

**Molecular analysis of
the inflorescence
architecture in
*Petunia hybrida***

The work presented in this thesis was performed at the department of Molecular Genetics, Vrije Universiteit, Amsterdam.

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Cover design by Kees en Geer RoobEEK.

Figure is based on the Greek mythology story the thread of Ariadne. Ariadne, the daughter of king Minos, helped her lover Theseus, to escape from the labyrinth with a thread, after he killed a Minotaurus, half man and half bull.

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VRIJE UNIVERSITEIT

**Molecular analysis of the inflorescence
architecture in *Petunia hybrida***

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door
Ilja Roobek
geboren te Alkmaar

promotor: prof.dr. R.E.Koes

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Chapter 1

Aim and outline of this thesis

Ilja RoobEEK

The genetic alterations that lead to the evolution of the different inflorescence architectures that we observe in nature are still poorly understood. To get a better understanding of how a cymose architecture is specified, we used *Petunia hybrida* to identify and study genes that are essential for the development of the inflorescence meristem. Comparative genetic analysis in species with different inflorescence architectures, like *Arabidopsis* and petunia, provides us insight into the genetic alterations that underlie their anatomic differences. However the development of cymes, such as the inflorescence of petunia or its close relative tomato, has not been studied as extensively as the raceme of *Arabidopsis* and therefore the knowledge about the development of a cyme is still fragmented.

In **Chapter 2**, we review all the genes that have a known function in the development of the *Petunia hybrida* inflorescence. The picture that emerges is that distinct inflorescence types are controlled by genes encoding conserved proteins.

In **Chapter 3**, we describe a new petunia mutant named *veggie*, which displays a delayed onset of flowering and a homeotic transformation of sympodial inflorescence units into vegetative shoots. The production of double mutants of *veggie* with the floral meristem identity genes *ABERRANT LEAF AND FLOWER*, *DOUBLE TOP*, *EVERGREEN* and the meristem maintenance gene *HERMIT* revealed that *VEGGIE* functions upstream of the floral meristem identity genes.

The isolation of *dTpH1* transposon tagged *veggie* mutants opened the way to isolate and characterize the *VEGGIE* gene. In **Chapter 4**, we describe the cloning of *VEGGIE* by a directed tagging approach. *VEGGIE* encodes a PEBP protein and is homologous to *FLOWERING LOCUS T (FT)* from *Arabidopsis*. *VEGGIE* shows rhythmic expression during the day that peaks at the end of the day only in long-day conditions. Grafting experiments revealed that *VEGGIE* is a mobile signal.

Although *FT* can substitute the function of *VEGGIE* in petunia and *VEGGIE* can induce early flowering in *Arabidopsis*, the function of *FT* in *Arabidopsis* is restricted to onset of flowering, whereas *veggie* mutants also show a defect in the development of the sympodial inflorescence meristem. To learn more about the function of *VEGGIE* in the sympodial inflorescence meristem, we performed a yeast two-hybrid cDNA library assay to identify binding partners of *VEGGIE*. In **Chapter 5** we describe the finding of several putative binding partners of

VEGGIE. Two of these are bZIP transcription factors that show high similarity to FD and were examined in more detail to unravel their function in cymose branching architecture.

In **Chapter 6**, we describe the identification of several *VEGGIE*-like genes, *PhBFT*, *PhIFT* and *PhMFT* and tried to unravel their phylogenetic relationships, expression patterns and studied their putative function in flowering time and growth architecture. Sub-functionalization of the various *VEGGIE*-like genes is argued for.

Chapter 1

Chapter 2

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.

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; Carroll 2005b), 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 the innovation or modification of gene products (proteins) has played an important role as well and argued that many of the cases that are cited as evidence for evolution via regulatory (*cis*) elements do not exclude alternative explanations (Hoekstra and Coyne 2007). Thus the issue is far from being 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 mentioned controversy is that transgenic experiments with non-model animal species are usually not 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 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 most dramatically

seen 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. 1).

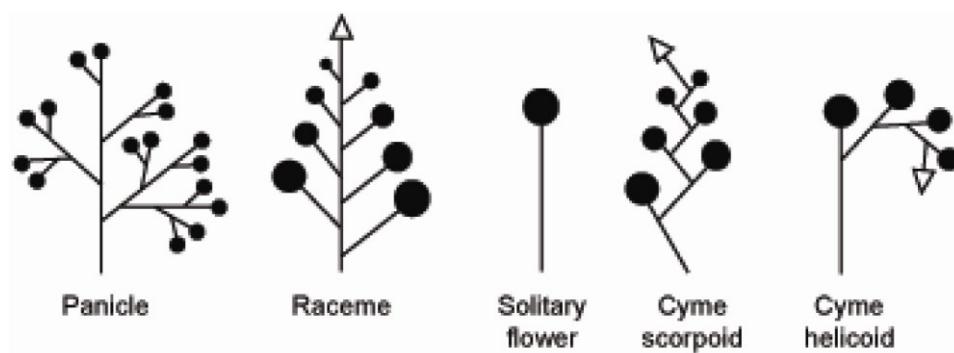


Figure 1. Various inflorescences observed in nature

Diagrams comparing two major types of inflorescence architecture growth, 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 of this are panicles and racemes (*Arabidopsis* and *Antirrhinum*). In species with sympodial growth, the apical meristem terminates in a flower (determinate), which can be a solitary flower (e.g. tulip) or a cymose inflorescence bearing many flowers (e.g. petunia). The circles indicate flowers and the triangles indicate the apical meristems.

Inflorescences can be divided into three major classes: racemes, cymes and panicles. 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 a lateral position that repeats this pattern. In panicles, all axes terminate in flowers. Each of these inflorescence types can be further divided into sub-classes based on differences in, for example, phylotactic patterns and lengths of internodes.

Recently, a theoretical model was put forward for the development and the 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 and a new lateral meristem.

Meristems initially have a vegetative (i.e., non-floral) 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 will be formed, respectively. Thus, changes in the spatio-temporal control of floral or non-floral 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*.

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 before they switch to the reproductive (flowering) phase. However, the timing of this switch to flowering can differ substantially between species. Distinct species may flower in different periods of the year (season) or at different plant ages. Some species (annuals) flower the same year in which they germinated and then die; others flower in the second year, after a cold winter period (biennials); 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 (photoperiod) is an important parameter by which plants measure progression through the seasons (Garner 1922; Garner 1933); that is, some plants ("long-day plants") flower only when the light period (day) is longer than a critical threshold value; 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 (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) signals received by distinct pathways. Ultimately, these integrators activate floral meristem identity genes such as *LEAFY* (*LFY*) and *APETALA1* (*AP1*), which promote the floral fate of meristems.

The three main floral pathway integrators in *Arabidopsis* are *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).

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 (Adams, Pearson, Hadley and Patefield 1999; Cathey and Campbell 1984; 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., feature 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 similar cues as 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 in 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 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 (Roobeek and Koes, unpublished), but also have thicker stems and altered inflorescence architecture. *VEGGIE* was recently

isolated and a detailed molecular analysis of its role in flowering is the topic of this thesis (Chapter 3).

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 it to be inactive. Expressed ubiquitously in Petunia, this 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*, *Fbp29* and *PFG*- that encode *AP1*-like MADS box proteins, although none of them appears to be a true ortholog of *AP1* or *SQUAMOSA* from Antirrhinum. Ubiquitous expression of *AP1* had no effect in Petunia (Kusters et al. unpublished). 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). As a *dTPH1* transposon insertion that interrupts 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 has a normal phenotype (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; 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. 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*.

Architecture and Development of the Petunia Inflorescence

The *Petunia* inflorescence has a typical cymose architecture and consists of an indefinite number of metamers (or anthoclaides), 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 (Napoli and Ruehle 1996; Souer et al. 1998) (Fig. 2). The two meristems in the bract axils (#2 and #3 in Fig. 2B) have a vegetative identity (i.e. upon outgrowth they form a number of leaves before they switch to flowering) and both are dormant, though to slightly different extents. That is, the vegetative meristem in the axil of the bract (meristem #2 in Figure 2B) forms a small shoot several millimeters in size before it becomes arrested, while vegetative shoot #3 in the axil of the bract remains smaller, not immediately evident to the untrained eye.

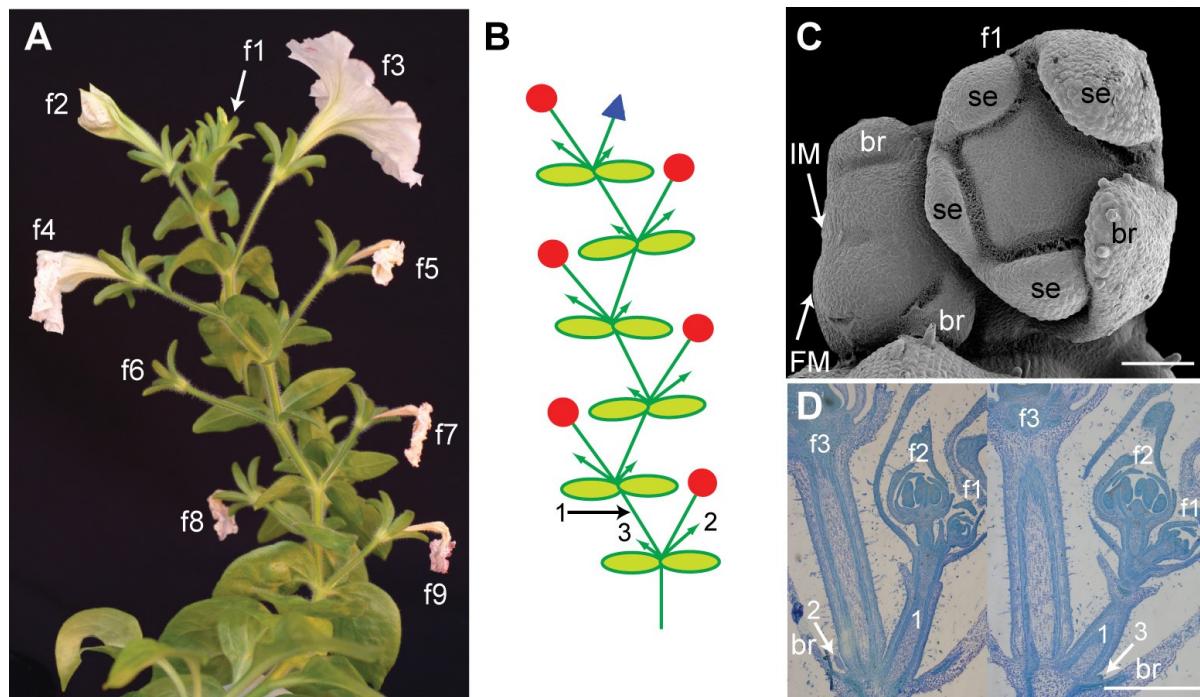


Figure 2. Petunia inflorescence architecture in detail.

A. Wild type W138 petunia with flowers from young to old (F1-F9). **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, the axillary on the side of the flowers (2) is less dormant than the one on the inflorescence shoot side (3). The circles indicate flowers and the triangle indicate the apical meristem. **C.** Scanning electron micrograph of an inflorescence apex, showing a flower (f1) where all the sepals (se) are already formed and the apex bearing 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 μ m. **D.** Two sections through an inflorescence apex showing three flowers from young to old (f1-f3) and the two bracts that support the older flower and the inflorescence shoot (s1). In the axil of the bract supporting the flower the less dormant axillary meristem forms the first leaves (2) while on the axil supporting the shoot #1, the axillary meristem forming the dormant vegetative shoot #3 is just visible. Scale bar 1 mm.

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). Note that in the next chapters of this thesis lateral inflorescences meristems (lateral IMs) are referred to as sympodial inflorescence meristems (SIMs). 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, which 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 the development of distinct inflorescences (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, a much taller line than W138 that was analyzed by others (Souer et al. 1998; Maes et al. 2001), and has in general longer internodes. In the mature V26 inflorescence the two bracts 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 the shoot #1, as an axillary shoot that is equivalent to that in the axil of the bract, but has a different identity (“sympodial” rather than vegetative) and growth rate (“accelerated”). Consequently, they view the vegetative shoot #3 in the axil of the 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 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. 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*), which is 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. The 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. 2C, 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 two weeks after the bracts are formed, by which time the apex has already generated several additional flowers (Fig. 2D). 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.

Genetic Control of Inflorescence Architecture

Correct development of the *Petunia* inflorescence depends critically on several developmental processes. First it requires that the apical meristem acquires 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.

Genes Specifying the Identity of the Floral Meristem.

Several mutants have been identified in which the apical FM fails to required for 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 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. During early vegetative development *LFY* is inactive; it 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, which occurs 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 is 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* from *Antirrhinum* and *UNUSUAL FLORAL ORGANS* (*UFO*) from *Arabidopsis* (Souer et al. 2008). Work in *Arabidopsis* suggested that *UFO* is a partially dispensable co-regulator 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), and 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 (Fig. 4E). Second, constitutive expression of *DOT* in Petunia leads to extremely early flowering, and converts the cymose inflorescence into a enlarged solitary 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 large flower. Furthermore, ubiquitous expression of *DOT* results in ectopic expression of floral organ identity genes and various homeotic transformations, including the partial conversion of leaves into petals. Thus, the 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 *DOT/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. In Petunia, however, *ALF* is expressed throughout the vegetative phase and *DOT* is inactive; hence flower formation is limited by the transcriptional regulation of *DOT* (Fig. 3B).

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 indicates 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 (Fig. 3A).

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

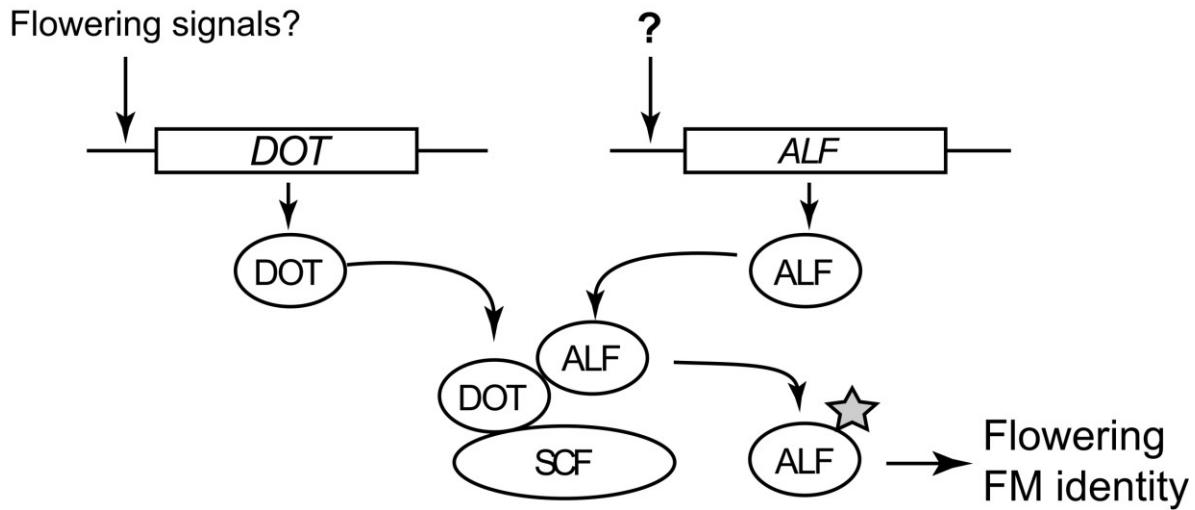
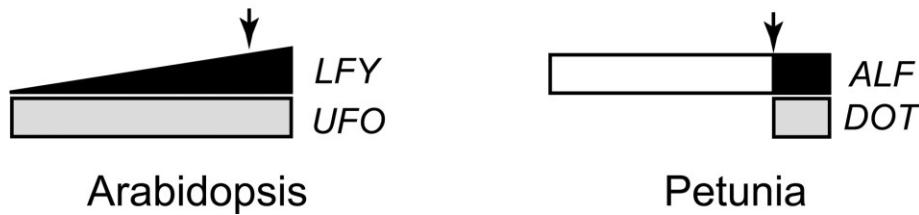
A**B**

Figure 3. Model for the function of ALF and DOT.

A. DOT expression is triggered by unknown flowering signals while ALF regulators are unknown. When ALF and DOT proteins are present in the same cells, they interact via a SCF E3-ubiquitin ligase complex which results on the post-transcriptional activation of ALF, possibly via ubiquitination (star attached to the ALF protein), resulting in the induction of flowering and specification of floral meristem identity. **B.** Graphs depicting gene expression (vertical axis) during plant development (horizontal axis) of the flower meristem identity genes *LFY* and *UFO*, from Arabidopsis, and *ALF* and *DOT*, from petunia. *UFO* is expressed since embryogenesis while *LFY* mRNA amounts rise during Arabidopsis development reaching a threshold upon which flowering is induced, hence *LFY* is the limiting factor for the transition to flowering. In petunia, *ALF* is expressed already during the vegetative phase, indicated by the white rectangle. Upon the start of *DOT* expression, ALF protein is activated (black square) and triggers flowering, suggesting that *DOT* is the petunia flowering limiting factor.

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.

Genes Specifying Identity of the Lateral Inflorescence Meristem.

Several mutations that disrupt the 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 (Fig. 4B and C) (Napoli and Ruehle 1996; Souer et al. 1998, Castel 2009). 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 (Castel 2009).

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 showed 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 (Castel 2009). 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. 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 “reversion” from reproductive to vegetative growth. However, since 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.



Figure 4. 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 again be solitary. **C.** *her* phenotype, similar to the *exp* mutant the bracts (br) support only one flower and the axillary meristems (ax). **D.** *alf* mutant phenotype, all the apical FMs have been converted into inflorescence meristem that behave like a lateral meristem making the next shoot and two new inflorescence meristems giving rise to a bushy phenotype. **E.** *dot* mutant phenotype, with a similar phenotype to *alf*. **F.** *evg* mutant phenotype. In addition to the transformation of flowers into inflorescence shoots, the inflorescence stems of an *evg* mutant fail to separate properly and grow fasciated.

Genes Specifying Initiation and Outgrowth of the Lateral Inflorescence Meristem

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 that 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. 4C), although *her* flowers display various aberrations, such as non-fused petals, petaloid anthers and malformed carpels, which are not seen in *exp/sym* flowers. Molecular and genetic evidence indicates that the *HER* plays a role in the initiation of the lateral IM similar to that which was initially (and incorrectly) proposed for *EXP* (Castel 2009)

The phenotype of *evg* resembles that of *alf* in that flowers are missing and bracts and meristems over-proliferate (Fig. 4F, Souer 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 (Fig. 4F). However, more detailed analysis of the *evg* phenotype and molecular analysis of the *EVG* gene indicated that it is, in fact, the other way around: the fasciation is a primary effect of the mutation, while the loss of FM-identity is a secondary, indirect, effect (Souer et al., 2008)

Molecular isolation and characterization of *EVG* showed that it encodes a transcriptional factor with a WUSCHEL-RELATED HOMEOBOX (WOX) domain (Souer et al. 2008). 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 (Souer et al. 2008). Moreover, the meristem domes in *evg* inflorescences are enlarged and often contain multiple domains that express *TERMINATOR*, 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 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* (Souer 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. Several 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 5 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

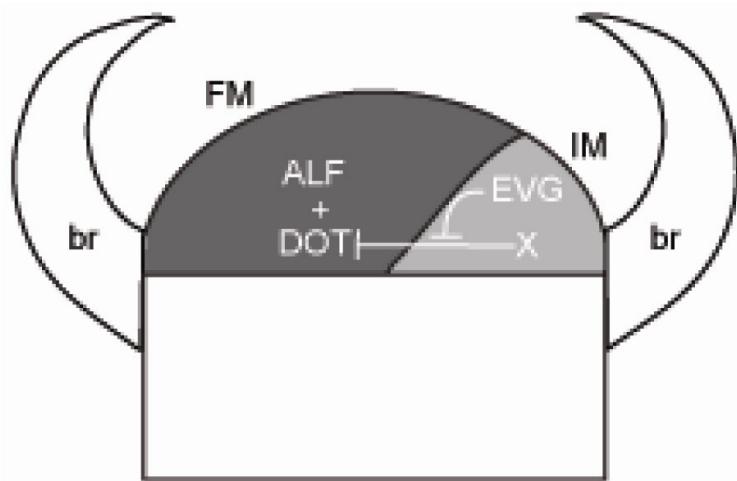


Figure 5. Model for the function of EVG on the inflorescence apex development.

The new lateral inflorescence meristem cells (IM, light grey) express *EVG* and a mobile X factor that prevents the expression of *DOT* in the apical floral meristem (FM, dark grey), where also *ALF* is 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* becomes expressed in the apical FM to specify, together with *ALF* floral fate.

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.

Concluding Remarks.

The picture emerging from 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 the latter remains to be proven. 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 2

Chapter 3

VEGGIE, a gene controlling flowering time and inflorescence architecture in *Petunia hybrida*

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Ronald Koes

Abstract

Angiosperms display a wide variation with regard to the moment that they start to flower as well as the architecture of the inflorescence that is produced. Both aspects represent important characters determining reproductive success and crop yield. The distinguishing feature of a cymose inflorescence is the emergence of a sympodial inflorescence meristem at the periphery of the apical floral meristem, by which inflorescence growth continues. We identified a new mutation in petunia, *veggie*, that strongly delays the onset of flowering and changes the cymose inflorescence into a solitary flower. Microscopic analysis showed that the defect in the inflorescence architecture results from the homeotic transformation of the (secondary) sympodial inflorescence unit into a vegetative shoot. To determine the function of *VEGGIE* in the inflorescence meristem, double mutants with known petunia meristem identity genes were produced. These findings revealed that *VEGGIE* is needed in indeterminate meristems, like the shoot apex and the sympodial shoots, to assign the inflorescence meristem identity before *VEGGIE* can act as a floral integrator to initiate the floral meristem identity genes.

Introduction

Plants species show a large diversity with regard to the time of the year (season) and/or age of the plant when they switch from vegetative growth to flowering. The correct timed flowering is essential for the reproductive success and for the survival of the plant species as it assures that all individuals flower synchronously, which is essential for cross-fertilization, and enables the species to occupy a specific ecological niche. Flowering is controlled by environmental cues like day length (photoperiod), preceding cold (winter) periods (vernalization), ambient temperature, to monitor the progression of the seasons, as well as endogenous factors like hormones. These different flowering signals are perceived and transduced by distinct pathways that ultimately converge to activate a handful of genes, known as "integrators", that promote the floral identity of meristems (Michaels, 2009; Turck et al., 2008).

Also the positions, and thereby the numbers, where flowers appear on the plant body varies amongst plant species. Some plants carry a solitary flower whereas others form clusters of flowers, known as inflorescences, with a wide variety of different architectures. Inflorescences fall into three major classes - named, panicles, cymes and racemes - with very different modes of development (Benlloch et al., 2007; Koes, 2008; Prenner et al., 2009; Prusinkiewicz et al., 2007). Inflorescence architecture is largely determined by the way that stem cells in the growing tips of branches (meristems) are patterned. In panicles, which are for example common among cereals, both the apical and lateral meristems have initially a vegetative identity and at some point all acquire simultaneously floral identity. In cymes, which are common among Solanaceae (nightshades, which includes petunia and tomato), the apical meristem rapidly acquires floral identity and subsequently terminates by forming a flower, while at its periphery a lateral or 'sympodial' inflorescence meristem (SIM) emerges, that will repeat the pattern by forming a new lateral SIM and an apical floral meristem (FM) (Lippman et al., 2008). In racemes, such as *Arabidopsis* and *Antirrhinum*, the apical meristem itself is indeterminate and grows indefinitely while flowers develop from lateral meristems that emerge on the flanks of the apical meristem .

Prusinkiewicz et al. (2007) constructed a model for inflorescence development that can generate the three major classes of inflorescences by

altering the kinetics by which apical and lateral meristems acquire floral identity (or loose vegetative identity). To establish a panicle inflorescence, both the apical and lateral meristems lose their vegetativeness with the same kinetics. In a cyme, the apical meristem acquires floral identity more quickly than the lateral meristem, whereas in a raceme the apical meristem loses vegetativeness more slowly than lateral meristems (Prusinkiewicz et al., 2007).

In the racemose *Arabidopsis* inflorescence lateral meristems acquire floral identity, or loose vegetativeness, by the expression of *LEAFY* (*LFY*) and *UNUSUAL FLORAL ORGANS* (*UFO*) (Weigel et al., 1992; Wilkinson and Haughn, 1995). In the cymose petunia inflorescence floral meristem identity is determined by the *LFY*-homolog *ABERANT LEAF AND FLOWER* (*ALF*) and the *UFO*-homolog *DOUBLE TOP* (*DOT*) (Souer et al., 2008; Souer et al., 1998). Although *ALF* and *LFY* as well as *DOT* and *UFO* encode similar and functionally exchangeable proteins they exhibit distinct spatial and temporal expression patterns, which can account for the distinct cymose and racemose architecture of the petunia and the *Arabidopsis* inflorescence (Souer et al., 2008).

To understand which genetic differences are responsible for the different development of cymes and racemes it is important to learn more about the initiation and maintenance of the SIM. Recently Castel et al. showed that the petunia gene *HERMIT* (*HER*), encoding a KNOTTED1-like homeobox (KNOX1) protein (Castel, 2009) similar to *SHOOT MERISTEMLESS* (*STM*) from *Arabidopsis* (Long et al., 1996), is expressed in all emerging meristems. In *her* mutants development of the inflorescence is arrested after the production of a solitary flower and the subsequent SIM does not emerge, which results in plants that bear a solitary flower at the end of a flowering branch. The petunia gene *EVERGREEN* (*EVG*), which encodes a transcription factor of the WUSCHEL homeobox (WOX) family, is exclusively expressed in the very young SIM and is thought to promote the proliferation of the SIM and the physical separation from the neighbouring apical FM (Rebocho, 2008). The latter is indirectly required for the activation of *DOT* in the apical FM and specification of its floral identity (Rebocho et al., 2008).

Although several genes have been identified in the cymose branching petunia that are essential for acquiring floral identity of a meristem and for reducing vegetativeness of the apical and lateral meristems, so far no genes are identified that are able to maintain the inflorescence capacity of the SIM. The

identification of a new petunia mutant, *veggie*, can help us to address the question of vegetativeness of meristems and of the specification of SIM inflorescence identity. *veggie* mutants show defects in the inflorescence architecture and in the control of flowering time resulting in plants that flower too late and bear solitary flowers instead of a wild type cymose truss. By microscopic analysis of *veggie* apices and by the production of double mutants with known petunia meristem identity genes we were able to show that *VEGGIE* is needed not only in the indeterminate meristems, like the SAM and the sympodial shoots, to acquire the inflorescence meristem identity, but is also acting as a floral inducer.

Results

The *veggie* mutant phenotype

During the vegetative growth of petunia, leaves are produced in a spiral pattern around the shoot apical meristem (SAM). Upon different endogenous stimuli, such as light, temperature and nutrients, the plant switches from vegetative to reproductive growth, and the vegetative shoot apical meristem (SAM) changes into an inflorescence meristem (IM). The IM is located between the primordia of two leaf-like structures, named bracts, and undergoes a transition into a floral meristem (FM) that develops into a flower. Simultaneously a sympodial inflorescence meristem (SIM) emerges in a lateral position at the periphery of the FM. This SIM reiterates the process of producing two bracts, an apical FM and a lateral SIM that together are a sympodial unit (SU), resulting eventually in a cymose, zig-zag shaped inflorescence that consists of numerous sympodial units that each terminate in a flower (Fig. 1A and D).

Among W138 progeny we identified a new spontaneous mutant, which we named *veggie*. *veggie* mutants have significantly thicker stems and firmer leaves compared to wild type plants and display a prolonged vegetative growth phase. Consequently, *veggie* mutants produce up to three times as much leaves as wild type plants, before the onset of flowering (25 ± 2 leaves in *veggie* mutants versus 9 ± 0.6 leaves in wild type W138 plants) (Fig. 1B).

Wild type petunia produces upon flowering an inflorescence consisting of a series of consecutive flowers, whereas *veggie* mutants generate a solitary flower and growth continues via a vegetative shoot that emerges from the axil of the

last formed bract. This shoot is initially vegetative and generates up to 20 leaves before producing again one flower and a new vegetative shoot resulting in a tall, late-flowering plant, in which flowers occur solitary and are separated by long vegetative shoots (Fig. 1C and D). Most flowers on *veggie* mutants in the W138 background displayed severe distortions in shape and dimension and have a reduced fertility. When we crossed these *veggie* mutants into a distinct genetic background (W115) the floral defects were abolished and nearly wild type, fully fertile flowers were formed. However, the defects in inflorescence architecture and flowering time remained as strong and pronounced as in the inbred W138

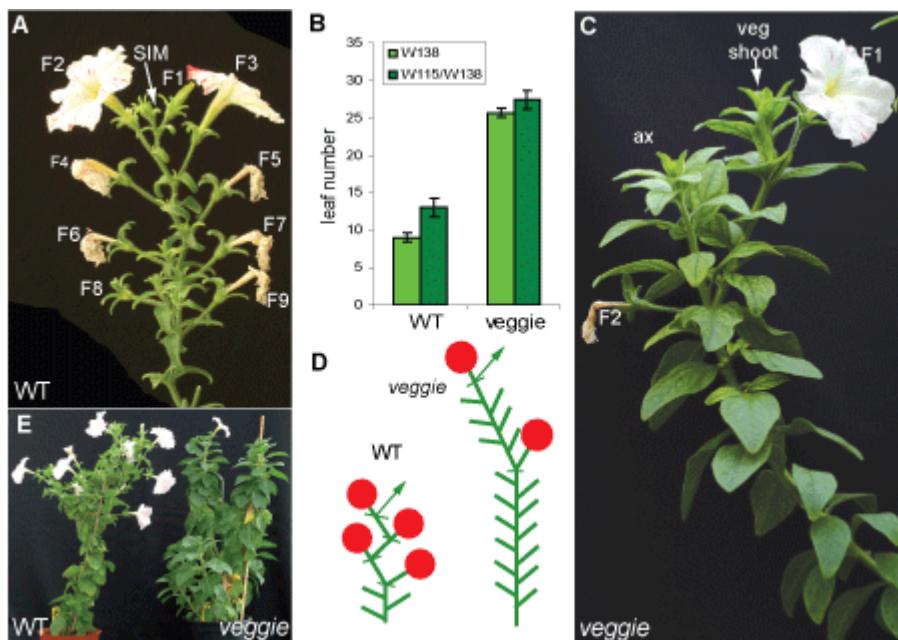


Figure 1. Phenotype of the *veggie* mutant.

A. Wild type W138 petunia. SIM indicated by arrow and flowers from young to old (F1-F9) between the bracts. **B.** Number of leaves produced before first floral transition in wild types and *veggie* mutants in either a W138 or W138/W115 background. **C.** *veggie*^{C3385} inflorescence. Vegetative shoot is indicated by arrow and flowers from young to old (F1 and F2). Note the long vegetative phase between the flowers. **D.** Schematic drawing of the wild type petunia and the *veggie* mutant (leaves are indicated as stripe at one side of the main stem, bracts occur in pairs and are indicated as short lines at both sides of the stem, flower as red dot and the SIM as arrow). **E.** Wild type and *veggie* mutant in the hybrid W138/W115 background.

background (Fig. 1E). That is, in the hybrid W138/W115 background *veggie* mutants produce 27.4 ± 1.2 leaves before they generate the first flower, whereas wild type flowers after producing 13 ± 1.2 leaves (Fig. 1B).

***veggie* homeotically transforms inflorescence units into vegetative shoots**

Wild type inflorescences have an apically placed determinate FM that terminates by forming a flower, and growth continues via a SIM that emerges in a lateral position and generates the subsequent sympodial unit that again consists of an apical flower and a lateral SIM, resulting in a continuous stacking of inflorescence sympodial units (ISU). To examine the ontogeny of the defects in the *veggie* inflorescence, we performed scanning electron microscopy (SEM) analysis. Therefore we dissected apices of *veggie* mutants that had already produced more than 20 leaves. The major fraction of these apices revealed no floral activity yet, as they were topped by a triangular-shaped meristem with leaves in a spiral pattern around it, and thus strongly resembled the vegetative SAM from wild types (Fig. 2A, B and C). Other apices had already undergone the transition to reproductive growth and were topped by an apically placed FM and a newly emerging meristem in a lateral position (Fig. 2D and E). In later developmental stages the apical FM subsequently initiates primordia for sepals, petals, stamens and carpels, while the SIM acquires a triangular shape (Fig. 2F and G) and generates leaf-like primordia in a spiral phylotaxis (Fig. 2H) instead of producing a flower and a SIM, as in wild type plants (Fig. 2I). These findings indicate that the *veggie* mutation does not abolish the formation of the SIM, but rather changes its identity into a vegetative meristem and thereby transforms the inflorescence sympodial unit into a vegetative shoot.

