Control of floral meristem determinacy in Petunia by MADS box transcription factors

Silvia Ferrario¹, Anna V. Shchennikova¹², John Franken¹, Richard G. H. Immink¹ and Gerco C. Angenent¹,*

¹Plant Research International, Business Unit Bioscience, P.O. Box 16, 6700 AA, Wageningen, The Netherlands.
²Centre of Bioengineering, Russian Academy of Sciences, 117312 Moscow, Russia.

• Corresponding author: Gerco.angenent@wur.nl
The shoot apical meristem (SAM), a small group of undifferentiated, dividing cells, is responsible for the continuous growth of plants. Several genes have been identified that control the development and maintenance of the SAM. Among these, WUSCHEL (WUS) from Arabidopsis is thought to be required for maintenance of a stem cell pool in the SAM. The MADS box gene AGAMOUS (AG), in combination with an unknown factor, has been proposed as a possible negative regulator of WUS, leading to the termination of meristematic activity within the floral meristem. Transgenic petunia plants were produced in which the E-type and D-type MADS box genes FBP2 and FBP11 respectively, are simultaneously overexpressed. These plants show an early arrest in development, at the cotyledon-stage. Molecular analysis of these transgenic plants revealed a possible combined action of FBP2 and FBP11 in repressing the petunia WUS homolog, TERMINATOR (TER). Furthermore, the ectopic upregulation of the C-type and D-type homeotic genes, FBP6 and FBP7 respectively, suggests that they may also participate in a complex, which causes the determinacy in the transgenic plants. These data support the model that a transcription factor complex consisting of C, D, and E-type of MADS box proteins control the stem cell population in the floral meristem.
Vascular plants continue to grow throughout their life cycle, a condition that does not hold for animals. This indeterminate growth is ensured by two regions of pluripotent, meristematic cells, which are located since early embryogenesis, at both ends of the plant body. The root apical meristem gives rise to the underground root system, while all the above-ground structures are initiated by the shoot apical meristem (SAM), which originates an orderly sequence of leaves, nodes and internodes. Upon floral induction the SAM turns into an inflorescence meristem and/or into a floral meristem, which will eventually cease its meristematic activity in order to allow the differentiation of a pistil, the formation of gametes and the completion of the life cycle.

Mutant and genetic analyses in *Arabidopsis* have revealed some of the regulatory circuits that perpetuate the delicate balance between stem cell accumulation and organ initiation (see for a review: Sharma et al., 2003; Williams and Fletcher, 2005). Meristem activity is promoted by the homeodomain proteins SHOOTMERISTEMLESS (STM) and WUSCHEL (WUS), whose loss-of-function mutants fail to maintain a population of stem cells. In these mutants the SAM is terminated at the end of embryogenesis and growth stops at the cotyledon stage (Barton and Poethig, 1993; Long et al., 1996; Laux et al., 1996; Mayer et al., 1998). Independent and complementary roles have been assigned to STM and WUS in SAM regulation: a WUS-dependent signal from the organizing center underlying the L1-L3 layers of the central zone (CZ), specifies the stem cell population in the CZ, while STM maintains cell proliferation in the peripheral zone (PZ) (Mayer et al., 1998; Long and Barton, 1998; Lenhard et al., 2002). The balance between stem cell maintenance and organogenesis in the SAM is tightly controlled by a regulatory circuit that involves the CLAVATA (CLV) and WUS proteins. WUS induces stem cell identity in the CZ and subsequent expression of the
CLV complex, which in turn activates a signaling pathway that feeds back from the stem cells to repress the transcription of WUS in the center of the meristem (Schoof et al., 2000; Brand et al., 2000). While organogenesis in the shoots can go on indefinitely, only a limited number of floral organs can be formed before the floral meristem ceases to perpetuate itself. Due to its central role in promoting stem cell identity, WUS is likely to be the target of pathways that lead to the termination of the floral meristem. It has recently been proposed that a negative feedback loop involving WUS, the floral meristem identity gene LEAFY (LFY) and the floral organ identity gene AGAMOUS (AG) takes place in the floral meristem and is responsible for its suppression (Lenhard et al., 2001; Lohmann et al., 2001). The combined action of LFY and WUS would activate AG in the floral meristem, which in turn represses WUS in the determinate flower. Furthermore, evidence of AG being a repressor of WUS is based on the opposite phenotypes of wus and ag mutants and on the expression patterns of the respective genes in wild type and mutant plants. The sporadic flowers that are formed in wus plants, lack carpels and most stamens, while ag flowers are indeterminate with an indefinite number of perianth organs, furthermore, WUS expression declines after stage 6 of wild type flowers but it persists in ag flowers. There are, however, some questions that remain to be resolved: first, AG alone is not sufficient to repress WUS since ectopic expression of AG causes termination of the inflorescence meristem, which forms a single terminal flower, but not of the shoot meristem. Second, expression of WUS persists till stage 6 of flower development, while AG mRNA can be detected from stage 3 onwards. The presence of another unknown factor, which acts together with AG in the flower to repress WUS, has been proposed as a possible explanation. Finally, it remains to be uncovered how conserved these mechanisms that regulate meristem self-maintenance and -termination are among different species.
Here we report about the relation between petunia MADS box proteins belonging to the C, D and E class and the WUS homologue, TERMINATOR (TER) (Stuurman et al., 2002) and propose a model how floral meristem determinacy is controlled in petunia.

