a subject of further research.

The analyses of the regulatory systems in *Petunia* floral development contribute to the elucidation of the mechanisms that have been at work in the evolutionary development of the flower as a sophisticated set of organs

that ensure successful reproduction. Moreover, further comparative research will enable us to better understand the molecular basis for the enormous diversity in floral (organ) development and function. One of the main forces in this process undoubtedly has been the high rate of gene duplications, resulting initially in a release of selection pressure as long as the original function is maintained by both duplicates. Subsequent divergence in gene sequence in either of the copies may lead to a shift in gene expression or a change in protein structure, thereby enabling a divergence in function. Both of these two overall mechanisms are probably important in causing functional divergence. Kramer *et al.* (2003), Kanno *et al.* (2003), and Nakamura *et al.* (2005) present several examples of how variations in gene expression patterns result in variations in floral forms. However, the diverged coding sequences of the different subfamilies within the MADS-box gene family also indicate that differences in coding sequence have a huge impact on gene function. In fact, one may conclude that it is not a matter of either/or: Nature itself provides the biggest laboratory, where all options we can think of (and more) have been and still are being tested.

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