To further characterize how *veggie* changes the development of the SIM, we examined the expression patterns of several marker genes in *veggie* mutants, such as the floral meristem identity genes *ALF*, *DOT* and *EVG*. In wild type plants *ALF* mRNA is expressed during the vegetative phase in leaf primordia, but is excluded from the central dome of the SAM, whereas in FMs and SIMs express *ALF* mRNA throughout the meristem (Fig. 3A). We observed that in the emerging SIM of *veggie* inflorescences *ALF* is expressed, but is excluded from the

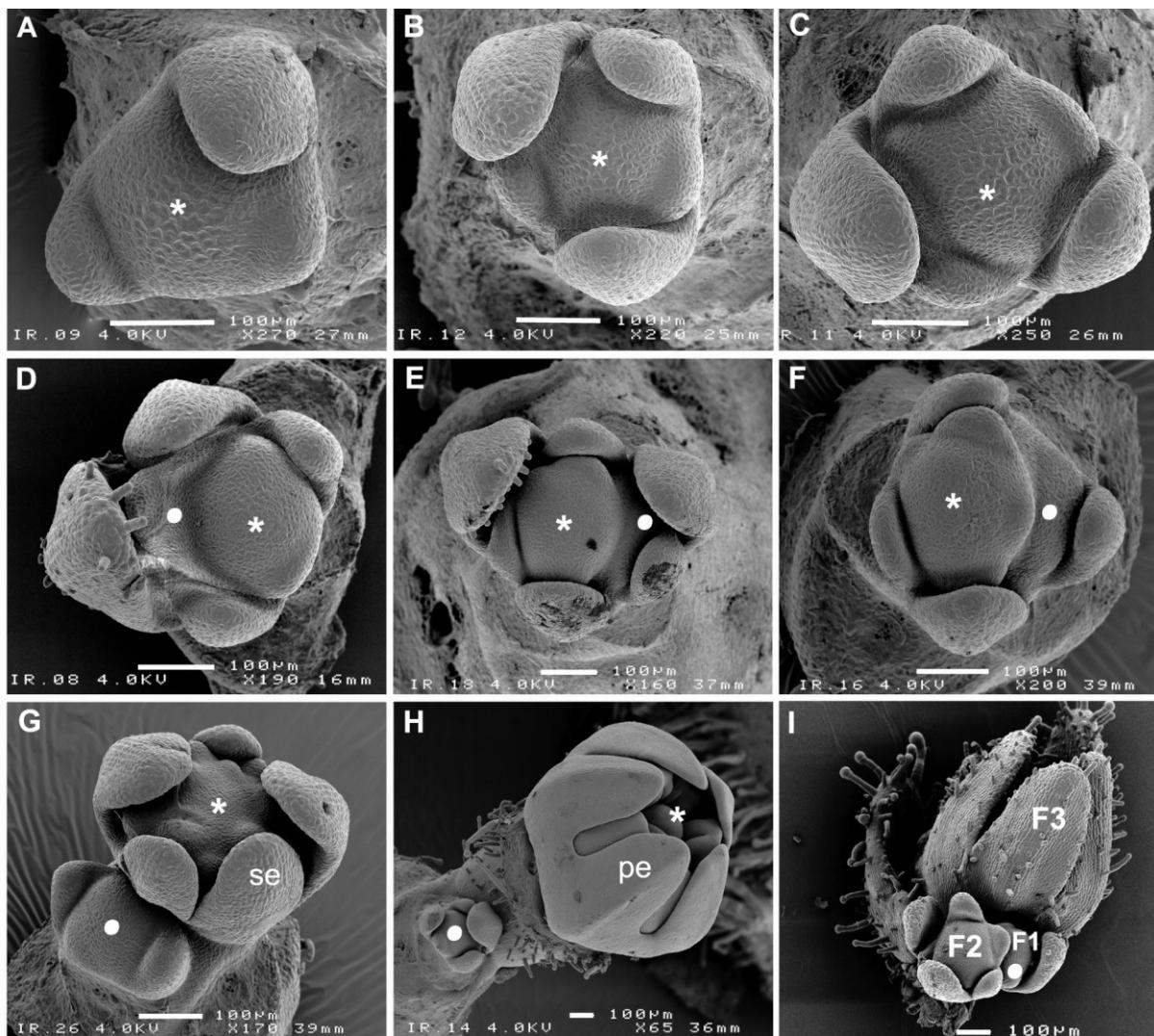


Figure 2. Scanning electron microscopy analysis of the *veggie* mutant inflorescence. SEM analysis of *veggie*^{C3385} apices. **A-C.** SEM micrographs of *veggie*^{C3385} apices just prior to the onset of flowering. The apical meristem has a triangular shape and the leaf primordia appear in a spiral pattern around the shoot apical meristem (SAM). **D-H.** SEM micrographs of a series of flowering *veggie*^{C3385} apices. **D,** *veggie* apex just after the switch to flowering. The SAM (*) is conferred to an FM and a lateral placed IM (•) emerges at the flank. **E.** The apical FM (*) starts to initiate sepal primordia, while the lateral meristem has enlarged and is now clearly visible. **F.** The sepals of the flower are starting to show. **G.** The flower has clearly recognizable primordia for sepals, petals and stamen. The SIM resembles a vegetative triangular-shaped meristem. **H.** The flower is fully developed (sepals are removed), but the SIM remained vegetative and generated leaf primordia in a spiral phytotaxy. **I.** Wild type inflorescence, which continuously produces flowers, a new FM and a SIM. * indicate the flowers and floral meristem and the • indicates the new IM. Scale bar is 100 μ m in all panels.

meristem dome itself, in a pattern similar to vegetative meristems of wild type, i.e. at the periphery of the meristem (coinciding with the leaf primordia), and is clearly different from the *ALF* expression observed in FMs of wild type plants (Fig. 3B and C).

In wild type *EVG* and *DOT* are inactive in vegetative meristems, but are both expressed in SIMs with a clearly different timing. *EVG* is only briefly expressed at the time that the young SIM emerges at the periphery of the apical meristem, while *DOT* is expressed later when the SIM acquires floral identity and initiates the first sepal primordium (Rebocho, 2008; Souer et al., 2008). By *in situ* hybridization we could not detect *EVG* or *DOT* mRNA in the emerging SIMs of *veggie* mutants, even though the same probes readily detected *EVG* and *DOT* mRNA in SIMs of wild type inflorescences. The lack of *EVG* and *DOT* expression is consistent with the idea that in *veggie* the SIM is transformed into a vegetative

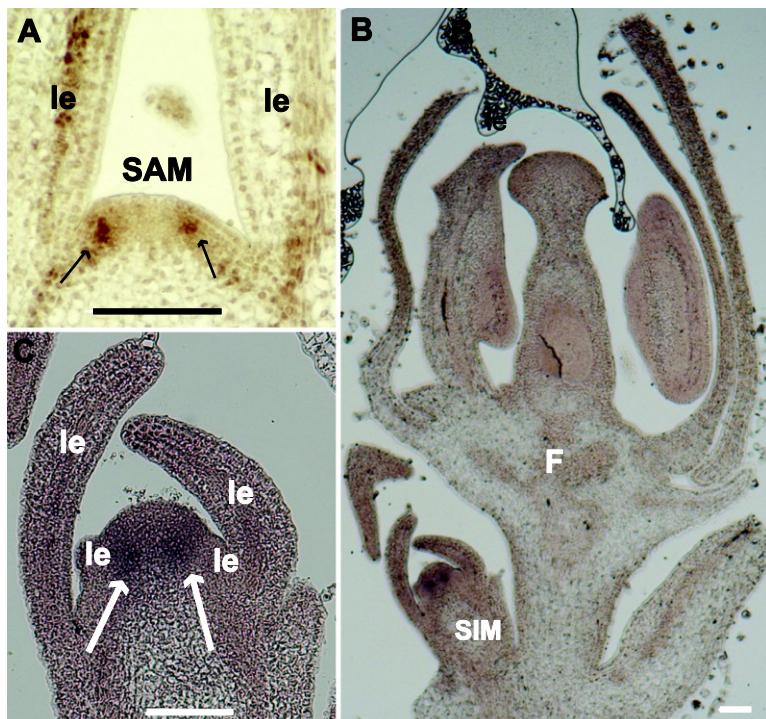


Figure 3. *ALF* expression in *veggie* mutant apices.

A. *ALF* expression (arrows) in incipient leaf primordia of a vegetative SAM in a 2-week old petunia seedling. **B.** *ALF* expression in the leaf primordia of a SIM of *veggie* mutant in W115/W138 hybrid background. Right the flower (F) and left the vegetative shoot (SIM). **C.** Detail of the vegetative *veggie* shoot (shown in B). *ALF* expression is present in a the leaf primordia pattern. Scale bar is 100 µm.

meristem. Taken together these findings imply that the *veggie* mutation homeotically transforms the SIM into a vegetative meristem.

Interaction of *VEGGIE* with meristem identity and maintenance genes

To better understand the role of *VEGGIE* and its genetic interactions with genes determining flowering time and/or inflorescence architecture we performed double mutant analysis.

***veggie alf* double mutants**

alf mutants are not affected in their vegetative growth or flowering time, but are unable to specify floral identity of the apical FM after the floral transition (Souer et al., 1998). To analyse the genetic interaction between *veggie* and *alf*, we generated *veggie alf* double mutants (Fig. 4A-D). Since *alf* mutants are completely sterile, we crossed *ALF^{+/−}* heterozygotes with *veggie* mutants, selected *ALF^{+/−}* F1 progeny by PCR and self-fertilized these *ALF^{+/−} VEGGIE^{+/−}* heterozygotes to obtain F2 progenies segregating for *alf* and *veggie*. Two F2 families were analyzed which segregated in 4 out of 17 and 8 out of 39 plants for the typical *veggie* phenotype during the vegetative growth phase. These plants were examined by PCR to determine the *alf* genotype. Three of these proved *alf^{m/m}* homozygous and thus were *veggie alf* double mutants. Phenotypical analysis of the three *veggie alf* double mutants showed they produced only leaf-like organs arranged around a vegetative, triangular-shaped, shoot apical meristem (Fig. 4D and Fig. 7). We followed the development of *veggie alf* double mutants for more than two years, during which they grew to over 2 metres in height, but observed no indications that they had switched to flowering, such as the formation of flowers, or the repeated sympodial branching that is typical of *alf* inflorescences.

The *veggie alf* phenotype might be explained by assuming that these double mutants are fully vegetative and do not switch to flowering or, alternatively that they switch to flowering but produce an inflorescence in which the formation of the SIM (e.g. due to *veggie*) and specification of floral identity is blocked (due to *alf*). The first hypothesis would predict that the leaf-like organs

at the tip of the primary *veggie alf* shoots have leaf-identity, whereas the latter hypothesis would predict that they have bract-identity.

Because it is difficult to discriminate leaves and bracts based on morphology alone, we examined the expression of the two MADS-box genes, *FBP13* and *FBP4*, in the leaf-like organs in the "inflorescences" of *veggie*, *alf*,

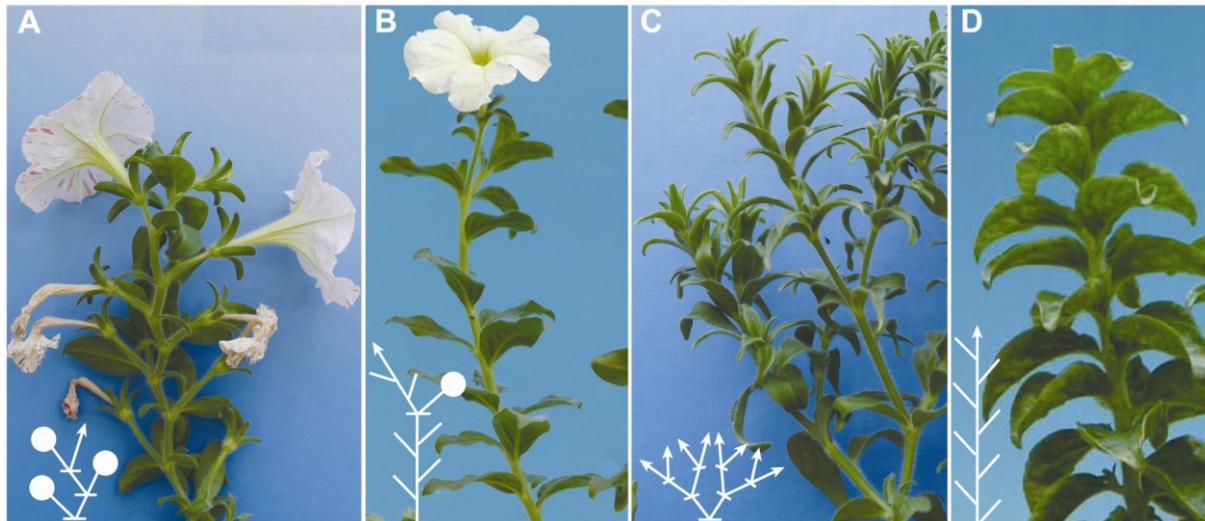


Figure 4. Double mutant *veggie alf* blocks flowering.

A. Wild type W138. Inset; schematic representation of floral growth structure of a wild type inflorescence. **B.** *veggie* mutant. Inset; schematic representation of floral growth structure of a *veggie* inflorescence. **C.** *alf* mutant. Inset; schematic representation of floral growth structure of a *alf* inflorescence. **D.** *veggie alf* double mutant. Inset; schematic representation of vegetative growth of a *veggie alf* vegetative apical meristem.

veggie alf and wild type plants from a segregating F2 family (Fig. 5A). *FBP13* is expressed in vegetative tissues only, whereas *FBP4* is transcribed in bracts and floral tissues, but not in leaves (Immink et al., 2003). We observed in bracts of wild type inflorescence sympodial units that *FBP4* and *FBP13* were both expressed (Fig. 5B). Also in the leaf-like organs in *alf* inflorescences *FBP4* and *FBP13* were expressed at similar levels, indicating that these leaf-like organs have bract-identity, which is consistent with the idea that *alf* does not block the onset of flowering, but homeotically transforms flowers into inflorescence shoots. In the leaf-like organs formed along the *veggie* inflorescence *FBP4* is hardly expressed, whereas *FBP13* is highly expressed. This confirmed that these organs

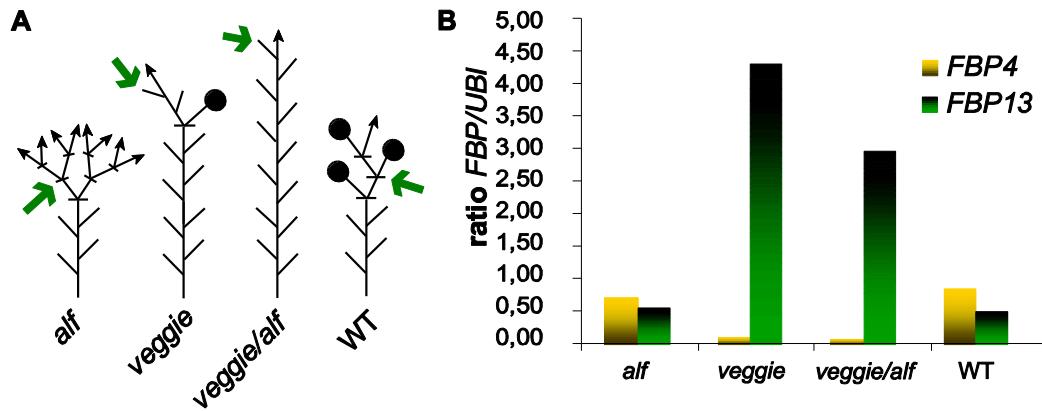


Figure 5. Determination of the identity of the “green organs” in *veggie* and *veggie alf* inflorescences.

A. Schematic drawing of flowering plants segregating for WT, veggie, alf and veggie alf mutants. RNA was isolated from the leaf-like organs formed shortly after the initial floral transition (arrows), veggie alf double mutants do not flower. **B.** *FBP4* and *FBP13* mRNA expression in leaf-like organs of the different genotypes, normalized against *UBIQUITIN*.

are leaves, which supports the idea that *veggie* fully transforms sympodial inflorescence units into vegetative shoots. The leaf-like organs at the tip of the primary *veggie alf* shoot/inflorescence express *FBP4* and *FBP13* in a similar way as *veggie* does, indicating that also these organs are leaves and that this structure is a vegetative shoot rather than an unbranched inflorescence. Thus, *veggie alf* double mutants remain vegetative and do not undergo the transition to flowering, suggesting that *VEGGIE* and *ALF* act in parallel partially redundant pathways that promote the onset to flowering.

***veggie dot* double mutant**

dot mutants switch from vegetative to reproductive growth (flowering) at the same time as wild type, but are unable to specify floral identity of the apical FM after the floral transition, which results in a pattern of repeated branching (Souer et al., 2008). The genetic interaction between *veggie* and *dot* mutants was studied in the *veggie dot* double mutants. Since *dot* mutants are completely

sterile, heterozygous *DOT^{+/m}* individuals were crossed with *veggie* mutants. The F1 population was screened by PCR for plants that were heterozygous for *DOT^{+/m}* and these plants were self-fertilized. We examined two F2 progenies, which segregated for 51:10 and 44:20 for wild type and the vegetative *veggie* phenotype respectively. Thirty *veggie* plants were analysed by PCR to identify *dot^{m/m}*, but none were found, suggesting that *DOT* and *VEGGIE* are linked. However, one *veggie* mutant was heterozygous for *dot* and harboured a cross-over chromosome. Self-fertilization of one line that contained a cross-over for *DOT⁺⁻*, resulted in a F3 family that contained 27 *veggie* and three *veggie dot* mutants. Phenotypical analysis of the *veggie dot* plants showed they flowered as late as *veggie* mutants and upon flowering produced two shoots similar to a *dot* mutant. Both these inflorescence shoots displayed vegetative characteristics and produced a number of leaves before each of them branched again into two vegetative shoots (Fig. 6A and Fig. 8). Thus, *veggie* and *dot* show additive effects indicating that they function in two separate pathways.



Figure 6. Genetic interaction of *veggie* with *dot* and *p35S::DOT*

A. *veggie* and *veggie dot* double mutants showing additive effects of *veggie* and *dot*. **B.** *p35S::DOT* in wild type (left) and in a *veggie* background (right). In wild type plants ectopic expression of *DOT* resulted in precocious formation of solitary flowers. The late flowering defect of *veggie* was not rescued by *p35S::DOT* expression. **C.** Close up of a *p35S::DOT* solitary flower. **D.** Close up of a solitary flower in *p35S::DOT veggie*.

veggie 35S::DOT double mutants

The constitutive expression of *DOT* (*p35S::DOT*) in wild type plants triggers early flowering and changes the cymose inflorescence into a solitary flower (Souer et al., 2008). To learn more about the function of *VEGGIE* and *DOT* in the onset of flowering and to see whether *DOT* acts downstream of *VEGGIE*, we crossed *p35S::DOT* plants to *veggie* mutants and selected three F1 plants for their *p35S::DOT* phenotype. These plants were backcrossed to *veggie*, which resulted in a B1 progeny that co-segregated 1:1 for the *p35S::DOT* phenotype and transgene. Among the *p35S::DOT* plants we observed a 1:1 segregation of the *veggie* and wild type, as judged by the phenotype during the vegetative growth stage (thick stem and amount of leaves). *p35S::DOT veggie* mutants flowered as late as *veggie* mutants, whereas *p35S::DOT* in wild type plants resulted in early flowering (Fig. 6B and Fig. 8).

The finding that *veggie* abolishes the precocious flowering of *p35S::DOT*, while *p35S::DOT* cannot rescue the flowering time defect of *veggie*, suggests that *VEGGIE* acts downstream or in parallel with *DOT*. Other effects of *p35S::DOT*, such as the curling of leaves and the partial transformation of leaves into petals do not require *VEGGIE* as they were also observed in *p35S::DOT veggie* plants.

When we examined the inflorescences of the *p35S::DOT veggie* mutants, we observed that they produced solitary flowers instead of the flower flanked by vegetative shoot as *veggie* single mutants, presumably because *p35S::DOT* blocks in a *veggie* background the development of the SIM (Fig. 6C and D), as it does in wild type (Souer et al., 2008). Thus, *veggie* is epistatic to *p35S::DOT* in the initial floral transition, but *p35S::DOT* is epistatic to *veggie* in specifying the identity of the inflorescence meristem.

veggie evg double mutants

To study the genetic interaction between *veggie* and *evg*, we were generated *veggie evg* double mutants. Since stable *evg* mutants are (almost) completely sterile, we crossed a heterozygous plant harbouring the stable *evg^{D2299}* allele (Rebocho, 2008) with *veggie* mutants. Plants were genotyped for *evg* by PCR and sequencing to select *EVG^{+/−}* heterozygotes and these *VEGGIE^{+/−}* *EVG^{+/−}* heterozygotes were self-fertilized. This resulted in two F2 families (28 and 39

plants) that contained 1 and 3 *veggie* mutants respectively. We analyzed these three *veggie* mutants for their *EVG* genotype by PCR and sequencing and found that two of them were *veggie evg* double mutants, one in each family. *evg* mutants develop normally during the vegetative phase, but after the switch to reproductive growth they fail to specify floral identity of the apical meristem, due to down regulation of *DOT*, while the SIM often fails to separate from the apical meristem, which results in fasciation (Rebocho et al., 2008). Phenotypical analysis of the *veggie evg* double mutants showed additive effects of both mutations (Fig. 7B and 8). The plants display a prolonged vegetative phase similar to *veggie* single mutants, and during the reproductive phase, they fail to form flowers and repeatedly produce two vegetative shoots that grow fasciated for some time before they split up. *veggie evg* mutants did on occasion produce flowers in a irregular pattern. Given that *evg* mutants in genetic backgrounds other than W138 can produce flowers (Rebocho et al., 2008), the flowers on *veggie evg* may be due to the mixed genetic background of the *veggie* parent that was used.

***veggie her* double mutants**

To analyse the genetic interaction between *veggie* and *her*, *veggie her* double mutants were generated. *veggie* mutants were crossed with *her* mutants resulting in double heterozygous F1 progeny. Self-fertilization of the F1 did not yield a double mutant in the F2, as determined by PCR, apparently due to linkage. From the F2 population 21 *veggie* mutants were analyzed by PCR to identify heterozygous *HER^{+/−}* individuals containing a cross-over chromosome. Five F2 plants heterozygous for *HER^{+/−}* were crossed among each other, which resulted in two F3 families that each segregated 3:1 for *veggie* and *veggie her* double mutants. All the *veggie hermit* double mutants ($n=10$) flowered at the same time as wild type after producing 10.6 ± 1.8 leaves, whereas sibling *veggie* single mutants flowered late after producing 35.4 ± 4.9 leaves. *veggie her* double mutants did not show a wild type cymose branching but a solitary malformed flower (Fig. 7C and 8), similar to *her*. The phenotype of the *veggie her* double mutants was unexpected because the strong flowering time effect was not observed before when *her* mutants were crossed to any of the meristem

identity mutants *alf*, *dot* or *evg* (Castel, 2009), and suggests *HER* has a role in the switch to flowering.



Figure 7. *veggie*, *veggie evg* and *veggie hermit* double mutant

A. *veggie* mutant. **B.** *veggie evg* double mutant. **C.** *veggie hermit* double mutant.

Discussion

We identified a new mutation, *veggie*, that alters the architecture of the cymose petunia inflorescence and in addition causes a strong delay in flowering. Mutation of *veggie* strongly delays the specification of floral identity of the primary shoot, resulting in a prolonged vegetative growth phase and a delay in the onset of flowering. Furthermore, the *veggie* mutation strongly delays the specification of

floral identity in the sympodial inflorescence shoot that emerges at the periphery of the apical flower, which now goes through a prolonged vegetative phase during which it initially generates leaves instead of producing an apical floral meristem and a new lateral SIM.

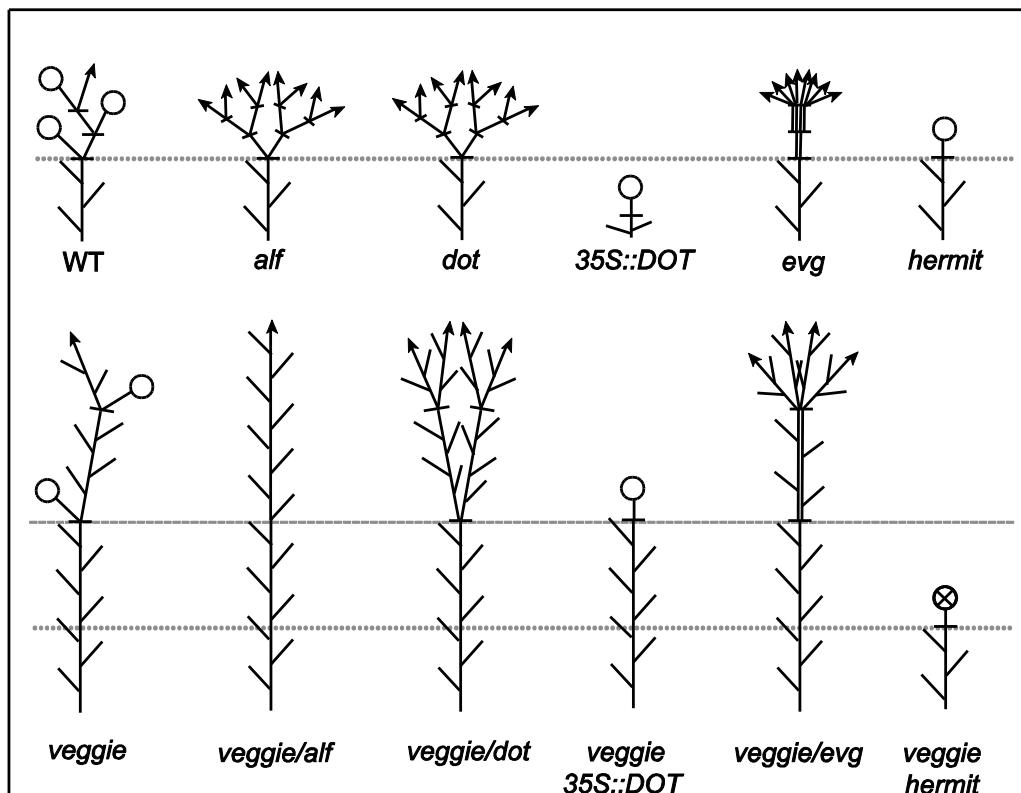


Figure 8. Overview of the phenotypes of *veggie* and double mutants.

Diagram of the phenotypes of different single mutants (*alf*, *dot*, *35S::DOT*, *evg* and *hermit*), wild type and the double mutants with *veggie*. Wild type plants first produce leaves (black stripes on one side of the main stem) and start to flower after the first set of bracts (black straight line on both sides of the stem) are formed, flower (circle), malformed flower (circle with cross) and IM (black arrow). Normal wild type timed floral transition is indicated by a dotted line (...) and late floral transition is indicated by a dashed line (---).

Role of *VEGGIE* in the onset of flowering

Genetic experiments in *Arabidopsis* and petunia indicated that the activation of LFY or ALF is major factor determining the onset of flowering (Souer et al., 2008; Weigel et al., 1992). In petunia, *ALF* is already expressed early during the vegetative phase in leaf primordia and after the onset of flowering in

floral meristems. The activity of the ALF protein is, however, restricted in space and time via the transcriptional control of another gene, *DOT*, that is first activated during the onset of flowering. *DOT* encodes the F-box protein component of a SCF-ubiquitin ligase (SCF^{DOT}) that promotes ALF activity by a post-translational mechanism that is poorly understood (Souer et al., 2008). Although *alf* and *dot* mutants do not display defects in the onset of flowering, constitutive expression of *DOT* is, together with endogenous ALF, sufficient to trigger precocious flowering. This suggests that *ALF* and *DOT* control the onset of flowering together with an independent pathway. Apparently *VEGGIE* is part of such an additional parallel pathway that induces flowering.

Flowering is delayed in *veggie* mutants rather than completely abolished, suggesting that *VEGGIE* is not fully essential for flowering but acts in parallel with one or more additional pathways to trigger flowering. One such a partially redundant gene is *ALF*, as in *alf veggie* double mutants flowering is completely abolished. The finding that constitutive expression of *DOT* does not rescue the flowering time defect of *veggie* is consistent with the idea that the *ALF-DOT* pathway acts in parallel with *VEGGIE*.

It is curious and unexpected that the switch to reproductive growth is fully blocked in *veggie alf* mutants, but not in *veggie dot*, as previous data (Souer et al., 2008) suggested that ALF and DOT proteins are interdependent. Interestingly, we observed a similar phenomenon in a genetic background containing a mutation in *EXTRAPETALS*, which affects the inflorescence architecture, but not flowering time. Both *exp* and *exp dot* double mutants switch to reproductive growth at a normal time, but in *exp alf* mutants flowering appears to be fully blocked (Castel, 2009). This suggests that not all activities of ALF require DOT, and that ALF has a *DOT*-independent role in the onset of flowering.

The inferred role of *VEGGIE* has some striking similarities with *FT* of *Arabidopsis* and *SINGLE FLOWER TRUSS (SFT)* of tomato. *FT* and *SFT* are homologs and encode a mobile protein that is expressed in the leaves in long-day conditions and that moves to the apex of the plant to trigger the formation of flowers (Corbesier et al., 2007; Jaeger and Wigge, 2007; Lifschitz et al., 2006; Lin et al., 2007; Mathieu et al., 2007). In *ft* and *sft* mutants flowering is delayed, as in *veggie*, while an additional mutation that inactivates *LFY* (*lfy ft*) or its tomato homolog *FALSIFLORA (FA)* (*fa sft*) abolishes flowering completely

(Molinero-Rosales et al., 2004; Ruiz-Garcia et al., 1997), as in *veggie alf* double mutants.

Role of *VEGGIE* in cymose inflorescence architecture

In wild type inflorescence the apical meristem acquires floral identity and simultaneously generates a SIM in a lateral position that repeats this process, resulting in the formation of a cymose inflorescence. Theoretical considerations indicate that in a cyme, floral identity is relatively quickly acquired in the apical meristem, whereas it should be delayed or transiently repressed in the SIM, to ensure that this meristem can generate the next lateral meristem, before it turns into a flower (Prusinkiewicz et al., 2007).

Previous data indicated that the identity of the flower is regulated by the *ALF-DOT* pathway, which promotes floral identity, and by *EXP*, which is thought to act as an antagonist that transiently inhibits floral fate and allows the SIM to form the next lateral meristem before it acquires floral identity (Castel, 2009). In *veggie* mutants the SIM generates some 20 leaves before it turns into a flower, indicating that *VEGGIE* acts in concert with *ALF* and *DOT* to promote floral fate of the SIM. This observation raises the question whether every newly formed SIM undergoes a similar switch from vegetative to floral identity as the primary apex, but which occurs so rapidly that it cannot be discerned in wild type plants. Although, there are in this respect some clear similarities in the regulation of the identity of the primary SAM and SIMs, there are also some notable differences. First, short-day conditions strongly delay the onset of flowering and thus the specification of floral identity in the SAM, However, short-day conditions do not affect the inflorescence architecture, suggesting that they do not affect identity or development of the SIM. Second, constitutive expression of *DOT* (*35S:DOT*) triggers precocious flowering, that is a premature switch from the primary SAM to a floral meristem, and converts the inflorescence into a solitary flower. The latter defect is thought to be due a premature acquisition of floral fate of the SIM, which thus becomes incorporated into the apical flower. The *35S:DOT veggie* phenotype indicates that in the absence of *veggie*, constitutive expression of *DOT* can precociously transform a SIM into an FM (hence resulting in a solitary flower), but not the primary vegetative SAM.

VEGGIE is essential for a normal timed floral transition and acts independently of the floral meristem identity genes *DOT* and *EVG*. The double

mutants only show additive phenotypes as is also seen in the double mutants of the tomato homologs of *DOT* (*ANANTHA* (*AN*)) and *EVG* (*COMPOUND INFLORESCENCE* (*S*)) with *sft* (Lippman et al., 2008).

HER is important for the diversification of distinct inflorescence architectures (Castel, 2009). In a *her* mutants the onset of flowering is not effected and is like wild type but its main apex ends in an aberrant flower and no sympodial is formed reducing the pertunia cyme to a single flower. *veggie her* mutants are indistinguishable of *her* mutants indicating that *her* fully suppresses the flowering time defect of *veggie*. It is possible that *VEGGIE* is not necessary at all in *her* mutants or that *HER* has a still unknown function in the floral transition. An alternative explanation is that the *veggie hermit* mutants are blocked in the SAM/IM transition although the exact why and how is still not understood.

Material and methods

Genetic materials and procedures

The *veggie*^{C3385} allele arose spontaneously among progeny of petunia line W138 and was maintained by inbreeding in the line W258. *alf*^{W2169} arose also in the W138 line and was maintained by inbreeding resulting in the line W229 (Souer et al., 1998). W258 (*veggie*^{C3385}) was crossed to W229 (*alf*^{W2169}) resulting in a heterozygote F1 that yielded upon self-fertilization in a segregating F2 family (J2019) that contained single and double mutant plants. The double mutant *veggie dot* originated from the self-fertilization of the F1 of a heterozygote for the inbred line M86 (*dot*^{B2413/+}) and W258 (*veggie*^{C3385}). To obtain the double mutant *veggie hermit*, W258 (*veggie*^{C3385}) was crossed to W261 (*hermit*^{D2413}). Self-fertilization of the F1 did not yield a double mutant, which is possibly caused by linkage. Therefore, we screened *veggie*^{C3385} plants by PCR analysis to identify cross-overs that were heterozygous for *her*. Self-fertilization of such a cross-over plant resulted in a F3 containing the double mutant *veggie hermit*. The *veggie*^{C3385} mutants in a W138 background had a very low fertility. To overcome the fertility problem the line W258 (*veggie*^{C3385}) was crossed to the unrelated wild type line W115; the homozygous *veggie*^{C3385} mutants that were obtained in the F2 and subsequent generations were fully fertile, but retained the late flowering and inflorescence architecture defect. *veggie*^{C3385} mutants from the hybrid background were crossed with the line W235 containing a stable recessive

evg allele with an 8-bp insertion (Rebocho, 2008) and the resulting F1 generation was genotyped by PCR and sequencing for the *evg*^{+8bp}. *VEGGIE*^{+/C3385} *EVG*^{+/D2299} individuals were self-fertilized to obtain an F2 that contained in the double mutant *veggie evg*. Transgenic *p35S::DOT* lines were described before (Souer et al., 2008).

SEM analysis

veggie apices were dissected under a stereo microscope, by removing only leaf material. Samples were fixed in 3,7% formaldehyde, 5% acetic acid and 50% ethanol for at least one night and then dehydrated via a graded ethanol series and critical point dried. Tops were mounted on stubs, coated with gold and viewed in a JSM-6301F (Field emission) scanning microscope (JEOL).

In situ hybridization

Inflorescence apices were fixed, embedded in paraffin, cut into 8 µm sections and used for in situ hybridization (Souer et al., 1998). The antisense digoxigenin labelled RNA probes were synthesized from full length cDNA clones by in vitro transcription using T7 polymerase and digoxigenin-11-UTP (Roche). RNA probes were hydrolyzed in 60mM Na₂CO₃ and 40mM NaHCO₃ to an average length of 100-150 bp prior to hybridization. The probes were detected with Western Blue stabilized AP substrate (Promega) which resulted in the formation of a brown precipitate.

PCR genotyping

To select petunia plants for the mutant allele of *alf*, *dot*, *evg* and *her* DNA was isolated from the leaves and used as template for PCR using gene-specific primers (Table 1) that flank the *dTPH1* insertions in the mutant alleles.

RNA Methodology

RNA was isolated from leaf material with TRIzol (Invitrogen). 2.5 µg RNA was used to make oligo d(T)¹⁸-RACE primed first strand cDNA with MLV-reverse transcriptase (Promega) and used for RT-PCR with gene-specific primers (Table 1). The PCR products were run on agarose gels, blotted on Hybond-N⁺

membranes (Amersham), hybridized with ^{32}P -labeled gene-specific probes and the signal was detected by a Phosphorimager (GE Healthcare).

Table 1. Primers used for genotyping and RT-PCR analysis.

| gene | fw/rev | sequence 5'-3' |
|---------------------|--------|-------------------------------|
| <i>ALF</i> | fw | CAGATGGAACTGCTTGTGGAG |
| | rev | GGGAATTCTTCTTGTGAGAGAGCATCAAG |
| <i>DOT</i> | fw | CTATTGACTTAGCTGTGGCTGG |
| | rev | CCGCATGGCGCTTGAATTAG |
| <i>EVGfootprint</i> | fw | CTTCTTCTTCGTCCCTCAAATAGT |
| | rev | GCTGAAGGAACAATGTAATCCATC |
| <i>EVGfootprint</i> | fw | CAGAGCAAATAGCATACTTGAAGC |
| | rev | GTAGAACCAACTGAATTGACTA |
| <i>HERMIT</i> | fw | GAGGAATTATGTGCCACGTCAGCAC |
| | rev | TCACACATTCTAAGAAACCAAGA |
| <i>FBP4</i> | fw | GGTGTTGCCTTCAGACATC |
| | rev | CCGAGTATATGCTATCATGCTC |
| <i>FBP13</i> | fw | GCCTCAACATGAGATTAAGC |
| | rev | CATTGCATGAAGTGTATCAC |
| <i>UBI</i> | fw | ATGGCTTCAAAGCGGATCTT |
| | rev | TCCATCTGTCCATGTTACACC |

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Chapter 3

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Chapter 4

VEGGIE encodes a homolog of FT and is required for sympodial inflorescence meristem identity

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Ronald Koes

Abstract

Flowering plant species display a large diversity with regard to the time that they start flowering and the positions where flowers form on the plant body. In racemes, like the well studied *Arabidopsis* inflorescence, flowers derive from lateral meristems. In cymes, which are common among Solanaceae, flowers derive from apical meristems and growth continues from a sympodial inflorescence meristem that emerges in a lateral position to generate the next apical flower and lateral sympodial meristem. In petunia, we identified a gene named *VEGGIE* that determines the identity of the sympodial inflorescence meristem. Here we show that *VEGGIE* encodes a phosphatidylethanolamine binding like protein (PEBP) that is structurally and functionally similar to FLOWERING LOCUS T (FT), a regulator of flowering time from *Arabidopsis*. Grafting experiments and expression analyses revealed *VEGGIE* is expressed in the vascular tissues of leaves and promotes the synthesis of a mobile signal, presumably *VEGGIE* protein, that is able to move through a graft junction and is needed throughout the flowering phase for the development of each sympodial inflorescence to maintain cymose architecture.