MATERIALS and METHODS

Plant material

*Petunia hybrida* lines W115 and W138 and transgenic plants were grown under normal greenhouse conditions (22°C, 14 h light/10 h dark).

In vitro germination was performed after surface-sterilization of petunia seeds by chlorine gas. Seeds were kept 3 hr in a desiccator with a mixture of 100ml commercial bleach and 3ml concentrated hydrochloric acid in a fume hood. Sterilized seeds were grown on half strength Murashige and Skoog medium (Murashige and Skoog, 1962) containing 0.5% (w/v) sucrose and 0.8% agarose, at 22°C in 16 h light/8 h dark.

The transgenic plant T46008 carrying the 35S::*FBP11* transgene was used in this study (Colombo et al., 1995).

In situ hybridization

In situ hybridizations were performed as described by Cañas et al. (Cañas et al., 1994). Digoxigenin-labelled RNA probes were synthesized by T7 polymerase-driven *in vitro* transcription, from the PCR fragments containing part of the *TER* ORF, the *PhSTM* ORF and the *FBP6* ORF, excluding the MADS box, according to the instructions of Boehringer Mannheim. The amplification products were obtained with the following forward and reverse primers: *TERfw* 5’-TGGAGAAGAGCTTTAGGG-3’, *TERrev* 5’-TAATACGACTCATATAGGG-3’, *PhSTMfw* 5’-TGGAAGACTCTAGGGATACGTAGTACATGGCC-3’, *PhSTMrv* 5’-GGCAAGATTATGGCTCATCC-3’,
PhSTMrev 5’-TAATACGACTCATATAGGGCATATCTTCTGGATGTTTCC-3’,

FBP6fw 5’-AGGTAC AAGAAACACCATGCCG-3’, FBP6rev 5’-

TAATACGACTCATATAGGGCATCAGACA AGCTGTAGAGCAG-3’, the T7 polymerase promoter site is underlined. FBP11 probe was prepared as described by Angenent et al. (1995).

Nomarski microscopy

Seedlings were cleared for 2 to 16 hours in a drop of Hoyer’s solution (7.5 g gum arabic, 100 g chloral hydrate, 5 ml glycerol in 30 ml water) on a microscope slide. Cleared seedlings were examined using a Nikon Optiphot microscope equipped with Nomarski optics.

RT-PCR analyses

Total RNA was extracted from single seedlings, pistils, young leaves and roots using the RNeasy Plant Mini Kit (QIAGen, Valencia, CA). cDNA was obtained from 1 µg of total RNA in a 50 µl reaction using the TaqMan reverse transcription kit (Applied Biosys
tem). Prior addition of the reverse transcriptase, 1 µl of DNase (1U/µl, Invitrogen) was added and the reaction was carried out for 30’ at 37°C. After heat inactivation of the DNase at 75°C for 5’, 10 µl were removed from the reaction and used in the PCR amplification step as a control for the absence of genomic DNA. 1 µl of reverse transcriptase was added to the remaining 40 µl and the reaction continued 10’ at 25°C, followed by 30’ at 37°C and 5’ at 95°C to inactivate the enzyme.

