Characterisation of plant MADS box transcription factor protein-protein interactions

Use of *Petunia hybrida* as a model system

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Contents

Chapter 1

Scope

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The MADS box family of proteins is one of the largest families of plant transcription factors. Members of this family are functional as molecular switches of important developmental regulatory pathways. The first members isolated from this family were all involved in floral development and based on their characterisation the well-known "ABC-model" of floral organ development was established. In the years after, many scientists realised the importance of these regulatory genes and started to characterise MADS box transcription factors from different species, which led to the identification of MADS box proteins with functions in the vegetative phase and an extension of the model for floral organ development to the "ABCDE-model" (Figure 1).

MADS box transcription factors are functional as dimers, either as homodimers or as heterodimers. For a few plant MADS box proteins dimerisation has been demonstrated and this dimerisation appeared to be essential for DNA binding. Recently, even higher order complexes of MADS box proteins were identified, which shows that they act in a combinatorial manner to establish their function.

The goal of the study presented here was to gain more insight in the molecular mechanisms underlying MADS box transcription factor functioning. As a model the MADS box family of the species *Petunia hybrida* has been studied. Genes belonging to this family are named *FLORAL BINDING PROTEINs* (*FBPs*) or petunia MADS (pMADS). Classical approaches to obtain information about gene functions are overexpression studies and gene knockouts. In the most optimal situation, overexpression of a specific gene results in a gain of function phenotype, which is complementary to the phenotype obtained by loss of function experiments. In such a case overexpression mutants shed light upon the function of the gene in a wild-type background. However, in overexpression plants the protein encoded by the gene is present in a different environment and therefore may lack its natural partner or other cofactors. Alternatively, the ectopically expressed protein interacts with another factor and affects its function, resulting in a dominant-negative phenotype that is not directly related to the natural function of the protein of interest (Chapter 4). On the other hand, gene knockouts often lack clear phenotypic alterations, either because the mutant phenotype becomes apparent under specific conditions or due to the presence of functional redundancy. Analysis of the genome sequence of *Arabidopsis* revealed that redundancy is common for members of the MADS box transcription factor family. In case of functional redundancy more than one gene represents a genetic function. Suppression of multiple genes can be achieved by cosuppression and this technology has been used to elucidate the function of one of the petunia MADS box transcription factors (Chapter 3). Another possibility is to generate dominant-

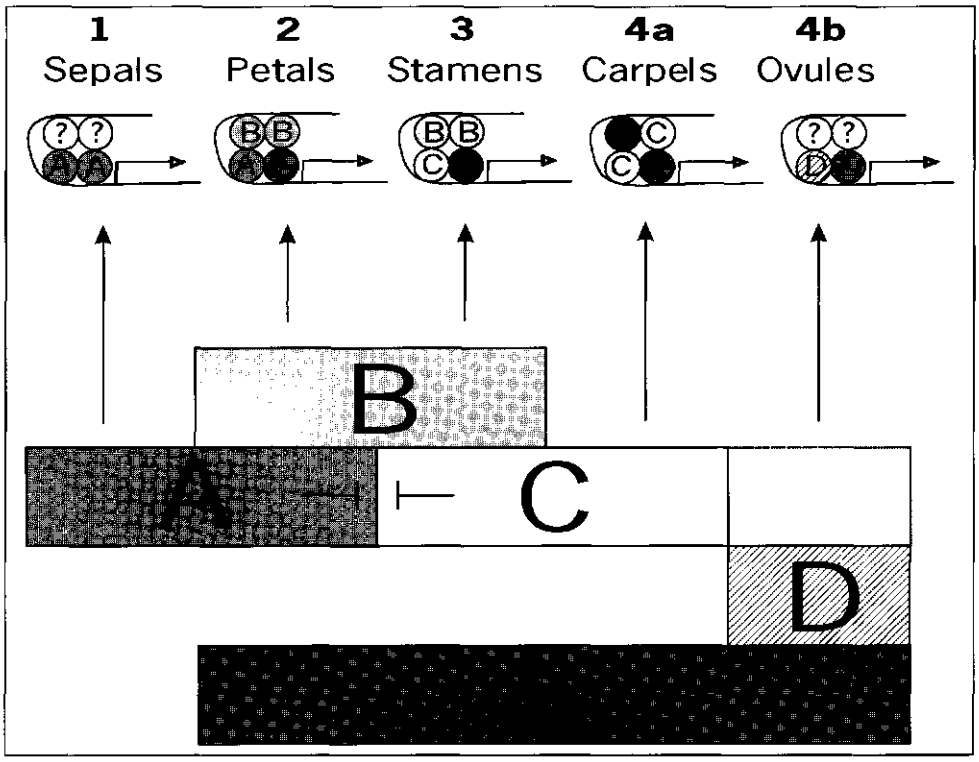

Figure 1: The "ABCDE-model" for floral organ development. Except for the A-type gene *APETALA2* from *Arabidopsis* all identified ABCDE genes belong to the MADS box transcription factor family. The proposed ternary and quaternary complexes that determine the specific organ identities are schematically drawn.

negative mutations by overexpression of a truncated or mutated protein that is able to interfere with the functioning of the endogenous protein. Also in the case of dominant-negative mutations redundancy can be circumvented because all functional homologues will be affected. In Chapter 5 of this study several strategies are described which were followed to obtain dominant-negative mutations for plant MADS box transcription factors.

As mentioned before, for some plant MADS box transcription factors specific homo- and heterodimerisation has been shown and this dimerisation appeared to be essential for their activity. Therefore, the characterisation of MADS box protein-protein interactions can be an alternative method to obtain insight in MADS box transcription factor functioning. Yeast two-hybrid systems have proven to be robust methods to determine protein-protein interactions in a high-throughput manner. In Chapter 2 the results from yeast two-hybrid analyses for 23 members of the petunia MADS box family are presented. Based on the obtained data functions could be predicted for some petunia and *Arabidopsis* MADS box

proteins and furthermore, knowledge has been gained about the presence of functional redundancy in the petunia MADS box transcription factor family. Although, yeast two-hybrid systems give first insight in interaction patterns, the results have to be interpreted with care, because these systems are artificial and sensitive to errors. Proteins, that never meet each other in a plant cell, are brought together in these systems and forced to interact by strong selection pressure. As a consequence, interactions identified by yeast systems need to be confirmed in a more natural environment and preferably *in planta*. Recently, two systems became available to identify protein-protein interactions in living plant cells. One system is based on reconstitution of a specific protein with a catalytic activity, upon a protein-protein interaction event. This system is called PCA (Protein Complementation Assay) and reliable results were obtained *in planta* making use of complementation of murine dihydrofolate reductase (mDHFR). The other system that has been reported is based on Fluorescent Resonance Energy Transfer (FRET), which is the process of energy transfer between two fluorescent dyes when they come in very close proximity. For this system two proteins of interest are fused with either Cyan Fluorescent Protein (CFP) or Yellow Fluorescent Protein (YFP) and expressed in plant cells. Upon a protein-protein interaction event CFP and YFP come close together resulting in FRET, which can be determined by Spectral Imaging Microscopy (SPIM) or Fluorescence Lifetime Imaging Microscopy (FLIM). The usefulness of this technology for the identification of MADS box transcription factor interactions is demonstrated in chapter 6.

In addition to functional analysis of protein families within a species, protein-protein interaction patterns can be used for comparative genomics analyses. Protein interactions appeared to be conserved even between non-related plant species. This hypothesis was tested by comparison of interaction maps obtained from different species and heterologous two-hybrid screenings as is demonstrated in chapter 7. These studies revealed that MADS box interaction patterns in *Arabidopsis*, *Antirrhinum* and petunia are very similar and *Arabidopsis* proteins interact with petunia proteins in a similar fashion as the homologous proteins do. Therefore, it was concluded that protein-protein interactions support a reliable comparison of gene functions and hence facilitate the transfer of genetic knowledge from model species to crop plants.

Chapter 2

Analysis of the petunia MADS box transcription factor family

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Abstract

Transcription factors are key regulators of plant development. One of the major groups of transcription factors is the MADS box family, which comprises at least 100 members in the *Arabidopsis* genome. In this study 23 members of the petunia MADS box transcription factor family were analysed by northern blot, phylogenetic and yeast two-hybrid analyses. Many of the characterised genes appeared to have one or more close relatives that share similar expression patterns. Comparison of protein-protein interaction patterns for the encoded proteins revealed that some have overlapping interaction patterns and hence are most likely paralogs, while others differ in interaction pattern, which demonstrates that they are not fully redundant. Furthermore, protein-protein interaction patterns in combination with expression patterns and phylogenetic classification appear to be good parameters for the identification of orthologs. Based on comparison of these data between petunia and *Arabidopsis*, functions could be predicted for a few MADS box transcription factors of both species.

Introduction

Transcription factors are abundantly present in genomes and many have important key regulatory functions in development. In *Arabidopsis thaliana*, over 5% of the annotated genes in the genome encodes for this type of proteins, which can be divided in at least 20 different families based on common conserved functional domains (Riechmann et al., 2000). One of the major classes of transcription factors is the MADS box family, from which members can be found in a diverse range of eukaryotic organisms, from yeast and plants to insects and mammals (Becker et al., 2000). The *Arabidopsis* MADS box family consists of at least 100 different members (The *Arabidopsis* Genome Initiative, 2000), which all share the N-terminally located MADS domain. The MADS domain is thought to be involved in DNA binding and dimerisation (Riechmann et al., 1996) and comparison of all known MADS domain sequences resulted in a subdivision of the family into two distinct classes. The type I lineage proteins are closer related to animal SRF-like MADS box proteins and the much better studied type II lineage proteins share homology with MEF2-like MADS box proteins (Alvarez-Buylla et al., 2000). The type II lineage proteins all have a second moderately conserved domain besides the MADS box, which is named K-box, after its homology to the coiled-coil structure of keratin (Ma et al., 1991). The K-box is found in plant MADS box proteins only and it is a key determinant for dimerisation specificity in conjunction with the

intervening region (I-region), which is located in-between the MADS and K-box (Davies et al., 1996 and Fan et al., 1997). The C-terminal part of plant MADS box proteins is the least conserved domain and little is known about its function. For the C-terminus of the *Arabidopsis* protein APETALA1 (AP1) and the SEPALLATA proteins (SEP1, SEP2 and SEP3) a transcriptional activation activity has been shown by transient assays (Goto et al., 2001) and furthermore, Egea-Cortines et al. (1999) demonstrated that the C-termini of the *Antirrhinum* proteins DEFICIENS (DEF), GLOBOSA (GLO) and SQUAMOSA (SQUA) are involved in ternary complex formation.

The first two MADS box genes isolated from plants were *AGAMOUS (AG)* from *Arabidopsis* (Yanofsky et al., 1990) and *DEF* from *Antirrhinum* (Sommer et al., 1990), both functional as homeotic selectors of floral organ identity. Subsequent detailed genetic and molecular analyses of floral development led to the establishment of the well-known ABC model (Coen and Meyerowitz, 1991), in which A function specifies sepals, A and B petals, B together with C stamens and the C-function alone carpels. Molecular analyses demonstrated that all three functions are represented by MADS box genes, except for *APETALA2* from *Arabidopsis* (Jofuku et al., 1994). In the years after, MADS box genes were isolated from numerous and distantly related species demonstrating a more complex regulation of floral organ identity. Analysis of the two very closely related *Petunia hybrida* MADS box genes *FLORAL BINDING PROTEIN7 (FBP7)* and *FBP11*, resulted in the addition of a D-function to the ABC model, controlling proper ovule formation (Colombo et al., 1995). Furthermore, isolation of A, B and C-type genes from other species revealed that small modifications of the ABC model exist for different species. In petunia for instance, the B-function is differently regulated. A mutation in *pMADS1,* which share similar sequence and expression pattern with *DEF* and *APETALA3*, causes homeotic conversion in whorl two only (Angenent et al., 1992; van der Krol et al., 1993; Tsuchimoto et al., 2000). Meanwhile, isolation and functional analyses of MADS box genes that based on expression pattern did not fit into the ABC model, revealed regulatory functions for MADS box genes throughout plant development. Some examples are fruit formation (Mandel and Yanofsky, 1995 and Flanagan et al., 1996), the control of root architecture (Zhang and Forde, 1998), the switch from the vegetative to the generative stage (Immink et al., 1999), seedpod dehiscence (Lijegren et al., 2000), abscission (Mao et al., 2000) and the regulation of flowering time (Michaels and Amasino, 1999, Sheldon et al., 1999, Samach et al., 2000, Hartmann et al., 2000 and Ratcliffe et al., 2001).

First insight in the molecular action of plant MADS box proteins came from in vitro DNA binding assays (Schwarz-Sommer et al., 1992), domain-swap

experiments (Krizek and Meyerowitz, 1996) and *in-vitro* immuno-precipitation experiments (Riechmann et al., 1996), which indicated that MADS box proteins are able to dimerise and that this dimerisation is essential for binding to specific target DNA sequences. Later, the yeast two-hybrid system was used to determine protein interaction patterns of plant MADS box proteins and specific homo- and heterodimerisation was demonstrated for a few MADS box proteins from different species (Davies et al., 1996; Fan et al., 1997; Davies et al., 1999; Moon et al., 1999a,b; Pelaz et al., 2001; Immink et al., 2002). Recently, Egea-Cortines et al. (1999) provided evidence that MADS box factors from *Antirrhinum* form higher-order complexes and they suggested an in vivo function for these ternary complexes. The final support for this hypothesis came from the work of Honma and Goto (2001), who proved that a ternary complex is indeed necessary for the action of MADS box genes in specifying flower organ identity. Based on the obtained results a new class of genes was introduced into the ABCD model, referred to as 'Identity mediating' (Im) or E function genes (Egea-Cortines and Davies, 2000; Theißen, 2001). These E function genes specify the identity of the inner three floral whorls in a combinatorial manner together with B and C class genes. Actually, the first evidence for this new class of genes, though, dates back to 1994, when the down-regulation of the *FBP2* and *TM5* genes caused very similar phenotypes in petunia and tomato plants, respectively, with homeotic conversions of whorls two, three and four simultaneously and loss of determinacy in the centre of the flower (Angenent et al., 1994; Pnueli et al., 1994).

The history of MADS box proteins in plants and the research flourishing around these factors, teach us that major breakthroughs in this field are the result of information collected from different species, and comparison between plants in search for analogies and differences. In this respect, our comprehensive study on the MADS box gene family in petunia that is presented here, should provide the information needed for a better understanding of plant development in this species as well as in others. For this purpose 23 MADS box genes of petunia all belonging to the type II lineage of MADS box transcription factors were investigated by phylogenetic, northern blot and two-hybrid analyses. Similarities and differences between the results obtained in this study and data available from other model species will be discussed.