Introduction

To establish their optimum flowering time plants monitor both environmental signals, such as day length and ambient temperature as well as endogenous cues, like hormones and plant age. These signals are perceived and transduced by distinct pathways that ultimately control the transcription of a small set of genes, floral integrators that promote flowering (Michaels, 2009; Turck et al., 2008).

Day length (photoperiod) was early recognized as an important parameter by which plants measure seasonal changes (Garner, 1933; Garner, 1922). Long-day (LD) plants such as *Arabidopsis*, wheat and barley flower in light periods longer than a certain threshold, whereas short-day (SD) plants such as rice and morning glory flower in light periods shorter than a certain threshold. Yet other species, like tomato for example, are day-neutral (ND) plant and flower independently from day length.

Grafting experiments revealed that a mobile signal, florigen, is produced in the leaves of flowering plants in response to day length that moves through the plant towards the shoot apex to induce flowering. This mobile signal is able to move from a flowering stock across a graft junction into a vegetative shoot and induce flowering (Zeevaart, 1976). Experiments in which SD plants were grafted on LD plants or vice versa, indicated that florigen is widely conserved and involved in the flowering of species with very different day length responses. The nature of this mobile signal remained unsolved for a long time.

The circadian clock is an autonomous system that regulates the biological rhythms of a plant in cycles of approximately 24 hours (Pittendrigh, 1964). Within this 24-hr time frame the circadian clock is sensitive to light so plants can respond to different day length regimes. *CONSTANS* (*CO*) from *Arabidopsis* is an output gene of the circadian clock that integrates circadian rhythms and light signals to promote flowering (Putterill et al., 1995; Samach et al., 2000; Suarez-Lopez et al., 2001). *CO* is a nuclear protein that contains two B-box zinc finger domains as well as a CCT domain (Ben-Naim et al., 2006; Wenkel et al., 2006). The B-box zinc finger domains are involved in protein-protein interactions and the CCT domain is found in proteins involved in either the circadian clock or photoperiod responses (Ben-Naim et al., 2006; Wenkel et al., 2006). *CO* mRNA is expressed in leaves at low levels in the morning, but its expression increases

in abundance during the day, in both long-day and short-day conditions (Ben-Naim et al., 2006; Wenkel et al., 2006). Because CO protein is stabilized in the light and degraded in the dark, CO protein accumulates only in LD conditions (Valverde et al., 2004).

CO protein causes the transcriptional activation *FLOWERING LOCUS T (FT)* at the end of the day in long-day conditions (Kardailsky et al., 1999; Kobayashi et al., 1999). *FT* encodes a small protein of 175 amino acids that is identical to or part of florigen and shows similarity to the mammalian phosphatidylethanolamine binding protein (PEBP) or Raf kinase inhibitor protein (RKIP) (Kardailsky et al., 1999; Kobayashi et al., 1999). *ft* mutants display delayed flowering (Koornneef et al., 1991) whereas constitutive expression of *FT* accelerated flowering resulting in the production of solitary flowers and, as a consequence, sprouting of axillary shoots (Kardailsky et al., 1999; Kobayashi et al., 1999). *FT* mRNA and protein is produced in the leaves and the FT protein is translocated via the phloem vascular tissues to the apex (Corbesier et al., 2007; Jaeger and Wigge, 2007; Kobayashi and Weigel, 2007; Mathieu et al., 2007; Notaguchi et al., 2008; Tamaki et al., 2007). In the apex the FT protein interacts with FLOWERING LOCUS D (FD), a bZIP transcription factor, to initiate flowering by direct activation of the floral meristem identity genes *APETALA 1 (AP1)* and *FRUITFULL (FUL)* (Abe et al., 2005; Wigge et al., 2005).

In *Arabidopsis* the flowers are formed in lateral positions, and the main apex grows in an indeterminate manner. This result in an inflorescence with a racemose architecture, that is a single axis (monopodium) with numerous lateral flowers. Petunia on the other hand displays a cymose inflorescence architecture meaning that the main axis is terminated by a flower and growth continues from a sympodial inflorescence meristem (SIM) that emerges in a lateral position at the periphery of the main apex. In petunia, a mutant named *veggie* displays a serious delay in the initial floral transition and produces twice as much leaves before floral transition as wild-type plants. Another remarkable feature of *veggie* mutants is that the SIM, which in wild type forms a new lateral SIM and then converts into a flower, is transformed into a vegetative meristem that generates up to 20 leaves before SIM identity is established and a flower with a new lateral SIM is formed. Hence, *veggie* mutants produce solitary flowers separated by long vegetative shoots. This mutant phenotype suggested an important function for *VEGGIE* in the shoot apex of the plant (Chapter 3). Double mutants of *veggie*

with various floral meristem identity genes point out that *VEGGIE* acts in a similar way as *FT* (Chapter 3).

Here we report the isolation and molecular analysis of *VEGGIE*. *VEGGIE* encodes a PEBP-like protein and is presumably orthologous to *FT* from *Arabidopsis* and *SINGLE FLOWER TRUSS (SFT)* from tomato. Expression analysis shows that *VEGGIE* is expressed in the leaves and that its expression is up regulated at the end of the day. Grafting experiments revealed that *VEGGIE* is a mobile signal that is transported from the leaves up to the shoot apex to perform its flower promoting function.

Results

Molecular cloning of *VEGGIE*

The majority of the mutants derived from W138 are unstable and result from insertions of *dTPH1* transposons (van Houwelingen et al., 1998). However, at low frequency also stable mutants carrying deletions have been recovered. The *veggie*^{C3385} allele arose spontaneously in the W138 background (Chapter 3), but did not appear unstable as no germinal revertants were found amongst 5000



Figure 1. *veggie* mutants obtained by directed transposon tagging.

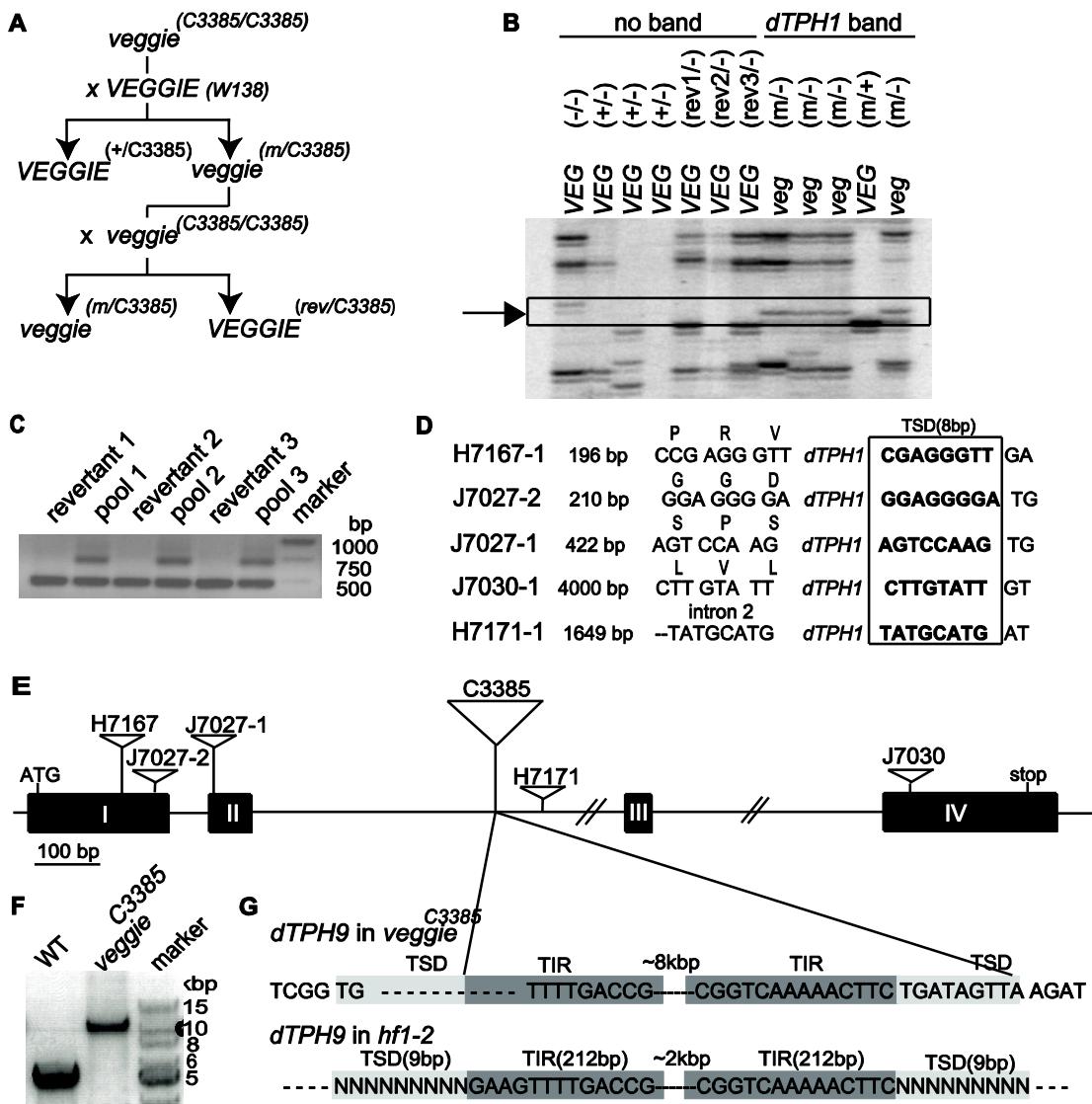
- A. Phenotype of the strong *veggie*^{C3385} (left), the *VEGGIE*^{+/+} wild-type W138 (middle) and the weak *veggie*^{H7171} mutant (right). B. *veggie*^{J7027-1}. C. *veggie*^{J7027-2}. D. *veggie*^{J7030}. E. *veggie*^{H7167}.

progeny plants that were grown. Therefore, it was unclear whether *veggie*^{C3385} was tagged by a *dTPH1* transposon, which precluded the isolation of *VEGGIE*.

To generate new unstable *dTPH1*-tagged *veggie* alleles, we crossed *veggie*^{C3385/C3385} mutants with wild-type (*VEGGIE*^{+/+}) W138 plants. The resulting progeny consisted of ~6000 plants with a wild-type phenotype (*VEGGIE*^{+/C3385}) and five mutants that displayed the typical *veggie* phenotype. That is, they were late flowering, had firm dark green stem and leaves and an aberrant inflorescence architecture due to the transformation of the sympodial inflorescence units into vegetative shoots. When these five mutant plants were backcrossed to stable *veggie*^{C3385/C3385} mutants, all the progeny had the *veggie* phenotype, confirming that they contained besides *veggie*^{C3385}, a new *veggie* allele (*veggie*^{H7167}, *veggie*^{J7027-1}, *veggie*^{J7027-2} and *veggie*^{J7030}) (Fig.1 A-E).

In addition, we identified one mutant *veggie*^{H7171} that displayed a weaker *veggie* phenotype. This *veggie*^{H7171/C3385} mutant is late flowering and has thick stems and firm leaves similar to the strong *veggie* mutants, but the lateral SIM produces only one or two leaves before it forms a flower, whereas strong *veggie* mutants produce up to twenty leaves before they generated one new flower (Fig. 1A). The backcross of the strong *veggie*^{H7167/C3385} mutant to *veggie*^{C3385/C3385}, resulted in ~1000 *veggie* mutants and three independent revertants that displayed a wild-type phenotype, indicating that *veggie*^{H7167} is an unstable allele that is possibly tagged by a *dTPH1* insertion (Fig. 2A). Self-fertilization of these three revertants gave in each case a 3:1 segregation for wild-type (revertant) and *veggie* mutants confirming that they were heterozygous for *veggie* and a germinal reversion allele (*VEGGIE*^{rev1} to *VEGGIE*^{rev3}).

Figure 2 (continued). introns (thin lines, intron 2 and 3 not to scale) and the various *dTPH1* insertions (triangle) in distinct *veggie* alleles. The original *veggie*^{C3385} allele contains a *dTPH9* insertion (big triangle). **F.** PCR analysis with primers complementary to the 5' and 3' UTR of *VEGGIE* results in a wild-type product of 5.5 kb and of *veggie*^{C3385} in a mutant product of about 12 kb. **G.** Schematic view of the *dTPH9* insertion in the second intron of *veggie* compared to the *dTPH9* insertion in *HF1*. The TSD at the 5' end of the *dTPH9* misses 7 of the 9 bp and the TIR at the 5' site misses 4 bp. The imperfect TIR of the *dTPH9* transposon resulted in a stable *veggie* mutant.

**Figure 2. Molecular analysis of VEGGIE**

A. Simplified pedigree of the unstable *veggie*^{H7167} allele, derived revertants and its progenitors. **B.** Detail of the transposon-display gel showing the *dTPH1* flanking sequence (box plus arrow) amplified from plants carrying the mutable *veggie*^{H7167} (indicated as *veggie*^m) and the stable recessive allele *veggie*^{C3385} (indicated as *veggie*^{+/−}). **C.** PCR analysis of the region surrounding the *dTPH1* insertion in *veggie*^{H7167/C3385}. The ~500-bp fragment derives from the *veggie*^{C3385} allele and the ~750-bp fragment from the *veggie*^{H7167} allele containing the *dTPH1* insertion. Revertants lack the *dTPH1* insertion, whereas the *dTPH1* insertion is present in the pooled mutant siblings. **D.** Sequences of *veggie*^{H7167} and the other *veggie* *dTPH1* insertion mutants. The target site duplication (TSD) is shown in bold. The *dTPH1* disrupts the reading frame except for *veggie*^{H7171}. **E.** Structure of *VEGGIE* and mutant alleles showing the exons (black rectangles),

To isolate the *VEGGIE* gene, we used transposon display analysis, which is an AFLP-based technique to visualize *dTPH1* flanking sequences (Maes et al., 1999), to compare the *dTPH1* insertions in *veggie*^{H7167/C3385} with those in its wild-type parents and derived germinal revertants (Fig. 2B). We identified a 156-bp fragment, consisting of 65 bp of the right end of *dTPH1* and 91 bp of flanking genomic DNA, which matched with the *VEGGIE* phenotype.

To prove that the isolated fragment is part of *VEGGIE*, we analyzed *veggie*^{H7167/C3385} mutants, related wild-type progenitors and revertants by PCR. PCR analysis of the *veggie*^{H7167/C3385} mutant using primers flanking the *dTPH1* element yielded two fragments of ~500 and ~750 bp respectively (Fig. 2C). The 500-bp fragment originates from the *veggie*^{C3385} allele and has a wild-type sequence, whereas the ~750-bp fragment originates from the *veggie*^{H7167} allele and contains the same sequence as the 500-bp fragment and included a *dTPH1* insertion. In the three revertants this *dTPH1* had excised, as PCR analysis yielded a 500-bp fragment only. Sequencing of these PCR products showed that they were homogeneous because no mixed sequences were seen, indicating that the revertant allele had the same sequence as the wild-type *VEGGIE* allele. This indicated that the excision of the *dTPH1* from the *veggie*^{H7167} allele did not result in the formation of a footprint and perfectly restored the wild-type sequence. Although *dTPH1* excisions usually result in the formation of a footprint, restoration of the wild-type sequence has also been observed, in particular when excision alleles were selected by phenotype (van Houwelingen et al., 1998).

Analysis of the cDNA and genomic sequences showed that the putative *VEGGIE* gene consists of four small exons (198, 62, 41 and 233 bp) and three introns (~100, ~2500 and ~1750 bp) (Fig. 2F). Next, we analyzed the four other independently isolated *veggie* alleles and found that they all contain *dTPH1* insertions in the same gene (Fig. 2E). In both *veggie*^{H7167} and *veggie*^{J7027-2} a *dTPH1* element was inserted in exon 1, in *veggie*^{J7027-1} a *dTPH1* was inserted in exon 2 and in *veggie*^{J7030} a *dTPH1* was inserted in the fourth exon (Fig. 2D). In *veggie*^{H7171} a *dTPH1* is inserted in the middle of intron 2, thereby explaining its weak *veggie* phenotype. Taken together these data show that the identified gene is identical to *VEGGIE*.

PCR analysis with primers complementary to the 5' and 3' end of the *VEGGIE* gene resulted in a fragment of 5.5 kb in wild-type plants and in a fragment of approximately 12 kb in *veggie*^{C3385} mutants suggesting that

veggie^{C3385} contained a large insertion of about 8.5 kb (Fig. 2F). Additional PCR experiments pinned down this insertion to the middle of intron 2. Sequencing from intron 2 into the insertion showed that termini of the insertion have high similarity to the transposon *dTPH9*. *dTPH9* was originally identified as a 2.1 kb insertion in the *HF1* gene (Matsubara et al., 2005) and is related to *MUTATOR* transposable elements described in maize (Kunze et al., 1997). Both have long terminal inverted repeats (TIR) of ~200 bp and generate a 9-bp target site duplication (TSD) upon insertion (Kunze et al., 1997; Matsubara et al., 2005).

The *dTPH9* insertion in *veggie*^{C3385}, however, lacks 4 bp from one of the TIRs and 7 bp of the TSD (Fig. 1G). We assume that either during the insertion or a subsequent excision attempt a few base pairs were deleted from the TSD and the TIR. Most likely the 4-bp deletion in the TIR immobilizes this *dTPH9* copy, thus explaining why *veggie*^{C3385} is a stable allele. The *dTPH9* insertion in *veggie* is much larger (~8 kb) than the known *dTPH9* inserted in *HF1-2* (2.1 kb) and therefore represents a new member of the *MUTATOR* family of transposable elements in petunia.

VEGGIE transcripts

To examine whether *veggie*^{C3385} mutants still produce *VEGGIE* mRNA, we analyzed *VEGGIE* transcripts. Although the full length *VEGGIE* transcript was only found in wild-type plants and never in *veggie*^{C3385} mutants, a transcript containing exon 1 and 2 could be detected in both *VEGGIE* and *veggie*^{C3385} plants (Fig. 3A). 3' RACE analysis on wild-type plants with primers complementary to the 5' end of the *VEGGIE* transcript and the poly-A-tail yielded a product of 698 bp, whereas *veggie*^{C3385} mutants yielded a shorter product of 663 bp that consisted of exon 1 and 2 of *VEGGIE* and a part of the *dTPH9* insertion (Fig. 3B). The aberrant *veggie*^{C3385} transcript consists of 262 bp of exon 1 and 2 from *VEGGIE* and 401 bp of the *dTPH9* (1675 bp- 1266 bp) an apparently originates from splicing of the 3' end of exon 2 to a cryptic splice site at position 1675 bp in *dTPH9* and poly-adenylation within the *dTPH9* sequence. From this *veggie*^{C3385} mRNA a truncated protein of 94 amino acids (87 amino acids encoded by exon 1 and 2 plus 7 amino acids encoded by the *dTPH9*) could be produced. Since *veggie*^{C3385} specifies an equally strong phenotype as other *veggie* alleles that are null (see below) we infer that this truncated protein is not functional. The alleles *veggie*^{J7027-1} and *veggie*^{J7027-2} are very likely null alleles because their transcripts

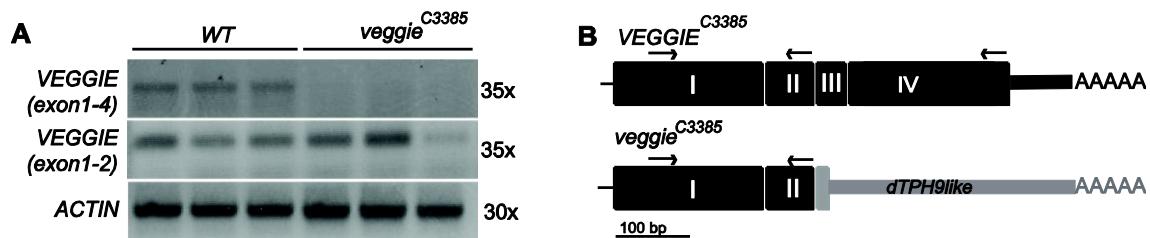


Figure 3. Characterization of *veggie*^{C3385}

A. Transcripts from W138 and *veggie*^{C3385} were amplified with primers complementary to exon 1 and 4 of *VEGGIE* and resulted in a 500-bp product for wild-type, but no product for *veggie* mutants (top panel). Amplification with primers complementary to exon 1 and 2 yielded a 200-bp fragment for both the wild-type and *veggie* mutants. *ACTIN* is used as an internal control. **B.** Structure of the *VEGGIE* transcript expressed in the wild type and *veggie*^{C3385} mutant. 3' RACE analysis on wild type cDNA yielded a 698-bp product that contained all four exons (black rectangles) of *VEGGIE* and the 3' untranslated region (black line). 3' RACE analysis of the *veggie*^{C3385} cDNA transcript yielded a product of 663 bp that contained exon 1 and 2 of *VEGGIE* (black rectangles), a part of a *dTPH9* transposable element (grey rectangle) plus 3' untranslated sequences derived from *dTPH9* (grey line).

are disrupted immediately downstream the transcription start by the *dTPH1* insertions. Also the phenotype of *veggie*^{J7030} is identical to that of the other *veggie* alleles and therefore we believe that except for *veggie*^{H7171} all the tagged *veggie* alleles are null alleles.

VEGGIE* shows similarity to the flowering time gene *FT* from *Arabidopsis

Comparison of the *VEGGIE* genomic and cDNA shows that *VEGGIE* consists of four exons and expresses a 698 bp mRNA encoding a protein of 178 amino acids. *VEGGIE* has similarity to the mammalian phosphatidylethanolamine binding protein (PEBP) or Raf kinase inhibitor protein (RKIP) (Banfield and Brady, 2000), and shows the highest similarity is to SINGLE FLOWER TRUSS (SFT) from tomato (Lifschitz et al., 2006) (sequence identity is 95.5%) and FLOWERING LOCUS T (FT) from *Arabidopsis* (74% identity). Both *SFT* and *FT* are identified as important floral inducers (Kardailsky et al., 1999; Kobayashi et al., 1999; Lifschitz et al., 2006). The FT protein is found throughout the plant kingdom and

is conserved among monocots like rice and barley and dicots like petunia, citrus, *Arabidopsis* and tomato (Carmel-Goren et al., 2003; Kobayashi et al., 1999; Tamaki et al., 2007; Yan et al., 2006}) (Fig. 4).

| | | |
|-----------|-------|---|
| PhVEGGI E | 1 | - - - M P R E R E P L V V G R V I G D V L D P F T R S I S L R V I Y R D R E V N N G C E L R P S Q V V N Q P R V E V G G |
| SI SP3D | 1 | - - - M P R E R D P L V V G R V V G D V L D P F T R T I G L R V I Y R D R E V N N G C E L R P S Q V I N Q P R V E V G G |
| Os Hd3a | 1 | M A G S G R D R D P L V V G R V V G D V L D A F V R S T N L K V T Y G S K T V S N G C E L K P S M V T H Q P R V E V G G |
| HvFT | 1 | - - - M A G R D R D P L V V G R V V G D V L D P F V R T T N L R V T F G N R A V S N G C E L K P S M V A Q Q P R V E V G G |
| Ci FT | 1 | - - - M S S R E R D P L I V G R V V G D V L D N F T R T I P M R I T Y S N K D V N N G R E L K P S E V L N Q P R A E I G G |
| At FT | 1 | - - - M S I N I R D P L I V S R V V G D V L D P F N R S I T L K V T Y G Q R E V T N G L D L R P S Q V Q N K P R V E I G G |
| PhVEGGI E | 5 8 | D D L R T F Y T L V M V D P D A P S P S D P N L R E Y L H W L V T D I P A T T G A S F G Q E I V C Y E S P R P S M G I H |
| SI SP3D | 5 8 | D D L R T F T L V M V D P D A P S P S D P N L R E Y L H W L V T D I P A T T G S S F G Q E I V S Y E S P R P S M G I H |
| Os Hd3a | 6 1 | N D M R T F Y T L V M V D P D A P S P S D P N L R E Y L H W L V T D I P G T T A A S F G Q E V M C Y E S P R P T M G I H |
| HvFT | 5 9 | N E M R T F Y T L V M V D P D A P S P S D P N L R E Y L H W L V T D I P G T T G A S F G Q E V M C Y E S P R P T M G I H |
| Ci FT | 5 9 | D D L R T F Y T L V M V D P D A P S P S D P S L R E Y L H W L V T D I P A T T G A S F G Q E I V N Y E S P R P T M G I H |
| At FT | 5 9 | E D L R N F Y T L V M V D P D V P S P S N P H L R E Y L H W L V T D I P A T T G T T F G N E I V C Y E N P S P T A G I H |
| PhVEGGI E | 1 1 8 | R F V L V L F R Q L G R Q T V Y A P G W R Q N F N T R D F A E L Y N L G L P V A A V Y F N C Q R E S G S G G R R R S A D |
| SI SP3D | 1 1 8 | R F V F V L F R Q L G R Q T V Y A P G W R Q N F N T R D F A E L Y N L G L P V A A V Y F N C Q R E S G S G G R R R S A D |
| Os Hd3a | 1 2 1 | R L V F V L F Q Q L G R Q T V Y A P G W R Q N F N T K D F A E L Y N L G S P V A A V Y F N C Q R E A G S G G R R V Y P - |
| HvFT | 1 1 9 | R F V L V L F Q Q L G R Q T V Y A P G W R Q N F N T R D F A E L Y N L G Q P V A A V Y F N C Q R E A G S G G R R M Y N - |
| Ci FT | 1 1 9 | R F V F V L F R Q L G R Q T V Y A P G W R Q N F S T R D F A E L Y N L G P P V A A V Y F N C Q R E S G S G G R P V R R - |
| At FT | 1 1 9 | R V V F L F R Q L G R Q T V Y A P G W R Q N F N T R E F A E Y N L G L P V A A V F Y N C Q R E S G C G G R R L - - - |

Figure 4. Alignment VEGGIE and homologs from other plant species.

Alignment of petunia VEGGIE, tomato SP3D, rice Hd3A, barley FT, citrus FT and *Arabidopsis* FT. Identical amino acids are marked in black, similar amino acids are indicated in grey.

To examine whether FT is able to substitute VEGGIE we expressed *FT* from the constitutive Cauliflower Mosiac Virus 35S promoter (*p35S::FT*) in both wild-type petunia and in *veggie* mutants. In wild-type plants we obtained only one *p35S::FT* line. This line showed accelerated flowering compared to non-expressing plants indicating that the expression of *FT* in wild-type petunia plants had an effect on flowering time (Fig. 5A). Transformation of *veggie* mutants with *p35S::FT* resulted in five independent lines that each displayed the same phenotype. *p35S::FT* *veggie* mutants flowered at a very early stage after having formed only 3 to 5 leaves. The expression of the *p35S::FT* transgene was sufficient to complement all the defects seen in *veggie* mutants. The firm stems and leaves as well as the late flowering and the vegetative identity of the lateral SIM of *veggie* mutants was restored, resulting in plants with a normal cymose inflorescence architecture. (Fig. 5B and C). When we backcrossed each of the independent *p35S::FT* lines to *veggie* mutants, the progeny segregated 1:1 for the *p35S::FT* and *veggie* phenotypes confirming that the near wild-type phenotype was due to the introduced transgene.



Figure 5. Ectopic expression of *FT* in petunia.

A. wild-type petunia (left) and wild-type plants transformed with *p35S::FT* (right). *p35S::FT* plant shows early flowering compared to wild-type plants. **B.** *veggie* (left) and *veggie p35S::FT* (right). Constitutive expressed *FT* restored the *veggie* phenotype. **C.** Detail of the *p35S::FT veggie* inflorescence structure, showing a normal cymose architecture.

To study the effect of *VEGGIE* in *Arabidopsis*, we introduced a *p35S::VEGGIE* transgene into wild-type *Arabidopsis* (Columbia). *Arabidopsis* plants transformed with *p35S::VEGGIE* ($n > 50$) flowered much earlier than wild-types, and produced instead of a indeterminate racemose inflorescence a determinate solitary flower. The loss of apical dominance resulted in the outgrowth of the axillary shoots similar to the expression of *p35S::FT* in *Arabidopsis* (Kardailsky et al., 1999; Kobayashi et al., 1999). Three *p35S::VEGGIE* lines were studied in more detail. Progeny obtained by self-fertilization of each of these lines (T2) showed a 3:1 segregation of the wild-type and the *p35S::VEGGIE* phenotype, indicating that each line contained one active

transgenic locus. These three lines were grown in both long-day (LD) and short-day (SD) conditions and monitored until flowering.

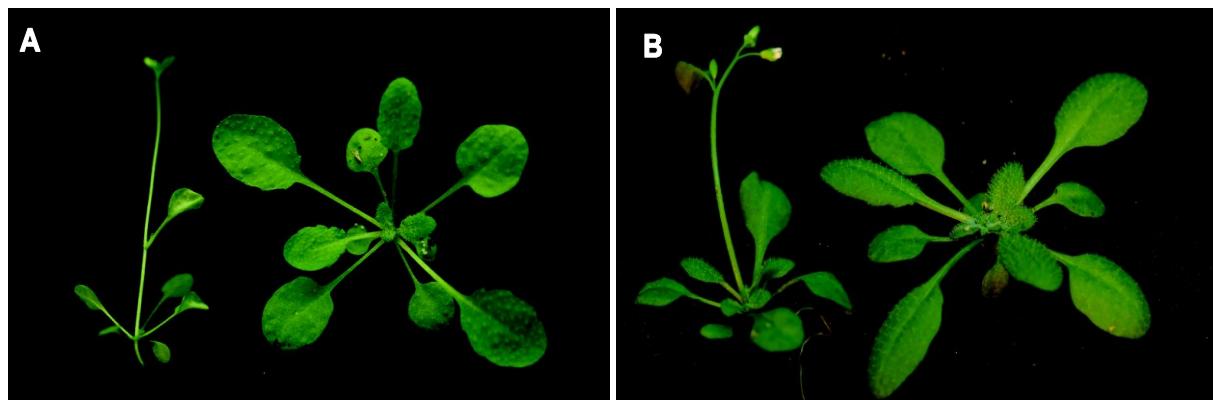


Figure 6. *p35S::VEGGIE* in *Arabidopsis* in long- and short-day conditions

A. *p35S::VEGGIE* (left) and wild-type plant (right) in LD conditions.

B. *p35S::VEGGIE* (left) and wild-type plant (right) in short-day (SD) conditions.

In LD conditions, *p35S::VEGGIE* lines flowered after producing 3.1 ± 0.5 ($n=50$) rosette leaves, whereas untransformed *Arabidopsis* siblings flowered later, after producing 14.2 ± 1.8 ($n=50$) rosette leaves (Fig. 6A and Table I). In SD conditions, *p35S::VEGGIE* plants flowered after producing 4.3 ± 0.5 ($n=55$) rosette leaves, whereas untransformed siblings flowered much later after producing 42.3 ± 3.0 ($n=47$) rosette leaves (Fig. 6B and Table I). Because, constitutive expression of *VEGGIE* in *Arabidopsis* caused the same phenotype as constitutive *FT* expression (Kardailsky et al., 1999; Kobayashi et al., 1999), and because *35S::FT* rescued *veggie* mutants, we concluded that *VEGGIE* is functionally equivalent to *FT*.

To test whether *p35S::VEGGIE* is able to complement the *veggie* mutation, we transformed *veggie* mutants with *p35S::VEGGIE*. Because transformations with *p35S::VEGGIE* to petunia never resulted in viable transgenic plants we suspect that the *35S* promoter is causing a too high expression of *VEGGIE* resulting in lethality of transformed plants.

Table 1: Flowering time of three *Arabidopsis* lines containing *35S::FT* and wild type siblings

| Family¹ | Genotype | Day-length² | Flowering time³ | | n |
|---------------------------|--------------------|-------------------------------|-----------------------------------|-----------------------|----------|
| | | | Rosette leaves | Cauline leaves | |
| M8101 | <i>35S::VEGGIE</i> | LD | 3.1 ± 0.7 (2-4) | 1.7 ± 0.5 (1-2) | 16 |
| | wild-type | LD | 13.8 ± 2.1 (10-16) | 2.7 ± 0.5 (2-3) | 6 |
| M8102 | <i>35S::VEGGIE</i> | LD | 3.2 ± 0.4 (3-4) | 1.3 ± 0.5 (1-2) | 17 |
| | wild-type | LD | 15.0 ± 1.7 (13-17) | 4.2 ± 1.3 (1-2) | 6 |
| M8103 | <i>35S::VEGGIE</i> | LD | 2.9 ± 0.2 (2-3) | 1.7 ± 0.5 (1-2) | 17 |
| | wild-type | LD | 13.7 ± 2.0 (12-17) | 3.1 ± 1.2 (1-2) | 7 |
| Columbia | wild-type | LD | 14.2 ± 1.6 (12-17) | 2.3 ± 1.2 (1-2) | 12 |
| M8101 | <i>35S::VEGGIE</i> | SD | 4.3 ± 0.6 (3-5) | 0.8 ± 0.5 (0-2) | 18 |
| | wild-type | SD | 41.0 ± 3.4 (10-16) | 8.6 ± 2.1 (8-11) | 7 |
| M8102 | <i>35S::VEGGIE</i> | SD | 4.4 ± 0.6 (3-4) | 1.1 ± 0.5 (1-2) | 18 |
| | wild-type | SD | 43.5 ± 3.4 (40-47) | 9.5 ± 1.0 (1-2) | 6 |
| M8103 | <i>35S::VEGGIE</i> | SD | 4.1 ± 0.4 (3-5) | 1.3 ± 0.5 (0-2) | 19 |
| | wild-type | SD | 43.6 ± 2.8 (40-47) | 8.7 ± 1.1 (7-10) | 7 |
| Columbia | wild-type | SD | 42.4 ± 3.0 (38-47) | 9.2 ± 0.8 (8-10) | 10 |

¹ The families M8101, M8102 and M8103 were progeny of three independent transformants that segregated for plants with (*35S::VEGGIE*) and without transgene (wild type)

² LD denotes long-days, SD denotes short-days

³ Flowering time was measured as number of rosette leaves and number of cauline leaves before flowering

Spatial expression pattern of *VEGGIE*

To determine the *VEGGIE* expression pattern in petunia we measured *VEGGIE* mRNA in various tissues of a wild-type petunia and found that *VEGGIE* mRNA is expressed in the early developmental stages of petal limb, flower tube, ovary, sepals, leaf, pistils and stems but is not in anthers and roots (Fig. 7). The absence of the *VEGGIE* expression in the leaves is due to the harvesting of the material in the morning.