RT-PCR amplification products were obtained using the following gene-specific forward and reverse primers: FBP2fw 5’-GCAAAGAACTTGAATCACTTGAAAGGC-3’, FBP2rev 5’-GCTTTCAAGGCAACCAGCC-3’, pMADS3fw 5’-CTGAATCTCAGAGATCTGAGG-3’, pMADS3rev 5’-CAAGGTCATAGCTAGAATCTCC-3’, FBP6fw 5’-GTACAGGATCTGTTT
CTGAAGC-3’, *FBP6*rev 5’-AATCTCCCTTTTGATGAGC-3’, *FBP7*fw 5’-CCCAAGTG
AAGTCCACATCG-3’, *FBP7*rev 5’-GCAAGAAAGCTTGAACACACC-3’, *FBP11*fw
5’-GTAAATTATTTGCAGCTGG-3’, *FBP11*rev GAGAAAAGCTTGAACGAGTTCACC-3’,
*PhGADPH*fw 5’-GTTTGAGAAAAGAGCCACC-3’, *PhGADPH*rev 5’-CGTTGTAGTACC
AAGACACG-3’.

The annealing temperature was 59°C for all the primer combinations used. *PhGADPH*
PCR products were visualized on a 2% agarose gel after 25 cycles of amplification. 30 cycles
were used in the PCR profile of all the MADS box genes.

**Three-hybrid analysis of petunia protein-protein interactions**

The full length *pMADS3* and *FBP6* coding regions were cloned into the pTFT1 vector
(Egea-Cortines et al., 1999). The three hybrid analyses were performed in the modified GAL4
yeast two-hybrid system as described by Ferrario et al. (2003). Growth of yeast and hence
complex formation, has been scored after 10 days incubation at room temperature (20 °C) on
a selective medium lacking His and supplemented with 1mM 3-amino-1,2,4-triazole (Sigma).

**RESULTS**

**Expression domains of C, D, E function genes and TER in the petunia flower**

Similar to *Arabidopsis*, petunia plants with impaired C or E functions, show loss of
determinancy within the flower, indicating a possible involvement of the corresponding genes
in floral meristem determination (Kapoor et al., 2002; Angenent et al., 1994; 2005; Ferrario et
al., 2003). The E function in petunia is accomplished by at least two genes, *FBP2* and *FBP5*,
as suggested by the floral phenotype of cosuppression plants. When these two genes are
simultaneously downregulated, homeotic conversion of the three inner whorls and loss of
determinacy occur within the flower (Angenent et al. 1994, Ferrario et al., 2003). The milder phenotype of the \textit{FBP2/FBP5} double knockout mutant, however, indicates the presence of at least one more gene partially redundant in the E function (Vandenbussche et al., 2003). In situ hybridization experiments revealed that \textit{FBP2} and \textit{FBP5} share a similar expression pattern from the floral meristem stage until complete maturity of the floral bud, where their expression is restricted to the three inner whorls (Ferrario et al., 2003; Fig 1A and 1B).

The fate of reproductive organs is controlled by class C genes belonging to the \textit{AGAMOUS} (\textit{AG}) clade, which have been duplicated during evolution in several plant species. In petunia, two genes share high sequence similarity with the Arabidopsis \textit{AG} gene, \textit{pMADS3} and \textit{FBP6} (Tsuchimoto et al., 1993; Kapoor et al., 2002; Kater et al., 1998). Similarly to \textit{AG}, \textit{FBP6} and \textit{pMADS3} transcripts are present in stamens and carpels from the appearance of their primordia till complete maturity of these organs (Fig. 1C and 1D; Kapoor et al., 2002). The phenotype of \textit{pMADS3} cosuppression plants, where homeotic conversion and loss of determinacy is observed in the third whorl only, support the idea that this gene may act redundantly with \textit{FBP6} in the determination of the fourth whorl (Kapoor et al., 2002).

\textit{TERMINATOR (TER)} is the petunia ortholog of \textit{WUSCHEL (WUS)}, a key player in meristem determination in Arabidopsis (Stuurman et al., 2002). We compared the expression domains of \textit{TER} with those of C and E class genes in wild type petunia plants, to obtain a preliminary indication about the functional relationships of these factors in the termination of the floral meristem. \textit{TER} expression, like its \textit{Arabidopsis} ortholog, is limited to a few cells in the central zone (CZ) of the SAM, as has been shown by in situ hybridization on shoot apexes of wild type seedlings (Stuurman et al., 2002; Fig. 2I). In the flower, \textit{TER} activity is still present in a limited number of cells in the central region at a stage when petal and stamen primordia are emerging (Fig. 1E), but it disappears at a stage during gynoecium development, just prior to
the fusion of the carpels (Fig. 1F). At that stage, TER expression remains in an area of the stamen that becomes the stomium.