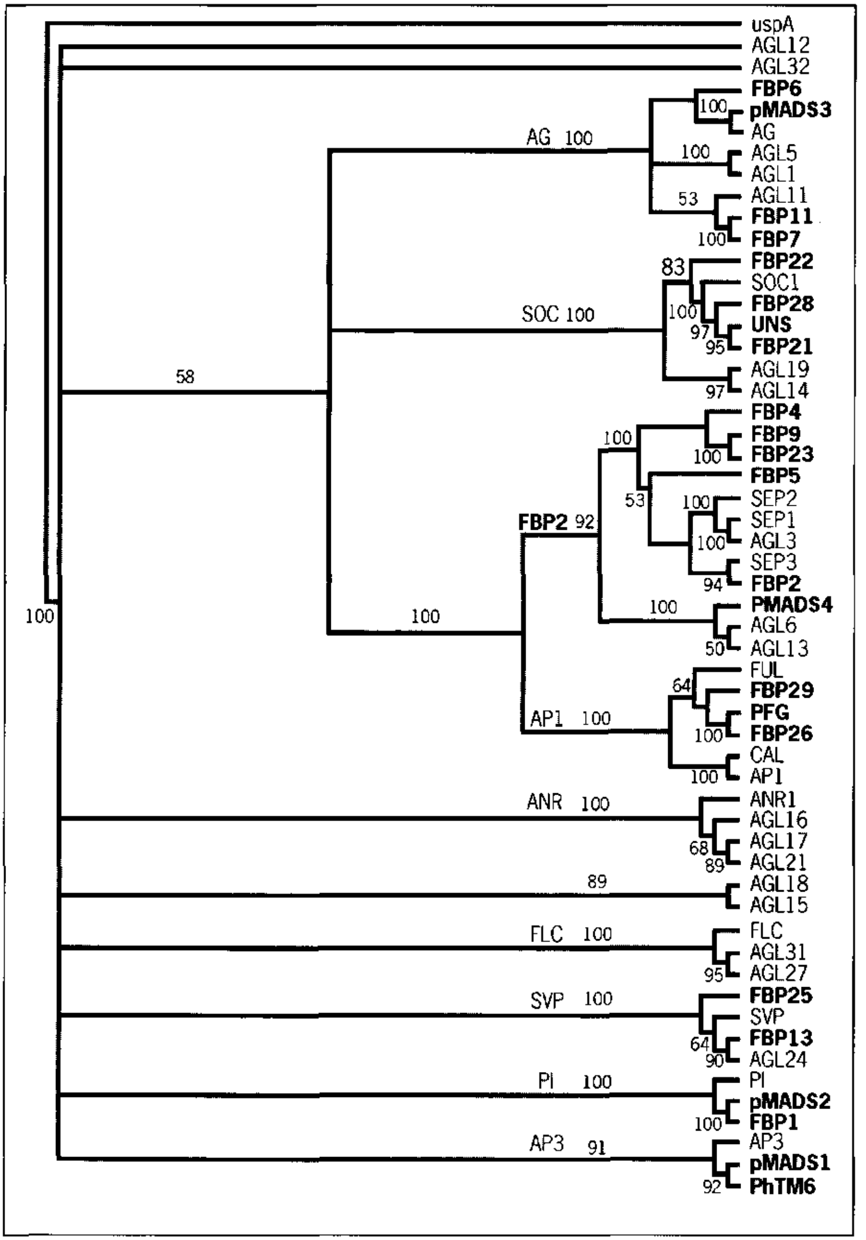

Figure 1: Dendrogram of petunia and *Arabidopsis* type II lineage MADS box proteins. The MADS box, I-region and K box were used for alignment and *E. coli* uspA was included as out-group. A bootstrap was carried out with 100 data sets, to assess the robustness of the branches. The numbers above each branch indicate the number of times each branch was found in 100 bootstrap replicas, branches with bootstrap values lower than 50 were collapsed. All petunia MADS box proteins are in bold. The representative gene for each clade is given in italic. The vertical lines next to the gene names illustrate putative paralogs.

Results

Isolation of new petunia MADS box genes

Full-length cDNA clones, corresponding to *FLORAL BINDING PROTEIN4 (FBP4), FBP13, FBP20 (UNSHAVEN,* Ferrario et al submitted*), FBP21, FBP22, FBP23, FBP26, FBP28* and *FBP29* petunia MADS box genes, were isolated by screening of a cDNA library derived from young inflorescences. This plant material included bracts, young leaves and floral buds. The screening was performed at low stringency using the MADS box regions of *FBP1* (Angenent et al., 1992), a petunia class B gene, and *FBP2* (Angenent et al., 1992), homologous to *Arabidopsis SEPALLATA3 (SEP3),* as a probe. Only petunia MADS box genes belonging to the type II lineage were isolated indicating that the hybridisation conditions used were not suitable to identify type I MADS box genes.

The dendrogram shown in figure 1 was generated using protein sequences that encompass the MADS box, the I region and the K box of all known petunia MADS box proteins and the *Arabidopsis* lineage II proteins (Alvarez-Buylla et al., 2000). The *E. coli* USPA MADS box protein was included as out-group. The organisation of the tree in clades is consistent with previous dendrograms (Alvarez-Buylla et al., 2000), while the presence of 23 petunia sequences strengthen the branches of most of the subfamilies in which they are present.

Expression analyses

Expression patterns of the petunia MADS box genes were determined by northern blot analyses using mRNA isolated from roots, seedlings, stems, leaves, bracts, sepals, petals, stamens, pistils, seeds and seedpods of wild-type petunia plants (line W115). The organs were dissected from mature flowers and the seeds and seedpods (without seeds) were isolated 10 days after pollination of the pistils. For some genes in situ hybridisation experiments were done to determine the expression pattern in ovules. The results of these experiments are shown in a schematic diagram in figure 2. The genes are displayed in the same sequence as they appear in the dendrogram, demonstrating that genes belonging to the same subfamily share not only sequence similarity but also have related expression patterns. This is most apparent for the B and C class homeotic genes, which have very specific and restricted expression domains, but it is also evident for genes belonging to the *SOC1* subfamily, that are mainly expressed in the vegetative tissues of the plant. Sequence similarities and comparable expression patterns are parameters often used to predict redundancy within a species and for the identification of functional homologues from different species. However, for large gene families such as the MADS box family more aspects have to be taken into account for a reliable

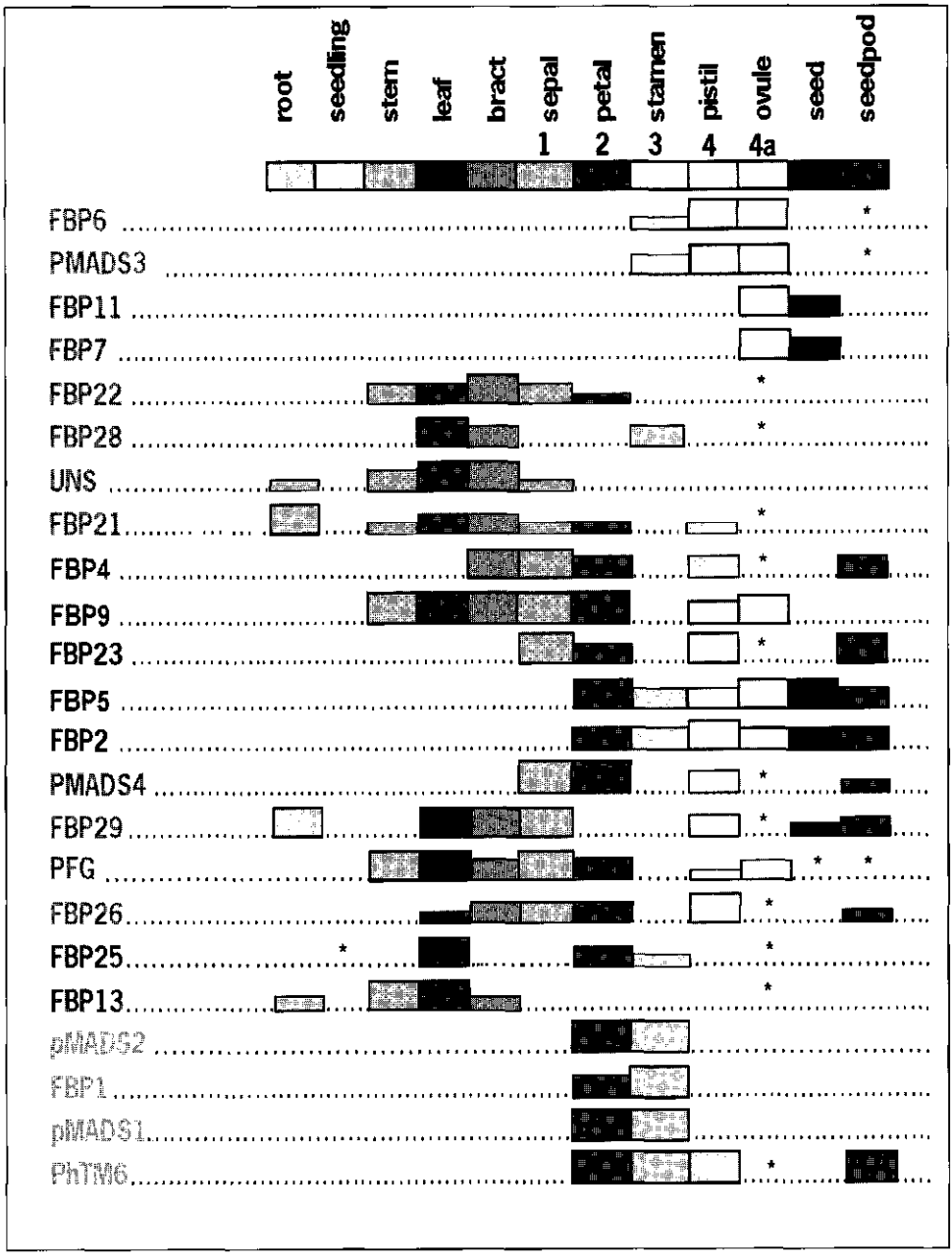

Figure 2: Schematic representation of petunia MADS box transcription factor expression patterns based on northern blot analyses. Relative expression levels in the various tissues are categorised in 3 different expression level groups for each gene. Genes are put in order according their phylogenetic grouping. For the *FBP25* blot polyA$^+$ RNA was used instead of total RNA. The ovule expression data were obtained by *in situ* hybridisation. * = not determined.

prediction of paralogues and orthologues. In the case of MADS box proteins, comparison of interaction patterns may provide additional clues about functional similarities among related proteins.

Determination of interaction patterns

To analyse the interaction patterns of petunia MADS box proteins, cDNA expression libraries were initially screened in the yeast two-hybrid GAL4 system (Fields and Song, 1989). FBP2 (Angenent et al., 1994), FBP4, FBP7 and FBP11 (Angenent et al., 1995), PETUNIA FLOWERING GENE (PFG, Immink et al., 1999) and UNS (Ferrario et al., submitted) were selected as bait proteins and tested for their ability to activate yeast reporter genes in the absence of the GAL4 activation domain. Both FBP2 and FBP4 appeared to give auto-activation of yeast reporter genes and hence could not be used as full-length bait proteins. The transcriptional activation domain of plant MADS box proteins is thought to be located in the C-terminal region of the protein (Riechmann and Meyerowitz, 1997; Cho et al., 1999) and therefore, this domain was deleted from both proteins, which appeared to be sufficient to abolish auto-activation. Subsequently, cDNA expression libraries derived from inflorescences and floral tissues were screened with the truncated FBP2 and FBP4 proteins and the other full-length baits without auto-activation activity. Except for the screening with the truncated FBP4 bait, which yielded no positives, all screenings resulted in the identification of a few MADS box proteins as heterodimerisation partner. It is noteworthy that truncated clones were isolated frequently, which often failed to interact as full-length proteins. Using PFG as bait, a truncated UNS protein lacking 77 Amino Acids at its N-terminus was isolated twice. In a subsequent direct screen for interaction between the full-length PFG and UNS proteins in the yeast two-hybrid GAL4 system, no activation of reporter genes was observed which demonstrated that the full-length proteins were not able to interact in yeast. In conclusion, library screenings in the yeast two-hybrid GAL4 system were labour intensive and less suitable for screenings with MADS box transcription factors due to auto-activation of reporter genes by intrinsic transcription activation domains. Moreover, library screenings resulted in the identification of false-positive interactions due to truncations of target proteins.

The recently developed Yeast two-hybrid CytoTrap system is a cytoplasmic system in which identification of protein-protein interactions is not based on transcriptional activation (Aronheim et al., 1997) and therefore a good alternative system to analyse MADS box transcription factor interactions. Twenty-three full-length petunia MADS box genes were cloned individually into the CytoTrap pSOSnes and pMYR vectors and transformed to the yeast *cdc25*H strains

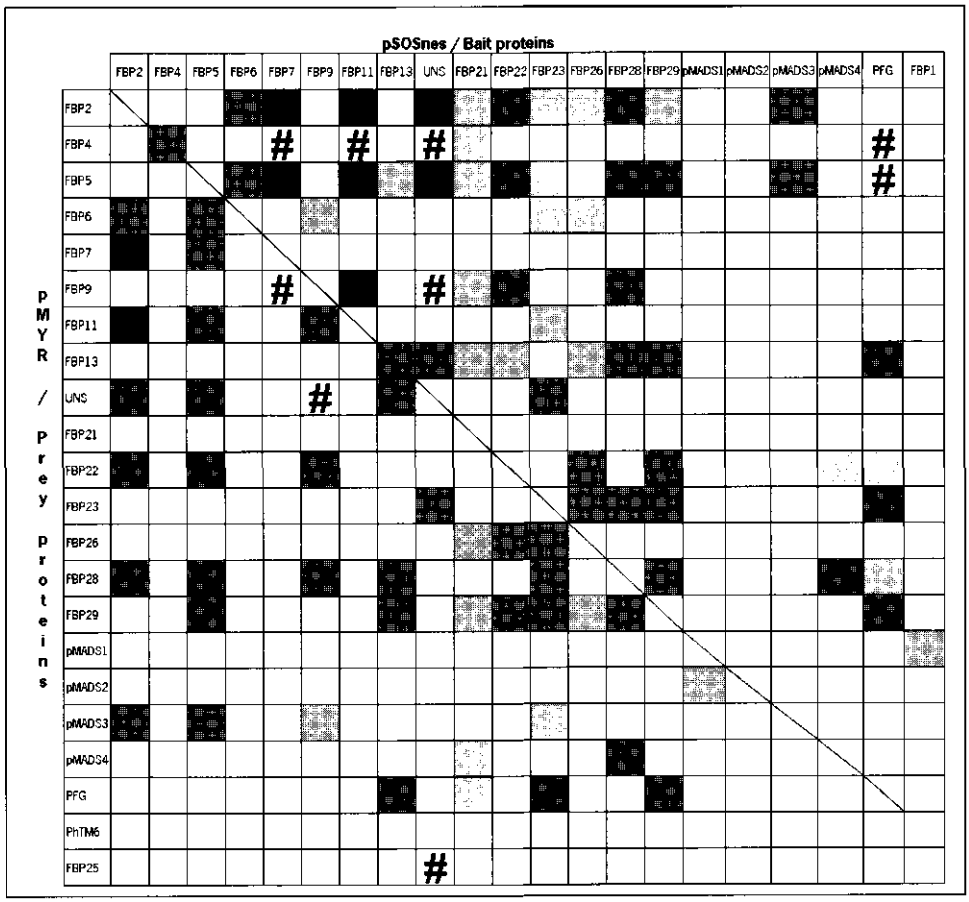

Figure 3: Petunia MADS box protein interactions identified by yeast two-hybrid systems. MADS box proteins expressed from pSOSnes are presented horizontally (bait proteins for GAL4) and MADS box proteins expressed from pMYR vertically (target proteins for GAL4).