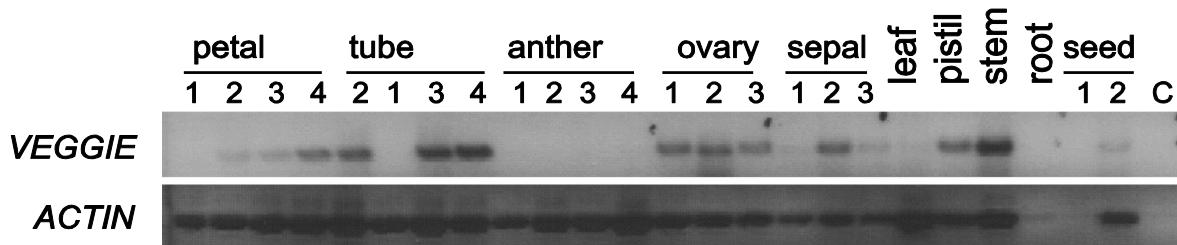


Figure 7. Expression pattern of *VEGGIE*.

Semi-quantitative RT-PCR analysis of the *VEGGIE* mRNA expression in various developing tissues of wild-type petunia (1 is the youngest developmental stage and 4 the oldest developmental stage).

To determine the spatial expression pattern of *VEGGIE* at higher resolution we used *in situ* hybridization. However, we were never able to detect *VEGGIE* mRNA in sections of a variety of tissues, possibly because the abundance of this mRNA is too low. As an alternative we fused a 2.25 kb *VEGGIE* promoter fragment to a chimaeric reporter gene encoding a translational fusion of GREEN FLUORESCENT PROTEIN (GFP) and β -GLUCURONIDASE (GUS) (*pV::GFP::GUS*). Of the seven transgenic lines obtained, three showed GUS expression in a similar pattern. The three expressors displayed only GUS activity in the phloem companion cells of the leaves (Fig. 8A-C), similar to the expression pattern of *pFT::GUS* and *pFT::FT::GUS* in *Arabidopsis* (Notaguchi et al., 2008), and *pSFT::GUS* in tomato (Lifschitz et al., 2006).

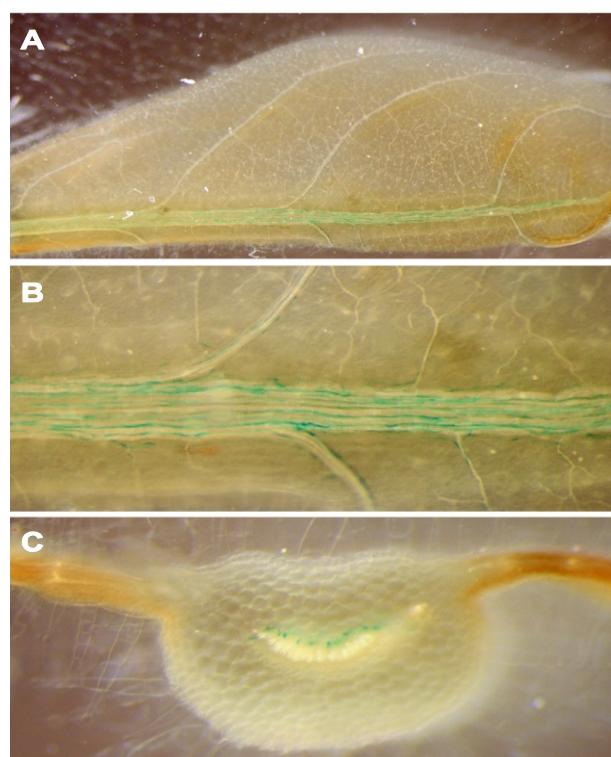


Figure 8. Expression pattern of *pVEGGIE::GUS*.

A. *pV::GFP::GUS* results in GUS activity in the phloem of a leaf. **B.** Close-up of the main vein of a *pV::GFP::GUS* leaf showing specific GUS activity of the phloem companion cells. **C.** Cross-section through a vein of a *pV::GFP::GUS* leaf.

No GUS expression was observed in the shoot apical region, suggesting the absence of *VEGGIE* expression in the shoot apex as also observed in *Arabidopsis* (Notaguchi et al., 2008). Due to the low expression of the *VEGGIE* promoter, no GFP signals were detected.

In T2 progeny obtained by self-fertilization of the three primary *pV::GFP:GUS* transformants, GUS expression could no longer be detected, suggesting that the transgenes were silenced. This precluded a more detailed analysis of *VEGGIE* expression during (early) vegetative growth stages.

Temporal expression of *VEGGIE*

To study the temporal expression of *VEGGIE* mRNA we harvested over a period of 24 hours leaf material from flowering petunia plants. We observed that *VEGGIE* mRNA expression is up-regulated during the day, peaks after 10-14 hr of light and subsequently decreases towards the end of the day when plants were grown in long-day (LD) conditions (Fig. 9A). This rhythmic cycling of *VEGGIE* mRNA is seen in various petunia lines (W137, W138, W115 and V26) and a hybrid (W115/W138) (data not shown). Because *VEGGIE* expression peaks after 10-14 hr of light, further sampling for *VEGGIE* mRNA analysis was done after 12 hr light induction in the subsequent experiments.

We observed that flowering of wild-type (W138 and W115/R27 hybrid) petunia lines in LD conditions was accelerated compared to short-day (SD) conditions. Petunias grown in LD conditions produce 10-15 leaves before the floral transition is initiated, whereas in SD conditions they produce up to 30 leaves before flowering (Fig. 9B), which is similar to the flowering time of *veggie* mutants grown in LDs (Chapter 3). This suggests that *VEGGIE* is a major component of the photoperiod pathway in petunia, like *FT* in *Arabidopsis*. When we grew *veggie* mutants in SD conditions they failed to flower at all, indicating that the photoperiod signal is relayed in part via *VEGGIE* and in part via a redundant pathway.

Importantly wild-type plants produce after the onset of flowering a normal cymose inflorescence, even in SD conditions, whereas *veggie* mutants do not. This indicates *VEGGIE* has additional functions during development of the inflorescence that are independent of day length, which implies that *VEGGIE* is expressed also in SD conditions. To test that hypothesis we monitored the expression of *VEGGIE* mRNA in developing petunia plantlets and observed that

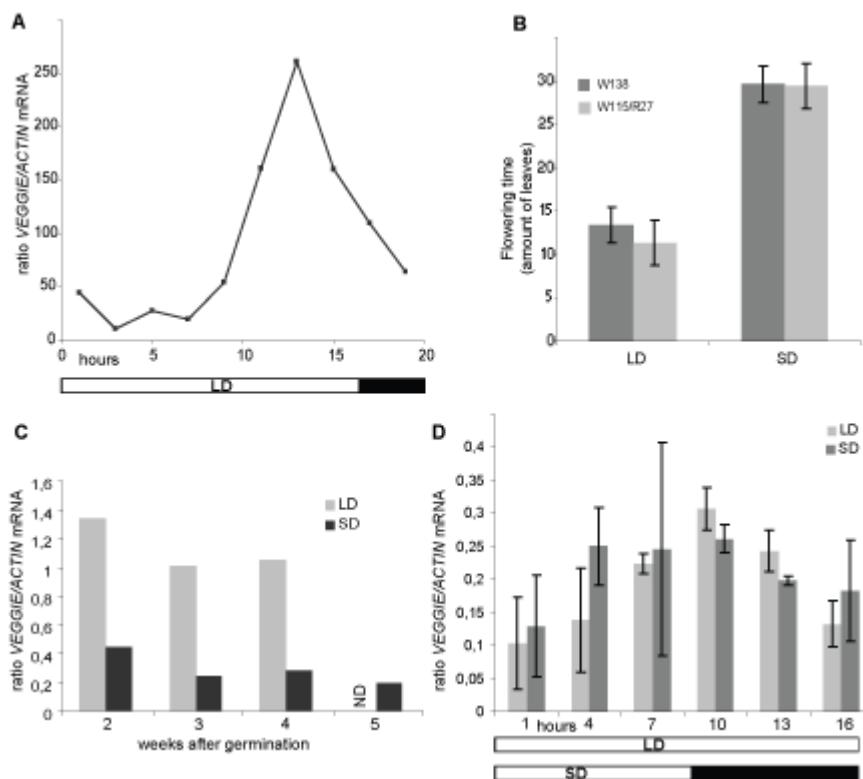


Figure 9. Expression of VEGGIE in long-day and short-day conditions and in developing plants.

A. Strong upregulation of *VEGGIE* mRNA expression at the end of the day in the leaves of flowering plants during long-day conditions (16 hours light, 8 hours dark). **B.** Amount of leaves generated before the floral transition in distinct wild-types (W258, hybrid W138/W115) grown in LD and SD (8 hours light, 16 hours dark) conditions. **C.** Expression of *VEGGIE* mRNA in the developing petunia seedlings in LD and SD conditions. **D.** Expression of *VEGGIE* mRNA in the leaves of 8-week-old plants during the day. Plants grown in LD conditions flowered already for 4 weeks, whereas SD-grown plants just started flowering. All the semi-quantitative RT-PCR measurements were normalized against *ACTIN*.

during the first 4 weeks, when both LD and SD grown plants were still vegetative, the plants grown in LD conditions expressed 5-fold more *VEGGIE* mRNA than siblings grown in SD conditions (Fig. 9C). Thus, during vegetative growth *VEGGIE* expression is dependent on day length. After 4 weeks the plantlets grown in LDs switched to flowering, whereas plantlets grown in SD were after 4 weeks still vegetative and needed up to 8 weeks before flowering. When we examined 8-week old plantlets grown in LD (which were by then flowering for

4 weeks) and plantlets grown in SD (which had just switched to flowering), we observed that *VEGGIE* was up-regulated at the end of the day to a similar level in both SD and LD conditions (Fig. 9D). This rather unexpected outcome might be the caused by the activation of *VEGGIE* by one of the other floral inductive pathways and confirms our assumption that *VEGGIE* is expressed in SD in wild-type plants.

Is a pulse of *FT* enough to induce flowering?

Arabidopsis transformed with *FT* and *GUS* both driven by a heat shock inducible promoter (*HSP*) from soybean (*pHSP::FT* and *pHSP::GUS*) showed that the activation of *FT* in a single leaf is sufficient to accelerate and maintain flowering (Notaguchi et al., 2008). To get a insight into the regulation of *VEGGIE* and to monitor possible auto-regulation, *veggie* mutants were co-transformed in one single step with two Agrobacterium cultures containing *pHSP::FT* and *pHSP::GUS* respectively. We obtained 13 transformants, three of which contained both transgenes. When these transformed plants were still in their vegetative growth phase, they were incubated overnight at 37 degrees to induce the *HSP* promoters and thereby the expression of *FT* and *GUS*. Although the plants expressed both *FT* and *GUS* mRNA as well as the *GUS* protein, the heat shock did not induce instant flowering. The induced plants did not show accelerated flowering, nor did they show any changes in the inflorescence architecture (data not shown). These data suggest that in contrast to *Arabidopsis*, a pulse of *FT* expression is not sufficient to induce flowering and that *VEGGIE/FT* has to be permanently expressed in petunia to maintain the cymose architecture.

Is *VEGGIE* a mobile signal?

VEGGIE is expressed in the phloem companion cells, but is essential in the apex to promote the transition to flowering and to specify the identity of newly formed sympodial meristems in the inflorescence. Several groups showed that in *Arabidopsis* the *FT* protein is transported from the leaves upwards to the shoot apex to promote flowering (Corbesier et al., 2007; Jaeger and Wigge, 2007; Kobayashi and Weigel, 2007; Mathieu et al., 2007; Notaguchi et al., 2008; Tamaki et al., 2007).

To test whether *VEGGIE* is, like *FT*, a mobile signal that moves upwards to the apex to promote flowering and to specify the identity of the sympodial

inflorescence meristems, vegetative shoots of *veggie* mutants were grafted on flowering wild-type donor plants. Of the twelve individual *veggie* grafts on four different flowering petunia stocks, eight remained small and failed to develop much further. Four grafts grew well and produced flowers in a cymose pattern similar to wild-type inflorescences (Fig. 10A and B).

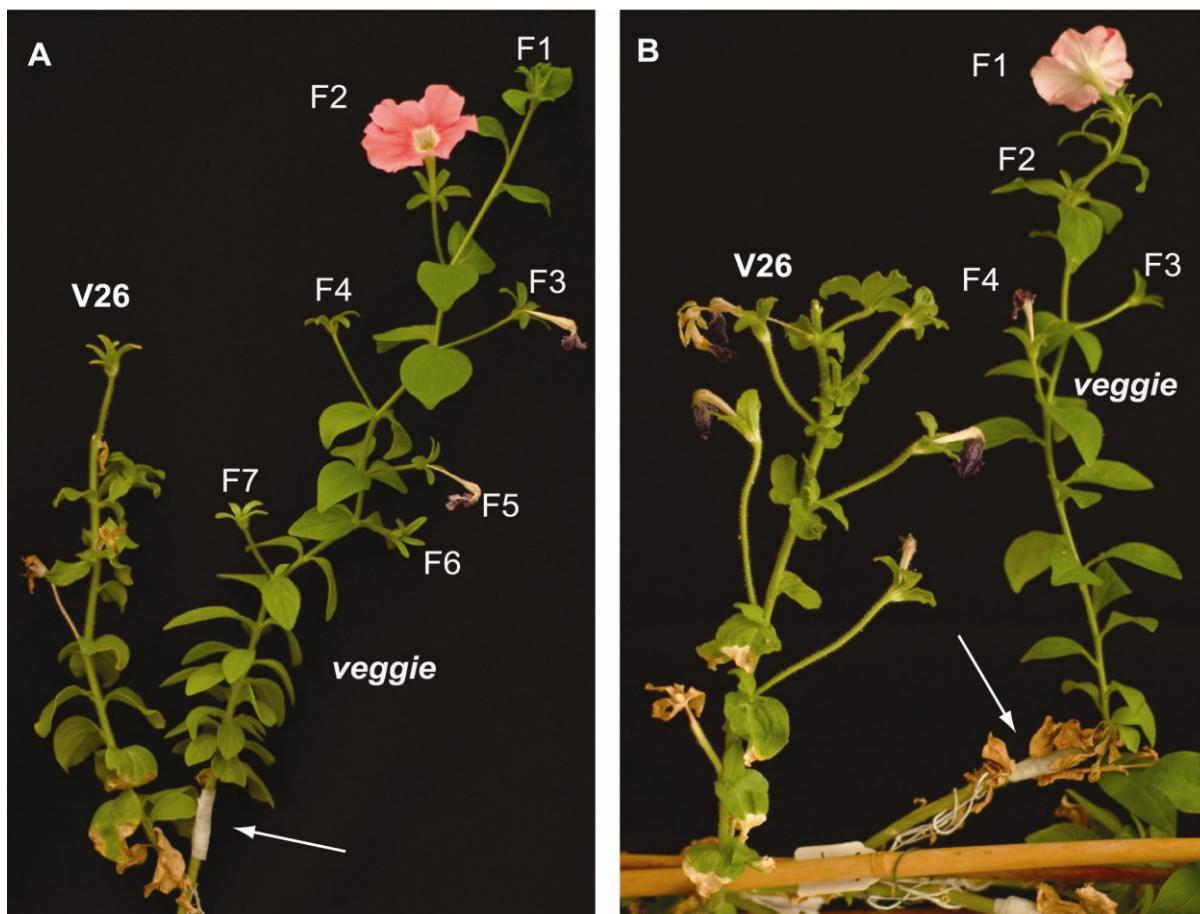


Figure 10. Rescue of *veggie* mutants by grafting on wild type stocks

A. The inflorescence of the V26 wild-type petunia donor shoot (left) shows the cymose wild-type architecture. The grafted *veggie* stock (right) acquired the cymose wild-type architecture and formed five flowers without intervening leaves (flower F2-F6), while formation of the next flower (F1) was preceded by the formation of two leaves, indicating that a weak *veggie* mutant reappears. Arrow indicates the graft junction, F = flower. **B.** Same as in panel A. but on a different V26 donor shoot.

The normal cymose branching in these *veggie* stocks was consistent for 3 to 6 flowers, but higher up in the *veggie* inflorescence the *veggie* phenotype started to reappear. That is, the sympodial inflorescence meristem of the *veggie*

stocks started to show vegetative characteristics again, i.e. production of leaves between the flowers. The amount of signal produced by the wild-type flowering stock is not sufficient enough to maintain the complementation of the *veggie* mutant phenotype or the floral signals originating from the donor shoot do not reach far enough into the *veggie* graft to reach the apex. To study whether the partial rescue of grafted *veggie* shoots, happened because of the grafting itself, we grafted *veggie* shoots on *veggie* stocks and wild-type shoots on a wild-type stocks, but saw no effects in the respective grafts (data not shown). Hence, floral promoting signals coming from a flowering wild-type donor plant, but not from a *veggie* stock, were able to rescue the phenotype of a *veggie* graft, indicating that *VEGGIE* is a mobile signal.

Discussion

***VEGGIE* is necessary for the inflorescence meristem identity**

We show that *VEGGIE* encodes a protein of the PEBP family and is highly similar, and probably orthologous, to *Arabidopsis FLOWERING LOCUS T (FT)* and tomato *SINGLE FLOWER TRUSS (SFT)*. Although *VEGGIE* and *FT* encode highly similar and functionally interchangeable proteins, they have partially distinct roles in the development of the racemose inflorescence of *Arabidopsis* and the cymose inflorescence of petunia. In *Arabidopsis*, *FT* is essential to make the switch from vegetative growth to flowering, but after the initial floral transition the apically placed IM continues growth in a indeterminate manner producing flowers in the lateral positions irrespective of the *FT* expression (Ruiz-Garcia et al., 1997). In petunia, *VEGGIE* is not only essential to promote the initial floral transition as in *Arabidopsis*, but remains necessary after the onset to flowering to specify the cymose inflorescence architecture. Petunia plants grow after the first floral transition in a determinate manner, as the main axis terminates by forming a flower while growth continues from a lateral (sympodial) meristem. Because in *veggie* mutants each SIM is (transiently) transformed into a vegetative meristem that produces some 20 leaves before it produces a flower and a new lateral SIM, it follows that *VEGGIE* is required throughout the flowering phase for the development of each sympodial inflorescence meristem.

Spatial and temporal expression of VEGGIE

The spatial expression of *VEGGIE* mRNA in the aerial parts of petunia resembles the spatial expression patterns of *FT* in *Arabidopsis* and *SFT* in tomato(Kardailsky et al., 1999; Kobayashi et al., 1999; Lifschitz et al., 2006). The results with the *VEGGIE* reporter gene showed that *VEGGIE* is like *FT* (Takada and Goto, 2003) and *SFT* (Lifschitz et al., 2006) mainly expressed in the main veins of the leaves. Whether the *VEGGIE::GUS* reporter fully reflects the *VEGGIE* expression pattern is not entirely clear, as introns and the 3' flanking region of *VEGGIE* may contain cis-regulatory elements that are absent in the reporter construct. This might be addressed by testing whether a *pVEGGIE::VEGGIE* construct can complement the *veggie* mutation. However, we consider this possibility unlikely as the expression of *VEGGIE* corresponds with the results of many research groups that showed that *FT* mRNA is produced in the leaves, and that the *FT* protein is translocated via the phloem vascular tissues to the apex (Corbesier et al., 2007; Jaeger and Wigge, 2007; Kobayashi and Weigel, 2007; Mathieu et al., 2007; Notaguchi et al., 2008; Tamaki et al., 2007). The expression of *SFT* in the flowers (sepals and petals) of mature tomato plants (Lifschitz et al., 2006) was also observed in petunia.

The temporal expression of *VEGGIE* shows the same rhythmic cycling during the day as *FT*, with a strong up regulation towards the end of the day in long-day growth conditions. Also in short-day growth conditions rhythmic cycling of *VEGGIE* was observed but during the vegetative growth phase the expression levels remained much lower than in long-days similar to *FT* in *Arabidopsis* (Kardailsky et al., 1999). *Petunia hybrida* is a facultative long-day plant meaning that flowering in SD is delayed, rather than completely abolished. The more rapid flowering in long-day conditions is apparently due to the higher *VEGGIE* expression in LD compared to SD. *veggie* loss-of-function mutants display a late flowering phenotype in LD conditions and produce the same number of leaves before flowering as wild-type does in SD conditions. The fact that *veggie* mutants in LD can still initiate flowers, even though no *VEGGIE* mRNA is produced is most likely due to the combined action of remaining floral inductive signals from the photoperiod pathway. When *veggie* mutants are grown in SD condition floral activity is completely abolished, resulting in fully vegetative plants, apparently because parallel pathways remains inactive.

VEGGIE is a graft-transmissible signal

A key feature of florigen is that it is a graft transmissible signal. Although the experiments with FT in *Arabidopsis* and SFT in tomato go a long way to demonstrate that FT/SFT is identical to, or part of, florigen, it is remarkable that the *ft* or *sft* phenotype can only be rescued by grafting mutant shoots on transgenic stocks that express FT or SFT from the constitutive 35S promoter, but not by grafting on wild type plants (Huang et al., 2005; Lifschitz et al., 2006; Shalit et al., 2009). Hence, FT and SFT differ in an important feature from the florigen signal. One explanation might be that florigen is actually a complex signalling compound that consists of mixture of *FT/SFT* and another unknown component, which can be substituted by increasing *FT/SFT* expression, but also other explanations can be envisaged. Our finding that *veggie* scions can be rescued by grafting them on wild type stocks, thus adds an important new piece of evidence showing that the endogenous *VEGGIE* protein (i.e. expressed at the normal sites at wild type levels) is a mobile signal that is similar or identical to florigen and is required for both the onset of flowering and specification of the identity of the SIM. The observation that the *veggie* phenotype reappears when the grafts grow taller, suggests that although *VEGGIE* is a long distance signal, its transport becomes limiting over distances that are more than five wild-type sympodial inflorescence meristems. Thus, as the inflorescence grows and generates more inflorescence units, the *VEGGIE* sources that specify the identity of the repeatedly appearing SIMs also move upwards. Overall it shows petunia is sensitive to the amount of *VEGGIE* mRNA present to initiate flowering and to maintain the cymose branching. *VEGGIE* is a mobile signal and therefore, we believe *VEGGIE* is a good candidate component to be the florigen of petunia.

VEGGIE is required continuously

Experiments with a heat-shock inducible *FT* transgene showed that in *Arabidopsis* the transient activation of *FT* in a single leaf by a 2 hr heat-shock is sufficient to accelerate flowering (Notaguchi et al., 2008). After the heat shock *FT* mRNA remains detectable for about 1 day and movement of *FT* protein across a graft junction could be detected after about 24 hrs. Yet, some ~2 weeks are needed before flowering is observed. This suggests that the *FT* pulse triggers the continuous expression of downstream factors, but not of *FT* itself, that promote flower formation. As a single pulse of *FT* expression in petunia is not sufficient to

trigger flowering, it appears that the network that is downstream of *VEGGIE/SFT* in petunia and *Arabidopsis* differs in some important aspects. This is in line with the observation that the transcription of homologous floral meristem identity genes in petunia and *Arabidopsis* is regulated by distinct networks (Souer et al., 2008).

The results of the grafting experiments further support that once flowering is initiated continued *VEGGIE* expression is required to specify the identity of the newly emerging SIMs. As *VEGGIE* travels over a long but limited distance, this implies that as the inflorescence grow taller, continuously new *VEGGIE* sources must be formed in the regions below the growing apex. This would suggests that the regulation of *VEGGIE* transcription during the flowering phase might be different regulated from its regulation during the vegetative phase. Before flowering *VEGGIE* expression cycles during the day, suggesting regulation by the clock, while expression levels are enhanced by long-day growth conditions, suggesting regulation by the photoperiod pathway. However, once flowering is initiated, *VEGGIE* mRNA still cycles over the day, but is no longer regulated by day length.

Material and methods

Genetic materials

The *veggie*^{C3385} allele arose spontaneously among progeny of the petunia line W138 and was maintained by inbreeding resulting in the line W258 (Chapter 3). The *veggie*^{C3385} mutants in a W138 background had a very low fertility. To solve the fertility problem of *veggie*^{C3385} F2 and F3 progeny of a cross between W258 (*veggie*^{C3385}) and the unrelated wild-type line W115 was used. In this hybrid background the flowering time and architectural defects of *veggie*^{C3385} were maintained, but fertility was restored so that crosses to W138 yielded sufficient seed to produce ~6000 F1 progeny, among which were 5 plants with the *veggie* phenotype (plants H7167-1, H7171-1, J7027-1, J7027-2 and J7030-1). Self-fertilization of the plant H7167-1 resulted in a thousand *veggie* mutants and three revertants (*VEGGIE* revertant1-3). Self-fertilization of each of the revertants resulted in a 3:1 ratio of wild-type and *veggie* progeny.

Transposon display

Genomic DNA was digested by MseI and ligated to MseI adaptors, generated by the annealing of primers MseI-1 and -2, in one single reaction and amplified by PCR with primers complementary to *dTPH1* and the Mse adaptor. The obtained PCR fragments were reamplified with a ³³P-labeled nested *dTPH1* primer and the Mse adapter primer extended with either an A, C, G or T, separated on a 6% polyacrylamide gel and visualized using a Phosphor Imager Scanner (Molecular Dynamics, Sunnyvale, CA). A 156-bp candidate *VEGGIE* fragment was cut from the display gel (MseI+C x out 13), reamplified by PCR, cloned in pGemT-easy (Promega) and sequenced with Big Dye terminator technology (Perkin Elmer).

Phylogenetic analyses

The protein alignment was constructed with a web-based version of ClustalW, using default settings and displayed in Boxshade 3.2.1 (http://www.ch.embnet.org/software/BOX_form.html). Genbank accession numbers: *Petunia hybrida* *VEGGIE* (no accession yet), *Lycopersicon esculentum* *SP3D* ([AAO31792](#)), *Oryza sativa* *Hd3A*(BAB61030), *Hordeum vulgare* *FT* ([ABI55203](#)), *Citrus unshiu* *FT* ([BAA77836.11](#)) and *Arabidopsis thaliana* *FT* ([BAA77839](#)).

DNA and RNA methodology

Full length genomic *VEGGIE* (5.5 kb) was amplified from W138 DNA with primers complementary to the 5' and 3' UTR and Phusion polymerase (BioKe). The *dTPH9* insertion in *veggie*^{C3385} mutants could be identified by PCR using primers complementary to *VEGGIE* intron 2 and *dTPH9* (Matsubara et al., 2005). RNAs were isolated from leaf tissue with TRIzol (Invitrogen) and were treated with DNaseI (Roche), and 2.5 µg RNA was used to make first strand oligo-d(T)¹⁸ cDNA with MLV-transcriptase (Promega) and the RT-PCRs were performed with gene-specific primers (Table 2). The PCR products were run on agarose gels, blotted on Hybond-N⁺ membranes (Amersham), hybridized with ³²P-labeled gene-specific probes and the signal was detected using a Phosphorimager (GE Healthcare). The full length *VEGGIE* cDNA was obtained via 5' and 3' Rapid Amplification on cDNA prepared from W138 inflorescence apices (First Choice RLM-RACE kit, Ambion) with *VEGGIE*-specific primers.

Gene constructs and plant transformation

The *VEGGIE* open reading framing was amplified from start- to stop-codon with primers containing NcoI and NotI restriction sites (digestion sites indicated in bold in the primers, listed in table 2), digested with NcoI and NotI and ligated into pEntry4 digested with NcoI and NotI. pEntry4-*VEGGIE* was recombined to pK7GW2 (Karimi et al., 2002) by GATEWAY LR Clonase II (Invitrogen) to yield *p35S::VEGGIE*.

A 2.25 kbp fragment upstream the ATG of *VEGGIE* was obtained by screening a genomic library of line W137. The *VEGGIE* promoter region was amplified from a phage lambda clone containing a genomic *VEGGIE* fragment with T7 pGEM TOPO primer complementary to the left phage arm and primer upstream the ATG (*VEGGIE*-ATG), inserted in pEntryD-TOPO by TOPO cloning and subsequently recombined into pKGWFS7,0 ((Karimi et al., 2002) to yield *pVEG::GUS*.

All the PCR amplifications used for cloning purposes were performed with Phusion polymerase (Bioke), with the proof reading activity. All the constructs were sequenced full length with Big Dye terminator technology (Perkin Elmer) before they were transformed into petunia line W115 by Agrobacterium-mediated leaf disc transformation (Spelt et al., 2000).

p35S::FT was kindly provided by Prof. Dr. Detlef Weigel and was introduced in *veggie* mutants (F2 hybrids of the petunia lines W115 and W258).

Fusions of the *HEAT SHOCK PROMOTER* (*HSP*) fused to *GUS* or *FT* were kindly provided by Prof. Dr. Ove Nilsson and the *pHSP::FT*, *pHSP::GUS* or both were introduced in *veggie* mutants (F2 hybrids of the petunia lines W115 and W258).

Plant transformation

Petunia plants were transformed by the Agrobacterium-mediated leaf disc transformation (Spelt et al., 2000). *Arabidopsis* plants were transformed by the floral dip method (Weigel, 2002).

Growth conditions

Growth conditions are the same for both *Arabidopsis Columbia* and *Petunia hybrida* plants. Long-day conditions: 16 hr light and 8 dark in greenhouse conditions (temp.>19 degrees, when needed artificial light). Short-day

conditions: 8 hr light and 16 hr dark in climate cabinet (temp. day 20 degrees, night 19 degrees, humidity 70%).

Table 2. Primers used for the transposon display and for amplification of *VEGGIE* products

| Primer | fw/rev | sequence 5'-3' |
|---------------------------------------|-------------|---|
| Out12 <i>dTPH1</i> | | CAGCATTGACACCCCTTC |
| Out13 <i>dTPH1</i> (nested on out 12) | | CAGTGTAAATTTGCGCAAA |
| MseI-1 ¹ | adaptor1 | GACGATGAGTCCTGAG |
| MseI-2 ¹ | adaptor2 | TACTCAGGACTCAT |
| Mse | Mse adaptor | GACGATGAGTCCTGAGTAA |
| Mse+CMse adaptor (nested) | | GACGATGAGTCCTGAGTAAC |
| <i>VEGGIE</i> atg | fw | TTACCAT GG ATATGCCAAGAGAACGTGAACAC |
| <i>VEGGIE</i> stop | rev | AGTACATAGG GC GG CC GC ATTAATCGGCAGAC |
| 5'UTR <i>VEGGIE</i> | fw | GAATACCAACATTGCTAGCCCC |
| 3' UTR <i>VEGGIE</i> | rev | GGGATGACATATTGCTAGTTAGC |
| <i>VEGGIE</i> exon1 | fw | CCCTTCACAAGATCCATAAG |
| <i>VEGGIE</i> exon2 | rev | GAGGTATTCTCTTAGATTGG |
| <i>VEGGIE</i> exon4 | rev | CAAGTCATTAATCGGCAGACC |
| <i>ACTIN</i> | fw | AGATCTGGCATCATACCTTCTACA |
| <i>ACTIN</i> | rev | CCMGCAGCTTCCATRCCAATCA |
| 3'RACE | rev | GCGAGCACAGAATTAAACGACT |
| 3'RACE nested | rev | CGCGGATCCGAATTAAACGACTCACTATAGG |
| <i>VEGGIE</i> intron 2 | fw | GATATCAACAGAACATGGATCG |
| <i>dTPH9</i> | fw | CTCAATTAGACTTGAGCAGCAGG |
| T7 pGEM TOPO | | CAC CTAATACGACTCACTATAGGGAG |
| <i>VEGGIE</i> -atg rev | | ATTAACGATAAAAAAATAGAGGAACTAC |

¹ MseI-1 and -2 were annealed to generate a double stranded Mse adaptor

Grafting

Vegetative *veggie* shoots (W258) were grafted on flowering V26 plants donor stocks. The grafts junction were sealed with Leukopor (Hansaplast, Germany) and young grafts were covered with plastic to prevent dehydration until the grafts started to grow. Controls were performed on the same plants.

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Chapter 4

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Chapter 5

The analysis of VEGGIE protein-protein interactions

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Abstract

VEGGIE has a dual role in petunia development not only to promote the switch from vegetative growth to flowering but also to specify the architecture of the cymose inflorescence, whereas the orthologous *FT* gene in *Arabidopsis* is only essential for the onset of flowering, but is not required to specify the architecture of the racemose inflorescence architecture. As *VEGGIE* and *FT* encode functionally similar proteins and are expressed in similar patterns, we aimed to identify and compare proteins that bind to *FT* and *VEGGIE* to understand how they acquired distinct roles in development of the inflorescence. In *Arabidopsis* the bZIP transcription factor FD interacts in the apex with *FT* and so promotes flowering. We observed that *VEGGIE* interacts in the petunia apex with various bZIP transcription factors that all belong to the same sub-group and focussed on the bZIP transcription factors that were most similar to FD to study their putative function in inflorescence architecture and their floral promoting potential. We confirm that these bZIP transcription factors are expressed in the inflorescence and they might be the FD homologs in petunia.

Introduction

Plants carefully control the moment when they switch from vegetative growth to flowering, which is of key importance for reproductive success. This developmental switch is controlled by endogenous factors as well as environmental factors, to ensure that distinct individuals flower simultaneously. A key parameter that is used by many species to monitor the progression of the seasons is day length. Some species flower in the spring or early summer when the day length exceeds a certain threshold (long day plants), whereas others flower later in the season, when day length drops below a certain threshold value (short day plants). Grafting experiments showed that day length is perceived in the leaves to generate a mobile signal, termed florigen, which moves to the plant apex, where it promotes the formation of flowers instead of leaves or other vegetative structures.

Recent work in *Arabidopsis* showed that the protein encoded by *FLOWERING LOCUS T (FT)* is a major component of florigen, or may even be identical to florigen. FT belongs to a small family of highly similar proteins that also includes *TERMINAL FLOWER 1 (TFL1)*, which acts antagonistically as a floral repressor (Ratcliffe et al., 1998). Structural analysis of a *TFL1* homolog from tobacco revealed that it contains a large central β -sheet with a strongly conserved binding pocket capable of binding phosphorylated proteins (Banfield and Brady, 2000). Remarkably, alterations of only a few amino acids in the binding pocket of *TFL1* are sufficient to change it from a floral repressor into a floral inducer, like *FT* (Ahn et al., 2006; Hanzawa et al., 2005). This minimal change in the protein suggests that *FT* has high structural similarity to *TFL1* and therefore might interact with similar kind of transcription factors and phosphorylated proteins (Ahn et al., 2006; Hanzawa et al., 2005).