According to the abovementioned expression data, C and E genes activities are overlapping in the center of the flower, in the region where also TER is active. Hence, similar as in *Arabidopsis*, the timing of C and E gene induction does not coincide with TER downregulation, which occurs at a later stage. It is therefore possible that another factor is responsible for the exact timing of TER downregulation. Good candidates for this function are the D type genes *FBP7* and *FBP11*, which determine ovule identity in petunia (Angenent et al., 1995; Colombo et al., 1995). The expression of these D type genes was analyzed by in situ hybridization using a labeled *FBP11* RNA probe, which should also detect the *FBP7* transcripts. The hybridization experiments (Fig. 1G and 1H) revealed that *FBP7/FBP11* transcripts approximately appear at a stage when TER expression is decreasing. Prior to the formation of carpel primordia, *FBP7/FBP11* mRNA is absent (Fig 1G), while expression of these MADS box genes appear in the cells located in the center of the flower and that differentiate into placental tissue (Fig 1H).

**Multimeric complexes among C-, D- and E-type proteins**

To investigate a potential combinatorial action of the C, D, and E in the floral meristem that could coincide with the down-regulation of TER expression, we analyzed the ability of the MADS box proteins to form multimeric complexes in yeast 2- and 3-hybrid experiments. Due to the transcriptional activation activity possessed by the C terminus of FBP2 in yeast, a truncated version of the protein (FBP2ΔC) lacking 59 aminoacids, was used, as described in Ferrario et al. (2003). It has already been shown in previous work that the E-type protein FBP2 interacts in yeast with both the C- and D- type proteins (Ferrario et al., 2003), whereas neither the C- nor the D- types are able to form heterodimers with each other (Immink et al.,
The presence of FBP2, however, is able to bring together the C- and D-type proteins in the same complex, as shown in Table 1. The formation of all possible complexes, which included FBP2 could be detected, although with different efficiency, as the interactions between FBP6, FBP7 and FBP2 and between FBP11, FBP7 and FBP2 appeared to be stronger in the yeast system. As a control, the combination of the D-type FBP11, the C-type pMADS3, and the B-type protein pMADS2 was tested, and appeared to be negative in all the conditions assayed. The formation of a multimeric complex involving C-, D- and E-type proteins may occur also in the developing flower, where it may induce the repression of the TER gene.

**Simultaneous ectopic expression of FBP2 and FBP11 arrests seedling development**

To examine whether the simultaneous ectopic expression of the abovementioned MADS box genes is sufficient to down-regulate TER expression outside the floral domain, we followed a transgenic approach to combine the constitutive expression of the MADS box genes. The full-length FBP2 cDNA under the control of the 35S constitutive promoter of the *Cauliflower Mosaic Virus (CaMV)* was introduced in petunia plants. Ectopic expression of FBP2 in plants carrying a copy of the transgene, which segregated as a single locus, was confirmed by northern blot hybridization (data not shown). No visible phenotype was observed in the transgenic plants at any stage of development. Conversely, overexpression of the ovule identity gene FBP11 did cause phenotypic alterations in the flower as previously reported by Colombo et al. (1995). Ectopic ovule formation was observed on the corolla tube and on the inner side of the sepals. Petals were also affected; the corolla at full maturity was nothing more than a tube that barely reached the length of the style. Plants homozygous for the FBP2 gene, coming from two independent lines, were subsequently crossed with transgenic plants hemizygous for FBP11. When seeds from three independent crosses were germinated *in vitro* approximately half of the seedlings showed a permanent arrest early in the
development, at a stage when only the two cotyledons were visible. A clear difference was observed between the two different seedling populations about six days after germination (Fig. 2A to 2C). Arrested seedlings and seedlings with a wild-type appearance were tested at DNA and mRNA levels. PCR amplification using primers specific for the FBP2 and FBP11 transgenes revealed that all the seedlings contained at least one copy of the FBP2 transgene, while only the small ones carried also the FBP11 transgene. The result was confirmed by Northern blot analysis: ectopic expression of both FBP2 and FBP11 was observed in the arrested seedlings only (data not shown).