Black: Analysed by both systems and positive

Dark Grey: Positive in CytoTrap™ two-hybrid system (reciprocal)

Light Grey: Positive in CytoTrap™ two-hybrid system (one direction)

#: Analysed by both systems but positive in just one of the two

FBP: FLORAL BINDING PROTEIN, PFG: PETUNIA FLOWERING GENE and pMADS: petunia MADS box protein. UNS: UNSHAVEN.

mating type a and alpha, respectively. All possible double combinations were generated by mating and tested for the ability to grow under selective circumstances. To obtain a reproducible result, the mating and screening were repeated three times with independent colonies obtained from the single transformations. This number of repetitions was necessary, because of the high percentage of revertants. The results of the two yeast two-hybrid analyses are summarised in figure 3. Three proteins were able to homodimerise in yeast and only PhTM6 failed to interact with any of the 23 MADS box proteins analysed. This latter MADS box protein aligns with the B-type proteins FBP1, pMADS1 and pMADS2, for which only one interaction partner for each protein has been identified. Probably PhTM6 has also just one interaction partner, which is not present in the collection of 23 analysed proteins. Surprisingly, both PhTM6 and FBP25 proteins resulted in auto-activation in the CytoTrap system, when expressed as SOSnes fusion proteins. Comparison of the two yeast two-hybrid systems reveals that the CytoTrap data mainly match those obtained by the GAL4 system. The few observed differences are indicated by specific colours in figure 3.

Discussion

In this study members of the MADS box transcription factor family of the solanaceous species *Petunia hybrida* have been studied. We estimate that the total number of type II MADS box genes in the genome of *Petunia hybrida* is roughly 60-70. This is based on the total number of type II MADS box genes present in the *Arabidopsis* genome (approximately 40; The *Arabidopsis* Genome initiative, 2000) and the many pairs of petunia MADS box genes, being most likely paralogs. The expression patterns of in total 23 members of this family were determined by northern blot analyses and the complete protein dimerisation scheme was obtained by yeast two-hybrid analyses. Comparison of these data with available information from species such as *Arabidopsis* enables a reliable prediction of paralogues and orthologues without further functional characterisation.

Correlation between expression and interaction patterns

Expression patterns give a first clue about possible functions of the corresponding genes. The analysed petunia MADS box genes are expressed throughout plant development in virtually all tissues and organs. Some genes appeared to have a very narrow expression range, such as the class B MADS box genes *FBP1*, *pMADS2* and *pMADS3* (Angenent et al., 1993), while others are expressed in almost all organs at different stages of plant development, like *PFG* and *FBP9*. Remarkably, it seems that MADS box proteins with overlapping expression

patterns are more preferred interaction partners than MADS box proteins that are not co-expressed. Recently, Ge et al (2001) compared the transcriptome and interactome mapping data from yeast, which corroborated this hypothesis that dimerisation capability is tightly linked with the co-expression of the proteins. Nevertheless, interactions were identified between MADS box proteins that based on northern blot analyses were not expressed in the same tissues, suggesting that they have no biological function. An example is the interaction between FBP5, which is expressed in the three inner whorls of the flower, and FBP13, which is expressed in vegetative tissues only. However, in spite of the lack of overlap in expression pattern as determined by northern blot analyses, it cannot be ruled out that the concerned genes are co-expressed in a very restricted area or cell type or under specific conditions. It is also possible that the proteins are not acting cell autonomously and are transported to adjacent tissues where they interact specifically with other proteins. Cell-to-cell trafficking has been shown for the *Antirrhinum* DEFICIENS and GLOBOSA MADS box proteins (Perbal et al., 1996).

Functional redundancy among plant MADS box proteins

MADS-box genes that are very similar in sequence and appear as pairs in the phylogenetic tree also share the same expression pattern suggesting that these genes be derived by recent duplications. Examples are FBP7/FBP11 (Angenent et al., 1995), FBP6/pMADS3 (Kater et al., 1998) and FBP2/FBP5 (Angenent et al., 1994; Ferrario and Angenent, unpublished results) and functional analyses of a number of these pairs indicate that they are indistinguishable. These duplicates are not caused by the hybrid nature of the modern *Petunia hybrida* varieties, because the separate genes already exists in the ancestors (Angenent et al., 1995; Kater et al., 1998), suggesting that they are derived from more ancient duplications. The existence of functional redundancy among plant MADS box proteins has also been demonstrated by mutational analyses of a few *Arabidopsis* MADS box genes (Reviewed by Smyth, 2000). In *Arabidopsis* approximately 50% of the MADS box genes appeared to have a close homologue (Riechmann et al., 2000), suggesting that functional redundancy is a common phenomenon among members of this class of transcription factors. Nevertheless, a high percentage of sequence similarity is not a guarantee for its occurrence. For instance, functional characterisation of the *Antirrhinum* MADS box genes *PLENA (PLE)* and *FARINELLI (FAR)*, two closely related MADS box genes, revealed that they are not mutually exclusive (Davies et al., 1999). In addition to small differences in expression levels a difference in protein-protein interaction pattern was found when comparing PLE and FAR. Because proteins with the same function should

have the same interaction partners, the identification of protein-protein interactions may provide a valuable additional tool to determine functional redundancy among MADS box proteins. Based on this parameter the petunia MADS box family was analysed. For this analysis only interactions found in both directions in the CytoTrap system and interactions which were confirmed by the yeast two-hybrid GAL4 system were taken into account. The yeast two-hybrid results show that FBP2 and FBP5, both of which are homologous to the *Arabidopsis* SEPALLATA3 protein (Pelaz et al., 2000 and Ferrario et al., in preparation) have overlapping interaction partners, which is in agreement with their supposed shared function. The same holds for the two highly homologous ovule specific MADS box proteins FBP7 and FBP11 (Angenent et al., 1995 and Ferrario et al, unpublished results), however in this case only for the yeast two-hybrid GAL4 results. The paralogs, identified based on phylogenetic analysis and expression pattern and interaction pattern comparison, are marked with a bar in figure 1.

Clear differences in interaction patterns on the other hand, may give clues about different functions for closely related MADS box genes. For example the two MADS box genes *PFG* and *FBP26*, with 73% identity at nucleotide level (84% at protein level), are both down-regulated in the *pfg* cosuppression mutant (Immink et al., 1999). Based on this observation it could be concluded that either both genes are redundant and down regulated by cosuppression or that *FBP26* is a target of *PFG*. The yeast two-hybrid results presented here, however, demonstrate that both proteins have different interaction partners. In combination with the differences observed in expression patterns, it demonstrates that *PFG* and *FBP26* are not fully functionally redundant.

Identification of functional homologues

Phylogenetic analyses, comparison of expression patterns and identified protein-protein interaction patterns allow a reliable prediction of orthologs of petunia MADS box genes in other species and vice versa without the analysis of mutants. In Table 1 an overview is given of petunia MADS box genes and their supposed *Arabidopsis* orthologs.

Mutational analyses performed in the past for the petunia gene *FBP1* (Angenent et al., 1993) and the *Arabidopsis* gene *PISTILLATA* (Bowman et al., 1989) e.g., demonstrate the reliability of the classification in Table 1. Based on this classification *AGL11* is the supposed *Arabidopsis* orthologue of the petunia D-type genes *FBP7* and *FBP11* (Angenent et al., 1995), although information about the function of *AGL11* is still pending. Similarly, *UNS* is classified as the putative petunia orthologue of the *Arabidopsis SOC1* gene (Samach et al., 2000; Ferrario et al., submitted). Functional classification of MADS box genes based on above-

mentioned criteria is however limited due to the lack of knowledge about MADS box protein-protein interactions in other species. Nevertheless, it is expected that yeast 2-hybrid and 3-hybrid analyses, which are in progress in large-scale functional genomics programmes, will yield this information for a number of species soon. In combination with the functional characterisation of these genes in *Arabidopsis* by loss- and gain-of-function mutants, this knowledge will provide a good starting point to predict the role of MADS box genes in other species, including petunia.

Petunia	*Arabidopsis*	Function
FBP1	*PISTILLATA*	B-type
pMADS2	*PISTILLATA*	B-type
FBP2	*SEPALLATA3*	E-type or IM-function
FBP5	*SEPALLATA3*	E-type or IM-function
FBP6	*AGAMOUS*	C-type
pMADS3	*AGAMOUS*	C-type
FBP7	*AGL11?*	D-type
FBP11	*AGL11?*	D-type
PFG	*?*	Flowering
UNS?	*SOC1*	Repression of flowering

Table1: Prediction of orthologs of MADS box genes based on phylogenetic analyses, expression patterns and interaction partners. B,C,D,E function according to Theißen (2001). IM = Identity Mediating.

Materials and methods

Screening of cDNA libraries and DNA sequence analysis

Full-length *FBP4, UNS, FBP21, FBP22, FBP23, FBP26, FBP28* and *FBP29* cDNA clones were isolated from a young-inflorescence-petunia-cDNA library, constructed in the Stratagene hybriZAP™ vector. *FBP5, FBP9* and *FBP13* were obtained from a pistil cDNA library in lambdaZAPII vector (Stratagene). Approximately 100,000 plaques from both libraries were screened with a mixed MADS box probe containing the 5' terminal sequence of *FBP1* (362bp) and *FBP2* (364bp) genes (Angenent et al., 1992). Hybridisation and washing of the Hybond N^+ membranes (Amersham) were done under low-stringency conditions (55°C hybridisation and wash with 2X SSC [1X SSC is 0.15M NaCl, 0.015M sodium citrate] at 55°C). The positive clones were isolated and purified, the pBluescript SK- and pAD-GAL4 phagemids were excised in vivo according to the Stratagene

protocol, and the inserts were sequenced (BigDye™ sequencing kit, Applied Biosystems).

Nucleotide and amino acid sequence comparisons were performed using the ClustalW multiple sequence alignment program (Thompson et al., 1994). Amino acid alignments including the MADS box, the I region and the K box, were used to obtain the dendrogram with the Protdist (using the Dayhoff PAM matrix, as distance matrix) and neighbour-joining (UPGMA method) programs of the PHYLIP 3.5c package (provided by Felsenstein J., Department of Genetics, University of Washington, Seattle, WA).

The accession numbers from the new petunia MADS box genes are as follows: *FLORAL BINDING PROTEIN4 (FBP4)*, AF335234; *FBP13* AF335237; *UNSHAVEN (UNS, FBP20)*, AF335238; *FBP21*, AF335239; *FBP22*, AF335240; *FBP23*, AF335241; *FBP25*, AF335243; *FBP28*, AF335244 and *FBP29*, AF335245.

RNA gel blot analysis

Total RNA was isolated from roots, seedlings, stems, leaves, bracts, sepals, petals, stamens, pistils, ovules, seeds and seedpods of wild-type W115 petunia plants according to Verwoerd et al. (1989). 10µg of each RNA sample, denaturated with 1.5M glyoxal, were fractionated on a 1.4% agarose gel and blotted onto Hybond N^+ membrane. For the *FBP25* blot $polyA^+$ RNA was isolated (Amersham columns) and further processed as described above. Gene specific fragments from all the genes tested, were used as probes for hybridisation. The probes were labelled by random oligonucleotide priming (Feinberg and Vogelstein, 1984) and blots were hybridised as described by Angenent et al. (1992).

Yeast two-hybrid GAL4 system

The open reading frames of *FBP2*, *FBP4*, *FBP7*, *FBP11*, *PFG* and *UNS* were generated by polymerase chain reaction (PCR) with gene specific primers (Isogen). Restriction enzyme sites (5' *EcoR* I and 3' *Sal* I sites) were introduced by the gene specific primers for in-frame cloning in the bait vector (pBD-GAL4). To obtain FBP4ΔC a second 3'-primer has been designed for this gene, annealing 87 nt upstream of the stop codon of the *FBP4* cDNA sequence and removing the C-terminal located amino acid residues. A new stop codon and a 3'-*Sal* I site were introduced as described above. All PCR products were subcloned in pGEM®-T-easy (Promega). *EcoR* I-*Sal* I MADS box gene fragments were obtained by digestion and subsequently ligated in the pBD-GAL4 bait vector. pBDGAL4-FBP2ΔC has been obtained by digestion of pBDGAL4-FBP2 with *Pst* I, which removes the DNA sequence from *FBP2* encoding for the last 59 C-terminal located

amino acid residues. All constructs were confirmed by sequencing (BigDye™ sequencing kit, Applied Biosystems).

Yeast strain PJ69-4A (James *et al.*, 1996) was used and transformed with the bait plasmids according the CLONTECH Yeast Protocols Handbook (Protocol # PT3024-1). Yeast colonies were checked for auto-activation of the yeast reporter genes, which was the case for both FBP2 and FBP4. Therefore, from these two baits C-terminal truncated versions were used for all further analyses. Subsequently, yeast cells containing the bait constructs were transformed with "inflorescence" (for FBP2ΔC, FBP4ΔC, PFG and FBP20) or "ovary" (for FBP2ΔC, FBP7 and FBP11) phagemid library DNA, according the CLONTECH protocol. At least 1 x 10^6 transformants were generated for the individual baits. Transformants were screened for activation of reporter genes and hence protein-protein interaction events according the manufacturers manual (Catalogue # 235601). Clones positive for all three yeast reporter genes were further analysed and plasmid DNA was isolated and transformed to *E. coli*. Finally, all positive clones were sequenced (BigDye™ sequencing kit, Applied Biosystems), to identify the cDNA-insert sequences.

Yeast two-hybrid CytoTrap system

The CytoTrap™ Vector Kit from Stratagene was used (Catalogue #217438). Instead of the enclosed pSOS vector, pSOSnes was used, containing a nuclear export signal (nes) fused in-frame between the SOS coding region and the Multiple Cloning Site (MCS). This vector and the yeast *cdc25*H mating type "a" strain were obtained from Stratagene US. The petunia MADS box cDNAs were cloned in the pMYR and pSOSnes vectors as described above for the yeast two-hybrid GAL4 system bait vector construction. For the in-frame cloning in pMYR *EcoR* I and *Sal* I restriction enzyme sites were used and for pSOS-NES, *BamH* I and *Sal* I restriction enzyme sites. All constructs were confirmed by sequencing (BigDye™ sequencing kit, Applied Biosystems).

All pMYR-petunia-MADS-box-gene constructs were transferred to yeast strain *cdc25*H mating type "a" and all pSOSnes-petunia-MADS-box-gene constructs to *cdc25*H mating type "alpha" according to the manual. For the mating 2 colonies of each single transformation were taken from fresh plates and inoculated in 0.5 ml of YPAD (Stratagene Manual, Catalogue #217438). Subsequently, 50 µl from the individual yeast "pMYR-MADS" suspensions and 50 µl from the yeast "pSOSnes-MADS" suspensions were combined in all possible combinations in 96-well plates, which were incubated at 25 °C (250 rpm). After approximately 16 hours, 10 µl from each combination was spotted on the selection plates and screened for protein-protein interaction events at 37°C as described by

the manufacturer. The mating has been repeated three times, using different individual colonies, to be able to score every combination for growth at 37°C without reversion of the yeast.