Transcription of *FT* takes places in vascular tissues of leaves when day length exceeds a certain threshold, and the FT protein was shown to travel to the apex of the plant, where it promotes the formation of flowers (Corbesier et al., 2007; Jaeger and Wigge, 2007; Kobayashi and Weigel, 2007; Mathieu et al., 2007; Notaguchi et al., 2008; Tamaki et al., 2007). Within the apex the FT protein is thought to bind to a bZIP transcription factor that is encoded by *FD*, to activate transcription of subordinate genes that specify floral fate (Abe et al., 2005; Wigge et al., 2005). *FD* directly controls the expression of the MADS-box

floral meristem identity gene *APETALA1* (*AP1*), in concert with a transcription factor encoded by the floral meristem identity gene *LEAFY* (*LFY*) (Abe et al., 2005; Wagner et al., 1999). In the *AP1* promoter motives are found that resemble binding-sites for bZIP transcription factors, supporting the claim FT-FD acts directly on *AP1* (Abe et al., 2005; Kaufmann et al., 2010; Wigge et al., 2005). In *lfy*, *ft* and *fd* mutants *AP1* expression is delayed, whereas in *lfy ft* and *lfy fd* double mutants the floral transition is blocked (Abe et al., 2005; Ruiz-Garcia et al., 1997; Wigge et al., 2005) indicating that LFY and FT-FD act in parallel pathways to promote floral cell fate and converge to induce *AP1* expression.

In *Arabidopsis* the apical inflorescence meristem is indeterminate as it grows indefinitely while producing at its periphery lateral meristems that develop into flowers. This results in an inflorescence with a racemose architecture, which is just one of the three major classes of inflorescences found in nature (Prusinkiewicz et al., 2007; Weberling, 1989). The nightshades (Solanaceae) tomato and petunia generate inflorescences of a distinct class that is named cyme. In cymes it is the apical meristem that forms the flower and growth continues from a lateral (also called sympodial) meristem that emerges at the periphery of the apical floral meristem (Castel et al., 2010). This sympodial inflorescence meristem (SIM) continues growth of the inflorescence by generating at its periphery the next SIM before it acquires floral identity and terminates by forming a flower.

The differences between the spatio-temporal control of floral meristem identity in cymes and racemes are at least in part due to alterations in the expression patterns of the floral meristem identity genes, such as *LFY* and its petunia ortholog *ABERRANT LEAF AND FLOWER* (*ALF*), as well as *UNUSUAL FLORAL ORGANS* and its petunia ortholog *DOUBLE TOP* (*DOT*) (Souer et al., 2008; Souer et al., 1998; Weigel et al., 1992; Wilkinson and Haughn, 1995). In the racemose *Arabidopsis* inflorescence *LFY*, *FD* and the downstream gene *AP1* are expressed in lateral meristems, while their expression in the apical inflorescence meristems is repressed by a pathway that involves *TFL1* (Conti and Bradley, 2007). In the cymose inflorescences of petunia and tomato, however, the floral identity genes *ALF* and *DOT* and their tomato homologs *FALSIFLORA* (*FA*) and *ANANTHA* (*AN*) are first expressed in apical meristems, while their

expression in the SIM is delayed or transiently repressed (Lippman et al., 2008; Molinero-Rosales et al., 1999; Souer et al., 2008).

Mutations in the tomato and petunia orthologs of *FT*, which are known as *SINGLE FLOWER TRUSS* (*SFT*) and *VEGGIE* respectively, cause a similar late flowering phenotype as *Arabidopsis ft* mutants, indicating that *SFT* and *VEGGIE* are like *FT* required for the onset of flowering (Chapter 4) and (Lifschitz et al., 2006). However, unlike *ft*, the *sft* and *veggie* mutations cause additional defects in the architecture of the inflorescence and the development of the SIM. That is, in *veggie* and *sft* mutants, the SIM initially develops as a vegetative meristem and generates leaves before it generates the next SIM and converts into a flower (chapter 3) and (Lifschitz et al., 2006). This defect in SIM development, a delayed specification of SIM/ floral identity, resembles the defect seen in the primary apical meristem (delayed onset of flowering).

Despite the clear differences in the role of *VEGGIE* and *SFT* during development as compared to *FT*, the three genes have highly similar spatio-temporal expression patterns and encode functionally interchangeable proteins (Chapter 4) (Lifschitz et al., 2006). This suggests that the functional diversification may result from differences in downstream genes, or in the expression patterns of proteins that interact with *VEGGIE*, *SFT* and *FT*. To specifically identify proteins that interact with *VEGGIE* in the apex of petunia and to learn more about the *VEGGIE* function in the cymose floral architecture we performed a yeast two-hybrid assay with *VEGGIE* as bait and a petunia inflorescence meristem library as prey. We show that *VEGGIE* interacts with various proteins, like bZIP transcription factors, and possibly with TCP proteins and a 14-3-3 protein. The bZIP transcription factors found show high similarity to FD from *Arabidopsis* and will be studied in more detail.

Results

Identification of proteins interacting with VEGGIE.

To understand the role of *VEGGIE* in the specification of the identity of apical and sympodial inflorescence meristems (SIMs), we sought for proteins that can interact with *VEGGIE* that might act as “receptors” and could add spatial specificity to the *VEGGIE* signal. Therefore, we used a yeast two-hybrid assay

and screened a cDNA library screen that was prepared from inflorescence apices with a VEGGIE bait. Before the actual yeast two-hybrid cDNA screen experiment we first addressed whether the VEGGIE bait would cause auto-activation of the reporter genes, or whether VEGGIE could form dimers. Full length VEGGIE (178 amino acids) fused to the DNA-binding domain of GAL4 (VEGGIE-GAL4^{BD}) was co-expressed with full length VEGGIE fused to the GAL4-activation domain (VEGGIE-GAL4^{AD}) or with the GAL4^{AD} domain alone and in either case no activation of the GAL4-regulated *HIS3*, *ADE2* and *lacZ* reporter genes was observed, indicating that VEGGIE lacks a transcription activation domain and cannot homo-dimerize (data not shown).

Next, we used the VEGGIE-GAL4^{BD} bait to screen a petunia R27 inflorescence meristem cDNA library (de Bruin, 2002) that was fused to the GAL4-activation domain. In total we screened 300.000 transformants for recombinants expressing a protein that can interact with VEGGIE and allow growth on drop-out media lacking leucine, tryptophan and histidine. This yielded 79 yeast transformants with a *HIS*⁺ phenotype: 40 of these were also *ADE*⁺ and could grow on plates lacking both histidine and adenine. Sequence determination of the prey plasmids in these 40 transformants revealed that they originated from 30 distinct mRNAs that encoded three different TCP transcription factors (11 clones), five different bZIP transcription factors (6 clones), a 14-3-3 protein (2 clones), while the remainder encoded either a protein with unknown function or contained multiple GAL4^{AD} plasmids with different inserts (21 clones) (Table 1).

TCP proteins comprise a family of plant-specific transcription factors that regulate plant growth and are named after their founder genes, ***TEOSINTE BRANCHED1*** (*TB1*) from maize, ***CYCLOIDEA*** (*CYC*) from *Antirrhinum* and ***PROLIFERATION CELL FACTOR*** (*PCF1*) from rice (Cubas et al., 1999). The TCP domain is a conserved basic-helix-loop-helix structure and is involved in dimerization and DNA-binding (Cubas et al., 1999). The three TCP proteins that were identified in the screen with VEGGIE bait, could activate the *HIS* and *ADE* reporter genes when co-expressed with VEGGIE-GAL^{BD} but also with GAL4^{BD} alone. Previous yeast two-hybrid screens of the same inflorescence cDNA library with distinct baits also yielded TCP proteins. Although the TCP proteins that were identified in the various screens were all different, they all gave a positive

Table 1: Putative interactors of VEGGIE identified by yeast two hybrid screen of an inflorescence cDNA library

| clone | confirm ¹ | equal to | SGN or NCBI | homology | Gene with highest similarity in Solanaceae data base or Genbank | |
|-----------------------|----------------------|-------------------|----------------|--|---|-------------|
| | | | | | species | E- value |
| TCP proteins | | | | | | |
| 10 | yes | 3,22,23,44, 58 | U279653 | TCP transcription factor TCP3 transcription factor | <i>Solanum tuberosum</i> | 3E-52 |
| 14 | yes | | U345840 | TCP3 transcription factor | <i>Solanum lycopersicum</i> | 9E-37 |
| 16 | yes | 43 | U211824 | TCP transcription factor | <i>Petunia hybrida</i> | 0E+00 |
| bZIP proteins | | | | | | |
| 19 | yes | | U283737 | AREB3, bZIP transcription factor self-pruning G box | <i>Solanum tuberosum</i> | 6E-56 |
| 37 | yes | | EF136919 | protein (SPGD) self-pruning G box | <i>Solanum lycopersicum</i> | 2E-62 |
| 56 | yes | 66 | ABL84199 | protein (SPGD) AREB2, bZIP | <i>Solanum lycopersicum</i> | 9E-22 |
| 62 | yes | | U321277 | transcription factor ABF3, bZIP | <i>Solanum lycopersicum</i> | 1E-134 |
| 78 | yes | | U284254 | transcription factor | <i>Solanum tuberosum</i> | 3E-61 |
| 14-3-3 protein | | | | | | |
| 15 | yes | 60 | U271901 | 14-3-3 protein GF14 omicron | <i>Solanum tuberosum</i> | 1E-171 |
| other proteins | | | | | | |
| 4 | no | 77 | U207762 | HMG (high mobility protein) Glycoside hydrolase | <i>Petunia hybrida</i> | 1E-146 |
| 8 | no | | U285800 | family 3 | <i>Solanum tuberosum</i> | 1E-118 |
| 31 | no | | U208858 | osmotin-like protein | <i>Petunia hybrida</i> | 1E-153 |
| 35 | no | | U207606 | glutamine synthase calmodulin-binding | <i>Petunia hybrida</i> | 3E-70 |
| 45 | no | | U344266 | family protein | <i>Solanum lycopersicum</i> | 4E-43 |
| 54 | no | | U208565 | histone H2A | <i>Petunia hybrida</i> | 4E-35 |
| 59 | no | | U315647 | heat shock protein 70 peptide chain release factor | <i>Solanum lycopersicum</i> | 1E-107 |
| 69 | no | | U217607 | | <i>Solanum lycopersicum</i> | ? |

¹For some clones the interaction of the encoded fusion protein as confirmed by retransforming the plasmid cloned in E. coli into the yeast two hybrid host.

response when co-expressed with the empty GAL4^{BD} vector (de Bruin, 2002) and were therefore excluded from further analysis.

14-3-3 proteins are important regulators of a wide variety of cell processes, including transcription (Schultz et al., 1998; van den Wijngaard et al., 2005; van Hemert et al., 2001). Two clones that interacted with VEGGIE encode the same 14-3-3 protein, PhY15, which shows high sequence similarity to 14-3-3 omicron from *Arabidopsis* (Q9S9Z8) and TFT7 from tomato (AAD46005). Yeast two-hybrid screens performed with the VEGGIE-homologs FT from *Arabidopsis*, SFT from tomato and Hd3A from rice also yielded 14-3-3 proteins (Abe et al., 2005; Pnueli et al., 2001; Purwestri et al., 2009; Wigge et al., 2005). The constitutive expression of the rice 14-3-3 protein GF14c resulted in a delay in flowering while knockout mutants displayed early flowering. These results suggested that GF14c acts as a negative regulator of flowering by binding to Hd3A (Purwestri et al., 2009). However, the interaction of VEGGIE with PhY15 was rather weak and could only be scored with the *HIS* reporter gene, but not with the more stringent *ADE* and *LacZ* reporter and was therefore not studied in more detail.

The most promising set of putative VEGGIE interactors were the bZIP transcription factors, because they specified a *HIS*⁺ *ADE*⁺ phenotype and could drive robust β-galactosidase expression when co-expressed with VEGGIE-GAL4^{BD}, but not with GAL4^{BD}. bZIP transcription factors contain a basic-leucine-zipper motif, N-7x-[RK]-9x-L-6x-L-6x-L, that is able to dimerize with the basic-leucine-zipper motif in other bZIP transcription factors and contains a specific DNA-binding domain in its N-terminal part. The *Arabidopsis* genome encodes 77 different bZIP transcription factors that can be classified in 13 groups, named A-L and S (Correa et al., 2008), based on conserved regions outside the bZIP domain. All the petunia bZIP transcription factors that interacted with VEGGIE have high similarity to the AREB/ABF/AB15 family (group A) from *Arabidopsis* (Choi et al., 2000; Uno et al., 2000). Most of these *Arabidopsis* A-type bZIP transcription factors are involved in ABA responses and fruit maturation or act in ABA-dependent signalling pathways that are involved in desiccation and salt stress (Jakoby et al., 2002), but two members of this A group bZIP transcription factors, FD and FDP (AtZIP14 and 27), interact with FT to induce flowering (Abe et al., 2005; Wigge et al., 2005).

VEGGIE interaction with bZIP transcription factors

The interaction of VEGGIE-GAL4^{BD} with GAL4^{AD} fusions of the full size bZIP transcription factors PhY19, PhY37, PhY56, PhY62, PhY78 from petunia, FD from *Arabidopsis* or, GAL4^{AD} alone was scored by assaying the expression of the *HIS*, *ADE* and *LacZ* reporter genes (Fig. 1). PhY37, PhY56 and PhY78 displayed a relatively strong interaction with VEGGIE as they conferred a *HIS*⁺ *ADE*⁺ phenotype and robust *LacZ* expression. PhY62 also interacts with VEGGIE, but this interaction was too weak to be detected with the *LacZ* reporter. We consider it unlikely that this weak response is due to a relatively low expression of PhY62-GAL4^{AD} as the same construct can efficiently activate *LacZ* when co-expressed with GAL4^{BD} fusions of VEGGIE paralogs (Chapter 6). PhY19 could activate the *HIS*, *ADE* and *LacZ* reporters when co-expressed with VEGGIE-GAL4^{BD} but also with GAL4^{BD} alone and was therefore discarded. The interaction of VEGGIE with FD from *Arabidopsis* was very weak and the activation of the *LacZ* reporter was barely detectable. This result was not expected, because FT and FD interact quite strongly in a yeast two hybrid assay (Abe et al., 2005) and

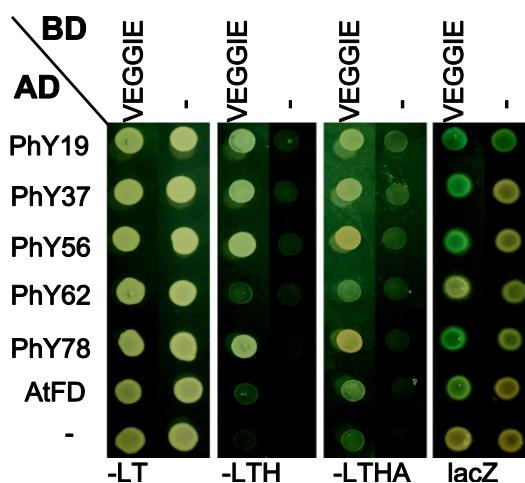


Figure 1. Yeast two hybrid interaction between VEGGIE and the different bZIP transcription factors. Double transformed yeasts (VEGGIE- GAL4^{BD} or empty- GAL4^{BD} with bZIP- GAL4^{AD}, FD- GAL4^{AD} or empty- GAL4^{AD}) on various selection media. Drop-out media lacking leucine and tryptophan (**-LT**, control), drop-out media lacking leucine, tryptophan and histidine (*HIS3* selection) (**-LTH**), drop-out media lacking leucine, tryptophan, histidine and adenine (*HIS3* and *ADE2* selection)(**-LTHA**) and test for β-galactosidase activity (**LacZ**).

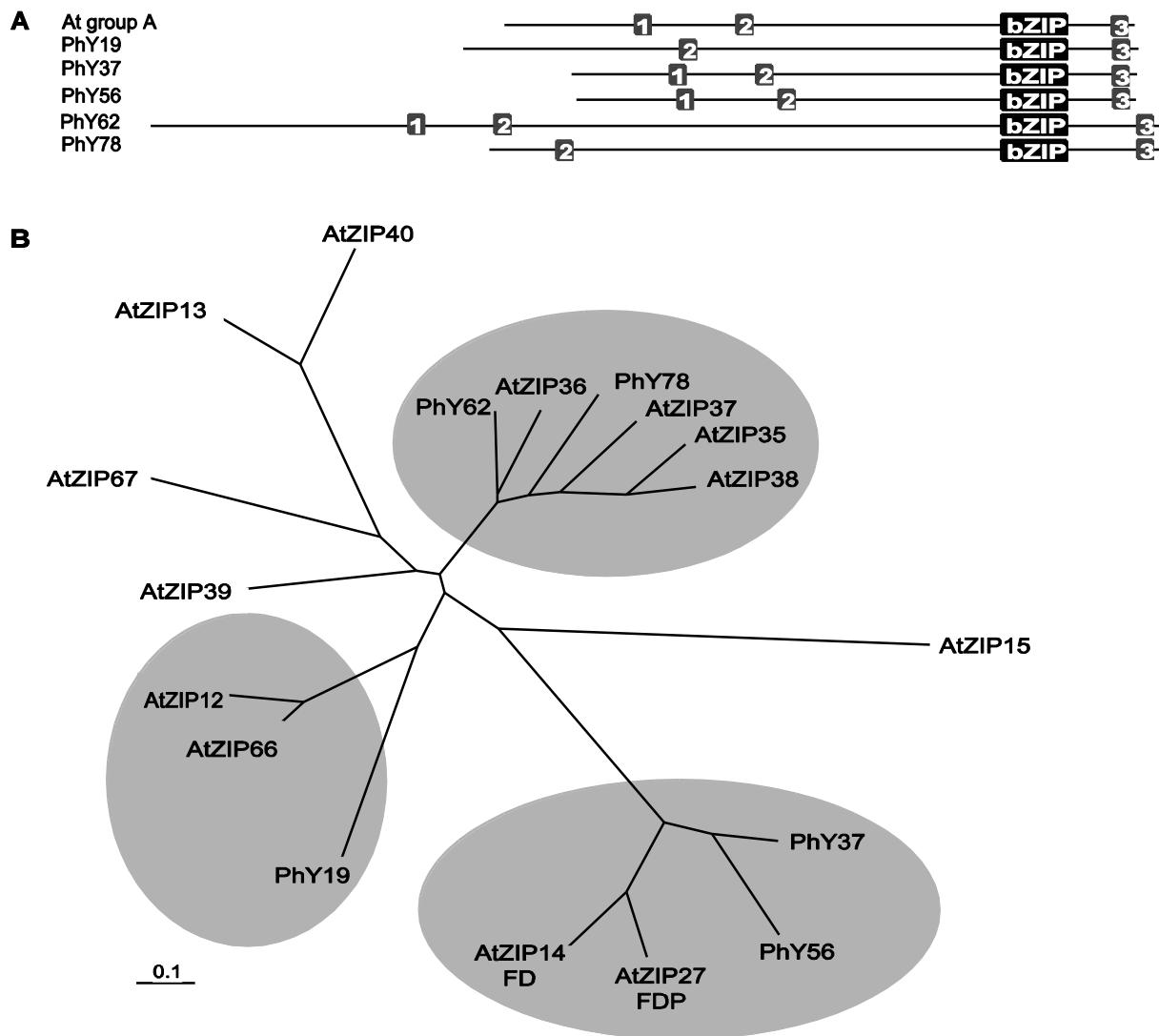


Figure 2. Petunia bZIP transcription factors interacting with VEGGIE.

A. Diagram depicting the position of diagnostic domains in an *Arabidopsis* group A bZIP transcription factor and the petunia bZIP transcription factors interacting with VEGGIE. The conserved domains 1, 2 and 3 are indicated by the grey boxes and the bZIP domain is indicated in black. **B.** Alignment of the conserved domains from the VEGGIE interacting bZIP transcription factors, *Arabidopsis* AtZIP14 (FD) and AtZIP27 (FDP) and the tomato bZIP, SPGB. For the alignment domain 1 [TSNR][VM][DEG][EDQ][VI]W, domain 2 [TS][LI][EF][DEQ][FLD][LF][LVAF], the bZIP domain N-7x-[RK]-9x-L-6x-L6x-L6x-L and domain 3 [LI]xRxx[ST] were used. **C.** Unrooted dendrogram of all the *Arabidopsis* bZIP transcription factors from group A, the petunia bZIP transcription factors that interact with VEGGIE, the tomato bZIP, SPGB, and the FD homologs TaDFL2 and ZmDFL1 from the monocots wheat and maize respectively.

because VEGGIE and FT can substitute each other's function when transformed into *Arabidopsis* and petunia respectively (Chapter 4).

The bZIP transcription factors that interact with VEGGIE contain three conserved motifs besides the basic-leucine-zipper domain as do the bZIP transcription factors from the A group from *Arabidopsis* (Fig. 2A). To study the similarity between the conserved domains of the petunia bZIP transcription factors we compared them to the two most related bZIP transcription factors from the *Arabidopsis* A-group, AtZIP14 (FD) and AtZIP27 (FDP), and a putative FD homolog from tomato (SPGB) (Fig. 2B). PhY37 and SPGB are most similar based on the four domains, as are FD, FDP and PhY56. The other three bZIP transcription factors, PhY19, PhY62 and PhY78, are less similar to FD and FDP than PhY37 and PhY56.

To assess the evolutionary relationships among the A-group bZIP transcription factors, we constructed a phylogenetic tree based on the alignment of the four conserved domains from the bZIP transcription factors from petunia, *Arabidopsis*, tomato and the putative FD homologs from the monocots, wheat *FD-LIKE 2 (FDL2)* and maize *DELAYED FLOWERING 1 (DFL1)* (Fig. 2C) (Li and Dubcovsky, 2008; Muszynski et al., 2006). The unrooted dendrogram shows that PhY19 is most similar to AtZIP12 and AtZIP66 (DPBF4 and AREB3 respectively) and to TaFDL2 and ZmDFL1, the FD homologs from the monocots, while PhY62 and PhY78 cluster with AtZIP35, AtZIP36, AtZIP37 and AtZIP38 (ABF1, ABF2, ABF3 and ABF4).

PhY37 and PhY56 form together with FD (AtZIP14), FDL (AtZIP27) and SPGB a separate clade within the A-group of bZIP transcription factors, suggesting that these proteins may be functionally equivalent and possibly orthologous. Moreover, a full length amino acid alignment of PhY37, PhY56, the *Arabidopsis* FD, FDP and the putative FD homolog of tomato, SPGB, revealed that these five proteins display similarity in several additional regions, besides the motifs 1-3 and the bZIP domain, even though the overall sequence identity is only ~ 24%. (Fig. 3). Interestingly PhY37 and FDP both contain a proline stretch in the middle of the protein suggesting that PhY37 might be the FDP homolog and PhY56 the FD homolog of petunia. Although, PhY37 and PhY56 are the most likely candidates to be the petunia FD and FDP homolog, we can at this stage not exclude that petunia encodes distinct bZIP transcription factors that are more closely related to FD.

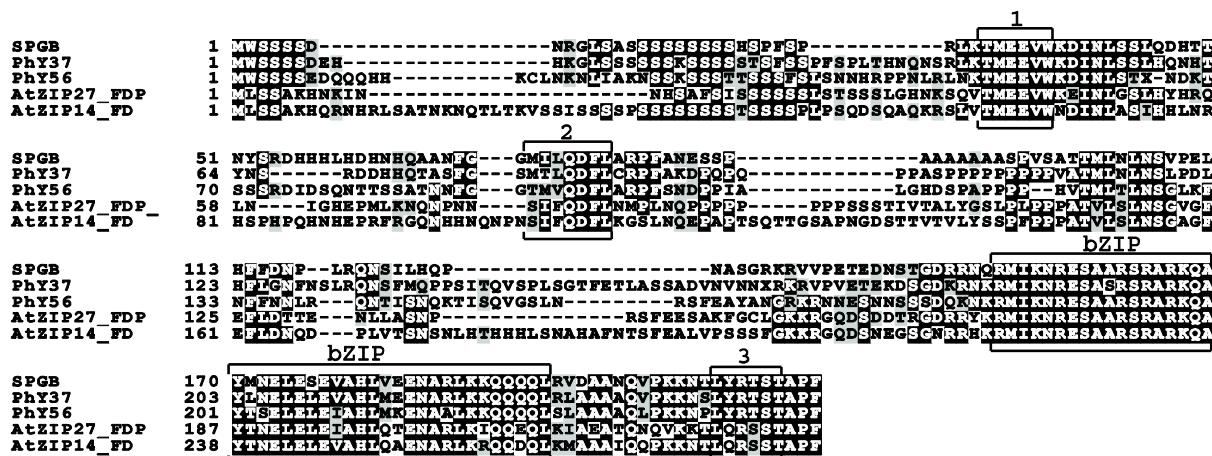


Figure 3. Alignment of SPGB of tomato, PhY37 and PhY56 of petunia and the FD and FDP of *Arabidopsis*. The conserved domains 1, 2, 3 and the bZIP domain are indicated by the boxes. Amino acid identity is indicated by black shading, and similarity by grey shading .

Expression of the petunia bZIP transcription factors PhY37 and PhY56 in *Arabidopsis*

To learn more about the function of PhY37 and PhY56, we constitutively expressed their cDNAs from the Cauliflower Mosiac Virus 35S promoter in *Arabidopsis* (Columbia). This resulted in 20 independent *p35S::PhY37* lines and 24 independent *p35S::PhY56* lines. The T2 progeny of three of the *p35S::PhY37* and *p35S::PhY56* lines were grown in both long-day (LD; 16hr light and 8hr dark) and short-day (SD; 8hr light and 16hr dark) conditions. Although the transgenes were highly expressed in each line, they did not cause any obvious aberrations in development and the transformed plants flowered at the same time as wild type (data not shown).

Abe et al (2005) showed that 1-week old *p35S::FD* *Arabidopsis* seedlings that were grown in long-day conditions already expressed *AP1*, whereas *p35S::FD* seedlings that were grown in short-day conditions and consequently do not express *FT* were unable to express *AP1* (Abe et al., 2005). To assess whether constitutive expression of *PhY37* or *PhY56* could precociously activate *AP1* in the same way as *FD*, we isolated RNA from 1-week old *p35S::PhY37* or *p35S::PhY56* seedlings that were grown in either LD or SD conditions. Both the *PhY37* and *PhY56* transgene were expressed in the *Arabidopsis* seedlings but they were

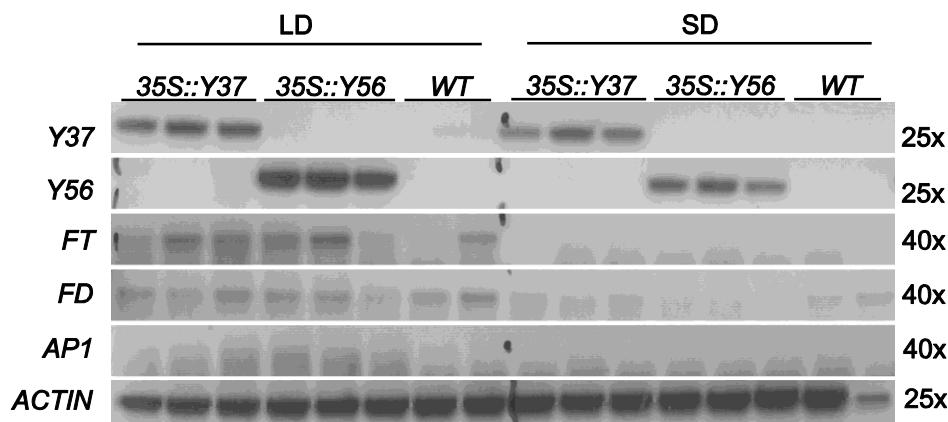


Figure 4. Can 35S::PhY37 and 35S::PhY56 induce flowering in *Arabidopsis* seedlings? RT-PCR analysis of the respective transgene, *FT*, *FD* and *AP1* and the internal control *ACTIN* in 1-week old *Arabidopsis* wild type (*WT*), 35S::*PhY37*, and 35S::*PhY56* seedlings grown in both long-day (LD) or short-day (SD) conditions. The number of amplification cycles used to detect the different transcripts is indicated on right side of the figure.

unable to induce *AP1* in LD conditions despite the fact that *FT* and, at lower levels, *FD* were expressed (Fig. 4). Why are *PhY37* and *PhY56* not able to precociously activate *AP1*? Are *PhY37* and/or *Ph56* not functionally equivalent to *FD* or are specific partners of *PhY37* and *PhY56* absent in *Arabidopsis*?

To examine whether *PhY37* or *PhY56* could substitute for the *FD* protein, a *fd* T-DNA insertion line (SALK_054421) was transformed with *p35S::PhY37* and *p35S::PhY56*. The T2 progenies of three independent *fd* *p35S::PhY37* and *fd* *p35S::PhY56* lines were selected for the presence of the transgene and were monitored for differences with homozygous *fd* mutants or heterozygous siblings. However, possibly due to the variable conditions in the greenhouse, we could not properly distinguish *fd* mutants and *FD* lines by phenotype and, hence, could not assess whether *PhY37* or *PhY56* could substitute for *FD*.

Expression pattern of the bZIP transcription factors in petunia.

To study the spatial expression patterns of the five different bZIP transcription factors within wild type inflorescences we performed *in situ* hybridization. Only the mRNA of *PhY62* was detectable in this way. Fig. 5 shows that *PhY62* is expressed in the stamens, but no expression could be detected in the young floral meristems (Fig. 5A) or in the SIM (Fig. 5B). Therefore we concluded that



Figure 5. In situ hybridization of *PhY62* mRNA in wild type inflorescence apices.

A. *PhY62* mRNA signal in the stamen of a full grown flower (F) (left), the floral meristem (FM) (right) shows no signal. **B.** *PhY62* mRNA signal in the stamen of a full grown flower (F) (left), but no signal in the inflorescence meristem (IM) (right). Scale bar is 100 μ M.

PhY62 is unlikely to be the gene that specifies the identity of the SIM by interaction with VEGGIE.

The expression patterns of *PhY37* and *PhY56* are the most interesting ones because they show high similarity with the *FD* and *FDP* of *Arabidopsis*. As we could not detect the corresponding mRNAs by in situ hybridization, we made reporter genes in which a 4.5 kb promoter of *PhY37* and 1.5 kb promoter of *PhY56* was placed in front of the fused coding regions of GFP and GUS

(*pPhY37::GFP:GUS* and *pPhY56::GFP:GUS*), and transformed into petunia plants. Three out of seven *pPhY37::GFP:GUS* transformants showed GUS activity. Although we observed differences in the strength of expression between transformants, the pattern of expression was identical. No GUS expression was seen in seedlings or vegetative tissues, but strong GUS expression was detected in floral tissues. In almost fully developed flowers, GUS expression was observed in the petals, at the base of stamen and in the developing carpel while in floral meristems (FM) GUS expression was observed in all emerging floral organ primordia (Fig. 6A). When we zoom in on the floral (FM) and inflorescence meristem (IM) the *PhY37* promoter driven GUS expression was observed in all floral tissues, but no activity was seen before or upon floral transition (Fig. 6B and C). In between the FM and the IM a specific dot of GUS expression was seen,

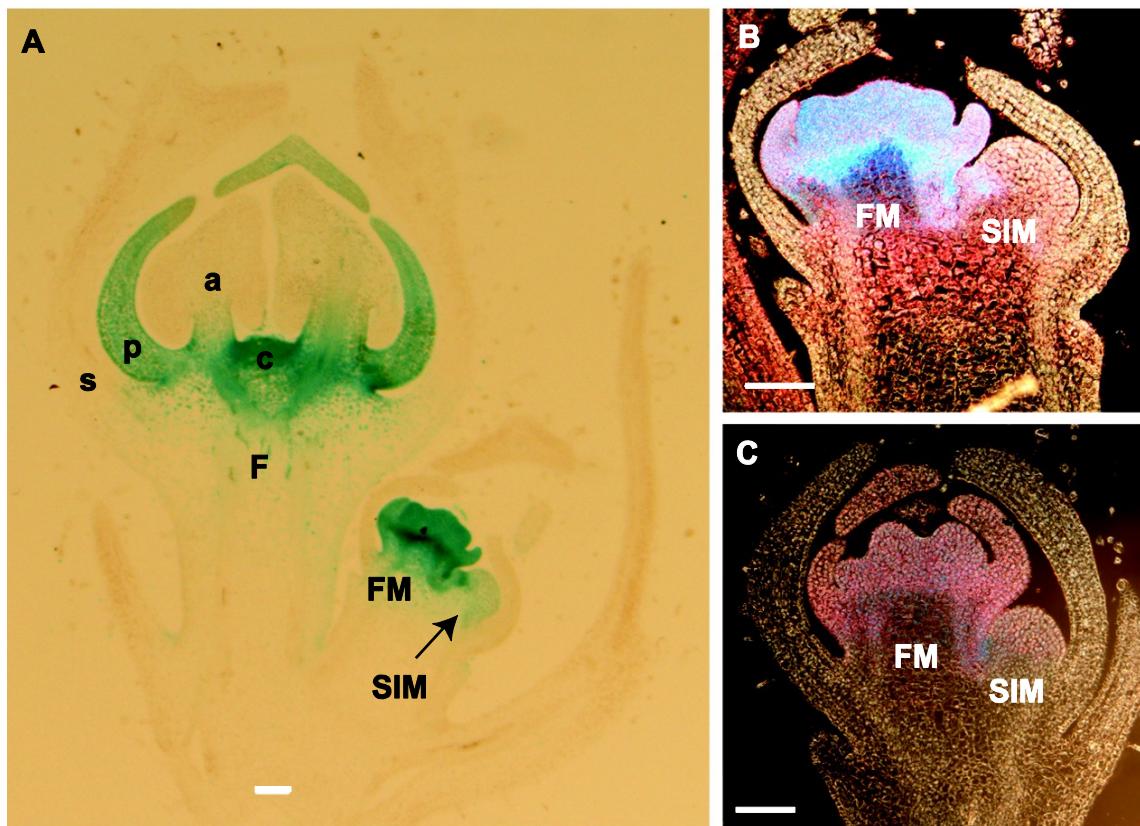


Figure 6. Expression analysis of the *pPhY37* promoter in wild type petunia plants.

A. Inflorescence of a *pPhY37::GFP:GUS* petunia. In the flower (F) GUS expression was observed in the petals (p), at the base of the anthers (a) and in the emerging carpel (c), but not in the sepals (s). In the floral meristem (FM) all floral organ primordia show GUS expression whereas no expression was seen in the inflorescence meristem (SIM). **B.** Detail of Fig. 6A, strong GUS expression was observed in the FM and hardly any in the IM. **C.** Another transgenic apex showed the same results, strong GUS expression in the FM and hardly any in the IM. Photos were taken in bright- and dark-field. Scale bar is 100 μ M.

but we could not adjudge this signal to any known plant organ based on the petunia -morphology (Castel et al., 2010).

Transformation of *pPhY56::GFP:GUS* in petunia yielded seven transgenic plants, of which only one showed weak GUS expression. GUS activity in this plant was limited to the young leaves and could not be seen in the vegetative meristem (Fig. 7A). After the floral transition GUS activity was detected in the base of full-grown flowers and again in the youngest bracts (Fig. 7B and C).



Figure 7. Expression analysis of the *PhY56* promoter in wild type petunia plants.