A more detailed phenotypic analysis of the small seedlings revealed that two to four leaf primordia were emerging from the SAM and the leaves produced remained always very small (Fig. 2D to 2F). To analyze the shape and size of the SAM, seedlings of wild-type plants and the double overexpressor were cleared and microscopically studied (Fig. 2G and 2H). This revealed that the size of the meristem was reduced in the seedlings of the double overexpressor compared to the wild-type. In addition, the SAM size of the single 35S::FBP2 overexpressor was microscopically analyzed and appeared to be unaffected when compared to the wild-type SAM (results not shown).

Generating plants that simultaneously overexpress either FBP6 or pMADS3 and any of the other MADS box genes was not possible by crossing, because it has been shown previously (Kater et al., 1998) that the 35S::FBP6 and 35S::pMADS3 transgenes failed to be transmitted to the progeny.

**TER expression is downregulated in seedlings that are arrested in development**

The arrest in development that was observed in the double overexpression lines suggested a failure in maintaining the stem cell pool in the shoot meristem, which could be caused by a decreased activity of the meristem gene TERMINATOR (TER; Stuurman et al., 2002).
Therefore, we analyzed the expression of *TER* in the SAM of the seedlings by in situ hybridizations on series of subsequent longitudinal sections. While *TER* transcripts were present in wild type seedlings in a small group of cells, we were not able to detect any signal in the CZ of the shoot apex of the arrested transgenic seedlings (Fig. 2I and 2J). The petunia ortholog of *STM* (*PhSTM*) (Stuurman et al., 2002), in contrast, was still expressed in the SAM of the transgenic seedlings (Fig. 2K and 2L), which is in line with the independent roles, assigned to *WUS* and *STM* in the determination of the *Arabidopsis* SAM.

**Upregulation of MADS box genes in the arrested seedlings**

Although the combined ectopic expression of *FBP2* and *FBP11* seemed sufficient to induce *TER* downregulation, we could not exclude an involvement of the C type genes. Therefore we analyzed *pMADS3* and *FBP6* (Tsuchimoto et al., 1993; Angenent et al., 1993) expression in the transgenic seedlings by means of RT-PCR. We also checked the expression of both D-type genes, *FBP7* and the endogenous *FBP11*, through the same technique. Although *FBP7* and *FBP11* share approximately 90% sequence similarity at protein level, it was possible to design PCR primers in the 3’ part of the sequence that were specific for each endogenous gene (Fig. 1, Supplemental Data).

PCR amplifications were performed on cDNA obtained from a single arrested seedling, a single wild-type seedling and a wild-type pistil. Surprisingly, *FBP6* but not *pMADS3* was upregulated to a detectable level in the double overexpression seedling, as shown in Figure 3A, indicating a possible involvement of only one of the two C-type genes in the phenotype observed. Noteworthy is that a basal, although very low expression of *FBP6* but not of *pMADS3* was detected in wild-type seedlings as well. Unfortunately the lack of an *fbp6* mutant makes it impossible to assess whether the same arrested phenotype could occur in the absence of the C-type gene product. On the other hand, both the endogenous *FBP7* and
FBP11 genes appeared to be upregulated in the transgenic seedlings (Fig. 3B), and the sequencing of the amplified fragments confirmed the identity of the two genes. Expression of FBP7 or FBP11 was never detected in wild-type seedlings. The petunia housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (PhGADPH), uniformly expressed in all tissues, was used as a control for the cDNA quantity in each tissue used (Fig. 3C).

Another indication that FBP7 is indeed activated by simultaneous overexpression of FBP2 and FBP11 came from the analysis of arrested seedlings in which the GUS reporter gene was expressed under the control of an FBP7 promoter fragment. GUS staining in the transgenic petunia plants from line T51013 was specifically observed in ovules (Fig. 4A), as it was previously described by Colombo and colleagues (Colombo et al., 1997). This line was used in crosses with 35S::FBP2 plants and the progeny plants, selected for the presence of both constructs, crossed again with 35S::FBP11 plants. The progeny of this second cross segregated arrested and wild-type seedlings, which were tested for GUS activity. All the wild type looking seedlings, which included also transgenic seedlings containing only one of the overexpression constructs and pFBP7::GUS did not show any GUS activity. On the other hand, all small seedlings containing the pFBP7::GUS construct showed clear GUS activity. The results are illustrated in Figures 4B and 4C and indicate that the increased expression of both FBP2 and FBP11 is necessary to activate the FBP7 promoter.