Chapter 3

A Petunia MADS box gene involved in the transition from vegetative to reproductive development

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Abstract

We have identified a novel petunia MADS box gene, *PETUNIA FLOWERING GENE (PFG)*, which is involved in the transition from vegetative to reproductive development. *PFG* is expressed in the entire plant except stamens, roots and seedlings. Highest expression levels of *PFG* are found in vegetative and inflorescence meristems. Inhibition of *PFG* expression in transgenic plants, using a cosuppression strategy, resulted in a unique non-flowering phenotype. Homozygous *pfg* cosuppression plants are blocked in the formation of inflorescences and maintain vegetative growth. In these mutants, the expression of both *PFG* and the MADS box gene *FLORAL BINDING PROTEIN26* (*FBP26*), the putative petunia homologue of *SQUAMOSA* from *Antirrhinum*, are down-regulated. In hemizygous *pfg* cosuppression plants initially a few flowers are formed, after which the meristem reverts to the vegetative phase. This reverted phenotype suggests that *PFG*, besides being required for floral transition, is also required to maintain the reproductive identity after this transition. The position of *PFG* in the hierarchy of genes controlling floral meristem development was investigated using a double mutant of the floral meristem identity mutant *aberrant leaf and flower* (*alf*) and the *pfg* cosuppression mutant. This analysis revealed that the *pfg* cosuppression phenotype is epistatic to the *alf* mutant phenotype, indicating that *PFG* acts early in the transition to flowering. These results suggest that the petunia MADS box gene, *PFG*, functions as an inflorescence meristem identity gene required for the transition of the vegetative shoot apex to the reproductive phase and the maintenance of reproductive identity.

Introduction

In flowering plants, the transition of the vegetative to the reproductive phase of growth is a critical developmental process, which is marked by a number of changes in the shoot apex at the molecular, physiological and morphological levels. Under the appropriate inductive environmental conditions and the ability to respond to these external factors, the vegetative meristem is transformed into a reproductive meristem, which either directly terminates into a flower or remains meristematic ('inflorescence meristem') and produces multiple flowers.

Genetic analysis in *Arabidopsis* has revealed at least 20 flowering mutants, which are affected in the transition to reproductive growth (reviewed in Levy & Dean, 1998). These mutants can be divided into two groups, the early and late

flowering mutants, which are either disturbed in sensing environmental signals or in the control of developmental processes. For example, mutations in the late flowering gene *CONSTANS (CO)* resulted in a late flowering phenotype under long day conditions, but not in short days, indicating that this gene is functioning under daylength control (Putterill et al., 1995). Another example of a gene involved in the timing of flowering is the gene encoded by *FLOWERING LOCUS C* (*FLC*-gene) (Michaels and Amasino, 1999), which appeared to be identical to the recently cloned *FLOWERING LOCUS F* (*FLF*-gene) (Sheldon et al., 1999). This novel MADS box gene acts as a repressor of flowering and its expression can be suppressed by both vernalisation and a decrease in genomic DNA methylation. Overexpression of this gene causes a significant delay in flowering. Two other MADS box genes with a supposed role in the transition from vegetative to reproductive growth are *SaMADS A* and *SaMADS B* from the long-day plant *Sinapis alba* (Menzel et al., 1996). Transcription of both genes is drastically increased in short-day grown plants shortly after the induction of flowering by a single long-day treatment. The only real non-flowering mutant described to date is the recessive pea *veg* mutant, obtained by X-irradiation of the late flowering, quantitative long-day cv. 'Dippes gelbe Viktoria' (Gottschalk, 1979). In the *veg* mutant lateral branches with shortened internodes are produced in stead of inflorescences. To date, no non-flowering mutants from *Arabidopsis* have been identified, suggesting that the autonomous floral induction pathway contains a high level of redundancy. Based on the characterisation of late- and early-flowering mutants, a model for the control of flowering time has been proposed by Martinez-Zapater, Coupland, Dean & Koornneef (1994). The hypothesis of this model is that flowering is a default stage, which is negatively controlled by a floral repressor. The late- and early-flowering genes that have been identified directly or indirectly affect this floral repressor.

The recently identified maize *INDETERMINATE1 (ID1)* gene, is the first example of a monocot gene, that is involved in the production or transmission of a flowering signal, and which is not a photoreceptor (Colasanti et al., 1998). *ID1* is expressed in young leaves, whereas it has its function in the shoot apical meristem, indicating that it is functioning in a non-cell-autonomous manner. In the *id1* mutant, the vegetative growth period is extended and eventually, aberrant inflorescences are produced with some vegetative characteristics. Thus, *ID1* seems to be involved in both the induction of flowering and the maintenance of the reproductive state.

Once the transition from the vegetative to the reproductive phase is initiated in plants, inflorescence structures are formed which are polymorphic, depending upon the timing and location of floral morphogenesis. A determinate

inflorescence meristem is characterised by the complete transformation of the meristem into a flower. An indeterminate meristem retains its inflorescence identity and has the potential to produce floral meristems throughout floral development (Weberling, 1989). *Arabidopsis*, *Antirrhinum*, and petunia all produce indeterminate inflorescences, whereas other species such as tulip produce a single terminal flower. The fate of the inflorescence meristem is controlled by two types of antagonistically acting genes. Genes such as *TERMINAL FLOWER* (*TFL*) (Shannon and Meeks-Wagner, 1991) and *CENTRORADIALIS* (*CEN*) (Bradley et al., 1996) in *Arabidopsis* and *Antirrhinum*, respectively, are essential to maintain the meristematic activity of the inflorescence meristem. In contrast, floral meristem identity genes such as *FLORICAULA* (*FLO*, Coen et al., 1990) and *LEAFY* (*LFY*, Weigel et al., 1992) promote the formation of flowers. The *FLO* gene from *Antirrhinum* was the first floral meristem identity gene cloned and its corresponding mutant is characterised by the homeotic transformation of floral meristems into inflorescences. Similar phenotypes were observed for mutants of the *LFY* gene from *Arabidopsis* and the *ABERRANT LEAF AND FLOWER* (*ALF*, Souer et al., 1998) gene from petunia. Other floral meristem identity genes are members of the extensive group of MADS box genes, several of which have a homeotic function. MADS box genes that play a role in establishing floral identity are *SQUAMOSA* (*SQUA*, Huyser et al., 1992) from *Antirrhinum* and *APETALA1* (*AP1*, Mandel et al., 1992), *CAULIFLOWER* (*CAL*, Kempin et al., 1995) and *AGAMOUS-LIKE8* (*AGL8*, Mandel and Yanofsky, 1995) from *Arabidopsis*.
This article reports the functional analysis of a novel petunia MADS box gene, designated *PETUNIA FLOWERING GENE* (*PFG*). Petunia plants, in which *PFG* was down-regulated, exhibit a unique non-flowering phenotype. The Shoot Apical Meristem (SAM) of this mutant has morphological characteristics of the vegetative phase. The phenotype of the mutant and the expression pattern of *PFG* strongly suggest that *PFG* is involved in the transition from the vegetative to reproductive phase of growth and in the maintenance of inflorescence meristem identity.

Results

Isolation and sequence of PETUNIA FLOWERING GENE (PFG) cDNA

To isolate MADS box cDNA clones, a lambda ZAP (Stratagene) cDNA library made from young petunia pistils (Angenent et al., 1995) was screened under low stringency hybridisation conditions using a mixed probe consisting of the MADS-box regions of *FLORAL BINDING PROTEIN* genes *FBP1* and *FBP2* (Angenent et al., 1992; 1995). Ten different clones containing a MADS box were identified, and analysed by cross-hybridisation. Clone *FBP10*, designated as *PETUNIA*

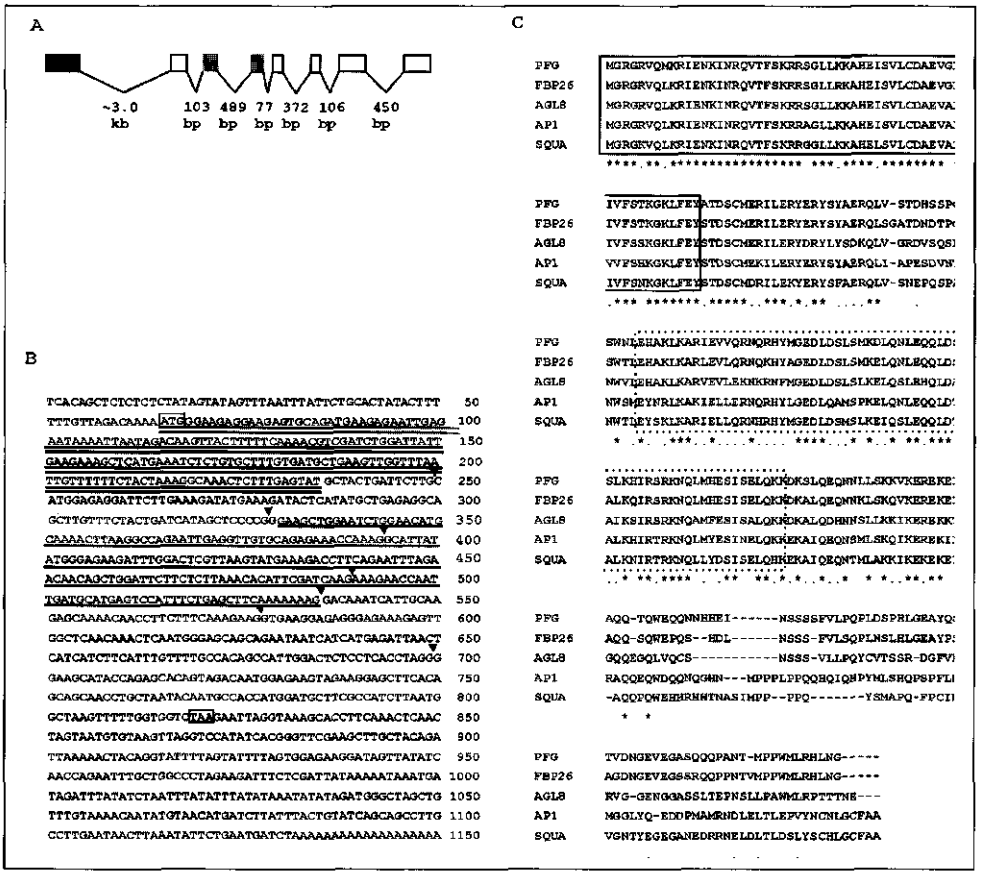

Figure 1: Characterisation of the *PFG* gene structure, cDNA sequence, and comparison of deduced amino acid sequence to PFG-related proteins. (A) Intron/exon structure of the *PFG* coding region. Boxes represent exons and thin lines show intron position with size indicated below. The black box represent the MADS box coding region and the 2 Gary boxes the K-box coding region. (B) cDNA sequence of *PFG*. The start and stop codons are outlined. The MADS box and K-box are double and single underlined, respectively. The positions of the introns are indicated by a closed triangle. (C) Comparison of the PFG amino acid sequence to that of petunia FBP26, *Arabidopsis* AGL8 (Mandel and Yanofsky, 1995) and AP1 (Mandel et al., 1992), and *Antirrhinum* SQUA (Huijser er al., 1992). The MADS box and K-box domains are outlined by a solid and broken line, respectively. Identical amino acid residues in all sequences are indicated by an asterisk. A dot means that all sequences have similar amino acids at that position.

FLOWERING GENE (PFG), was sequenced and appeared to be full length (Fig. 1B). PFG contains a N-terminally located MADS box domain of 56 amino acids and an amphipathic α-helical structure (Fig. 1C) typical of plant MADS box proteins. This domain is called the K box and is involved in establishing protein-protein interactions among MADS box proteins (Davies and Schwarz-Sommer, 1994; Davies et al., 1996; Fan et al., 1997).

The genomic structure of *PFG* was determined by amplification of genomic DNA fragments using primers specific to the cDNA sequence. The *PFG* gene consists of 8 exons (Fig. 1A,B), separated from each other by short introns except for a large intron of about 3 kb, located just downstream of the MADS box domain. This structure is conserved among different members of the MADS box gene families from various species (Ma et al., 1991 and Huijser et al., 1992).

A comparison of the amino acid sequence of PFG and related sequences of the *Arabidopsis* proteins AGAMOUS LIKE8 (AGL8, Mandel and Yanofsky, 1995) and APETALA1 (AP1, Mandel et al., 1992), the *Antirrhinum* protein SQUAMOSA (SQUA, Huijser et al., 1992) and the petunia protein FLORAL BINDING PROTEIN26 (FBP26), is presented in Fig. 1C. *FBP26* cDNA was isolated from an inflorescence cDNA library, using the *Antirrhinum SQUA* cDNA as probe for the hybridisation. DNA sequence comparison revealed that *FBP26* most likely belongs to the *AP1/AGL8/SQUA* group of MADS box genes, involved in specification and maintenance of floral meristem identity (Rounsley et al., 1995). Overall amino acid sequence identity matches with PFG are 64% for AGL8, 63% for AP1, 61% for SQUA and 84% for FBP26.

Expression of PFG in wild-type petunia

Northern blot hybridisation experiments were performed to examine the *PFG* expression pattern. RNA was isolated from various organs and hybridised with a 3'-terminal *PFG*-specific DNA probe. The RNA gel blot shown in Figure 2, reveals that *PFG* transcripts are present in leaves, stems, bracts, and all floral organs except stamens. No *PFG* expression was detected in roots and seedlings. To determine the expression pattern of *PFG* in the various meristems of the plant, *in situ* hybridisation experiments were done using a digoxygenin-labelled antisense RNA probe generated from a 3' *PFG*-specific cDNA fragment. The strongest hybridising signals were observed in the tunica and corpus layers of vegetative (Fig. 3A) and inflorescence (Fig. 3B) meristems. Lower levels of signal are also detectable in newly formed leaves and in vascular tissue including the procambium (Fig. 3A). *PFG* is strongly expressed in floral meristems (Fig. 3C) and its expression persists during early floral organ development. At late flower developmental stages, during pistil and ovule development *PFG* is expressed in

ovules and the ovary wall (Fig. 3D). No hybridising signal was detected using sense digoxygenin-labelled *PFG* RNA as a probe (Fig. 3E).

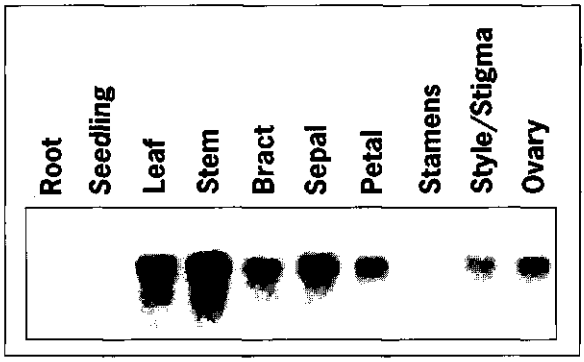

Figure 2: Expression of PFG in wild-type petunia (W115). Northern blot analysis of total RNA isolated from root, seedling, leaf, stem, bract, sepal, petal, stamen, style/stigma and the ovary. Each lane contains 10 µg of total RNA. Blots were probed with a ^{32}P labelled gene-specific *PFG* fragment.