A. Expression of *pY56::GFP:GUS* in the apex of a vegetative petunia plantlet. **B.** GUS expression in the inflorescence of a *pY56::GFP:GUS* petunia plant. From left to right, a young developing flower behind a FM/IM meristem and a full grown flower. **C.** A young developing flower at the back, a FM (left) and a IM (right) in front. Photos taken in bright-field. Scale bar is 100 µM.

Dominant negative bZIP transcription factor phenotype?

We screened available collections of transposon insertion mutants (Vandenbussche et al., 2008) but could not identify mutants in which a gene encoding one of the identified bZIP proteins was disrupted by a *dTPH1* transposon insertion. As an alternative we attempted to create loss of function phenotypes by expressing a fusion of *PhY37* or *PhY56* to the ERF2 motif from tobacco, which is expected to create a dominant negative phenotype. ERF (for EAR- associated amphiphilic repression) is plant-specific motif that acts as a transcription repression domain (Ohta et al., 2001) that upon fusion to a transcription factor converts it into a dominant repressor that can specify a loss of function phenotype (Hiratsu et al., 2003). The transformation of *p35S::PhY37:EAR* resulted in 15 plants of which 5 showed expression of the *PhY37:EAR* mRNA. The latter 5 primary transformants did, however, not display any obvious phenotypical changes. It could be that the phenotype is very subtle and that small differences in flowering time were not noticed.

Transformation of *p35S::PhY56:EAR* in petunia resulted in 12 transgenic plants. Four plants could not be analyzed because they died at an early stage, when they were still very small. Of the 7 remaining plants mRNA was isolated and analyzed for the expression of the transgene but no expression was observed. Maybe the expression from the *p35S::PhY56:EAR* is too strong and therefore lethal, or the transformation did not yield any *p35S::PhY56:EAR* expressing plants. Therefore the question whether *p35S::PhY56:EAR* could have a function in the apex of petunia remains unsolved.

Is VEGGIE interacting with TCP proteins?

Although the interaction of VEGGIE with the TCP proteins is not very convincing due to the binding of the TCP-GAL4^{AD} to the GAL4^{BD} control, it is remarkable that a third of all the found VEGGIE interactors were TCP proteins. The identified TCP proteins encoded only for three different TCPs and were not identified in other screens of the same inflorescence meristem cDNA library with distinct baits. Therefore, we decided to perform some experiments to find out whether these TCP proteins have functions that overlap with VEGGIE. *PhY10* shows high sequence similarity to the *Arabidopsis TCP3* protein. *PhY14* encodes a TCP protein of which only the TCP domain shows high similarity to that of *LANCEOLATE (LA)* from tomato (Ori et al., 2007) and *PhY16* encodes a TCP protein that is throughout the entire sequence highly similar to LA.

Because *la* mutants have a flowering time phenotype (Ori et al., 2007), we decided to study the expression of *PhY16* in petunia. Semi-quantitative RT-PCR analysis revealed that *PhY16* mRNA is expressed in various tissues of wild type petunia, in all the early developmental stages of the sepals, petals, ovary and leaf (Fig. 8).

In tomato *LA*, is needed to make compound leaves and is negatively regulated by the microRNA *miRNA319a*. *miRNA319a* inhibits mRNA expression, thereby reducing the complexity of the leaves and delaying the flowering time (Ori et al., 2007). Interestingly, both the TCP domain and the *miRNA319a* binding site of *LA* are identical to that in *PhY16*. To study the role of *PhY16* in petunia development, *p35S::PhY16:EAR* and *p35S::miRNA319a* plants were produced. We expected that petunia plants expressing the *p35S::PhY16:EAR* construct would have a dominant negative phenotype that should be similar to the phenotype of plants expressing *miRNA319a*. Of 6 transgenic *p35S::PhY16:EAR*

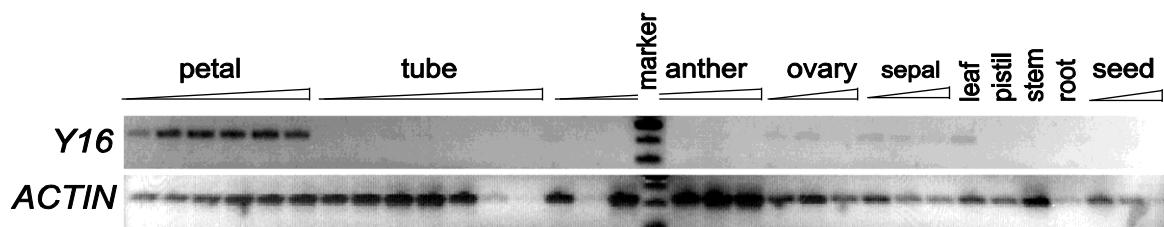


Figure 8. Expression pattern of *PhY16*. RT-PCR analysis of *PhY16* mRNA expression in early developmental stages of various plant organs.

transformants that were raised, two expressed the transgene and both showed a similar phenotype (Fig. 9A). These plants displayed curled, bubbly leaves with necrotic leaf tips (Fig. 9C) and grew much more slowly than the siblings that did not express the transgene. However, they produced a similar number of leaves before the initial floral transition, suggesting that the switch from vegetative growth to flowering was not altered.

Of the five *p35S::miRNA319a* transformants, two plants expressed the transgene, which resulted in down-regulation of *PhY16* mRNA. These two *p35S::miRNA319a* expressors displayed the same defects as the *p35S::PhY16:EAR* plants. That is, they had curled, bubbly, pointy leaves with necrotic leaf tips, and grew slowly compared to siblings that did not express the transgene and generated flowers with smaller petal limbs and a longer and more coloured petal tubes (Fig. 9C and D).

To study whether these transgenes interfered with *VEGGIE* expression, we analysed by semi-quantitative RT-PCR, the expression of *VEGGIE* mRNA in a family segregating for *p35S::miRNA319a*. We observed that *VEGGIE* mRNA was down-regulated in plants that constitutively expressed *miRNA319a* compared to wild type siblings plants (Fig. 9E). Whether the reduced expression of *VEGGIE* results from suppression of *PhY16* alone or from suppression of other TCP genes by the *miRNA319a* remains unclear. It is also possible that the reduction of *VEGGIE* mRNA in the *miRNA319a* plants compared to wild type is simply due to anatomical differences. The aberrant leaves of the *miRNA319a* expressing plants might just have problems producing or accumulating enough *VEGGIE* transcripts.

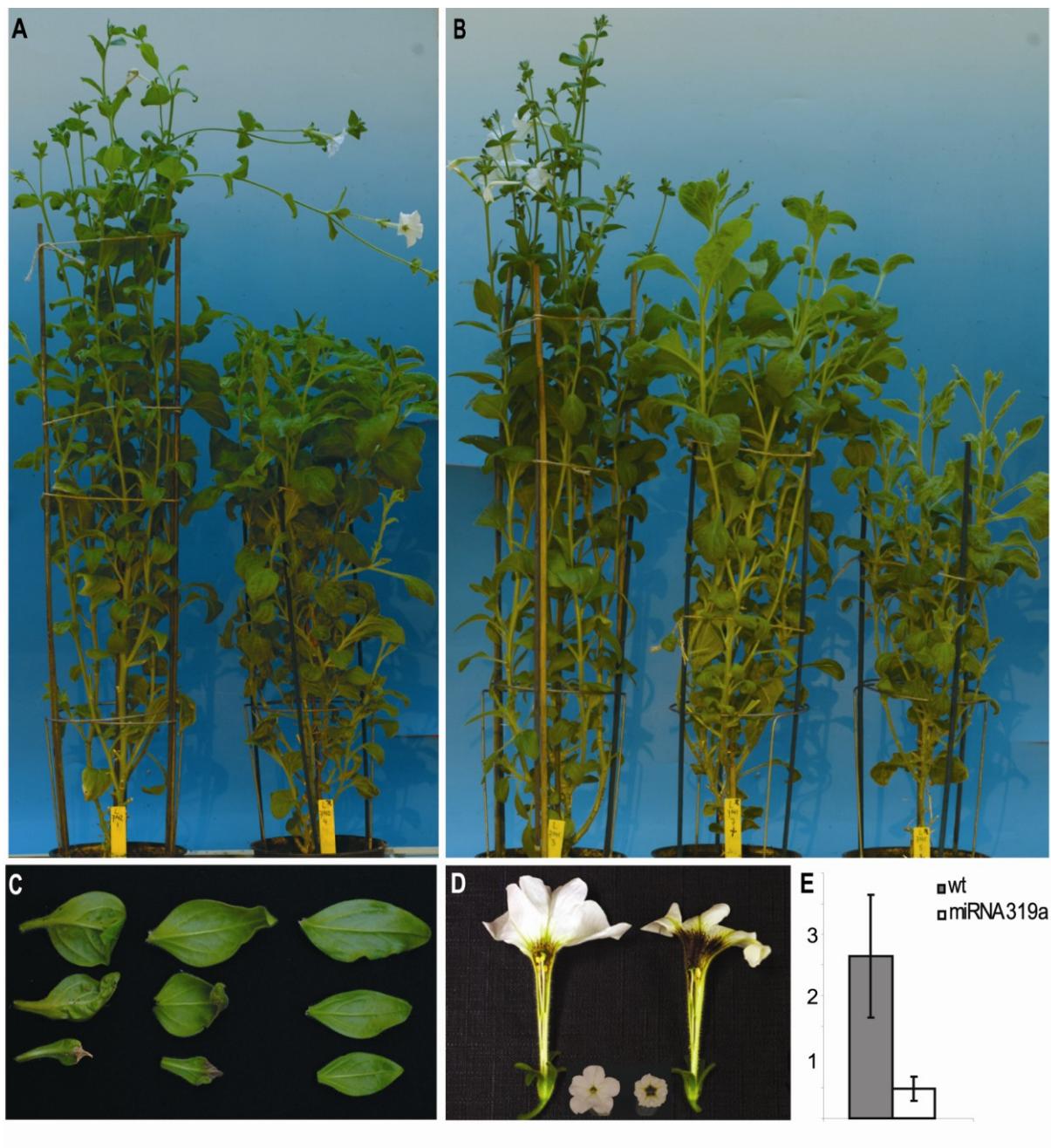


Figure 9. *PhY16:EAR* and *miRNA319a* plants

A. Transgenic *p35S::PhY16:EAR* petunia plants. Left a non-expressor, right an expressor. **B.** Transgenic *p35S::miRNA319a* petunia plants. Left a non-expressor, in the middle a low expressor and at the right a higher expressor. **C.** From left to right: leaves of lines expressing *PhY16:EAR*, *miRNA319a* and a wild type line. **D.** Flowers of wild type and a *miRNA319a* expressor: part of the corolla was removed to view the inner organs . Inset shows a top view of the corolla of a wild type and a *miRNA319a* flower. **E.** Relative expression level of *VEGGIE* in *miRNA319a* plants and its wild type siblings (shown as average \pm SD, n=3), normalized against *ACTIN*.

Discussion

VEGGIE is expressed in leaves and some downstream product, presumably the *VEGGIE* protein, moves to the apex to regulate the development to specific meristems within the apex of the plant, similar to *FT* in *Arabidopsis*. Within the apex *FT* needs to interact with the bZIP protein *FD* to activate the downstream gene *AP1* and others to specify floral identity (Abe et al., 2005). Because *FD* expression is restricted to lateral (floral) meristems in the apex, the expression of *FD* is thought to be the major factor controlling the regional specificity of *FT* action (Abe et al., 2005; Wigge et al., 2005). The action of *VEGGIE* resembles that of *FT* in that it promotes the switch from vegetative to reproductive development in the apical shoot meristem, but differs from *FT* in that *VEGGIE* specifies during subsequent inflorescence development the identity of the consecutively emerging (lateral) SIMs (Chapter 3 and 4). This suggests that also *VEGGIE* activity is regulated in time and space, but in a different way than *FT* in *Arabidopsis*. To unravel how *VEGGIE* activity is regulated and how that diverged from *Arabidopsis*, we searched for proteins that can bind to *VEGGIE* and might act as a “receptor” that provides spatial specificity, like *FD* does in *Arabidopsis*.

The yeast two-hybrid screen of an petunia inflorescence library with *VEGGIE* bait yielded two bZIP transcription factors, *PhY37* and *PhY56*, that are highly similar to *FD* and the closely related paralog *FDP*. In *Arabidopsis* the *FD* mRNA is strongly expressed in emerging lateral (floral) meristems, but not in the apical meristem, consistent with the idea that *FD* provides spatial specificity (Abe et al., 2005; Wigge et al., 2005). However, in the petunia inflorescence both *PhY37* and *PhY56* are expressed at very low levels that could not be detected by *in situ* hybridization. Using reporter genes we could detect expression of *PhY37* and *PhY56* in various tissues, but not in emerging SIMs or early floral meristems. The *PhY37* promoter is activated in floral tissues only and is therefore expressed too late in development to be the *VEGGIE*-binding partner needed to specify the floral identity of the young SIM. The *PhY56* promoter is activated in vegetative meristems just before flowering, and could be the *VEGGIE*-binding partner that is needed for the onset of flowering. Constitutive expression of *FD* (*Arabidopsis*) and *FDL2* (wheat) in *Arabidopsis* resulted in earlier flowering plants compared to non-transformed plants (Abe et al., 2005; Li and Dubcovsky, 2008; Yan et al., 2006). However, constitutive expression of either *PhY37* or *PhY56* in *Arabidopsis*

did not alter flowering time or induce the curling of leaves as seen in *35S::FD* *Arabidopsis* plants (Wigge et al., 2005) suggesting that PhY37 and PhY56 fail to activate some or all target genes of FD.

In *Arabidopsis* and wheat the respective FD-homologs bind to specific FD-response elements in the promoter of the floral meristem identity gene *AP1* from *Arabidopsis* and *VRN1* from wheat (Li and Dubcovsky, 2008; Wigge et al., 2005) to promote flowering. Although several MADS box genes belonging to the *AP1*-clade have been identified in petunia, none of them appears to be the true *AP1* ortholog, so we were unable to assess whether PhY37 or PhY56 can promote expression of *PhAP1*, or bind to its promoter. It is noteworthy that constitutive expression of either *AP1* or related MADS box genes in petunia does not affect flowering time or inflorescence architecture, suggesting that VEGGIE does not act via an *AP1*-like gene (Kusters, unpublished). The constitutive expression of PhY37 and PhY56 in *Arabidopsis* was ineffective in activating *AP1* expression, suggesting that if FT interacts with either PhY37 or PhY56 the formed complex is not able to bind and/or activate the *AP1* promoter to induce flowering. Although PhY37 and PhY56 and the FD and FDP from *Arabidopsis* show more than 80% amino acid similarity within the four conserved domains, the overall similarity between these two petunia bZIPs and the two *Arabidopsis* bZIPs is only 24%, which might be the cause of the inefficient formation and stabilization of an active FT-PhY37 and FT-PhY56 protein complex that is able to induce flowering as efficient as FT-FD. This hypothesis is supported by the observation that the interaction of VEGGIE with FD was weak. It might be that other factors or partners are involved that stabilize such a complex in the meristem, which are absent in the yeast. VEGGIE and FT are able to substitute each other when constitutively expressed in *Arabidopsis* and petunia respectively (Chapter 4) as seems to be the case for FT homologs from a variety of different plant species (Carmona et al., 2007; Hayama et al., 2007; Igasaki et al., 2008; Lin et al., 2007). This would suggest that the FT-homologs are conserved among different plant species but that the mechanism to activate the floral inductive pathway is changed or needs additional unknown partners to function properly.

Whether or not PhY37 and PhY56 have FD-like functions in petunia cannot be concluded from the current data. *Arabidopsis* plants just need a pulse of FT to induce flowering, whereas in petunia VEGGIE needs to be expressed throughout the reproductive phase to specify the identity of the consecutively appearing

SIMs in the inflorescence. Possibly petunia requires PhY37 and PhY56 only in small amounts or for short periods to induce the floral transition in each new sympodial inflorescence shoot. It is conceivable that VEGGIE interactors may be required at the place of action for a short period only and can interact with VEGGIE instantly. Such a transient expression of PhY37 and PhY56 could explain the difficulties to detect their mRNAs by *in situ* hybridizations.

Yeast two-hybrid screens in tomato, a Solanaceous species closely related to petunia, were only performed with the floral repressor *SELF PRUNING (SP)* as bait (Pnueli et al., 2001) and not with the floral inducer *SINGLE FLOWER TRUSS (SFT)* as bait. The floral repressor *SP* has homology to *TERMINAL FLOWER 1 (TFL1)* from *Arabidopsis* and yielded in the yeast two hybrid only one bZIP protein, SBPG. Because the screen was performed with a leaf cDNA library as prey, any proteins that are solely expressed in the apex were missed. SBPG was also able to bind *SFT* (Lifschitz et al., 2006), but its spatial expression was observed in the leaves and in the SAMs and it was therefore assumed that another bZIP transcription factor is likely to exist that is solely functional in the SAM (Lifschitz and Eshed, 2006) and that the FD homolog of tomato is not identified till now.

Material and methods

Yeast two-hybrid assay

For all two-hybrid assays we used the yeast strain PJ69, which contains the *HIS3*, *ADE2* and *lacZ* reporter genes driven by GAL4-responsive promoters (James et al., 1996), and the vectors pBD-GAL4 (Cam) and the pAD-GAL4 (Amp)(Stratagene). Yeast transformations were done using the lithium acetate method (Gietz and Woods, 2006) and cells transformed with pBD-GAL4 or pAD-GAL4 vectors were selected by plating on drop-out media lacking leucin and tryptophan. LacZ activity was measured using a semi-quantitative overlay assay (Weigel, 2002).

Yeast two-hybrid cDNA library screen

The yeast expression cDNA library was made from young inflorescence meristems of the petunia line W138 and the HybriZAP Two-Hybrid cDNA Gigapack Cloning Kit (Stratagene) (de Bruin, 2002). Candidate clones were

checked for size and double insertions by PCR with specific primers complementary to pAD-GAL4 sequences flanking the insertion. Plasmids from yeast cells were transformed into E. coli (DH10 cells) to clone the single plasmids for sequencing and to reintroduce in yeast PJ69 cells expressing VEGGIE-GAL4^{BD} to confirm that this (cloned) plasmid was indeed responsible for the positive two-hybrid response.

Phylogenetic analyses

Unrooted phylogenetic trees were constructed using the neighbour-joining method and bootstrap analysis (1000 replicas) using PHYLIP and visualized with Treeviewer version 1.6.6. Genbank accessions: The *Arabidopsis* bZIP transcription factor A group exists of AtZIP12 (AF334209), AtZIP13 (BN000023), AtZIP14 (BN000021), AtZIP15 (AJ419599), AtZIP27 (BN000022), AtZIP35 (AF0935474), AtZIP36 (AF093545), AtZIP37 (AF093546), AtZIP38 (AF093547), AtZIP39 (AF334206), AtZIP40 (U01823), AtZIP66 (AB017162) and AtZIP67 (AJ419600). Various putative FD homologs have been identified in other species: tomato SPGB (ABL84199), wheat DFL2 (ABZ91908), maize DFL1 (ABK91939). The tomato TCP protein is LANCEOLATE (ABM65600). Multiple sequence alignments were produced with a web-version of ClustalW (<http://clustalw.ddbj.nig.ac.jp/top-e.html>).

DNA and RNA Methodology

Plasmid inserts encoding candidate VEGGIE interactors originating from the petunia R27 inflorescence meristem cDNA library (de Bruin, 2002) were sequenced with the Big-Dye terminator kit (Perkin Elmer) with the primers complementary to the GAL4^{AD} coding sequence and the T7 promoter on the pAD-GAL4 vector. Full length cDNA clones of these genes were obtained by Rapid Amplification of 5' and 3' cDNA ends of W138 inflorescence meristems (First Choice RLM-RACE kit, Ambion) with gene-specific primers.

Gene constructs

-VEGGIE-GAL4^{BD} and VEGGIE-GAL4^{AD} were constructed by amplifying the coding sequence of VEGGIE from cDNA prepared from W138 inflorescence apices with primers complementary to the start and stop of the VEGGIE gene which were extended with several nucleotides to contain sites for the restriction

enzymes, EcoRI and PstI resp. (Table 2). Amplification products were digested with EcoRI and PstI and ligated in between the EcoRI and PstI sites of pBD-GAL4. and pAD-GAL4 respectively

-FD-GAL4^{AD} was constructed by amplifying the coding sequence of *FD* from cDNA of whole young *Arabidopsis* plants with primers that are complementary to the start and stop of the *FD* gene which were extended with several basepairs to contain sites for the restriction enzymes EcoRI and PstI resp. (Table 2). The amplified product was digested with EcoRI and PstI and ligated between the EcoRI and PstI sites of pAD-GAL4.

-p35S::PhY37 and p35S::PhY56 were constructed by amplifying the coding region of *PhY37* or *PhY56* from cDNA prepared from inflorescence apices of the petunia line W138 with primers that were complementary to the start and stop codon of the *PhY37* or *PhY56* and extended with the TOPO isomerise recognition site on the forward primer (Table 2). These amplified fragments were introduced in pEntryD-TOPO (Invitrogen) by TOPO cloning and recombined into pB7GW2 (<http://www.psb.ugent.be/gateway/index.php>) using GATEWAY LR Clonase II (Invitrogen).

-p35S::PhY37:EAR and p35S::Y56:EAR were constructed by the recombination of the *pEntryD-TOPO-PhY37* and -*PhY56* into the homemade pB7EARWG2,0 by GATEWAY LR Clonase II (Invitrogen).

-pPhY37::GFP:GUS and pPhY56::GFP:GUS were constructed by amplification of 4.5kb promoter region *PhY37* and 1.5 kb promoter region of *PhY56*. These upstream regions were identified by screening a W137/W138 genomic library with the ³²P-labelled coding regions of *PhY37* and *PhY56*. The phage that contained *PhY37* upstream region consisted of a large promoter fragment (4.5kb) and a part of exon 1 of *PhY37*, was isolated and digested with EcoRI and ligated between the EcoRI sites of the pBS plasmid. The *PhY37* promoter region was amplified from the cloned genomic fragment with primers complementary to the T7 promoter on the pBS plasmid and to the start of the coding region of the *PhY37* and extended with the TOPO isomerise recognition site on the forward T7 primer (Table 2). By TOPO cloning the *PhY37* promoter fragment was introduced in pEntryD-TOPO and recombined into pKGWFS7,0 (<http://www.psb.ugent.be/gateway/index.php>, Gent) using GATEWAY LR Clonase II (Invitrogen). The *PhY56* promoter region could be amplified directly from the identified phage with primers complementary to the T7 promoter on the pBS

plasmid and to the start of the coding region of the *PhY56* and extended with the TOPO isomerase recognition site on the forward T7 primer (Table 2). By TOPO cloning the *PhY56* promoter fragment was introduced in pEntryD-TOPO and recombined into pKGWFS7,0 using GATEWAY LR Clonase II (Invitrogen).

-p35S::PhY16:EAR was constructed by amplifying of the coding region of *PhY16* from W138 IFM cDNA with primers complementary to the start and stop of the *PhY16* gene which were extended with several nucleotides to contain sites for the restriction enzyme, EcoRI (Table 2). The amplified product was digested with EcoRI and ligated between the EcoRI sites of the homemade pEntry 4-EAR and recombined by GATEWAY LR Clonase II (Invitrogen) into pK7GW2.

All the regions that were used for cloning purposes were amplified with a polymerase, Phusion polymerase (Bioke), with the proof reading activity. All the constructs were fully sequenced with Big Dye terminator technology (Perkin Elmer) before they were transformed into the line W115 by Agrobacterium-mediated leaf disc transformation (Spelt et al., 2000).

Expression analysis

RNAs were isolated from plant material with Trizol (Invitrogen). 2.5 µg RNA, was first treated with DNaseI (Roche) before it was used to make first strand cDNA with MLV-transcriptase (Promega). The cDNAs were used for RT-PCRs that were performed with primers that are complementary to genes of interest and the internal control *ACTIN* (Table 2). The PCR products were separated on agarose gels, blotted on Hybond-N⁺ membranes (Amersham), hybridized with ³²P-labeled gene-specific probes and the signal was detected by a Phosphorimager (GE Healthcare).

In situ hybridization

Wild type apices were fixed, embedded in paraffin, cut into 8 µm sections and used for in situ hybridization (Souer et al., 1998). The antisense dioxigenin labelled RNA probes were synthesized from full length cDNA clones by in vitro transcription using T7 polymerase and digoxigenin-11-UTP (Roche). RNA probes were hydrolyzed in 60mM Na₂CO₃ and 40mM NaHCO₃ to an average length of 100-150 bp prior to hybridization. The probes were detected with Western Blue stabilized AP substrate (Promega) which resulted in the formation of a brown precipitate.

Table 2: sequences of primers used for PCR amplification

| Gene | | orientation¹ sequences |
|--------------|--------|--|
| pAD-GAL4 | - | TACCACTACAATGGATGATGTA |
| T7 | - | GTAATACGACTCACTATAGGG |
| VEGGIE-BD | fw | ATC GAATT CATGCCAAGAGAACGTGAACAC |
| | rev | GGA ACTGCAG TTAATCGGCAGACCTTCT |
| VEGGIE-AD | fw | TTAT CTCGAG TATGCCAAGAGAACGTGAAC |
| | rev | CAAG TCTAGA ATCGGCAGACCTTCTGCGTC |
| FD-AD | fw | CT CTCGAG TATGTTGTCATCAGCTAAG |
| | rev | GTAT TCTAGA TCAAATGGAGCTGTGGA |
| Y37 TOPO | atg | CACC ATGTGGTCATCAAGTAGTGATGAG |
| | stop | AAATGGCGCAGTTGACGTCCG |
| pY37 | atg | GGTGCCTCATCACTACTTGATGACCAC |
| | atg | AGAGAACGAGCAAGAGAGAAA |
| Y56 | stop | AAATGGCGCAGTTGACGTTCG |
| | atg | AGAGAACGAGCAAGAGAGAAA |
| T7 TOPO | - | CAC CTAATACGACTCACTATAGGGAG |
| | rev | CCGGATCCAATTCCATAGGTGGAGCAAGGTTAAG |
| Y16 | fw | GGAGAATT CATGGCAGAACGTTGGTTGC |
| | rev | GCT GAATT CATGGCGAGAACATCAGAGGAAG |
| Y37 RT-PCR | fw | ATGTGGTCATCCAGTAGTGATG |
| | RT-PCR | CTAGAGTTATAAGTGTGGTTCTG |
| Y56 RT-PCR | fw | TGGAATTCATGAAAAATAATTCACACTTC |
| | RT-PCR | TGGTAGCACTAGAACAGTAGTATT |
| Y16 RT-PCR | fw | GGCCCTTTACAGGGTTG |
| | RT-PCR | GCTGAATTCATGGCGAGAACATCAGAGGAAG |
| ACTIN RT-PCR | fw | AGATCTGGCATCATACCTTCTACA |
| | RT-PCR | CCMGCAGCTTCCATRCCAATCA |

¹ Forward (fw) indicates primers that are identical to the top strand of the gene

GUS staining

The histochemical staining of GUS was executed as described (Weigel, 2002). The X-Gluc concentration was 2mM. After staining the plant material was dehydrated by emerging the material through a serie of washes with increasing percentage of ter-butanol: water. To saturated the material with paraffin it was emerged via a graded paraffin: ter-butanol series, casted in paraffin blocks and

sectioned with the microtome to a thickness of 10 µm. Photos were taken with a Zeiss microscope using bright light and dark-field illumination.

Plant transformation and tissue culture

Petunia plants were transformed according to the leaf disk transformation protocol (Tobena-Santamaria et al., 2002). *Arabidopsis* plants were transformed by the dipping method and selected by spraying with Basta (Weigel, 2002).

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Chapter 6

Molecular characterization of the *VEGGIE* gene family from *Petunia* *hybrida*

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Abstract

The *FLOWERING LOCUS T/ TERMINAL FLOWER 1 (FT/TFL1)* gene family from *Arabidopsis* encodes proteins with similarity to phosphatidylethanolamine-binding proteins (PEBP) that act as floral inducers or repressors. *FT/TFL* homologs have been identified in many flowering plants which shows that this gene family is highly conserved. In *Petunia hybrida*, the *FT*-homolog *VEGGIE* promotes the onset of flowering and is required to specify the architecture of the cymose inflorescence. Three additional members of the *VEGGIE* family from petunia, *PhBFT*, *PhFTL* and *PhMFT* were identified and analyzed for their genomic structure, phylogenetic relationships and expression patterns. We could show that *PhBFT* is regulated by the circadian clock in both long- and short-day conditions, and that *PhMFT* is expressed during seed development.

Introduction

Plants display a wide variation with regard to flowering time and the architecture of their inflorescences. Not surprisingly the genetic regulation and evolutionary divergence of both features are linked, as both rely on (alterations in) the spatio-temporal expression of genes that promote or inhibit the formation of flowers. Day length is in many species an important parameter by which plants monitor the progression of the seasons in order to commence flowering at a specific point in time. Early studies revealed that day length is perceived in the leaves to trigger the synthesis of a mobile compound, florigen, that travels to the apex of the shoot to promote the floral identity of newly formed meristem and the development of flowers.

Genetic data indicate that the product of the *FT* gene of *Arabidopsis* is a major component of florigen or completely identical to it. The *FT* protein is synthesized in the leaf and travels to the apex where it interacts with a bZIP transcription factor encoded by the *FD* gene, to promote the expression of floral meristem identity genes (Abe et al., 2005; Wigge et al., 2005).

FT is a member of a small gene family that in *Arabidopsis* consists of six genes, *FT*, *TWIN SISTER OF FT (TSF)*, *ARABIDOPSIS THALIANA RELATIVE TO CENTRORADIALIS (ATC)*, *TERMINAL FLOWER LOCUS 1 (TFL1)*, *BROTHER OF FT (BFT)* and *MOTHER OF FT (MFT)* (Kobayashi et al., 1999; Mimida et al., 2001). The members of this family show high similarity at amino acid level and they all contain a phosphatidylethanolamine-binding domain (PEBP). This PEBP domain is wide spread and found in prokaryotes, archaea and eukaryotes (Banfield et al., 1998). Animals also contain multiple PEBP proteins some of which were shown to act as Raf kinase inhibitors in intracellular signalling cascades (Banfield et al., 1998; Odabaei et al., 2004; Serre et al., 1998).

The *FT* family of *Arabidopsis* can be divided in four distinct phylogenetic groups. The paralogs *FT* and *TSF* act as inducers of flowering, whereas the paralogs *TFL1* and *ATC* act as repressors (Mimida et al., 2001). The function of *BFT* and *MFT*, single representatives of two additional groups, is not well understood yet. *TSF* is the most closely related paralog of *FT* and is, like *FT*, activated by the transcription factor CONSTANS (CO) (Kardailsky et al., 1999; Kobayashi et al., 1999; Samach et al., 2000; Yamaguchi et al., 2005). *TSF* expression shows the same diurnal oscillation, with a peak of expression at the

end of the day, and photoperiod response as *FT*, i.e. high expression in long-day conditions (LD) compared to short-days (SD) (Yamaguchi et al., 2005). *tsf* mutants show delayed flowering in SD conditions, but not in LD, conditions, whereas the *ft tsf* double mutants are extremely late flowering in both LD and SD conditions, suggesting that *TSF* acts redundantly with *FT* in LD, but also contributes to flowering in SD. Constitutive expression of *TSF* resulted in early flowering, also in an *ft* or *co* mutant background, indicating that *FT* and *TSF* act independently to promote flowering (Yamaguchi et al., 2005). To test the requirement of *FT* and *TSF* mRNA in the flowering process an artificial microRNA was made that targeted both *FT* and *TSF* (Mathieu et al., 2007). Ectopic expression of this amiRNA repressed *FT* and *TSF* mRNA and made these transgenic plants flower as late as *ft tsf* double mutants. Flowering was also strongly delayed when the amiRNA was expressed from the phloem-specific *SUC2* promoter. However, expression from the meristem-specific *FD* promoter did not result in earlier flowering indicating that *FT* and *TSF* mRNA is needed in the phloem companion cells and not in the meristem. Thus, *TSF* is most likely able to regulate flowering by the same mechanism as *FT* (Mathieu et al., 2007).

TFL1 performs the opposite function of *FT* and acts as a floral repressor. In *tfl1* mutants the apical meristem, which is normally indeterminate, terminates by forming a flower (Bradley et al., 1997),. Moreover, in *35S::TFL1* plants flowering is delayed, as in *ft* mutants (Ratcliffe et al., 1998). *TFL1* mRNA is expressed in the centre of the SAM and its expression is elevated upon floral transition (Ratcliffe et al., 1999; Simon et al., 1996), whereas the *TFL1* protein is found throughout the meristem, indicating that *TFL1* protein can move between cells (Conti and Bradley, 2007). The change of only a single amino acid is sufficient to change the entrance of the ligand-binding pocket and to convert *TFL1* from a floral repressor into a promoter of flowering (Ahn et al., 2006; Hanzawa et al., 2005). Whether *TFL1* represses flower formation by competing with *FT* for binding to FLOWERING LOCUS D (FD) has remained unclear so far (Abe et al., 2005; Wigge et al., 2005).

The functions of *ATC*, *BFT* and *MFT* are not resolved yet. *ATC* and *BFT* encode very similar proteins as *TFL1* and *35S:ATC* or *35S:BFT* plants display the same late flowering phenotype as *35S:TFL* (Mimida et al., 2001), suggesting that the encoded proteins are also functionally similar. However, loss of function *atc* and *bft* mutants do not display an aberrant phenotype (Mimida et al., 2001). *MFT*

has floral promoting activities when constitutively expressed, but *mft* loss of function mutants have no obvious phenotype (Yoo et al., 2004).