**Gene activation and repression in the flower of 35S::FBP11 transgenic plants**

The strong activation of FBP2 and FBP11 genes seems to be sufficient to trigger the repression of TER, as it has been shown in the double overexpressor seedlings. However, this occurs in the SAM where these genes are normally not expressed. Therefore, we wondered whether TER expression was also affected in the floral meristem (FM) of 35S::FBP11 plants, where both FBP2 (endogenous) and FBP11 (35S::FBP11) are strongly active. Indeed, in situ
experiments performed on flower buds confirmed the downregulation of TER expression in 35S::FBP11 FMs at a stage when it is normally still active in a wild type flower (compare Fig. 1E and 5B). TER expression could still be detected in a transgenic FM at a very early stage of flower development, when floral organ primordia are not apparent yet (Fig. 5A) and FBP6 transcripts are still absent (Fig. 5D). TER repression occurs only later in development, after petal primordia have appeared, at a stage when also the C-type gene FBP6 is strongly activated (Fig. 1C). The precocious downregulation of TER in the transgenic FM, when FBP2 (Fig. 1A), FBP11 (35S::FBP11) and FBP6 (Fig. 1C) are simultaneously highly expressed, strongly suggests that the three gene activities are together required for TER repression. The hypothesis that FBP11 may take part in TER downregulation is also indicated by the lack of TER expression in the stomium area of the transgenic stamens (Fig. 5C). In wild type flowers TER expression could still be detected in stamens where normally FBP2 and FBP6 are also present (arrows in Fig. 1F), but its expression is abolished in the 35S::FBP11 stamens. Although a precocious downregulation of TER occurs in 35S::FBP11 plants, the transgenic flowers can still develop a complete pistil (Colombo et al., 1995). This could be explained by the fact that TER downregulation in these plants occurs at a stage when sepals are formed, petal primordia are arising and the number of meristematic cells present is most likely sufficient to ensure the completion of the remaining inner floral organs.

We also tested whether the ectopic expression of FBP11 alone could trigger the upregulation of the set of genes that was observed in the double overexpressors. RT-PCR performed on wild type leaves and carpels and on transgenic leaves with different sets of gene specific primers showed the activation of FBP2 and FBP7 genes only, whereas transcripts of the C-type genes FBP6 and pMADS3 were never detected (Fig. 6). The lack of FBP6 upregulation in transgenic leaves could be explained by the absence of an FBP6 basal expression in these organs. The absence of an arrested phenotype in 35S::FBP11 plants
despite the upregulation of *FBP2*, could be either due to a lack of *FBP6* or to an insufficient amount of FBP2 protein produced by *FBP2* activation.

**DISCUSSION**

In contrast to the shoot apical meristem (SAM), the floral meristem has to be terminated in order to allow the determinate structure of the flower to be completed. This occurs in *Arabidopsis* via a feedback loop involving the C-type gene *AG*, which is activated by *WUS* and in turn promotes its suppression (Lenhard et al., 2001; Lohmann et al., 2001). Mutant and expression analyses on both genes, however, indicate that at least one other unknown factor is necessary to downregulate *WUS* together with *AG*. With the evidence produced in this study we suggest that the C-type gene in petunia acts together with the E- and D-types in the downregulation of the *WUS* ortholog and the termination of the floral meristem.

The involvement of E-type genes in floral meristem identity has been well documented by mutant studies in petunia, tomato, gerbera and *Arabidopsis* (Angenent et al., 1994; Pnueli et al., 1994; Pelaz et al., 2000; Uimari et al., 2004; Ditta et al., 2004). In all mutants in which the E function was impaired, floral determinacy was affected, demonstrating their involvement in the termination of the flower. However, the petunia C- and E-type genes *FBP6/pMADS3* and *FBP2* (as well as their *Arabidopsis* counterparts *AG* and *SEPALLATA*) are activated within the flower at a stage when *TER* transcript is still detectable, suggesting the presence of another factor whose later appearance is finally responsible for *TER* downregulation. Possible candidates for this role are the D-type genes *FBP7* and *FBP11* whose expression coincides with the downregulation of *WUS*-like genes, which ultimately leads to loss of meristematic activity. Furthermore, at later developmental stages *WUS* is activated in nucellar cells of the ovule, where expression of the D-type gene is lacking (Angenent et al., 1995; Gross-Hardt et
al., 2002). Although no direct evidence is available about the mode of action of the abovementioned MADS box genes, the yeast three-hybrid experiments we performed leads us to suggest that the corresponding proteins, which interact in yeast, may form a multimeric complex responsible for TER repression within the flower.