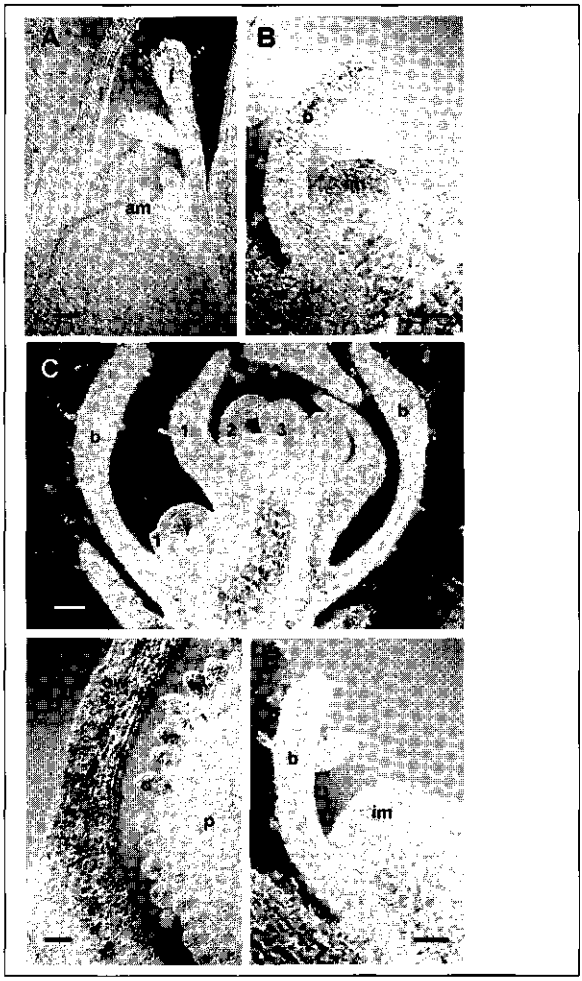

Figure 3*: Localisation of *PFG* mRNA in vegetative and inflorescence meristems, floral buds and carpels of wild-type petunia plants (W115). Longitudinal sections were hybridised with an antisense digoxygenin-labelled *PFG* RNA-probe (A) to (D) or to a sense digoxygenin-labelled *PFG* RNA-probe as a negative control (E). (A) Vegetative shoot apex with emerging leaves on its flanks. (B) and (E) Inflorescence meristem in the axil of a bract. (C) Longitudinal section through an inflorescence with a floral bud and floral meristem. Sepal primordia start to emerge from the floral meristem. The floral whorls are indicated: sepal (1), petal (2), and stamen(3). (D) Part of the bottom part of an ovary showing hybridisation signals in ovules and ovary walls. am, vegetative apical meristem; b, bract; ow, ovary wall; f, floral meristem; im, inflorescence meristem; l, leaf; o, ovule; p, placenta. Bars = 100 µm.

*) ***Figure in colour in appendix, page I.***

Inhibition of PFG expression results in plants affected in the phase switch form vegetative to generative development

To inhibit *PFG* expression for analysis of its function, sense cosuppression transgenic plants were produced. A binary vector containing the full length *PFG* cDNA in the sense orientation, downstream of the CaMV35S promoter, was introduced into *Petunia hybrida* line W115. Eighteen independent transgenic petunia plants were generated and examined for morphological alterations. Wild-type petunia plants form indeterminate inflorescences that give rise to the formation of two bracts on their flanks (Fig. 4A). These two bracts have an opposite arrangement compared to the spiral phyllotaxy of vegetative leaves. Once flowering is induced, the inflorescence meristem is maintained indefinitely and will not revert to the vegetative phase (Prior, 1957). Two primary transgenic plants (T30.09 and T30.12) were selected which showed aberrant inflorescence structures. These selections produced normal flowers but, occasionally, the inflorescence reverted to the vegetative phase and vegetative shoots arise (Fig. 4B). These two plants were self-pollinated and the progeny were analysed. The offspring population of T30.09 (24 plants) could be divided into three groups: plants with a wild-type phenotype (9 plants), plants with a phenotype similar to the primary transformants (10 plants), and five severely altered plants (T30.09S) in which the switch to generative development is not made at all. These plants continued their vegetative growth independently. Occasionally, a single terminal, normal flower was produced which might be explained by a low level of functional *PFG* transcript accumulation in some shoot tips temporally establishing the inflorescence meristem identity. Normal inflorescences were never produced in these severely affected transgenic plants (Fig. 4C). Back crosses with wild type revealed that the latter class of severely affected plants are homozygous for the transgenes that cause the cosuppression phenotype. The progeny of primary transformant T30.12 contain wild-type plants and plants that phenocopied the primary transformant, indicating that the mutant phenotype is inherited but is not more pronounced. Two additional transgenic lines (T103.01 and T103.05) show a similar phenotype as transformants T30.12 and T30.09, with partial reversions to vegetative growth, however this phenotype was only observed in plants homozygous for the transgene. Thus, in total 4 independent lines were obtained having these aberrant inflorescences. The 14 other kanamycin resistant lines showed no phenotypical alterations, most likely due to an absence of cosuppression. All further analyses focused on T30.09 and T30.09S.

A detailed phenotypical analysis of the *pfg* cosuppression mutant T30.09S was performed by using Scanning Electron Microscopy (SEM). Comparison of apical shoot tips from the transformant and vegetative and flowering wild-type

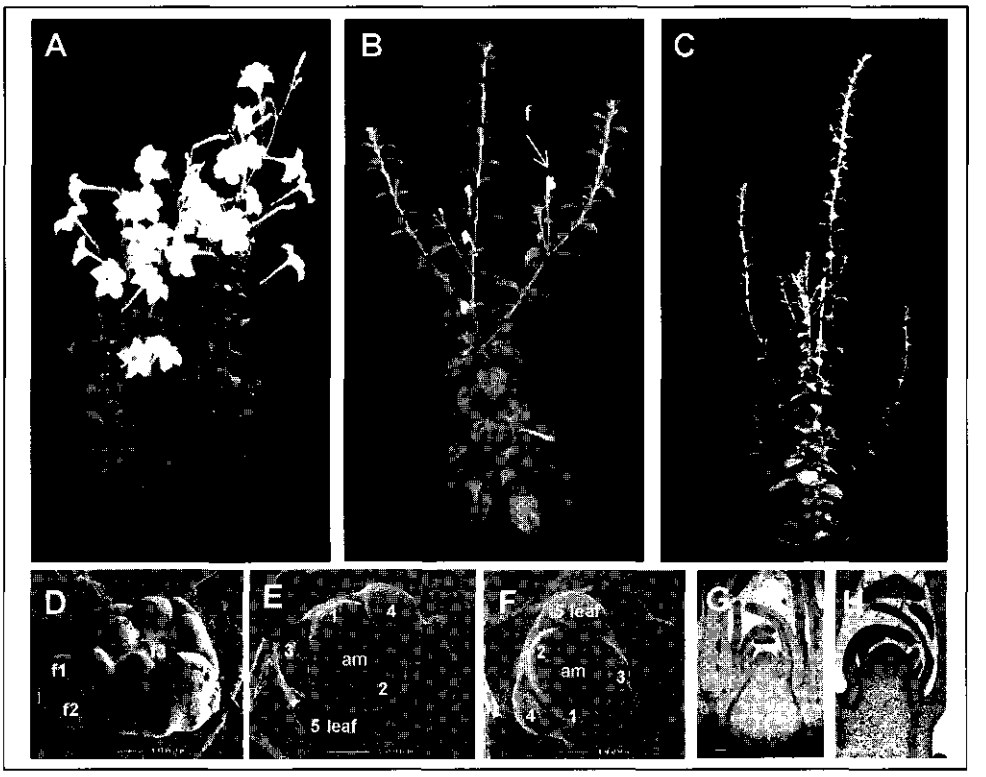

Figure 4*: Comparison of morphology and development of petunia wild-type plants (W115) and *pfg* cosuppression plants. (A) Petunia wild-type plant. After flower induction flowers are continuously produced from the apical inflorescence meristem. (B) Primary *pfg* cosuppression transformant in which the switch to generative development is initially made and some flowers (f) are formed, whereafter it reverts to vegetative growth. (C) Severe *pfg* cosuppression plant completely blocked in the switch from vegetative to generative development. (D) Scanning Electron Microscopy (SEM) image of a wild-type inflorescence. The inflorescence meristem (i) has generated three floral meristems (f1 to f3), from which the oldest one (f3) has initiated five stamen primordia (third whorl) and five petal primordia (second whorl). The sepals that enclose the flower partly at this stage were removed except for one. (E) SEM image of shoot apex from a vegetative wild type plant. Leaves are generated in a spiral phyllotaxy, characteristic for vegetative development. Leaves are numbered from 1 (youngest) to 5 (oldest) in order they are generated from the apical meristem (am). (F) SEM micrograph of shoot apex from an elongated shoot of a severe *pfg* cosuppression plant at a stage, when wild-type plants are already flowering. Leaves are numbered as described for (E). (G) Light micrograph of longitudinal section of a vegetative wild-type shoot apex. (H) Light micrograph of longitudinal section of an elongated shoot apex of a severe *pfg* cosuppression plant. Bars = 100 µm.

) Figure in colour in appendix, page II.

plants clearly shows that the apical meristem of the mutant has a vegetative character (Fig. 4D, E, F). The shoots produce small leaves arranged in a spiral phyllotaxy, in contrast to the opposite arrangement of bracts in an inflorescence meristem. In addition, longitudinal sections through apical meristems of a non-flowering wild-type petunia plant (Fig. 4G) and of T30.09S (Fig. 4H) were compared to each other. No clear differences in structure between these two longitudinal sections were observed. Thus, the formation of elongated vegetative shoots instead of indeterminate inflorescences, implies a block in the switch from the vegetative to the inflorescent stage.

Molecular analysis of pfg cosuppression plants

Northern blot hybridisation was performed on a segregating T2 population of plant T30.09 to confirm the linkage between the suppression of *PFG* expression and the aberrant phenotype (data not shown). Nine out of 24 offspring plants were indistinguishable from wild type plants and all have normal *PFG* mRNA levels. In plants with an intermediate phenotype similar to the primary transformant, very low transcript levels were detectable. No *PFG* transcripts were detected in leaves, bracts or in rarely formed flowers from the 5 non-flowering plants (T30.09S, Fig. 5), indicating that the inhibition of *PFG* expression is associated with the non-flowering phenotype. In the mild mutant plants of line T30.12, T103.01 and T103.05 *PFG* transcript levels are reduced substantially, but always detectable with northern hybridisation.

Expression of six other petunia MADS box genes and the floral meristem identity gene *ABERRANT LEAF AND FLOWER (ALF,* Souer et al., 1998) was examined in T30.09S to analyse if more MADS box genes were down-regulated and to gain more insight into the molecular mechanisms leading to the altered phenotype in the cosuppression mutant. The six MADS box genes were selected from 28 petunia MADS box genes known to date (Ferrario et al., unpublished results), based on their high level of sequence match with *PFG* on full-length cDNA level and in the MADS box respectively (*FBP4,* 50.5% (75.4%), *FBP20,* 48.6% (71.9%), *FBP21,* 48.9% (72.5%), *FBP22,* 46.0% (71.7%), *FBP23,* 51.3% (74.6%) and *FBP26,* 73.9% (88.0%)). The expression of none of these genes was down-regulated in the *pfg* mutant except for the putative petunia *SQUA* homologue *FBP26* (Fig. 5). *FBP26* is expressed in bracts, floral buds, sepals, petals and carpels of wildtype petunia plants. No expression of *FBP26* is found in wildtype seedlings, stems and leaves (not shown). In the *pfg* cosuppression plant T30.09S *FBP26* mRNA levels are dramatically reduced in bracts and flowers (Fig. 5). In contrast, expression of the floral meristem identity gene, *ALF,* was not affected by the *PFG* down-regulation (Fig. 5).

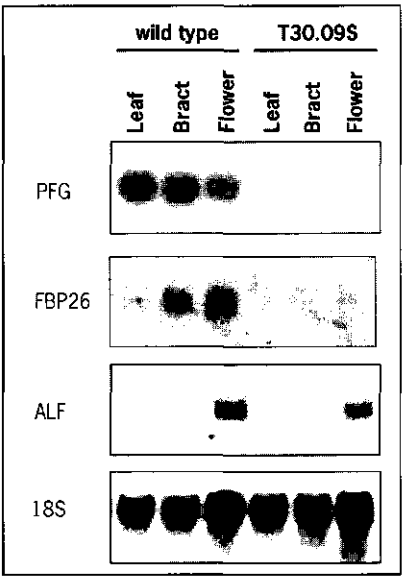

Figure 5: Comparison of expression of *PFG*, *FBP26* and *ALF* in wild-type petunia plants (W115) and *pfg* cosuppression plants. Northern blot analysis of total RNA isolated from leaves, bracts and flowers (rarely formed in T30.09S mutant). Each lane contains 10 µg of total RNA. Identical blots were probed with ^{32}P labelled gene specific fragments for *PFG*, *FBP26* and *ALF* (Souer et al., 1998). To show equal loading of RNA in each lane, blots were reprobed with 18S ribosomal DNA sequences. One of these control blots, representative for all, is shown.

Genetic interaction between PFG and ALF

To clarify the genetic interaction between *PFG* and the floral meristem identity gene *ALF*, double mutants between T30.09S and *alf* were made. In the *alf* single mutant the development of the inflorescence is altered. After bifurcation of the inflorescence meristem, both meristems continue to develop as indeterminate inflorescences generating bracts on their flanks and divide again to form new inflorescence meristems (Souer et al., 1998, Fig. 6A, E, F, G). Upon bifurcation of the wild type meristem, one meristem develops into a determinate flower, while the other meristem maintains its inflorescence identity and continues to develop as an indeterminate inflorescence (Fig. 6A, B, C, D). Double mutants were obtained by crossing a hemizygous *alf* transposon insertion mutant (*alf-S3018*) with the hemizygous *pfg* cosuppression mutant T30.09. F1 progeny plants with a weak *pfg* cosuppression phenotype and still containing the transposon insertion, were selected and self fertilised. Phenotypic and molecular analysis was performed on the F2 offspring plants. From forty F2 plants three had a wild-type phenotype, thirty-two had a weak or severe *pfg* cosuppression phenotype (Fig 6 H,I) and five plants showed characteristics of both single mutants (Fig 6 J,K). No plants with an *alf* mutant phenotype alone were identified, most likely due to the low number of plants analysed. The presence of the *alf* mutant allele was confirmed by using PCR amplification of an *ALF*-specific fragment containing the transposon insertion site. The *pfg* cosuppression trait was confirmed by northern blot analysis. These molecular analyses confirmed that the latter class of plants showing both single mutant features are *alf*⁻ mutants with reduced *PFG* mRNA levels. Based on the

residual *PFG* transcripts and the phenotype observed, these plants are most likely hemizygous for the *PFG* cosuppression transgene. In hemizygous *pfg* cosuppression mutants, the switch from vegetative to generative development is made initially, as demonstrated by the formation of a few flowers. Subsequently, the inflorescence meristem reverts to a vegetative developmental pathway and vegetative shoots arise (see Fig. 4B). In an *alf*⁻ background, these hemizygous *pfg* mutants produce inflorescence meristems instead of flowers, which after a few bifurcations eventually revert to vegetative shoots (Fig. 6J, K). At later developmental stages, the *pfg* cosuppression phenotype becomes apparent, as in the single *pfg* mutants, and the *alf* mutant phenotype is masked. Molecular analysis of the F2 plants showing a severe *pfg* mutant phenotype (Fig. 6H, I) revealed that three out of 32 were also homozygous for the *alf* insertion allele. Phenotypically these three plants are completely identical to homozygous *pfg* cosuppression mutants. Inflorescence meristems are not formed in these double mutants and consequently, the *alf* mutant phenotype does not become apparent.