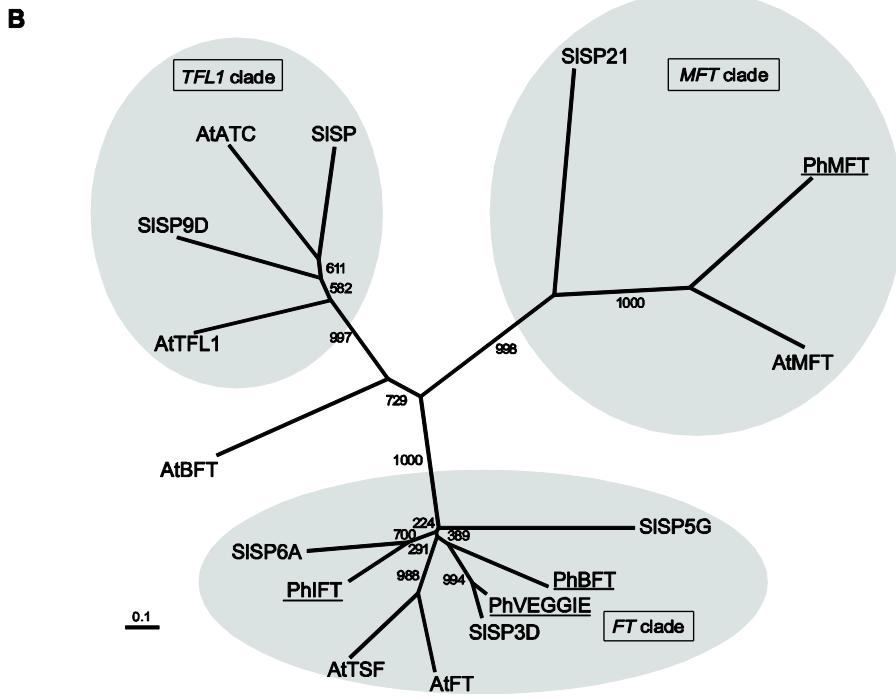
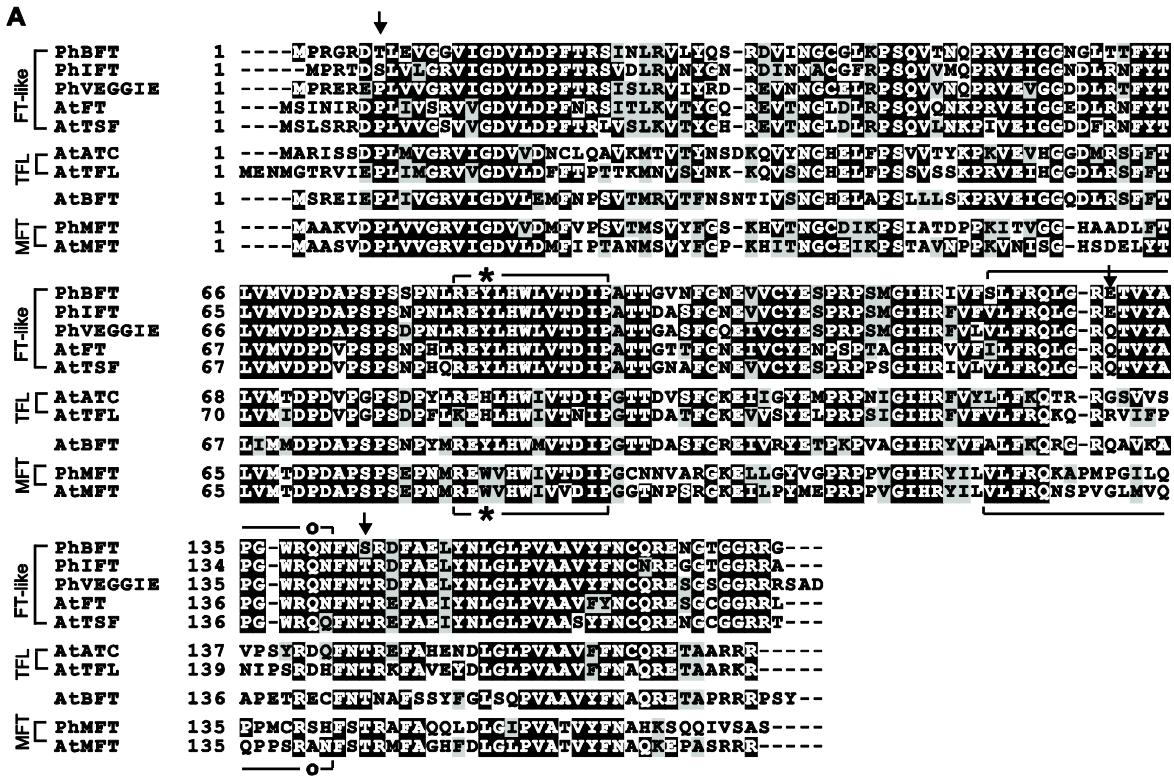
Tomato and petunia are closely related species, both belonging to the Solanaceae, and bear inflorescences with a cymose architecture that develop very different from the racemose *Arabidopsis* inflorescence. The primary difference is that in *Arabidopsis* flowers develop from lateral meristems that are formed at the flank of the indeterminate apical meristem, while in cymes, like tomato and petunia, flowers develop from the (determinate) apical meristem and the lateral meristem, also called sympodial inflorescence meristem (SIM), grows out to generate a next flower plus a flanking SIM (Chapter 3). Mutations in the tomato *FT*-homolog *SINGLE FLOWER TRUSS* (*SFT*) or the petunia homolog *VEGGIE* delay not only the onset of flowering, but also convert the SIM into a vegetative meristem that first generates a series of leaves before it forms a flower and a new SIM (Chapter 3) (Lifschitz et al., 2006). Transgenic experiments indicated that *VEGGIE* and *FT* are functionally exchangeable proteins, suggesting that the distinct roles of these proteins in *Arabidopsis* and petunia development are due to other genes, possibly genes encoding proteins binding to *VEGGIE/FT* or downstream genes that are regulated by *VEGGIE/FT*.

Since in *sft* as well as *veggie* mutants the formation of flowers is delayed rather than being fully abolished the question arose whether these genes are partially redundant. To study the possible role of additional *VEGGIE* paralogs in cymose growth we isolated three such genes, analyzed their genomic structure, phylogenetic relationships and expression patterns and studied their putative function in the control of flowering time and inflorescence architecture.

Results

Cloning and identification of genes of the petunia *VEGGIE* gene family.

To identify homologs of *VEGGIE* in petunia, we used several approaches. RT-PCR on RNA isolated from inflorescence apices with primers complementary to conserved regions in exon 3 and 4 of the *SELF-PRUNING* genes, *SP3D*, *SP5G* and



SP6A from tomato (Carmel-Goren et al., 2003) yielded one cDNA fragment, which derived from new gene that we named *ILJA'S FLOWERING LOCUS T-LIKE (PhIFT)*. We isolated the 5' and 3' end of this RNA by RACE-PCR which resulted in a cDNA that measures 954 bp and comprises of an open reading frame of 519 bp, encoding a 173 amino acid protein, and 5' and 3' untranslated regions (UTRs) of 204 bp and 228 bp respectively.

In a separate approach, we used low-stringency hybridization to screen a petunia leaf cDNA library. With *PhIFT* as a probe we identified a new gene that we named *BART'S FLOWERING LOCUS T (PhBFT)*. Its 5' and 3' end and the open reading frame were obtained by RACE-PCR and resulted in a cDNA of 909 bp long that consists of an open reading frame of 522 bp, encoding a protein of 174 amino acids, and a 5' and 3' UTR of 199 bp and 185 bp respectively.

When we screened the database of *dTPH1* transposon flanking sequences (Vandenbussche et al., 2008), we identified two different fragments that originated from a distinct *VEGGIE* homolog that we named *MICHEL'S FLOWERING LOCUS (PhMFT)*. The 5' and 3' end and the open reading frame of the corresponding cDNA were obtained by RACE-PCR. The *PhMFT* cDNA measures 753 bp and comprises an open reading frame of 522 bp, encoding a protein of 174 amino acids, and 60 bp 5' UTR and 168 bp 3' UTR.

Figure 1. Alignment of proteins encoded by VEGGIE and paralogs and homologs from *Arabidopsis*.

A. The proteins BFT, IFT, VEGGIE and MFT of petunia show high similarity to the *Arabidopsis* PEBP protein family. Amino acids in black are identical and in grey are similar. The binding pocket and the external loop are indicated by the lined boxes and the essential amino acids that pair to form the loop of the ligand binding pocket for floral promoting/ repressing activity, are Tyr85 (Y) - Gln140 (Q) (indicated by an asterisk) and His88 (H) - Asp144 (D) (indicated by a circle) for members of the FT or TFL clade respectively (Ahn et al., 2006 ; Hanzawa et al., 2005). The arrows point out the amino acids that are different between PhBFT and VEGGIE, FT and TSF. **B.** Unrooted tree for the members of the FT/TFL1 family of *Arabidopsis thaliana* (*At*), *Solanum lycopersicum* (*Sl*) and *Petunia hybrida* (*Ph*). The different clades are indicated by the grey ovals.

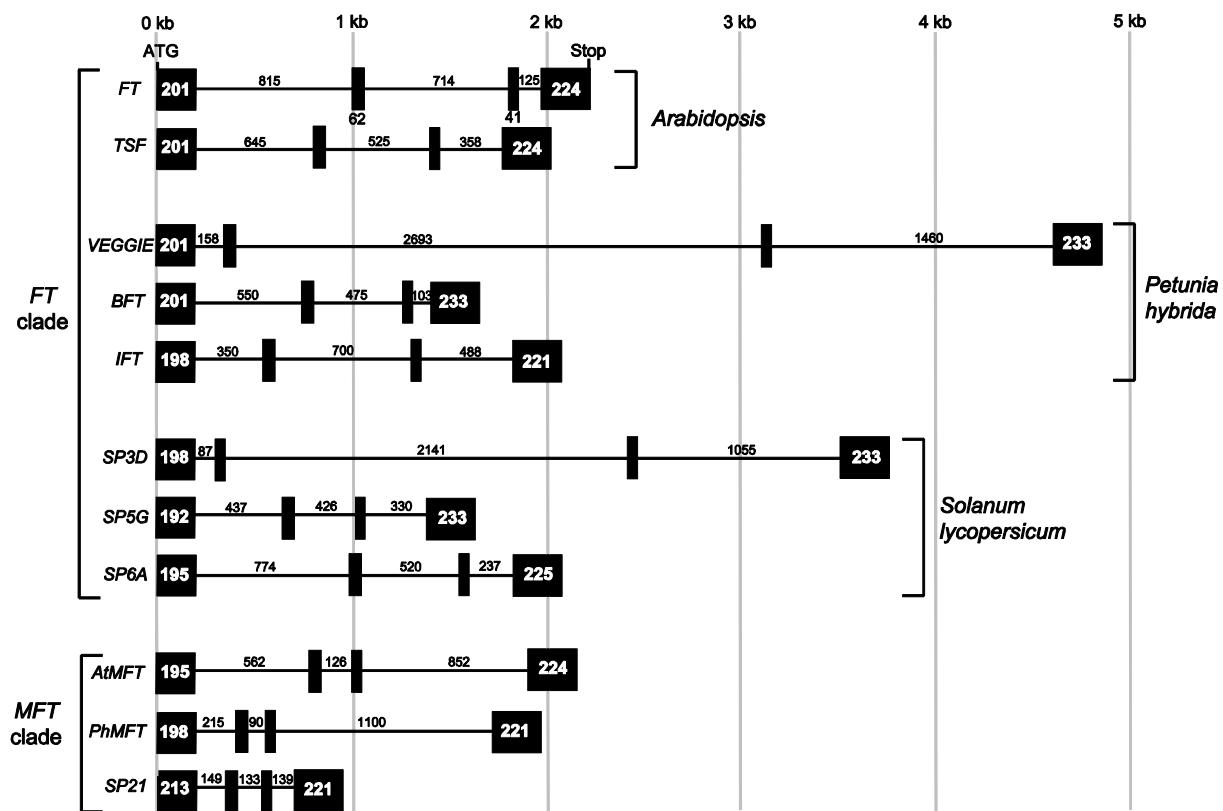


Figure 2. The genomic organization of the *FT* and *MFT* family members in *Arabidopsis*, *petunia* and *tomato*.

Exons indicated as black rectangles and the introns are represented by lines. The length of exons and introns is in basepairs. Size of exon 2 and 3 is conserved in all shown genes (62 bp and 41 bp respectively).

Alignment of the three VEGGIE homologs showed that they are highly similar to VEGGIE and the FT/TFL1 family of *Arabidopsis* (Fig. 1A). To establish the evolutionary relationships among VEGGIE, PhIFT, PhBFT and PhMFT, we performed phylogenetic analysis in which we included the FT/TFL1 family from *Arabidopsis* and tomato. The *FT/TFL1* family of tomato consists of six genes, *SELF PRUNING* (*SP*), *SP3D*, *SP5G*, *SP6A*, *SP9D* and *SP21* (Carmel-Goren et al., 2003) as in *Arabidopsis*. Neighbour-joining distance analysis of the entire amino acid sequences, generated an unrooted tree with four different clades (Fig. 1B). VEGGIE, PhBFT and PhIFT cluster with FT and TSF from *Arabidopsis* and SP3D, SP5G and SP6A from tomato. PhMFT clusters with MFT in a separate clade. Unfortunately no petunia homologs belonging to the TFL1 or BFT clade were found.

Analysis of the genomic organization of *VEGGIE* and the three petunia homologs showed that they all contained four exons and three introns (Fig. 2). Exons 1 and 4 vary in length from 198-201 bp and 221-233 bp respectively, while exon 2 measures in all the genes 62 bp and exon 3 always 41 bp. Although intron sizes differ considerably, their positions are fully conserved in the *VEGGIE* paralogs from petunia and homologs from tomato and *Arabidopsis*.

Expression pattern of *PhBFT*.

VEGGIE is expressed in the leaves in a rhythmic pattern with maximal expression at the end of long-days (Chapter 4). We performed semi-quantitative RT-PCR experiments to study the *PhBFT* and *PhIFT* mRNA expression during long-day conditions. These experiments revealed that *PhIFT* mRNA is barely detectable in leaf tissue, whereas *PhBFT* mRNA is, like *VEGGIE*, rhythmically expressed in leaves and peaks towards the end of the day after a light period of about 10-14 hours (Fig. 3). The rhythmic *PhBFT* expression, together with the observation that PhBFT and VEGGIE have highly similar sequences and interact with similar proteins, suggests that *PhBFT* might play a redundant role in the floral induction and specification of floral meristem identity.

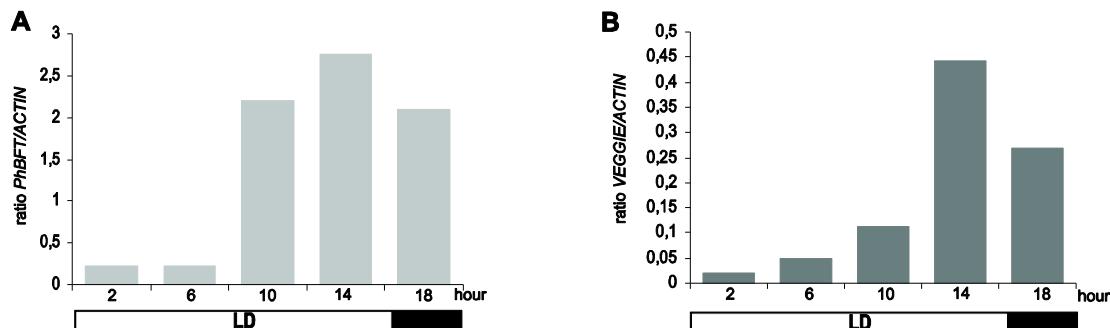


Figure 3. *PhBFT* and *VEGGIE* mRNA expression in the leaf during the day.

Semi-quantitative RT-PCR analysis of *PhBFT* (A) and *VEGGIE* (B) in leaf tissue during the day, normalized against *ACTIN*.

To assess whether expression of *PhBFT* is, like *VEGGIE*, sensitive to day length we isolated RNA from two-week old seedlings that had been grown in either long-day (LD) or short-day (SD) conditions. Semi-quantitative RT-PCR analysis showed that the *PhBFT* mRNA is up-regulated towards the end of the

day to the same levels in LD and SD conditions (Fig. 4), suggesting that the expression of *PhBFT* is not under the control of the photoperiod pathway.

To study the spatial *PhBFT* expression pattern, we fused a 1.75 kb *PhBFT* promoter fragment to a reporter gene consisting of a translational fusion of the GFP and GUS coding sequences (*pPhBFT::GFP:GUS*) and transformed it into wild type petunia plants. Seven transgenic plants were produced, which all showed GUS expression in a similar pattern. GUS activity was visible in the vascular tissues of the leaves (Fig. 5A and 5B), in the stem as a ring where the vascular bundles are located (Fig. 5C), but also in floral tissues especially in the base of the flower, in the petals and the abscission zone (Fig. 5D). Thus, compared to the *VEGGIE* promoter, which is only active in vascular tissue of the leaves (Chapter 4), the *PhBFT* promoter shows a much wider expression pattern.

Progeny of the self-fertilized *pPhBFT::GFP:GUS* transformants were grown in LD and SD conditions to study the effect of different light regimes on the activity of the *PhBFT* promoter. In one-week old seedlings grown in LD, we observed GUS expression in the first true leaves, but not in the cotyledons (Fig. 6A and B), in the vascular bundle close to the conjunction with the shoot (Fig. 6C), and in the roots in initiating lateral root meristems (Fig. 6D).

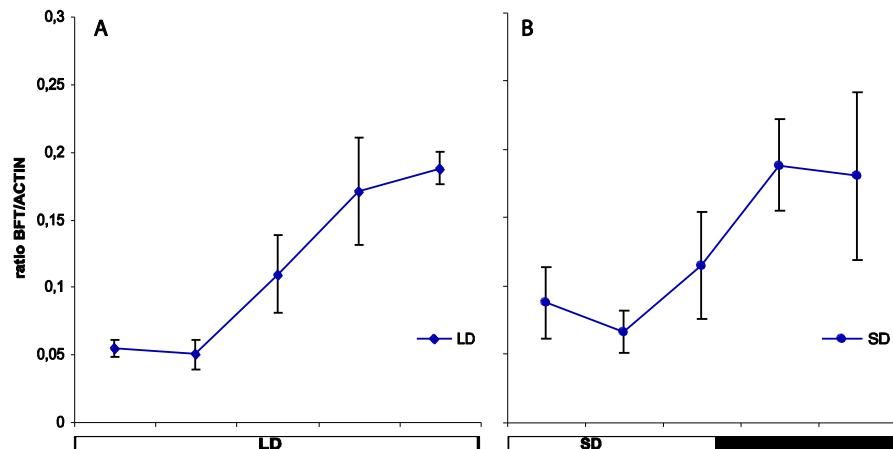


Figure 4. Figure 4. Expression pattern of *PhBFT* during the day in plants grown in long-day and short-day conditions. Semi-quantitative RT-PCR analysis of *PhBFT* mRNA expression during the day in two week old petunia seedlings normalized against *ACTIN* **A.** in LD. **B.** in SD.

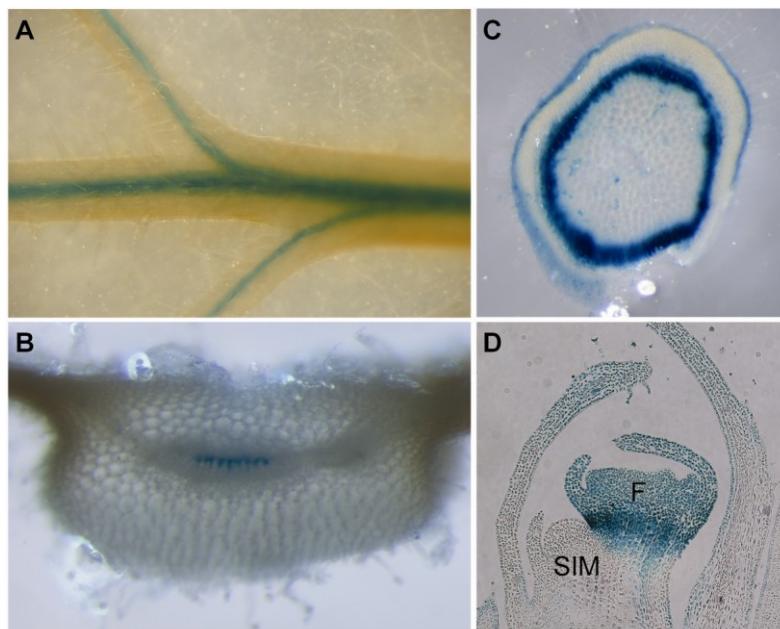


Figure 5. *PhBFT::GFP:GUS* expression pattern.

A. GUS expression in the veins of a *pPhBFT::GFP:GUS* leaf. **B.** Cross section of the main vein shows GUS expression in the phloem companion cells. **C.** Strong GUS expression is seen in the stem as a ring indicating the location of the phloem vascular tissue. Staining is also seen in (sub)epidermal cells. **D.** At the base of the flower (F) strong GUS expression is observed but little or nothing in the SIM.

In two-week old seedlings GUS expression was observed in the youngest leaves and petioles, but not in the meristem (Fig. 6E and 6F), and in roots in the vascular tissues near the junction with the shoot (Fig. 6G and 6H). In three-week old seedlings we found GUS expression in the stems, in the youngest leaves (Fig. 6I) and in the vascular bundles coming from the leaves (Fig. 6J). SD-grown seedlings grow slower as siblings grown in LD, but nevertheless show a similar GUS pattern when they are at the same developmental stage (data not shown).

To learn more about the expression of *PhBFT* in relation to *VEGGIE* we monitored the expression of *PhBFT* in the leaves of flowering *veggie* mutants during the day. In wild type plants we observed the rhythmic cycling of *PhBFT* mRNA during the day, which peaks after 14 hours of light and is followed by a rapid decrease towards the end of the day. In *veggie* mutants, however, *PhBFT* still shows the rhythmic expression but at a much lower level. In previous experiments we showed that wild type seedlings express *PhBFT* in short-days conditions at the same level and in the same rhythm as in long-days (Fig. 4).

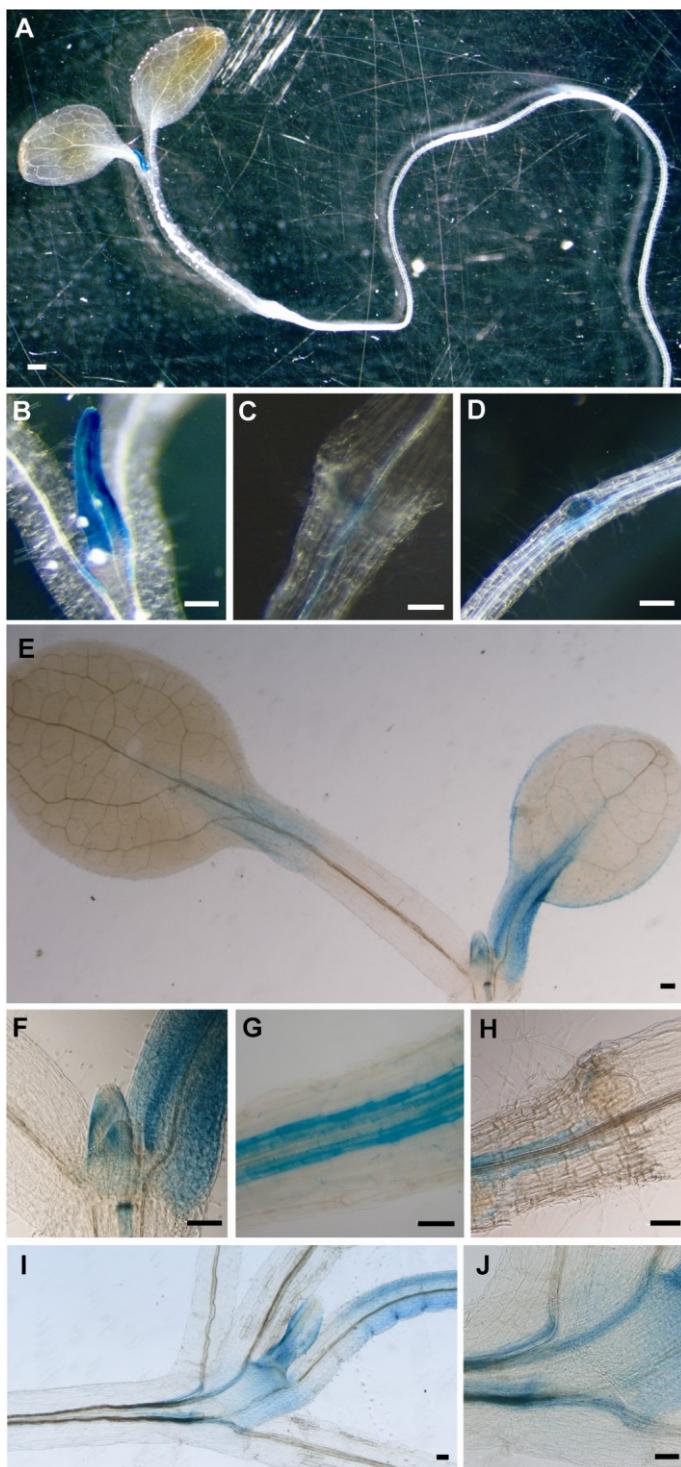


Figure 6. Expression analysis in *pPhBFT::GUS* in developing seedlings in long-day conditions.

A-D. One-week old *pPhBFT::GFP:GUS* seedling **A.** overview of GUS expression. **B.** Detail of the young developing leaves. **C.** GUS signal around the vascular bundles at the root to shoot junction. **D.** GUS signal at the start of the lateral root. **E-H.** Two-week old *pPhBFT::GFP:GUS* seedling **E.** overview of GUS expression in the leaves. **F.** Detail of the youngest developing leaf. **G.** GUS signal in the vascular

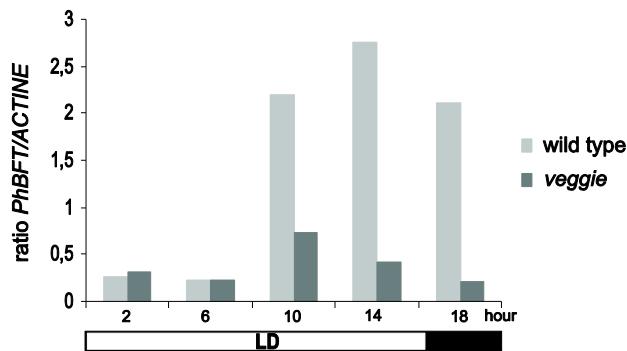


Figure 7. *PhBFT* mRNA expression in leaves during long-day conditions in flowering wild type and *veggie* plants.

In wild type plants in short-day conditions *VEGGIE* expression is repressed but not completely abolished as is the case in *veggie* mutants. The complete depletion of any *VEGGIE* transcript reduces the *PhBFT* expression suggesting that *PhBFT* expression is controlled by *VEGGIE* (Fig. 7).

Functional differences between BFT and VEGGIE

Because of the high similarity between the *VEGGIE* and *PhBFT* amino acid sequences (~85% similarity) we were interested whether *VEGGIE* and *PhBFT* might be functionally similar and involved in similar protein-protein interactions. *VEGGIE* is able to interact in yeast two-hybrid assays with several bZIP transcription factors of petunia known as, PhY19, PhY37, PhY56, PhY62 and PhY78 (Chapter 5). To test whether *PhBFT* can interact with any of these five different bZIP transcription factors or with the *Arabidopsis* bZIP transcription factor FD, a yeast two hybrid assay was performed. We transformed yeast strain PJ69 with plasmids expressing either *VEGGIE* or *PhBFT* fused to the DNA-binding domain of GAL4 (GAL4^{BD}) and fusions of the bZIP transcription factors to the activation domain of GAL4 (GAL4^{AD}) and assayed whether this activated the

Figure 6 (continued)

bundles of the root. **H.** GUS signal around the vascular bundles at the root to shoot junction. Three-week old *pPhBFT::GFP:GUS* seedling (**I and J**). **I.** GUS staining in the vascular bundle in the aerial parts of the seedling. **J.** GUS signal in the vascular bundles into the leaves. Scale bar is 100 μM .

GAL4-responsive *HIS3*, *ADE2* and *LacZ* reporter genes. PhBFT was able to interact with the bZIP transcription factors and these interactions resembled the ones shown earlier between VEGGIE and the bZIPs, as judged by the activation of the *HIS* and *ADE* reporter genes (Fig. 3). PhY37, PhY56, PhY78 and AtFD interact equally strong with VEGGIE and PhBFT. The observation that the pair PhBFT-PhY56 restores histidine and adenine auxotrophy, but does not show detectable β-galactosidase activity indicates that VEGGIE interacts stronger with PhY56 than PhBFT. PhY19 interacted more efficiently with VEGGIE than with PhBFT and PhY62 interacted more efficiently with PhBFT than with VEGGIE. This data show that VEGGIE and its homologs can interact with similar partners, but do so with different affinities, suggesting functional diversification.

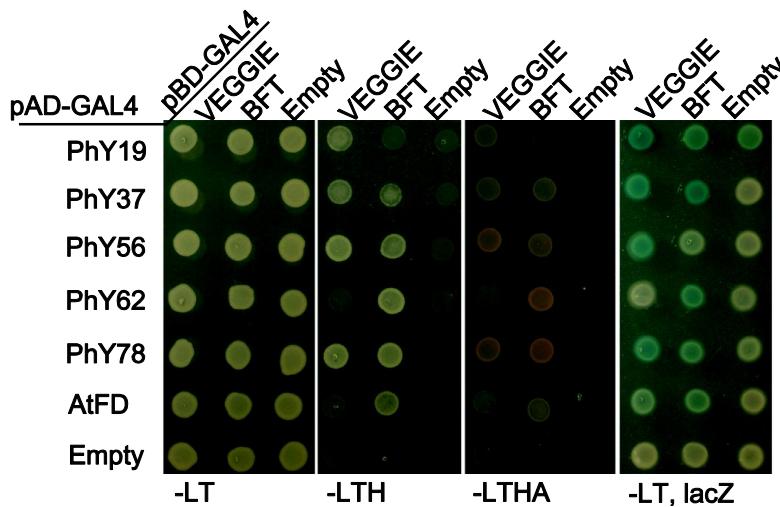


Figure 8. Interaction of PhBFT with different bZIP transcription factors using yeast two hybrid analysis. Yeast two hybrid assay of strains expressing GAL4^{BD} fusions of PhBFT and VEGGIE or GAL4^{BD} alone, with GAL4^{AD} fusions of PhY19, PhY37, PhY56, PhY62 ,and PhY78 from petunia, FD from *Arabidopsis* or GAL4AD alone on drop-out media lacking leucine and tryptophan (-LT), leucine, tryptophan and histidine (-LTH), leucine, tryptophan, histidine and adenine (-LTHA) and leucine and tryptophan and coloured in a β-galactosidase activity assay (**LacZ**).

To determine whether PhBFT is involved in the initiation and maintenance of flowering and/or functionally exchangeable with VEGGIE, the ORF of *PhBFT* was expressed under the control of the Cauliflower Mosaic Virus 35S promoter (*p35S::PhBFT*) in *veggie* mutants. We obtained six transgenic plants that

constitutively expressed *PhBFT*, as judged by RT-PCR analysis, but neither accelerated flowering nor a change in the inflorescence architecture was observed. Given that a 35S:*VEGGIE* or 35S:*FT* gene can rescue the *veggie* phenotype, while 35S:*PhBFT* cannot, this suggests that despite the high similarity PhBFT is unable to replace VEGGIE.

Constitutive expression of *PhIFT*

To examine the function of *PhIFT* in petunia, we transformed wild type petunia plants with a *PhIFT* construct driven by 35S promoter (*p35S::PhIFT*). This transformation resulted in three transgenic plants of which only one showed *PhIFT* mRNA expression. Constitutive expression of *PhIFT* showed to have no effect on flowering time or floral architecture.

Expression pattern of *PhMFT*

Expression of *PhMFT* mRNA is very restricted and was only found in inflorescence apices and in seeds (Hedman et al., 2009; Yoo et al., 2004)). We determined the expression levels of *PhMFT* during seed development by semi-quantitative RT-PCR analysis on inflorescence apices, unfertilized ovules and ovaries at 2, 5, 8, 10, 12, 14 and 16 days after pollination (DAP) (5 DAP represents the 1 cell embryo stage, 8 DAP the 8 cell embryo stage, 10 DAP the globular stage, 12 and 14 DAP represent the early and late heart stage and 16 DAP represents the torpedo stage of the developing embryos). (Fig. 9A).

We found that the mRNA expression of *PhMFT* is low in inflorescence apices and ovules compared to the expression levels observed in developing embryos. When the embryos are at the globular stage (10 DAP) and start to form a meristem the *PhMFT* expression increases until the embryos develop into the heart stage (12 to 14 DAP). When the embryos reach the torpedo stage (16 DAP) the *PhMFT* mRNA expression level decreases again.

To study the spatial expression pattern of *PhMFT* in more detail we localized its mRNA in the inflorescence and developing seeds by *in situ* hybridization. *PhMFT* is expressed in the endosperm surrounding embryos in the early and late heart stage (Fig. 9B and C). In inflorescence meristems and floral meristems *PhMFT* is expressed in the organ primordia that will develop into the style and stigma (Fig. 9D) and in the developing flower high *PhMFT* expression is observed at the stigma on the tip of the carpel (Fig. 9E and F).

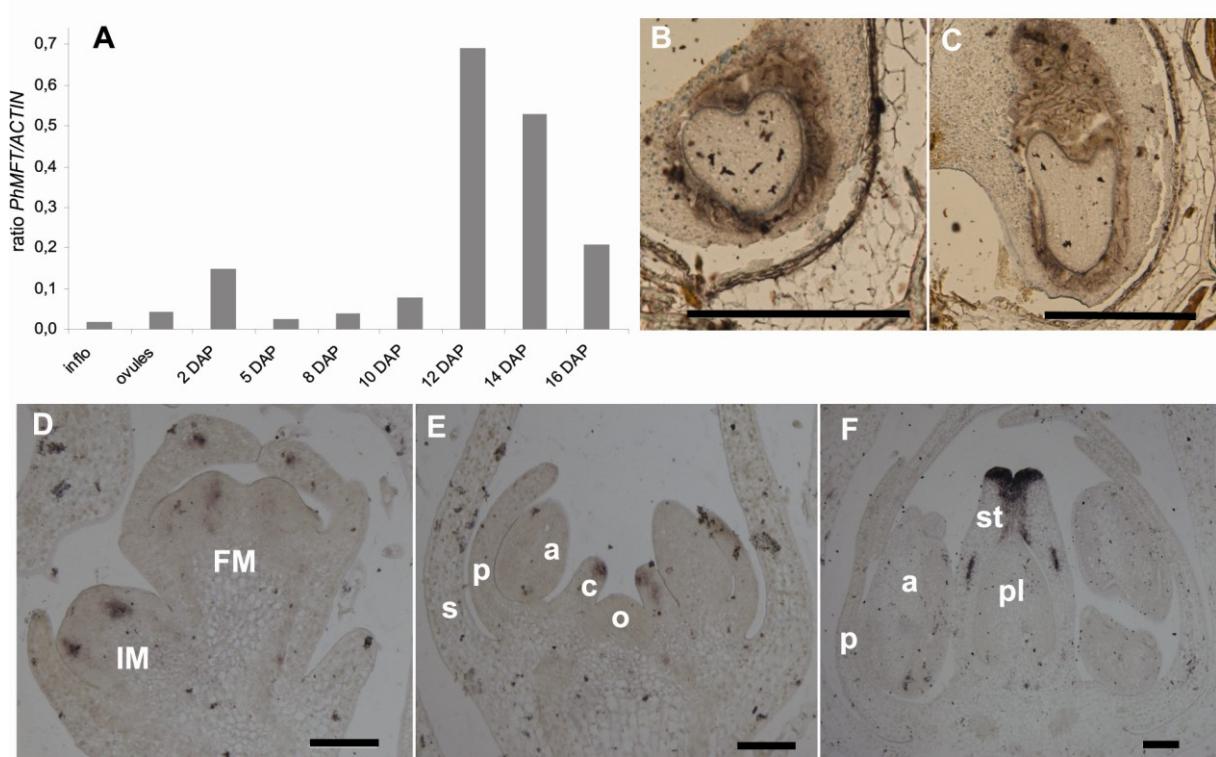


Figure 9. The temporal and spatial *PhMFT* expression during embryo development.