Thus involvement of \( FBP2, FBP11 \) and \( FBP6 \) in blocking meristematic activity via TER downregulation is also indicated by the arrested phenotype of the petunia seedlings in which \( FBP2 \) and \( FBP11 \) were overexpressed. The ectopic expression of the two genes caused a significant upregulation of \( FBP6 \), bringing together in the same cell the three components of the hypothetical complex that acts in the flower. Also the endogenous D-function MADS box genes \( FBP7 \) and \( FBP11 \) themselves, were strongly upregulated; although, unlike \( FBP6 \), no transcripts were ever detected in wild-type seedlings. Whether these feedback loops are controlled by dimers or higher order protein complexes containing the target gene remains to be solved. In a similar way, the B type genes \( PISTILLATA (PI) \) and \( APETALA3 (AP3) \) from Arabidopsis and \( DEFICIENS (DEF) \) and \( GLOBOSA (GLO) \) from Antirrhinum are autoregulated by complexes containing the AP3-PI or DEF-GLO heterodimers respectively (Schwarz-Sommer et al., 1992; Tröbner et al., 1992; Goto and Meyerowitz, 1994; Zachgo et al., 1995; Krizek and Meyerowitz, 1996b; Samach et al., 1997). The expression of two other MADS box genes, \( AGL15 \) and \( AG \) in two separate studies have been recently shown to respond to the accumulation of their own product (Zhu and Perry, 2005; Gómez-Mena et al., 2005). Although direct evidence is at present still limited, there are indeed several indications that MADS box genes are regulated by members of the same family and that for some of them a mechanism of autoregulation may be responsible for the maintenance of their own expression, (deFolter et al., 2005).

There are however some discrepancies between the double overexpressor phenotype and a \( ter \) knock-out mutant. Like \( wus \) mutants, \( ter \) plants are not completely arrested in
development, they form adventitious meristems which terminate prematurely, leading to the characteristic bushy phenotype (Laux et al., 1996; Stuurman et al., 2002). Therefore, it has been postulated that other factors may act redundantly with WUS to promote stem cell activity (Fletcher, 2002). Furthermore, a recent work in Arabidopsis revealed that WUS may not be necessary for stem cell maintenance in the shoot apex (Green et al., 2005). Therefore, we hypothesize that in our double overexpressor plants the high concentration of the transgenic proteins may interfere also with the pathway that acts in parallel with WUS/TER, leading to a complete arrest of any meristematic activity.

A role of the D-type genes FBP7/11 in determinacy is in contrast to the FBP7/11 cosuppression phenotype, because a knock-down of these genes did not cause indeterminacy in the flower (Angenent et al., 1995). However, in this study we demonstrated that when FBP11 is activated in floral tissues where normally only FBP2 and FBP6 are present, a premature TER downregulation occurs. In fact, the ectopic expression of FBP11 alone caused TER repression in the floral meristem and stomium area of the transgenic stamens only at a stage when both FBP2 and FBP6 were highly active. The lack of indeterminacy in FBP7/11 cosuppression plants could possibly be explained by the presence of another MADS box gene that acts redundantly with FBP7/11 in TER downregulation or by a residual amount of FBP7/FBP11 protein in the cosuppression plants not detectable by northern blot analysis but still sufficient to cause TER repression.

Although overexpression phenotypes should be considered cautiously due to the high level of transgenic protein produced, the phenotypes generated in the double overexpressors are consistent with the results obtained in the single overexpressors and supported by the expression data of several endogenous genes in wild type and transgenic plants.

In conclusion, our study with simultaneous activation of different MADS box genes in petunia and consequent repression of the meristem gene TER, suggests the requirement of C-,
D- and E-type genes in floral meristem determination, most likely through the formation of an active multimeric complex involving SEP-, AG- and SEEDSTICK (STK)-like proteins. A similar regulation of floral determinacy might occur in Arabidopsis as well, where genetic and molecular interaction among SEP, AG and STK were also documented (Favaro et al., 2003).

ACKNOWLEDGEMENT

This work was supported by a ‘VENI’ grant from The Dutch Organisation for Research (NWO) to R.G.H.I.