Discussion

The onset of flowering is controlled by both endogenous and environmental signals. The cells of the shoot meristem become competent to receive flower-inducing stimuli, which are produced in the leaves and promote the transition from vegetative to reproductive growth. Despite the numerous physiological and genetic studies of the last decades, conclusive evidence for the existence of a genetic or hormonal factor acting as the floral stimulus, often referred to as "florigen", is still lacking. Recent genetic studies with *Arabidopsis* have revealed a number of genes required for the correct timing of flowering (for review see Levy and Dean, 1998), nevertheless, these genes are not essential for the floral transition itself. This important process in the life cycle of a flowering plant is still a mystery and the genetic factors involved remain elusive. In this report, we describe a novel petunia MADS box gene, *PETUNIA FLOWERING GENE* (*PFG*), which is involved in the floral transition. *PFG* is not a floral meristem identity gene because its action is earlier, before or at the time of flower induction. In addition, the mutant phenotype and the expression pattern of *PFG* suggest that it is also necessary to maintain the reproductive identity of the meristem after the phase switch. Down-regulation of *PFG* in transgenic petunia plants, using a sense cosuppression strategy, resulted in non-flowering plants. Molecular analysis of these transgenic plants revealed that both *PFG* and *FBP26*, the putative homologue of the floral meristem identity genes *SQUAMOSA* from *Antirrhinum* (*SQUA*, Huyser et al., 1992) and *APETALA1* (*AP1*), *FRUITFULL* (*FUL*) and *CAULIFLOWER* (*CAL*) from *Arabidopsis*

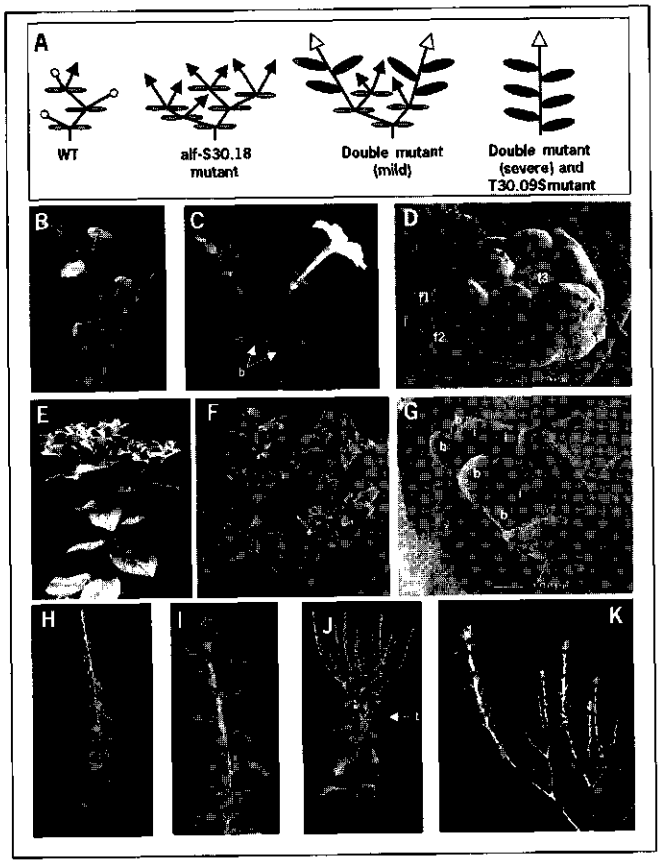

Figure 6*: Comparison of phenotypes and development of wild-type petunia plants (W115), *alf-S3018* mutant plants and *pfg alf* double mutant plants. (A) Schematic representation of inflorescence structures of a wild-type plant, *alf-S3018* mutant and mild and severe *pfg alf* double mutants. Bracts and leaves are indicated by small light green and big dark green ovals, respectively. Vegetative meristems and inflorescence meristems are shown by open and closed triangles, respectively. Open circles are flowers. (B) Wild-type petunia plant (W115). (C) Structure of a wild-type petunia inflorescence. An indeterminate inflorescence meristem and a flower meristem develop in the axils of two bracts (b) which are arranged in an opposite position. (D) Scanning Electron Microscopic (SEM) image of a wild-type inflorescence apex. The inflorescence has generated three floral meristems (f1 to f3), from which the oldest one (f3) has initiated five stamen primordia (third whorl) and five petal primordia (second whorl). The sepals that enclose the flower partly at this stage were removed except for one. (E) Side view of an *alf-S3018* inflorescence in a W115/W138 background. Bifurcation of the *alf* inflorescence meristem is similar to that in wild-type inflorescences. However, both meristems behave as inflorescence meristems and continue to develop bracts on their flanks and divide again to form new inflorescence meristems, finally giving rise to a highly branched structure. (F) Top view of an *alf-S3018* inflorescence. (G) SEM image of *alf-S3018* inflorescence. i, inflorescence meristem; b, bract. (H) Severe *pfg alf* double mutant, in which the switch from vegetative to generative development is abolished. This double mutant phenotype is indistinguishable from that of a *pfg* single mutant. (I) Elongated shoot of a severe *pfg alf* double mutant, with leaves arranged in a spiral phyllotaxy. (J) Mild *pfg alf* double mutant. The switch from vegetative to generative development (t) is initially made and the *alf* mutant phenotype becomes apparent. After a few bifurcations the inflorescence reverts to vegetative growth with spirally arranged leaves like in the *pfg* single mutant. (K) Detail of (J).

) Figure in colour in appendix, page III.

(Rounsley et al., 1995) are down-regulated. In addition to the analysis of the cosuppression mutants, the interaction between *PFG* and the floral meristem identity gene *ABERRANT LEAF AND FLOWER (ALF)* was studied in *pfg* cosuppression/*alf* double mutants, which provided information about the position of PFG in the flowering process.

The pfg cosuppression mutants have a unique non-flowering phenotype

Four cosuppression mutant lines were identified, which were affected in the phase change from vegetative to reproductive. The most severe mutant shows a non-flowering phenotype, marked by elongated vegetative shoots. Because vegetative petunia shoots are normally not maintained for extended periods of growth without flowering, the shoot manifests itself in a compensatory form of growth distinguished by elongation and a reduction in leaf size relative to the wild type. The phyllotaxy of the cosuppression mutants is maintained in a spiral arrangement, characteristic of vegetative development (Prior, 1957). Another morphological marker for vegetative development of petunia is apical dominance (Prior, 1957). Apical dominance is maintained during the vegetative phase of growth and lateral meristems are inhibited. After flower induction, apical dominance is lost, lateral vegetative shoots grow out, and several floral buds form on the inflorescence with no cessation of meristematic activity until the flower is completely formed (Prior, 1957). Reminiscent with vegetative growth, the shoots of the cosuppression mutant plants are all strongly apical dominant. Taken together, these results show that the shoots of the *pfg* cosuppression mutants have a number of vegetative hallmarks demonstrating that these mutants are blocked in vegetative to reproductive transition, resulting in the unique non-flowering phenotype.

PFG and FBP26 are down-regulated in the pfg cosuppression mutants

Cosuppression is a phenomenon first described for the *CHALCONE SYNTHASE (CHS)* gene in petunia (Napoli et al., 1990 and van der Krol et al., 1990) and occurs in a certain percentage of plants, when an introduced transgene is partly homologous or identical to an endogenous gene. Previous studies using an identical approach to inhibit the expression of MADS box genes involved in specification of floral meristem identity (Angenent et al., 1994), floral organ identity (Angenent et al., 1993) and ovule identity (Angenent et al., 1995) have shown that this strategy is highly gene specific, although it can not be ruled out that other MADS box genes are down-regulated as well. To determine whether more MADS box genes besides *PFG* are suppressed in the *pfg* mutant (T30.09), expression of *FBP4, FBP20, FBP21, FBP22, FBP23 and FBP26* was investigated by

northern blot analysis. These five MADS box genes were selected from 28 petunia MADS box genes known to date (Ferrario et al., unpublished results), because they were the closest in sequence match to *PFG*.

Northern blot analysis for *PFG* has demonstrated that the suppression of this gene is linked to the observed phenotype in the cosuppression mutant. From the other MADS box genes, only expression of *FBP26* was down-regulated. Based on the level of sequence match, the suppression could be explained by cosuppression, or alternatively, *FBP26* expression is regulated by *PFG*. Because *FBP26* is expressed at later developmental stages than *PFG*, this gene is most likely acting downstream *PFG* and hence not involved in the phase switch to generative development. Nevertheless, we can not exclude the possibility that there are unidentified MADS box genes present in petunia, which are more homologous to *PFG* than the five we tested and that are also down-regulated in the mutant plants.

Position PFG in the flowering pathway

To get insight in the position of *PFG* in the processes that finally give rise to flowering, the interaction between *PFG* and the floral meristem identity gene *ALF* was studied by analysing *ALF* expression in the *pfg* cosuppression mutant and double mutants.

Northern hybridisation revealed that in contrast to *PFG*, *ALF* is expressed in rarely formed flowers of *pfg* cosuppression mutants at a comparable level as in wild type flowers, indicating that the expression pattern of *ALF* is not affected. Considering this result, it is most likely that *ALF* is not in the same signal transduction pathway as *PFG*.

Double mutants, obtained by crossing of *alf* mutant *S3018* (Souer et al., 1998) and the *pfg* cosuppression plant (S30.09), have a phenotype identical to the phenotype of the single *pfg* cosuppression mutants. This indicates that the *pfg* cosuppression phenotype is epistatic to the *alf* mutant phenotype. Double mutants, which are hemizygous for the *PFG* transgene have an intermediate phenotype with both *pfg* and *alf* mutant characteristics. In these plants an inflorescence meristem is initiated, which bifurcates into two identical inflorescence structures. Later the meristem reverts to a vegetative shoot with a spiral phyllotaxis. These reversions also occur in single mutants hemizygous for the *PFG* transgene indicating that a certain threshold level of *PFG* is required to maintain the reproductive identity. The idea of a threshold level is consistent with the observation that occasionally a normal flower appears in the severe *pfg* cosuppression mutants. These ectopic flowers are most likely produced when the threshold level for *PFG* is reached in a certain meristem. The fact that these flowers are indistinguishable from wild-type

flowers demonstrates that *PFG* is not responsible for the further development of a flower, despite its expression at later developmental stages. This late expression is in agreement with the proposed additional role of *PFG* in maintaining the florally determined state and preventing the reversion to vegetative growth.

Evolutionary conservation of floral induction

The floral induction process is controlled by a complex of environmental and endogenous signals (Bernier, 1988; McDaniel et al., 1992) and is most likely controlled by redundant pathways, which is consistent with the fact that a non-flowering single mutant has never been found for *Arabidopsis* (Weigel, 1995). Also the non-flowering mutant described in this paper might be due to cosuppression of both *PFG* and *FBP26*, although we can not exclude the possibility that the inhibition of *FBP26* is indirect.

Recently, the characterisation and functional analysis of the maize *INDETERMINATE1* gene (*ID1*), which is also involved in the transition of the shoot apex from vegetative to reproductive growth has been described (Colasanti et al., 1998). The expression pattern of *ID1* overlaps in part that of *PFG*. Early in development, similar to *PFG*, *ID1* is expressed in immature leaves and vegetative shoots (Colasanti et al., 1998) and its expression increases just before the floral transition. However, no expression of *ID1* is found in the apical meristem itself, whereas *PFG* is highly expressed in these meristematic cells just before the phase switch.

This expression pattern of *ID1* suggests that it acts in a non-cell-autonomous manner. ID1 is produced in the leaves and regulates the production of a transmissible signal, which triggers the transition to reproductive development in the shoot apical meristem. In contrast, PFG may act directly at the site of transition, although it is unlikely that the PFG protein itself is involved in triggering the flower induction process. First, because *PFG* is almost ubiquitously expressed, which makes it not a very likely candidate for the inducing component. Secondly, numerous physiological studies of the last century have revealed that floral transition is initiated by a signal that originates in the leaves and not in the apex (Bernier, 1988). In the *id1* mutant flowering is delayed and when eventually the switch to generative development is made, the shoot apex is converted to an inflorescence like structure with reversions to vegetative growth. This observed phenotype is comparable to the phenotype of the hemizygous *pfg* cosuppression plants. In contrast to the severe *pfg* cosuppression plant, a complete block in the phase transition has not been observed in *id1* mutants.

Another gene, which is supposed to be involved in floral transition and shows similarities to *PFG* in sequence and expression pattern, is the *Arabidopsis*

FRUITFULL (FUL) MADS box gene, formerly known as *AGAMOUS-LIKE8 (AGL8,* Mandel and Yanofsky, 1995). *FUL* is strongly expressed in the inflorescence meristem, the inflorescence stem and cauline leaves (Mandel and Yanofsky, 1995). In contrast to *PFG* expression, no accumulation of *FUL* transcript was detected in the vegetative apical meristem (Gu et al., 1998). Although *ful* single mutants have abnormal fruits, a striking phenotype associated with the transition to flowering was only observed when the *ful* mutation was combined with mutations in *CAL* and *AP1*. In these triple *ful cal ap1* mutants the switch to reproductive development is initially made, followed by a proliferation of inflorescence meristems and leaves (Yanofsky, personal communication). Therefore, despite the similarities in sequence and expression pattern, *PFG* and *FUL* seems to be MADS box genes with different functions.

Conclusions

Recent studies in maize, *Arabidopsis* and this study in petunia revealed that the first genes have now been cloned which are essential for floral transition and floral identity maintenance. This class of genes is clearly distinct from the early- and late- flowering genes, which are in general involved in perception and signalling of the floral stimulus.

In the petunia *pfg* cosuppression mutant the phase transition to reproductive growth is completely abolished. Our results indicate that PFG is most likely not the inducing signal for flowering and is therefore not a candidate for the "florigen" which is supposed to be the diffusable factor that migrates from the leaves to the apex in response to environmental and/or developmental cues. *PFG* may be essential to establish the competence of the apical meristem to receive the floral stimulus and to promote the physiological changes that occur when the vegetative meristem undergoes the transition to the reproductive phase. More likely, *PFG* can be regarded as a homeotic gene, which is in line with the function of several other members of the MADS box gene family. Then, *PFG* is an inflorescence meristem identity gene, which is essential for determining the identity of the inflorescence meristem during the transition of the vegetative shoot apex to an inflorescence meristem.

Materials and Methods

Plant material

The *Petunia hybrida* line W115, the transgenic *pfg* cosuppression plants and the *pfg alf* double mutants were grown under normal greenhouse conditions.

Screening a cDNA library and DNA sequence analysis

The *PFG* cDNA clone was isolated from a petunia pistil cDNA library (Angenent et al., 1995) constructed in lambda ZAP (Stratagene). The *PFG* clone was identified by hybridisation with MADS box DNA sequences from *FBP1* and *FBP2* (Angenent et al., 1992) as probe under low stringency conditions. A population of 10 different MADS box clones, among which *PFG*, was isolated. *In vivo* excision was used to isolate and purify double-stranded Bluescript SK $^-$ plasmid containing the *PFG* insert. The sequence of this clone was obtained using the *Taq* polymerase sequencing kit of Perkin-Elmer. Nucleotide and amino acid sequence comparisons were performed using the Clustal W multiple sequence alignment program, Version 1.7 June 1997 (Thompson et al., 1994).