A. Semi-quantitative RT-PCR analysis of *PhMFT* normalized against *ACTIN* mRNA in inflorescence apices, unfertilized ovules and in ovaries 2, 5, 8, 10, 12, 14 and 16 days after pollination (DAP). In situ hybridization with *PhMFT* in developing embryos and in emerging flowers (**B-F**). **B.** *PhMFT* expression around an early heart shape embryo (12 DAP). **C.** *PhMFT* expression around a late heart shape embryo (14 DAP). **D.** *PhMFT* expression in the inflorescence meristem (IM) and in the floral meristem (FM). **E.** Expression of *PhMFT* in the tip of carpel (c) tissue in a developing flower. S (sepal), p (petal), a (anther) and o (ovary). **F.** Expression of *PhMFT* at the tip of the emerging style and stigma tissue (st) and around the placenta (pl). Scale bar is 100 µm.

Alterations in *MFT* expression do not cause an obvious phenotype

We screened a population of ~1000 petunia plants for mutants with a *dTPH1* insertion in *PhMFT* (Vandenbussche et al., 2008), and identified two individuals harbouring two distinct insertion alleles, *mft*^{M2128} and *mft*^{L2257}. The *mft*^{M2128} allele contains a *dTPH1* insertion in exon 1 and *mft*^{L2157} contains an *dTPH1* insertion in

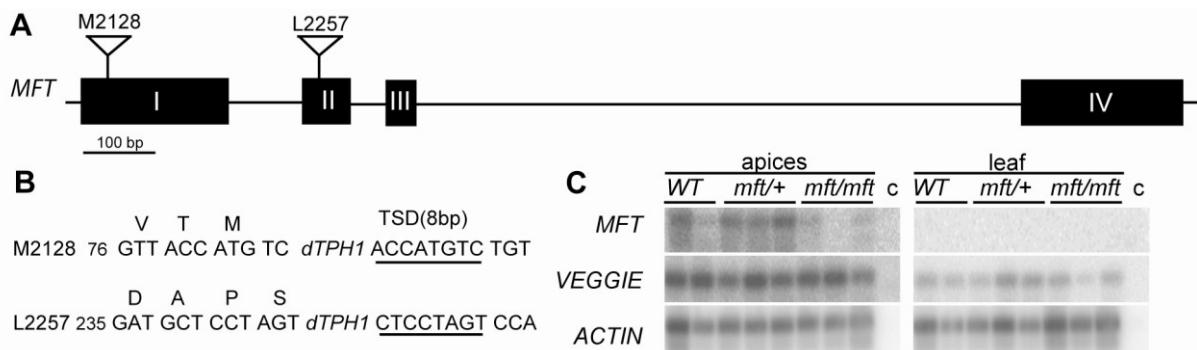


Figure 10. *mft* mutant alleles and expression analysis.

A. Schematic drawing of the genomic organization of *PhMFT*. Exons are represented by the black rectangles and the introns by lines. The *dTPH1* insertions of the different *mft* alleles are indicated by triangles. **B.** *dTPH1* flanking sequences of the two *mft* alleles, *mft*^{L2257} and *mft*^{M2128}. Both alleles have a target duplication site (TSD) of 8 bp. **C.** Semi-quantitative RT-PCR analysis shows *PhMFT* mRNA expression in inflorescences apices but not in leaves. No *PhMFT* expression can be detected in the *mft*^{M2128}. *VEGGIE* mRNA expression is not affected by the down-regulation of *MFT* in the mutants. *ACTIN* is internal control.

exon 2 (Fig. 10A). Both the independent *dTPH1* insertions introduced a target site duplication (TSD) of 8 bp (Fig. 10B) and disrupted the reading frame.

To examine whether the *dTPH1* insertion compromised the expression of *PhMFT* mRNA, we analysed the RNA from leaf and inflorescence apices of plants segregating for the *mft*^{M2128} allele by RT-PCR using primers complementary to *PhMFT*, *VEGGIE* and *ACTIN* (Fig. 10C). Although *VEGGIE* and *ACTIN* were equally expressed in different leaf samples, no expression of *PhMFT* was seen in either the wild type or the *mft* leaves confirming that *PhMFT* is not expressed in leaves. In the inflorescence apices *VEGGIE* and *ACTIN* were equally expressed in different genotypes, but *PhMFT* was only expressed in the wild type and heterozygous *PhMFT* plants. Since no *PhMFT* transcript was observed in homozygous mutants, we concluded that the *mft*^{M2128} is a null allele (Fig. 10C).

Even though *mft*^{M2128} is a null allele we did not observe a clear defect in homozygous mutants. Because the expression of *PhMFT* is the highest in the maternal material that is surrounding the developing embryos we examined whether the *mft* mutant plants were able to self-fertilize and set viable seeds. Although serious effort was made to self-fertilize homozygous *mft* mutants we

were unsuccessful in producing any seeds. Note that the mft^{M2128} and mft^{L2257} allele are in the W138 background and it is known that this line is not easy to propagate.

To study the effect of mft in a *veggie* background double mutants were produced, but these double mutants showed besides the *veggie* phenotype no additional defects.

Next, we produced gain of function mutants in which *PhMFT* was constitutively expressed from the 35S promoter. In total 24 transgenic plants were obtained, of which only one plants showed ectopic *PhMFT* mRNA expression. This *p35S::MFT* plant did not show any effect on flowering time, flower morphology or inflorescence architecture. Analysis of progeny of the backcross of this transgenic *p35S::MFT* plant with a wild type plant resulted in a 1:1 segregating population for the *p35S::MFT* transgene, but these plants were undistinguishable from wild type plants based on phenotype.

Discussion

We report the identification and partial characterization of three new petunia genes belonging to the *VEGGIE/FT* family. Two of these, *PhBFT* and *PhIFT*, show high sequence similarity to the floral integrator *FT* while the third one, *PhMFT*, is most similar to *MFT* from *Arabidopsis* (Yoo et al., 2004). Whether petunia contains besides *PhBFT*, *PhIFT* and *VEGGIE* additional genes that belong to the *FT* subclade will remain uncertain until the whole petunia genome is sequenced. In tomato, a nightshade that is closely related to petunia, so far three genes have been identified that belong to the *FT*-clade (Carmel-Goren et al., 2003). Therefore, we consider it likely that we have identified all the *VEGGIE* paralogs that are located in the *FT*-clade. Less related eudicot species that are fully sequenced contain 2 *FT* members for *Arabidopsis* and 5 *FT* homologs for the perennial *Lombardy Poplar* (Igasaki et al., 2008). That the number of *FT* paralogs differs amongst plant species can be explained by assuming that each *FT* homolog has a specialized inductive function. Annual plants make the floral switch once and continue flowering for the rest of the season, whereas perennial plants like poplar first undergo a long juvenile phase of several years, before they start seasonal flower initiation (Danilevskaya et al., 2008; Hsu et al., 2006).

Monocots have many more *FT*-homologs than dicots, up to 19 in wheat and 13 in maize. These *FT*-homologs can be subdivided into two groups of which the *FT*-like II group appears to be monocot-specific (Chardon and Damerval, 2005; Danilevskaya et al., 2008)). The high number of *FT*-homologs in maize can be explained by an ancient tetraploid ancestor (Bruggmann et al., 2006).

The fact that phylogenetic analysis of the *FT/TFL1* families of many different plant species revealed always four *FT/TFL1* clades suggests that the last common ancestor of eudicots and monocots contained at least four genes encoding PEBP proteins. Also the observation that the sizes of the exons 2 and 3 (62 and 41 bp) are conserved among all the studied *FT/TFL1* genes points towards a high conservation of these genes throughout the plant kingdom.

FT from *Arabidopsis* has 83% amino acid similarity to VEGGIE and can complement *veggie* mutants when constitutively expressed and is most likely orthologous to VEGGIE (Chapter 4). Constitutive expression of *TSF* in *Arabidopsis*, the closest paralog of *FT* (89% amino acid similarity), results in early flowering in wild type, *co* and *ft* mutant backgrounds indicating that *FT* and *SFT* are interchangeable and act redundantly (Jang et al., 2009; Yamaguchi et al., 2005). Within the petunia *FT*-clade we distinguished three members, VEGGIE, PhBFT and PhIFT, of which PhBFT is most similar to VEGGIE (85% amino acid similarity). Despite this high sequence similarity, the constitutive expression of *PhBFT* in wild type and *veggie* mutants is not sufficient to induce early flowering or to rescue the *veggie* phenotype. The distinct activity of PhBFT was unexpected and might be due to the change of one of the four amino acids that are different between PhBFT on one hand and VEGGIE, *FT* and *TSF* on the other. In PhBFT the residues Pro7 (P), Arg43 (A), Gln130 (Q) and Thr143 (T) are changed into Thr7 (T), Lys43 (K), Glu130 (E) and Ser 143 (S) respectively (Fig. 1A). The difference in activity between PhBFT and VEGGIE, *FT/TSF* is most probably caused by the amino acid change Gln130 (Q) to Glu130 (E) that is located in the binding groove of PhBFT. Whether this particular amino acid or any of the other three different amino acids is responsible for the floral inductive qualities of PhBFT remains to be investigated. Also the yeast two-hybrid experiment showed that VEGGIE and BFT interact with the same set of bZIP transcription factors, but do so with a different affinities consistent with their functional diversification.

PhBFT is like *VEGGIE* regulated by the circadian clock based on the observation that *PhBFT* mRNA expression is oscillating during the day and peaks at the end of the day in both long- and short-day conditions. Although *PhBFT* is unable to act redundantly with *VEGGIE* in LD, the rhythmic oscillation of *PhBFT* might contribute to flowering in SD as both the *pPhBFT::GUS* as the *PhBFT* mRNA is expressed in seedlings grown in SD. The suppression of *PhBFT* expression in a *veggie* mutant background suggests that *PhBFT* is at least partly under the control of *VEGGIE* and thus the photoperiod pathway.

The *PhMFT* gene of petunia is likely to be the most ancient PEBP protein because in the basal plant species *Physcomitrella patens* (bryophyte, a moss) and *Selaginella moellendorffii* (lycophytes, a fern) four and two MFT-like genes were identified respectively, but no FT or TFL-like genes could be found (Hedman et al., 2009). The absence of the FT- and TFL-clade in these primitive plants suggested that these arose later during the evolution of seed plants (Hedman et al., 2009). Analysis of MFT and homologues in flowering plants revealed that many of the studied eudicots (*Arabidopsis*, petunia, tomato, poplar, grapevine) have only one MFT homolog, whereas monocots as rice and maize have two or more MFT-paralogs due to duplication events (Danilevskaya et al., 2008).

Ectopically expressed *MFT* from *Arabidopsis* slightly exhilarates flowering, but ectopic expression of *PhMFT* in petunia or *PnTFL4* from poplar in *Arabidopsis* has no obvious effect on flowering (Igasaki et al., 2008; Yoo et al., 2004). It could be that the effect of the constitutive expression of *PhMFT* is so mild that it escaped our attention in the plants grown in the semi-controlled greenhouse conditions that we used. Loss of function mutants of *PhMFT* and *MFT* do not show phenotypical alterations, although they are null alleles (Yoo et al., 2004). Most likely *MFT*-genes in eudicots do not play a role in determining the inflorescence architecture or flowering time. Jang et al (2009) showed that the triple mutant *ft tsf mft* flowered as late as the double mutant *ft tsf* and concluded that MFT was not acting redundant in the flowering time process (Jang et al., 2009). Yoo et al (2004) did observe an restricted expression during the seed development (Yoo et al., 2004) and this was also observed in the basal plant species *Physcomitrella patens* and *Selaginella moellendorffii* which express *MFT* in the gametangia and sporophytes (Hedman et al., 2009). Also in higher plant species like *Arabidopsis* (*MFT*), poplar (*PnTFL4*) and maize (*ZCN9/10 and 11*) *MFT* is expressed during

seed setting and in seeds (Danilevskaya et al., 2008; Igasaki et al., 2008; Yoo et al., 2004).

We also observed *PhMFT* expression in seeds and were able to show its spatial expression pattern in the endosperm in a layer that is surrounding the developing embryo. Possibly the *PhMFT* expression is needed to produce viable seeds because we were unable to self-fertilize *mft* mutants. The remarkable strong expression of *PhMFT* observed in developing style and stigma might be important for pollen to stick to the stigma and for pollination of the plant. *mft* mutants, however, did not show any problem in cross-pollination and seed production and also did not show any visual defects in the stigma, style or ovaries. Although it is still unclear what is the function of *PhMFT* in the style and stigma, it adds a new interesting feature to the expanding knowledge about the PEBP genes in plants

Material and methods

Isolation of *PhIFT*, *PhBFT* and *PhMFT* cDNA and genomic DNA sequences.

A 241 bp *PhIFT* fragment was amplified by PCR from W138 leaf cDNA with primers that are complementary to exon 3 and 4 of the tomato *SP3D*, *SP5G* and *SP6A* (CACTGGYTGGTCACAGATATCC and CCACCTTCCCTATTGCAATTG) was cloned in pGemT-easy (Promega) and sequenced with vector-specific primers. The full-length cDNA sequence was obtained by 5' and 3' end amplification on cDNAs of W138 inflorescence apices (First Choice RLM-RACE, Ambion). The *PhIFT* cDNA was used as a P^{32} -labelled PCR probe (dCTP^{32} , Amersham) to hybridize 60.000 plaques of a W115 leaf cDNA library. Low stringency washing (2xSSC, 0,1%SDS, 65°C) resulted in three hybridizing plaques that were purified, excised and sequenced with vector specific primers. Only one plaque contained a fragment of an *FT* homolog, named *PhBFT*. The full length *PhBFT* cDNA sequence was obtained by 5' and 3'end amplification of W138 leaf cDNA. Genomic sequences of both *PhIFT* and *PhBFT* were obtained via PCR fragments on W138 genomic DNA using primers complementary to the ends of the *PhIFT* and *PhBFT* cDNAs. Two *PhMFT* fragments were identified in a database of *dTPH1* flanking sequences (Vandenbussche et al., 2008). The full length *PhMFT* cDNA sequence was obtained by 5' and 3' end amplification of W138 inflorescence meristem cDNA library. The whole genomic sequence of *PhMFT* was

obtained from W138 genomic DNA using primers complementary to the *PhMFT* cDNA.

Sequence alignment and construction of a phylogenetic tree

Amino acid alignments were made using ClustalW (<http://clustalw.ddbj.nig.ac.jp/top-e.html>) and shaded using Boxshade 3.21 (http://www.ch.embnet.org/software/BOX_form.html). The dendrogram was constructed by the Neighbour-joining method. The phylogenetic unrooted tree is displayed in Treeviewer (<http://taxonmy.zoology.gla.ac.uk/rod/rod.html>). Sequences are obtained from the following accession numbers *Arabidopsis thaliana*, ATC BAA75932.1, BFT (NP201010.1), MFT (NP173250.1), TFL1 (NP196004.1), FT (AAF03936), TSF (NP 193770) and *Lycopersicum esculentum*, SP (AAC2616.1), SP21 (AAO31791.1), SP3D (AAO31792.1), SP5G (AAO31793.1), SP6A (AAO31794.1), SP9D (AAO31795.1).

Analysis of gene expression

RNAs were isolated from leaf and inflorescence apices with Trizol (Invitrogen) and from developing seeds with a hot-borate method (Wan and Wilkins, 1994). 2.5 µg RNA was used to make oligo d(T)¹⁸-RACE primed first strand cDNA with MLV-reverse transcriptase (Promega) and was used to perform semi-quantitative RT-PCR analysis with primers specific for *PhBFT* (CATGCCAAGAGGAAGGGACAC and ATTATCCTCGCGACCACCG), *PhIFT* (ATGCCAACAGATTCTTG and CGGCAGCACGTATTATAATG), *PhMFT* (ATGGCTGCAAAGTTGATCCAT and AACAGAGACAGAGAAAGTAGAATC), *VEGGIE* (CCCTTCACAAGATCCATAAG and CAAGTCATTAATCGGCAGACC) and *ACTIN* (AGATCTGGCATCATACTTCTACA and CCMGCAGCTTCCATRCCAATCA). The PCR products were run on agarose gels, blotted on Hybond N⁺ membranes (Amersham), hybridized with ³²P-labeled gene-specific probes and the signal was detected by a Phosphorimager (GE Healthcare).

Gene constructs and plant transformation

PhIFT and *PhBFT* were amplified from W138 leaf cDNA by the primers (fw GCT**CCATGG**TATGCCAACAGATTCTTG; NcoI in bold letters and rev TATGTATTAG**CGGCCG**CTTATGCACGACGGC; NotI in bold letters) and (fw TCC**CCATGG**TATGCCAACAGAGGAAGGGACAC (NcoI) and rev TAATTGAGAT**CGGC**

CGCTTAAATTATCCTCG (NotI)) respectively. PCR fragments were digested with restriction enzymes and ligated in pEntry4 (NcoI/NotI).

PhMFT was amplified from W138 inflorescences apices cDNA with the primers (fw **CACCATGGCTGCAAAAGTTGATCCATTGG** and stop **GCTAGCAGACTCGATCAGCTA** GC) and by TOPO cloning introduced in pEntryD-TOPO (Invitrogen).

pEntry4-*PhIFT*, pEntry-*PhBFT* and pEntryD-TOPO-*PhMFT* were recombined into pK7GW2 (<http://www.psb.ugent.be/gateway/>) by GATEWAY LR Clonase II (Invitrogen).

A 1.75 kb promoter region of *PhBFT* was obtained by SOTI-PCR (genome walking with the help of *dTPH1* transposons) (Rebocho, 2008). The whole 1.75 kb of *PhBFT* promoter region was amplified from W138 genomic DNA with the primers (fw **CACCGCTAAGGTGGCAAGGTTTC** and rev **GACGAAATGGATGCTAGTGTAT** AAATTAAGC) and by TOPO cloning introduced in pEntryD-TOPO (Invitrogen). pEntryD-TOPO-*pPhBFT* was recombined into pKGWFS7,0 (<http://www.psb.ugent.be/gateway/>).

For yeast two hybrid purposes *PhBFT* was amplified from W138 leaf cDNA with the primers (fw **CATGAATTCATGCCAAGAGGAAGGGACACG** (EcoRI) and rev **GATTCTGCAGTTAAATTATCCTCGCGACCA** (PstI)) digested with restriction enzymes and ligated in pBD-GAL4 (EcoRI/PstI).

All the PCR amplifications used for cloning purposes were performed with Phusion polymerase (Bioke), with the proof reading activity. All the constructs were sequenced full length with Big Dye terminator technology (Perkin Elmer) before they were transformed into yeast or the line W115 by Agrobacterium-mediated leaf disc transformation (Spelt et al., 2000).

GUS staining

The histochemical staining of GUS was executed as described (Weigel, 2002). The X-Gluc concentration was 2mM. After staining the plant material was dehydrated by emerging the material through a series of washes with increasing percentage of ter-butanol: water. To saturate the material with paraffin it was emerged via a graded paraffin: ter-butanol series, casted in paraffin blocks and sectioned with the microtome to a thickness of 10µm. Paraffin was removed with ter-butanol. Photos were taken with a Zeiss microscope in bright light or dark-field.

Yeast two-hybrid assay

The yeast strain used is PJ69, which contains the *HIS3*, *ADE2* and *lacZ* reporter genes driven by the GAL4-responsive *GAL4*, *GAL1* and *GAL7* promoter (James et al., 1996). Yeast transformations were done using the lithium acetate method (Gietz and Woods, 2006). The pBD-GAL4 (Cam) and the pAD-GAL4 (Amp) (Stratagene) were fused to the full-length open reading frame of *VEGGIE*, *PhBFT* and *FD*. The yeast cells transformed with pBD-GAL4 or pAD-GAL4 vectors were selected on plates lacking either leucine or tryptophan or both. Re-transformation of PJ69 yeast was done by adding the appropriate BD and AD plasmid (1-2 µg each) together with 500µl of PJ69 yeast cells (overnight culture) and 50 µl of sonificated salmon sperm DNA (carrier) and plating on selective media.

To perform a LacZ overlay assay yeast double transformants were spotted and grown on SD-LT and permeabilized by chloroform. After evaporation of the chloroform the yeast was covered with topagar (0,7 % topagar in 0,1M KPO₄ pH=7 with 20mg X-gal) and incubate at 37 degrees for several hours before staining is observed.

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Chapter 6

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Summary

Fertilization is in both animals and plants the start of life and the development of the embryo will eventually result in a full grown organism. Whereas animals develop their complete body plan during embryogenesis, plants only establish a rudimentary plan and most adult structures will develop after embryogenesis. During development cells differentiate and acquire specified functions, but some small groups of cells, stem cells, are maintained both in plants and in animals, in an undifferentiated state to provide tissues with new cells. Intercellular signals are needed to maintain the stem cells and plants are able to establish new stem cells regions more easily than animals and use different signals.

When a plant seed germinates the aerial part consists of a stem (hypocotyl) with two leaves (cotyledons) and in between the cotyledons a stem cell niche, a meristem, named the shoot apical meristem (SAM). From this SAM the plant continues growth and develops more stem and leaf tissue and new emerging vegetative meristems in a geometrically highly organized indeterminate manner. After the vegetative phase plants switch to the reproductive phase during which the SAM obtains the ability to transform into an inflorescence meristem (IFM). This IFM is able to produce one or multiple flowers resulting in the enormous diversity of floral branches that we observe in the plant kingdom. The reproductive phase can be induced in different seasons and/or in different years. Some plant species start to flower in spring when the days are getting longer and light intensity gets higher whereas other prefer to flowering in declining day length and light intensity. Annual plants can undergo the floral transition only once whereas perennial plants have the capacity to reiterate the reproductive phase every year.

Plant species that display all their flowers at the same time are highly depending on good growth conditions to be able to set seeds to preserve their progeny whereas other plant species spread the risk and develop their flowers after each other over a longer period of time. The sequential production of flowers over an extended time period increases the chance of the plant to produce viable seeds and to overcome less favourable conditions during the reproductive season. The above mentioned strategies that plants display in the production, position and timing of flower formation results in three distinct inflorescence types that are named panicle, cyme and raceme respectively. Plant

species that display a panicle inflorescence form all their flowers at the same time, whereas plants that show a cymose branching inflorescence develop an apical floral meristem and at the periphery an inflorescence meristem to continue growth, resulting in a zig-zag stack of newly formed flowers. Plants that show a racemose branching pattern display an indeterminate growth of the apical meristem and flowers emerge sequentially in lateral positions.

Angiosperms display a wide variation of different inflorescence types but not much is known about the genes that determine that variety. To study the similarities and the differences between the different inflorescences it is essential to learn more about the spatial and temporal expression of the genes that specify the cymose inflorescence of *Petunia hybrida* and the raceme inflorescence of *Arabidopsis*. Although more genetic data about the cymose branching of petunia and other Solenaceous species like tomato (*Solanum lycopersicum*) is getting available, it is still fragmented and most of the genetic information known is obtained from the raceme branching *Arabidopsis*. The function of known and newly identified genes that are important for the initiation and maintenance of the cymose inflorescence meristem and their putative role in the onset of flowering will be studied in **Chapter 2**. The most distinguishing feature of a cymose inflorescence is the emergence of a sympodial inflorescence meristem at the periphery of the apical floral meristem, by which inflorescence growth continues. The identification of a new mutation in petunia, *veggie*, that strongly delays the onset of flowering and changes the cymose inflorescence into a solitary flower is described in **Chapter 3**. Phenotypical analysis of this mutant showed that the defect in the inflorescence architecture resulted from the homeotic transformation of the (secondary) sympodial inflorescence unit into a vegetative shoot. The production of the double mutant of *VEGGIE* with the meristem identity gene *ABBERRANT LEAF AND FLOWER* (*ALF*), revealed that *VEGGIE* is needed in indeterminate meristems, like the shoot apex and the sympodial shoots, to assign the inflorescence meristem identity and acts as a floral integrator to initiate the floral meristem identity genes. Also the double mutants of *VEGGIE* with the meristem identity genes *DOUBLE TOP* (*DOT*), *EVERGREEN* (*EVG*) and *HERMIT* (*HER*) were created and studied in detail.

In **Chapter 4**, we present the isolation and molecular characterization of *VEGGIE*. We found that *VEGGIE* encodes a phosphatidylethanolamine-binding protein (PEBP) that is structurally and functionally similar to FLOWERING LOCUS

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T (FT), a regulator of flowering time from *Arabidopsis*. Grafting experiments and expression analyses revealed *VEGGIE* is expressed in the vascular tissues of leaves and promotes the synthesis of a mobile signal, presumably VEGGIE protein, that is able to move through a graft junction and is needed throughout the flowering phase for the development of each sympodial inflorescence to maintain cymose architecture.

VEGGIE has a dual role in petunia development to promote the switch from vegetative growth to flowering and to specify the architecture of the cymose inflorescence, whereas the orthologous *FT* gene in *Arabidopsis* is only essential for the onset of flowering but is not required to specify the architecture of the racemose inflorescence. As *VEGGIE* and *FT* encode functionally similar proteins and are expressed in similar patterns, we aimed to identify and compare proteins that bind to *FT* and *VEGGIE* to understand how they acquired distinct roles in development of the inflorescence. In *Arabidopsis* the bZIP transcription factor FD, interacts in the apex with *FT* and so promotes flowering. In **Chapter 5**, we report that *VEGGIE* interacts in the petunia apex with various bZIP transcription factors that all belong to the same sub-group and focussed on two bZIP transcription factors that were most similar to FD to study their putative function in inflorescence architecture and their floral promoting potential.

The *FLOWERING LOCUS T/ TERMINAL FLOWER 1 (FT/TFL1)* gene family from *Arabidopsis* encodes proteins with similarity to PEBP proteins that act as floral inducers or repressors. *FT/TFL* homologs have been identified in many flowering plants showing that this gene family is widely conserved. In **Chapter 6**, we identified three additional members of the *VEGGIE* family from petunia, *PhBFT*, *PhIFT* and *PhMFT* and analyzed their highly similar genomic structure, phylogenetic relationships and expression patterns. *PhBFT* and *PhIFT* cluster together with *VEGGIE* and we were able to show that *PhBFT* is rhythmic regulated during the day as is *VEGGIE* and that *PhBFT* expression can be observed in both long- and short-day conditions. *PhMFT* shows high sequence homology to *MFT* from *Arabidopsis* and is expressed at the tip of the developing stigma and during seed development. The different functions that were displayed by the *VEGGIE* family members suggest that these genes act not only as floral inducer or repressors but can have additional functions that are still to be discovered.

Samenvatting van het proefschrift getiteld: "De moleculair biologische analyse van het inflorescence meristeem van Petunia hybrida"

Na de bevruchting ontstaat nieuw leven, een embryo, dat zich ontwikkelt en uitgroeit tot een volwaardig organisme. In het dierenrijk wordt tijdens de embryogenese de complete opbouw van het organisme vastgelegd terwijl planten starten met een eenvoudige opbouw en hun plant onderdelen pas in een veel later stadium ontwikkelen. Gedurende de ontwikkeling en groei van het organisme differentiëren cellen en verkrijgen deze een specifieke functie, een kleine groep cellen, de stamcellen, worden echter in een ongedifferentieerde staat gehouden en deze cellen kunnen in een later stadium alsnog een specifieke functie krijgen. Hoe deze stamcellen in stand worden gehouden, wordt in planten en dieren door andere intercellulaire signalen bepaald en zijn planten veel gemakkelijker dan dieren in staat om nieuwe regio's voor stamcel productie aan te leggen.

Zodra een plantenzaad ontkiemt, bestaat de zaailing uit een ondergronds deel, de wortel en een bovengronds deel, dat bestaat uit een stengel (hypocotyl), een of twee zaadbladeren (cotyledons) en tussen de cotyledons een stamcel regio, het meristeem. Vanuit dit meristeem kan de plant groeien en ontwikkelen hij stengel, bladeren en nieuwe vegetatieve meristemen in een strak geometrisch georganiseerde structuur. Na deze vegetatieve fase kunnen planten overschakelen naar de reproductieve fase waarin het vegetatieve meristeem kan worden omgezet in een bloem of een bloeiwijze. De bloei kan in een verschillend seizoen en/of in een verschillend jaar worden ingezet. Zo zijn er planten die bloei induceren in het voorjaar wanneer de dagen langer worden en de lichtintensiteit hoger terwijl andere planten pas gaan bloeien bij afnemende daglengte en lichtintensiteit. Eenjarige planten kunnen slechts eenmalig tot bloei komen terwijl meerjarige planten in staat zijn elk jaar opnieuw te bloeien. Kortom, planten hebben verschillende strategieën ontwikkeld om zich te handhaven.

Niet alleen in de tijd maar ook in opbouw van de bloeiwijze hebben planten verschillende tactieken ontwikkeld. Planten die al hun bloemen tegelijkertijd laten bloeien zijn in hoge mate afhankelijk van goede groeicondities voor

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zaadproductie en instandhouding van de soort terwijl andere planten het risico van bloei spreiden door bloemen te produceren gedurende een langere periode. Door de bloemen na elkaar te ontwikkelen verhoogt de plant de kans om levensvatbare zaden te produceren en kan op deze wijze slechte groeiconditie gedurende het seizoen omzeilen. De verschillen in de productie, positie en timing van bloemontwikkeling aan de plant resulteert in drie verschillende bloeiwijzen (inflorescences), genaamd panicle, cymose en raceme. Planten met een panicle inflorescence vormt al hun bloemen tegelijkertijd terwijl planten met een cymose inflorescence de stengel met het meristeem wordt beëindigd met een bloem en groei wordt gecontinueerd door een nieuw inflorescence meristeem (sympodial inflorescence meristeem) dat zich net onder de bloem ontwikkeld. Deze cymose bloeiwijze resulteert uiteindelijk in een zigzag structuur van telkens nieuw gevormde bloemen. Voor planten met een raceme inflorescence groeit de stengel met het meristeem in de top door en worden de bloemen stuk voor stuk geïnitieerd aan de zijkant van de stengel.

In bloeiende planten vind je een enorme variëteit in bloeiwijzen maar de betrokken genen zijn veelal onbekend. Om de verschillen en overeenkomsten tussen de inflorescence types te bestuderen is het essentieel om te onderzoeken waar en wanneer genen specifiek tot expressie komen. Hiervoor hebben wij gebruik gemaakt van de raceme vertakkende *Arabidopsis thaliana*, de zandraket, en de cymose vertakkende *Petunia hybrida*. Helaas is de genetische informatie van petunia en andere Solanaceae familieleden zoals tomaat nog gefragmenteerd en wordt de meeste genetische data verkregen in Arabidopsis. In **hoofdstuk 2** worden bekende en nieuw geïdentificeerde petunia genen besproken die belangrijk zijn voor de aanleg en onderhoud van het inflorescence meristeem.

Het meest opvallende aan de cymose inflorescence is het ontstaan van het sympodial inflorescence meristeem waar vanuit de groei van de plant wordt voortgezet. In **hoofdstuk 3** beschrijf ik de identificatie van een nieuwe mutant in petunia, genaamd *veggie*. De *veggie* mutant laat een sterk verlate bloei zien en tevens is de cymose vertakking verdwenen wat resulteert in een laat bloeiende plant met een solitaire bloem. Fenotypische analyse van deze mutant laat zien dat het defect in de inflorescence is ontstaan door een homeotische verandering (een verandering waarbij één plantdeel vervangen wordt door een structuur die normaal elders in de plant voorkomt) van de sympodial inflorescence unit naar een vegetatieve scheut. Door de productie van de

dubbele mutant van *veggie* met het bekende meristeem identiteits gen *ABERANT LEAF AND FLOWER* werd het duidelijk dat *VEGGIE* nodig is het vegetatieve meristeem om deze een inflorescence identiteit toe te bedelen en de bloem ontwikkeling te initiëren. Ook de dubbel mutanten van *veggie* met de meristeem identiteits genen *DOUBLE TOP*, *EVERGREEN* en *HERMIT* zijn vervaardigd en zij leveren waardevolle informatie over de functie die elk van de bovengenoemde genen heeft in de totstandkoming van de cymose inflorescence.

De isolatie en de moleculaire karakterisatie van *VEGGIE* staat beschreven in **hoofdstuk 4**. We hebben gevonden dat *VEGGIE* een fosfotidylethanolamine (PEBP) bindend eiwit is dat qua structuur en functie gelijk is aan *FLOWERING LOCUS T* (FT). FT is een positieve regulator van bloeitijd in *Arabidopsis*. Door expressie studies uit te voeren en *veggie* mutanten scheutten te enten op bloeiende wild type planten hebben we kunnen aantonen dat *VEGGIE* tot expressie komt in het vasculaire weefsel van het blad en het de synthese van een mobiel signaal, het florigen, promoot. Mogelijk is het het *VEGGIE* eiwit dat zich door de plant kan bewegen om zo gedurende de hele bloeiperiode elk van de sympodiaal inflorescence meristemen te voeden en op deze wijze de cymose inflorescence structuur te behouden.

VEGGIE is niet alleen belangrijk voor de bloeitijd maar ook voor de specificaties van de cymose inflorescence van de petunia terwijl *FT* in *Arabidopsis* enkel een functie heeft tijdens de start van de bloei. *VEGGIE* en *FT* coderen voor gelijke eiwitten en laten gelijke expressie profielen zien maar door de identificatie van eiwitten die binden aan *FT* en *VEGGIE* proberen wij te achterhalen hoe zij bijdrage aan de ontwikkeling van het raceme en cymose inflorescence. In *Arabidopsis* is een bZIP transcriptie factor, genaamd FD, geïdentificeerd die interactie aangaat met *FT* en op deze wijze bloei initieert. In **hoofdstuk 5**, laten we zien dat *VEGGIE* kan binden aan verscheidende bZIP transcriptie factoren die allemaal behoren tot dezelfde subgroep waartoe ook FD behoort. Voor de verdere analyse hebben wij ons gefocust op de twee bZIP transcriptie factoren die het meest op FD lijken en hebben wij hun mogelijke functie in de inflorescence architectuur en bloei potentiaal bestudeerd.

De genen van de *FLOWERING LOCUS T/TERMINAL FLOWER 1* (*FT/TFL1*) familie van *Arabidopsis* coderen voor eiwitten die gelijkenis vertonen met PEBP eiwitten en ze werken als bloei activator of remmer. *FT/TFL* homologen zijn geïdentificeerd in veel verschillende bloeiende planten wat er op wijst dat het om

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een geconserveerde gen familie gaat. In **hoofdstuk 6**, laten wij de identificatie van drie *VEGGIE* familieleden, *PHBFT*, *PHIFT* en *PHMFT*, zien en analyseren hun genomische structuur, fylogenetische relaties en expressie patronen. *PHBFT* en *PHIFT* clusteren met *VEGGIE* en we hebben kunnen aantonen dat *PHBFT* net zoals *VEGGIE* ritmisch gereguleerd is gedurende de dag en dat *PhBFT* zowel in korte- als in lange-dag condities tot expressie komt. *PhMFT* heeft een hoge sequence homologie met *MFT* van *Arabidopsis* en komt tot expressie op de tip van het stigma en gedurende zaad ontwikkeling. De verschillende functies die de *VEGGIE* familieleden tentoongespreiden suggereert dat deze genen mogelijk niet alleen werken als bloei activators of remmers maar dat er mogelijk nog andere onbekende functies zijn.

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Dankwoord

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