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**Figure Legends**

**Fig. 1.** In situ localization of petunia gene expression in wild type flower buds. A and B, *FBP2* expression in young (A) and older (B) flower buds; C and D, *FBP6* expression in flower buds at the same stage as in A and B; E and F, *TER* expression is detected in a limited number of cells in the meristematic tissue of young flower buds (E). At a later stage *TER* expression appears in the stomium area of the stamens (arrows in F). G and H, *FBP11* is only expressed in flower buds at a later stage, in the placenta tissue inside the developing pistil; Sections C, E and F were viewed using bright-field microscopy, the other sections were viewed using dark-field microscopy. B: bract; C: carpel; P: petal; PP: petal primordium; S: sepal; SP: sepal primordium; St: stamen. Bars = 500μm
**Fig. 2.** Wild type and 35S::FBP2/FBP11 double overexpression seedlings. A, progeny of a cross between 35S::FBP2 and 35S::FBP11 transgenic plants, 6 days after germination; the double over expression seedlings are indicated by the arrows; B and D, wild type seedlings 6 and 13 days after germination, respectively; C, E and F double overexpression seedlings 6 and 13 days after germination.; pictures D and E were taken with the same magnification, whereas F is a magnification of E; cleared shoot apical meristems (SAM) of 35S::FBP2/FBP11 (G) and wild type (H) seedlings; pictures were taken with the same magnification. The dome of the SAM is indicated with a broken line; I and K, in situ hybridization of wild type seedlings using TER and PhSTM as a probe, respectively; the same probes were used to hybridize 35S::FBP2/FBP11 double overexpression seedlings in sections J and L, respectively. LP: leaf primordium; C: cotyledon. Bars = 200µm

**Fig. 3.** RT-PCR amplification products of different genes in wild type and transgenic tissues. 
(A) Upregulation of the C-type gene FBP6 but not of pMADS3 in 35S::FBP2/FBP11 transgenic seedlings (TS).
(B) Upregulation of the D-type genes FBP7 and FBP11 in 35S::FBP2/FBP11 transgenic seedlings (TS).
(C) PhGADPH RT-PCR products in wild type and transgenic tissues.
Pi, pistil; TS, 35S::FBP2/ FBP11 transgenic seedling; Sdl, wild type seedling; M, 1 Kb DNA size marker.

**Fig. 4.** FBP7::GUS expression in wild type and transgenic tissues.
(A) Cross section through an FBP7::GUS ovary showing GUS staining in the ovules.
(B) GUS staining of a 35S::FBP2/FBP11, FBP7::GUS transgenic (left) and wild type seedling (right).
(C) GUS staining of 35S::FBP2/FBP11 seedlings segregating for the FBP7::GUS locus.

**Fig. 5.** In situ hybridization of 35S::FBP11 (A to C) wild type (D) and flower buds. Sections A to C were hybridized with a TER specific probe. An FBP6 probe was used for section D. B: bract; Cp: carpel; FM, floral meristem; IM, inflorescence meristem; P: petal; PP: petal primordium; S: sepal; St: stamen. Bars = 200µm in (A) and (D) and 500µm in (B) and (C).

**Fig. 6.** RT-PCR amplification products of different genes in wild type and 35S::FBP11 transgenic tissues.

(A) Upregulation of FBP2 in 35S::FBP11 transgenic leaves.

(B) Upregulation of FBP7 in 35S::FBP11 transgenic leaves.

(C) and (D) FBP6 and pMADS3 transcripts were not detected by RT-PCR in 35S::FBP11 transgenic leaves.

(E) *PhGADPH* RT-PCR products in wild type and transgenic tissues.

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**Table 1.** Higher order complex formation as determined by a yeast GAL4 three-hybrid assay.
Selection for complex formation was performed at room temperature (RT). + + indicates strong interaction, + weak interaction and – indicates no growth of the yeast cells on the selective medium. Because of the transcriptional activation activity possessed by the C terminus of FBP2 in yeast, a truncated version of the protein (FBP2ΔC) lacking 59 aminoacids, was used, as described in Ferrario et al. (2003). H, histidine; L, leucine; T, threonine, 3AT, 3-amino-1,2,4-triazole
Fig. 3

(A) M Pi TS Sdl Pi TS Sdl

PMADS3 FBP6

(B) M Pi TS Sdl Pi TS Sdl

FBP7 FBP11

(C) M Pi TS Sdl

PhGADPH