The *FBP26* cDNA clone was isolated from a petunia inflorescence cDNA library constructed in Lambda HybriZAP (Stratagene). As probe for hybridisation, the full-length *Antirrhinum SQUAMOSA* cDNA was used (Huijser et al., 1992). Purification and final sequence analysis were performed as described above. The sequence of *PFG* and *FBP26* cDNAs are deposited in GenBank under accession numbers: AF176782 and AF176783, respectively.

Construction of binary vector and plant transformation

The complete coding sequence of *PFG* cDNA was inserted as a *Bam*HI - *Bgl*II fragment into the binary vector pFB21 (Angenent et al., 1993) containing the CaMV 35S promoter, the alcohol dehydrogenase intron, multiple cloning site, and the nopaline synthase terminator derived from the plasmid pBI101 (Jefferson et al., 1987). The *PFG* cDNA fragment containing the 5'-*Bam*HI and 3'-*Bgl*II restriction sites was generated using PCR.

The chimeric construct was transferred to *Agrobacterium tumefaciens* strain LBA4404 by triparental mating (Rogers et al., 1986). *Agrobacterium* conjugates were used to transform *Petunia hybrida* line W115 plants using the standard leaf disk transformation method (Horsch et al., 1985). The transformants were regenerated as described by van Tunen et *al.* (1989). After shoot and root induction on kanamycin media, plants were planted in soil and transferred to the greenhouse.

RNA gel blot analysis

The isolation of total RNA was performed according to Verwoerd et *al.* (1989). For RNA gel blot analysis, 10 µg of total RNA was denatured with 1.5 M glyoxal before electrophoresis. Equivalent loading of each RNA sample was verified by visualising ethidium bromide-stained bands in the gel or by reprobing the blot with 18S ribosomal DNA sequences. A 300 bp *XhoI/NcoI* fragment from the 3'-end of the *PFG* cDNA was used as a probe for hybridisations. From *FBP4, FBP20, FBP21, FBP22, FBP23, FBP26* (Ferrario et al., unpublished results) and *ALF* (Souer et al., 1998) gene specific cDNA fragments were used as probes for hybridisation. The probes were labelled by random oligonucleotide priming (Feinberg and Vogelstein, 1984) and blots were hybridised and washed as described previously (Angenent et al., 1992).

In situ RNA hybridisations

Vegetative and inflorescence shoot tips and floral buds were fixed and embedded in paraffin, and 10-µm sections were prepared as described by Canas et al. (1994). Digoxygenin-labelled RNA probes were synthesised by in vitro transcription using the pSPT18/19 vectors (Dig RNA labellings kit (SP6/T7), cat. no. 1 175 025, Boehringer Mannheim). For the synthesis of antisense RNA, we cloned the *XhoI/SmaI* 3'-fragment of *PFG* (0.8 kb) into pSPT18 or pSPT19. Transcripts were partially hydrolysed by incubation at 60° C in 0.1 M $Na_2CO_3 \cdot NaCHO_3$ buffer, pH 10.2 for 45 min. Hybridisation and immunological detection were performed as described by Canas et al. (1994).

Microscopy

Longitudinal sections of apical meristems from transgenic T30.09S plants and from W115 wild-type petunia plants were prepared and microscopically analysed as described in Angenent et al. (1993).

For cryoscanning electron microscopy (SEM), the samples were mounted on a stub and subsequently frozen in liquid nitrogen. The samples were coated and observed as described in Angenent et al. (1995).

alf pfg double mutants

alf pfg double mutants were obtained by crossing the hemizygous *alf* transposon insertion mutant *alf-S3018* (Souer et al., 1998) with the hemizygous *pfg* cosuppression mutant T30.09. F1 progeny plants with a weak *pfg* cosuppression phenotype and still containing the transposon insertion, verified by PCR as described below, were selfed. The resulting F2 population contains wild-type

plants, single *pfg* mutants and double mutants. This F2 population was used for phenotypical and molecular analyses.

Identification of alf mutants

The presence of the *alf* mutant allele was identified by PCR amplification of an *ALF* specific fragment containing the S3018 transposon insertion. Primers flo5 and flo6 (Souer et al., 1998) were used for amplification.

Acknowledgements

We thank Dr. Erik Souer for kindly providing the petunia *alf-S3018* transposon insertion mutant and primers flo5 and flo6; Marty Yanofsky to communicate unpublished results on MADS box gene *AGL8* (*FUL*); Adriaan van Aelst for assistance with the Scanning Electron Microscopy experiments; and Gerrit Stunnenberg for care of the plants.

Chapter 4

Ectopic expression of the petunia MADS box gene *UNSHAVEN* confers leaf-like characteristics to floral organs in a dominant-negative way

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Submitted for publication

Abstract

Several genes belonging to the MADS box transcription factor family have been shown to be involved in the transition from vegetative to reproductive growth. The petunia MADS box gene *UNSHAVEN (UNS)* is expressed in vegetative tissues and is down regulated upon floral initiation and the formation of floral meristems. To understand the role of *UNS* in the flowering process, knockout mutants were identified and *UNS* was expressed ectopically in petunia and *Arabidopsis*. No phenotype was observed in petunia plants in which *UNS* was disrupted by transposon insertion indicating that its function is redundant. Constitutive expression of *UNS* leads to the unshaven phenotype, which is characterised by ectopic trichome formation on floral organs and conversion of petals into organs with leaf-like features. The same floral phenotype was obtained when a truncated version of UNS, lacking the MADS box domain was introduced. We show that this protein was not translocated to the nucleus. This indicates that the unshaven phenotype is due to a dominant negative action of UNS, most likely suppressing the activity of factors required for the maintenance of floral identity.

Introduction

The role of MADS box proteins as key regulators in many steps of development and differentiation has been acknowledged frequently (see for a review: Riechmann and Meyerowitz, 1997). The first discovered members of this family, *AGAMOUS (AG)* and *DEFICIENS (DEF)*, were identified as flower homeotic genes in the model species *Arabidopsis* and Snapdragon respectively, more than a decade ago (Yanofsky et al., 1990; Sommer et al., 1990).

The origin of this DNA binding superfamily of proteins predates the divergence of plants and animals: conserved MADS box structures also occur in yeast and mammalian cells, where they are involved in the conversion of external signals into developmental or metabolic responses (Shore and Sharrocks, 1995), indicating ancient regulatory functions not related to differentiation and organogenesis.

In the plant field, studies on flower homeotic mutants in different species have led to the isolation of more MADS box genes and to the extrapolation of a simple model which explains the identity of each flower organ according to the single or combined action of specific homeotic genes. The ABC model was formulated in 1991 (Coen and Meyerowitz, 1991), and became the exemplification

of a common genetic program that seems to underlie flower development in all angiosperm species. Nevertheless, extensive studies and mutant analysis provided evidence that a number of *Arabidopsis* MADS box genes have regulatory functions in processes other than flower organogenesis. *ANR1* is a root-specific, NO_3^- inducible MADS box gene which encodes a component of the signal transduction pathway linking the external NO_3^- supply to an increased rate of lateral root elongation (Zhang and Forde, 1998). Other members of the *ANR1* subfamily are *AGAMOUS-LIKE17 (AGL17)*, another root specific MADS box gene with unknown function (Rounsley et al., 1995) and *NMHC5*, an alfalfa gene specifically expressed in root nodules (Heard et al., 1997). *SHATTERPROOF1 (SHP1)* and *SHATTERPROOF2 (SHP2)*, previously known as *AGL1* and *AGL5*, encode functionally redundant proteins that are required for the specification of the valve margin and to direct dehiscence zone development in mature *Arabidopsis* fruits (Liljegren et al., 2000). A gene that interacts antagonistically with the previous two is *FRUITFULL (FUL)* (formerly known as *AGL8*) which promotes valve cell differentiation and cell expansion after fertilisation (Gu et al., 1998). MADS box genes have also been recruited within the complex genetic network that regulates the transition to flowering: the switch between vegetative and generative growth is determined by the activation of interacting groups of genes that are responding to a specific combination of environmental and endogenous factors. The MADS box gene *FLOWERING LOCUS C (FLC)*, together with *FRIGIDA (FRI)*, accounts for most of the differences between early and late flowering ecotypes in *Arabidopsis*, being a repressor of flowering regulated by vernalisation (Burn et al., 1993; Lee et al., 1993; Clarke and Dean, 1994; Sheldon et al., 1999). Another MADS box gene, *AGL20*, later renamed as *SOC1 (SUPPRESSOR OF OVEREXPRESSION OF CONSTANS 1)*, has recently been shown to be an early target of *CONSTANS*, a putative transcription factor that acts in the long-day pathway as a promoter of flowering (Simon et al., 1996; Samach et al., 2000). Non-flowering petunia plants obtained by suppression of the MADS box gene *PETUNIA FLOWERING GENE (PFG)*, also demonstrate that MADS box proteins are required for the switch from vegetative to generative growth (Immink et al., 1999).

MADS box proteins are composed of a conserved DNA binding domain, the MADS box, which is located at the N-terminus; furthermore, most of the plant MADS box proteins have an additional conserved region that resembles the coiled-coil segment of keratin (therefore named K-box; Ma et al., 1991), located in the middle of the protein, and separated from the MADS box by the highly variable I region (see for a review: Riechmann and Meyerowitz, 1997). A number of studies using both *in vitro* and *in vivo* systems, have been conducted in order to identify

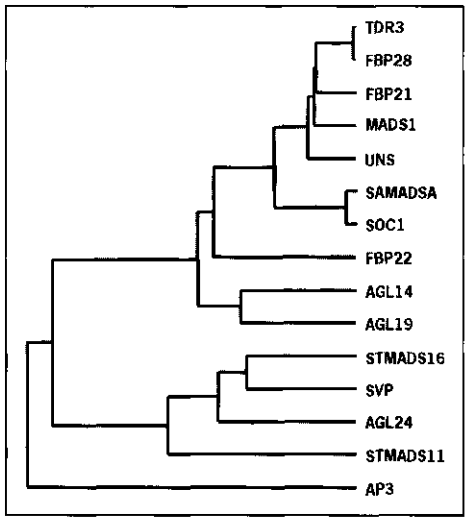

Figure 1: Dendrogram of predicted MADS box protein sequences from several species. Comparisons were made using the MADS box, the I region and the K box, of the genes from the plant species *Arabidopsis* (SOC1, AGL14, AGL19, SVP, AGL24, AP3), petunia (FBP28, FBP21, UNS, FBP22), potato (STMADS16, STMADS11), *Sinapis alba* (SAMADSA), tobacco (MADS1) and tomato (TDR3).

the regions responsible for DNA binding, dimerisation, transactivation and interaction with co-accessory regulatory factors. Given the complexity of regulatory networks, homo-, heterodimerisation or even multimeric complex formation efficiently create a large collection of transcription activation complexes, originating from a relatively limited number of proteins.

The presence of the MADS box and an adjacent short region is sufficient to mediate homodimerisation as well as DNA binding in mammalian SRF and MEF2, and in yeast MCM1 (Mueller and Nordheim, 1991; Pellegrini et al., 1995; Tan et al., 1998). Similarly the MADS domain and the I region of *APETALA1 (AP1)* and *AGAMOUS (AG)* in *Arabidopsis* and of *SQUAMOSA (SQUA)* and *PLENA (PLE)* in Snapdragon are necessary and sufficient to guarantee homodimer formation and a proper DNA binding of the protein complex (Riechmann et al., 1996a; Riechmann et al., 1996b; West et al., 1998). For a specific heterodimerisation with the correct partner, the K-box may also be required. This has been proven for the APETALA3 (AP3)/PISTILLATA (PI) dimer, as well as for heterodimers between AG and different AGLs (Riechmann et al., 1996a; Fan et al., 1997). The presumed activation domain is located at the C-terminus, although it has been suggested that some heterodimer complexes need additional (MADS box) factors for transcriptional activation (Egea-Cortines et al., 1999).

Dominant inhibitory proteins are potentially useful in generating loss-of-function phenotypes. Therefore, MADS box proteins lacking the activation domain have been produced to act as dominant negative isoforms. Human SRF and MEF2 proteins, lacking the activation domain, have been used successfully in several experiments to confirm their role in differentiation pathways (Belaguli et

al., 1997; Ornatsky at al., 1997; Okamoto et al., 2000). Similar mutant phenotypes can be obtained when a transcription factor is altered in its DNA binding ability, while it still retains its dimerisation capacity. A mutated form of the avian SRF, in which a triple point mutation was introduced in the MADS domain, competes successfully with binding by the endogenous SRF to its DNA target site (Johansen and Prywes, 1993; Croissant et al., 1996). Although much research has been done on dominant negative mutations in MADS box proteins in vertebrates, only two examples have been described of loss-of-function phenotypes due to such mutations in plants, each of them belonging to one of the two above-mentioned categories. A truncated form of the AG protein, lacking the C terminal region, generates an *ag* mutant phenotype, when ectopically expressed in *Arabidopsis* plants (Mizukami et al., 1996). In rice, the naturally occurring mutant *leafy hull sterile1* is caused by a point mutation in the MADS box of the *OsMADS1* gene, which inhibits the binding of the transcription factor to its DNA target site (Jeon et al., 2000).

In this study, we demonstrate that a petunia MADS box gene, *FLORAL BINDING PROTEIN (FBP)* gene *20*, which was recently renamed as *UNSHAVEN* (*UNS*), is expressed in vegetative tissues only and can act as a dominant negative inhibitor when mutated. The gene confers leaf-like characteristics to floral organs when expressed ectopically. An identical mutant phenotype is obtained by overexpression of *UNS*, lacking the MADS domain and the I region. These results, which are partly confirmed in *Arabidopsis*, strongly suggest a function for UNS in suppressing the activity of a factor required for the maintenance of floral identity.

Results

Isolation and sequence analysis of UNS

A full-length cDNA clone, corresponding to the *UNSHAVEN* (*UNS*, formerly known as *FBP20*) MADS box gene, has been isolated from a cDNA library obtained from young petunia inflorescences. The plant material used for preparing the library included also vegetative tissues, such as bracts and young leaves. The screening was performed at low stringency using the MADS box regions of *FBP1* and *FBP2* as a probe (Angenent et al., 1992). Among five new MADS box gene clones designated *FBP18*, *UNS*, *FBP21*, *FBP22* and *FBP28*, full-length clones of *UNS* were further investigated.

The *UNS* cDNA encodes a putative protein of 217 amino acids, which has a high sequence similarity with the *Arabidopsis* SOC1 protein (Samach et al., 2000) and other related sequences from tobacco (MADS1; Mandel et al., 1994), *Sinapis alba* (SaMADS A; Menzel et al., 1996) and tomato (TDR3; Pnueli et al., 1991).

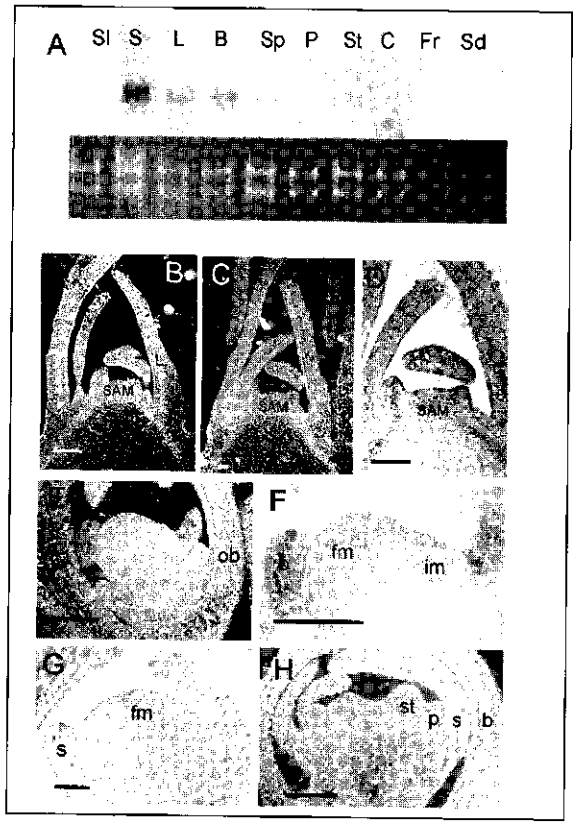

Figure 2*: Expression of *UNS* in wild type tissues of petunia. **(A)** RNA gel blot using RNA from seedlings (Sl), stems (S), leaves (L), bracts (B), sepals (Sp), petals (P), stamens (St), carpels (C), fruits (Fr) and seeds (Sd) of wild type petunia hybridised with an *UNS* specific probe. Below the blot is a picture of the gel prior to blotting, stained with ethidium bromide, illustrating equal loading of the samples. **(B-H)** RNA *in situ* localisation of *UNS* in shoot apical meristems and developing petunia inflorescences. Longitudinal sections were hybridised with a digoxygenin-labelled sense *UNS* probe **(B)** or antisense UNS RNA **(C-H).** Abbreviations used are: L, leaf; SAM, shoot apical meristem; ob, old bract; b, bract; fm, floral meristem; im, inflorescence meristem; s, sepal; p, petal; st, stamen. **(B-D)** Shoot apical meristems with emerging leaves on their flanks. The *UNS* mRNA is equally distributed throughout the apex and the emerging young leaves. **D** shows a higher magnification of **C**. **(E-F)** Inflorescence apex with developing floral meristem. **F** is a higher magnification of **E**. The expression is strong in young bracts, but weak hybridisation signals are also present in the floral and inflorescence meristems. **(G)** Floral meristem with developing sepals. **(H)** Young flower bud with petal and stamen primordia. Bars represent 200um in **B-F** and 500um in **G** and **H.** ****) Figure in colour in appendix, page IV.***

UNS and SOC1 share 65% sequence identity, when the full-length proteins are compared, but this raises to 89% in the MADS box domain.

Figure 1 shows a dendrogram, generated using the MADS box, the I region and the K box of UNS and related *Arabidopsis* protein sequences present in the databases (Alvarez-Buylla et al., 2000). We included also the sequences from tobacco, *Sinapis alba* and tomato, related to UNS, and STMADS11 and STMADS16 (Carmona et al., 1998; Garcia-Maroto et al., 2000) from potato. Although the potato MADS box proteins are not as closely related as those are from the other species, we included them in our analysis because of the overexpression phenotype that *STMADS16* produces in tobacco plants, as

discussed later. The dendrogram reveals that UNS, SOC1, MADS1, SaMADS A and TDR3 group together in the same subfamily, while StMADS11 and StMADS16 belong to another, unrelated clade together with SHORT VEGETATIVE PHASE (SVP) and AGL24.

Expression analysis of UNS in wild-type petunia

RNA gel blot analysis was performed to analyse the expression of *UNS* in wild type petunia tissues. An *UNS* specific probe without the MADS box region was used to hybridise blots containing RNA isolated from seedlings at two cotyledon stage, stems, leaves, bracts, sepals, petals, stamens, pistils, fruits and developing seeds at 10 days after fertilisation. *UNS* expression is predominantly confined to the vegetative tissues and bracts, while in the flower low expression was detectable in sepals only (Figure 2A). A more detailed picture of the *UNS* expression pattern was obtained by in situ hybridisation of shoot apical (SAM), inflorescence and floral meristems (Figures 2B-H). The mRNA of *UNS* is uniformly distributed throughout the vegetative meristem and the emerging young leaves (Figures 2C and D). The expression pattern of *UNS* is similar to that of *SOC1* in *Arabidopsis* plants that have been induced to flowering by 16h long day (LD) conditions (Samach et al., 2000). However, unlike *Arabidopsis SOC1*, *UNS* transcript was already present in the SAM and emerging leaves at developmental stages, long before the transition to flowering, indicating that the expression of *UNS* is not directly linked to the switch from vegetative to generative phase. *UNS* is highly expressed in young bracts and at a very low level in inflorescence meristems (Figures 2E and F). Hardly any hybridisation signal was detectable in the flower meristem and in the emerging floral organ primordia (Figure 2G and H). mRNA levels rapidly declined with further development of the flower organs (Figure 2, results not shown).

Ectopic expression of UNS and UNSΔMI generate the same phenotype in petunia

Transgenic petunia plants carrying the full-length *UNS* cDNA under the control of the constitutive 35S Cauliflower Mosaic Virus (CaMV) promoter, were generated by *Agrobacterium* mediated transformation. Thirty-eight independent regenerants were obtained, of which 14 showed transgene expression at variable levels, as determined by RNA gel blot analysis (data not shown). Phenotypic alterations of the transgenic plants were only apparent in flower organs where *UNS* transcript is normally not present. The overall architecture of the petunia inflorescence was not affected, and also no significant change in flowering time was observed. Leaves, bracts, sepals and anthers did not reveal obvious changes in their morphology,

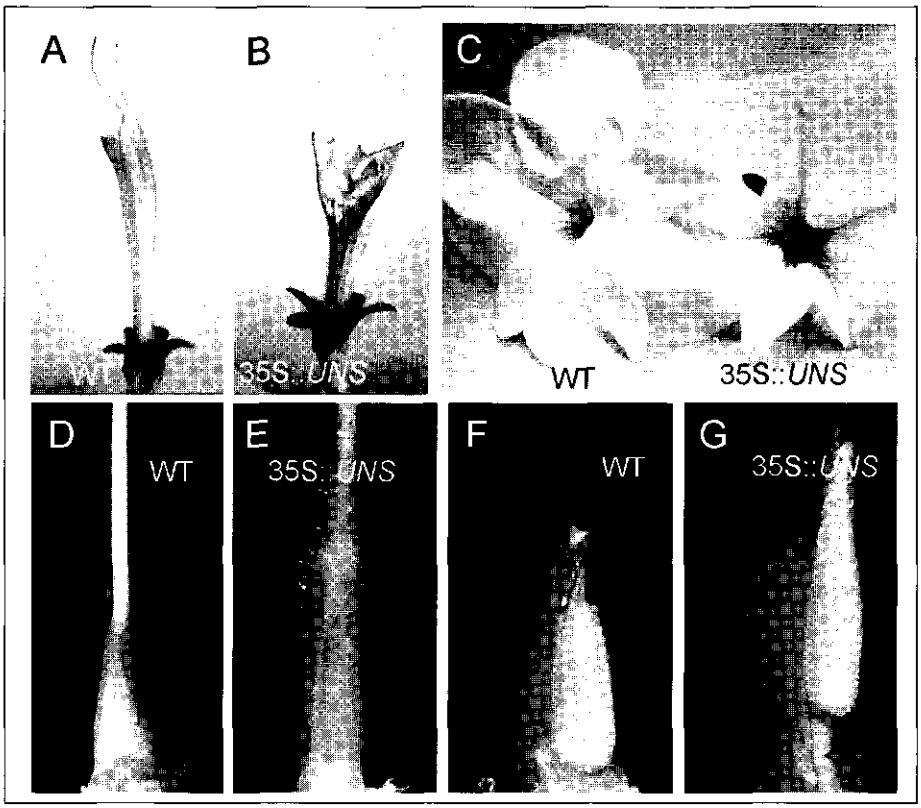

Figure 3*: Flowers and ovaries of wild type and 35S::*UNS* petunia plants. **(A-B)** wild type (WT) and transgenic (35S::*UNS*) petunia buds at the same stage of development. The transgenic bud is significantly shorter and greener than a bud of WT plants. **(C)** WT and 35S::*UNS* mature flowers. The reduced dimensions are maintained till full maturity of the transgenic flower, which does not open completely. **(D-G)** Ovaries of WT **(D, F)** and 35S::*UNS* **(E, G)** plants. The transgenic ovaries are significantly longer than those from WT. The outer epidermis is covered with trichomes and a stem-like structure develops at the basis of the septum (arrow in **G**). One carpel is removed from the ovaries in **F** and **G**.

) Figure in colour in appendix, page IV.

while modifications in petals and ovaries could be seen in all 14 plants in which ectopic expression of *UNS* was observed.

As shown in Figure 3, transgenic floral buds are significantly shorter and greener than wild type buds. The green hue of the petals in overexpression plants is maintained till full maturity of the flower, in contrast with the white colour of the fully developed wild type flowers. Transgenic flowers are reduced in size and the corolla does not open completely (Figure 3C). Petals of wild type and mutant flowers were analysed in detail by Scanning Electron Microscopy (SEM, Figure 4).

These analyses revealed that trichomes develop on both abaxial and adaxial sides of transgenic petals, equally distributed over the whole surface. In contrast, petals of wild type plants have only a few trichomes on the abaxial side, predominantly along the mid-vein (Figures 4A and B). The shape of the petal cells was also affected: epidermal cells at both sides of the petals in overexpression lines were flatter than those of wild type plants, which have conically shaped cells (Figures 4C-D and 4I-J). Petals of overexpressor plants exhibit features of leaves and bracts of both wild type and mutant lines which have flat epidermal cells and are covered by many trichomes (Figures 4E-F).

Pistils of the overexpression lines were also changed in morphology. The ovary was elongated compared to wild type ovaries (Figures 3D-G and 5A-D) and a stem-like structure developed at the basis of the septum inside the ovary (arrows in Figures 3G and 5D). Like on transgenic petals, trichomes developed ectopically on the surface of the transgenic ovaries and over the whole style till the basis of the stigma, while they are completely absent on wild type pistils (Figures 5F-H). Even inside the ovary, trichomes develop occasionally on the surface of the septum (Figure 5E). Because the presence of many trichomes on different organs was one of the most striking features of the transgenic plants, we renamed *FBP20* in *UNSHAVEN (UNS).*

In order to assess the identity of the modified floral organs in the 35S:: *UNS* plants, we used the expression of two different petunia MADS box genes, *FBP28* and *FBP29*, as molecular markers. Both are expressed in leaves and bracts of wild type petunia plants, while their patterns of expression differ in the floral organs (Figure 6). *FBP29* mRNA is detectable in sepals and carpels, but it is excluded from whorls 2 and 3. *FBP28* mRNA, in contrast, is present only in stamens and absent from the other three floral whorls. In the 35S:: *UNS* plant the expression of *FBP29* is extended to petals, where also *FBP28* is upregulated. Furthermore, *FBP28* mRNA could also be detected in 35S:: *UNS* carpels, the other floral organ that undergoes drastic changes in the mutant plants. These observations indicate that petals and carpels of the mutant plants are being converted into leaf-like organs rather than having a sepalloid identity.

Taken together, the phenotypic changes due to *UNS* overexpression suggest a role for this transcription factor in activating a vegetative program in floral organs where it is normally suppressed. The phenotype that we obtained in transgenic plants overexpressing a truncated version of *UNS* however provided evidence against an activating role of this gene. When *UNS* lacking the MADS box and the I region (*UNSΔMI*), was ectopically expressed in petunia plants, the same phenotypic alterations were observed in the flowers, as seen in transgenic plants overexpressing the full-length cDNA. Greenish petals covered with

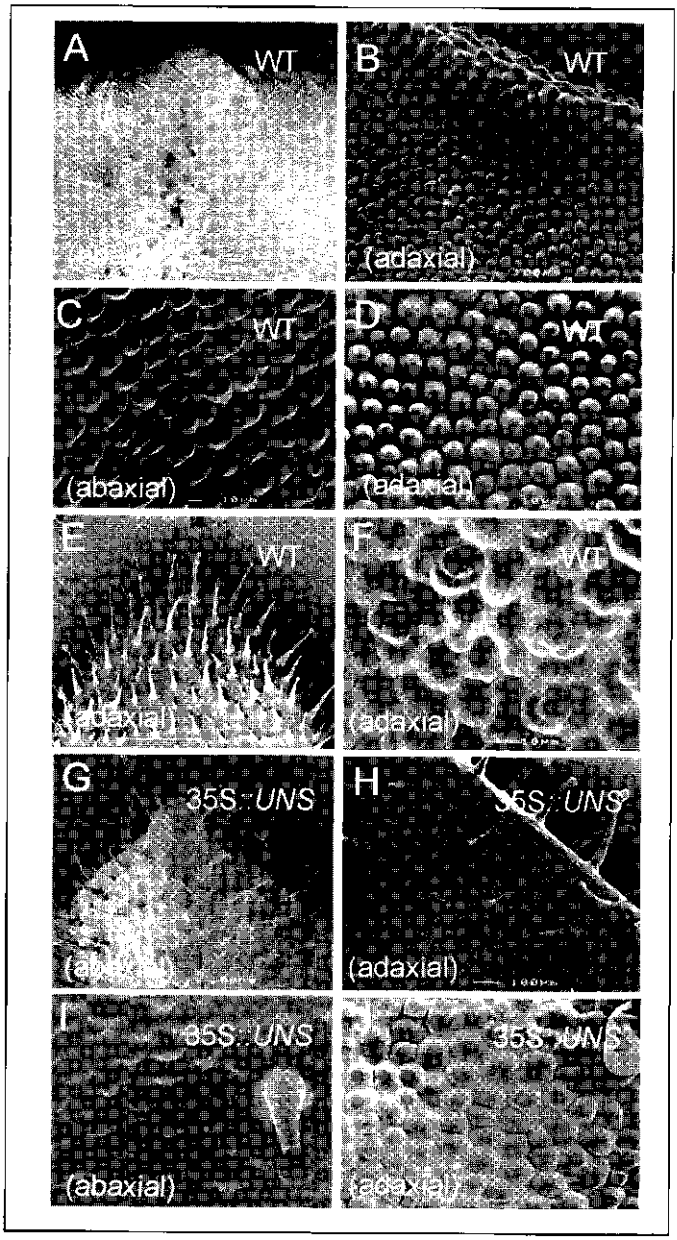

Figure 4: Scanning Electron Microscopy (SEM) of wild type (WT) petal and bract, and 35S::*UNS* petals. **(A-D)** Abaxial and adaxial side of a wild type petal. A few trichomes are visible along the mid-vein at the abaxial side only **(A).** A higher magnification shows the conical shaped cells that are present on both sides **(B-D). (E-F)** Adaxial side of a wild type bract, which is covered with trichomes **(E)** and stomata **(F). (G-J)** Abaxial and adaxial sides of a 35S::*UNS* line. Numerous trichomes are growing on both sides of the petals and the cells are flatter compared to the wild type **(I-J). I** and **J** are higher magnifications of **G** and **H,** respectively.

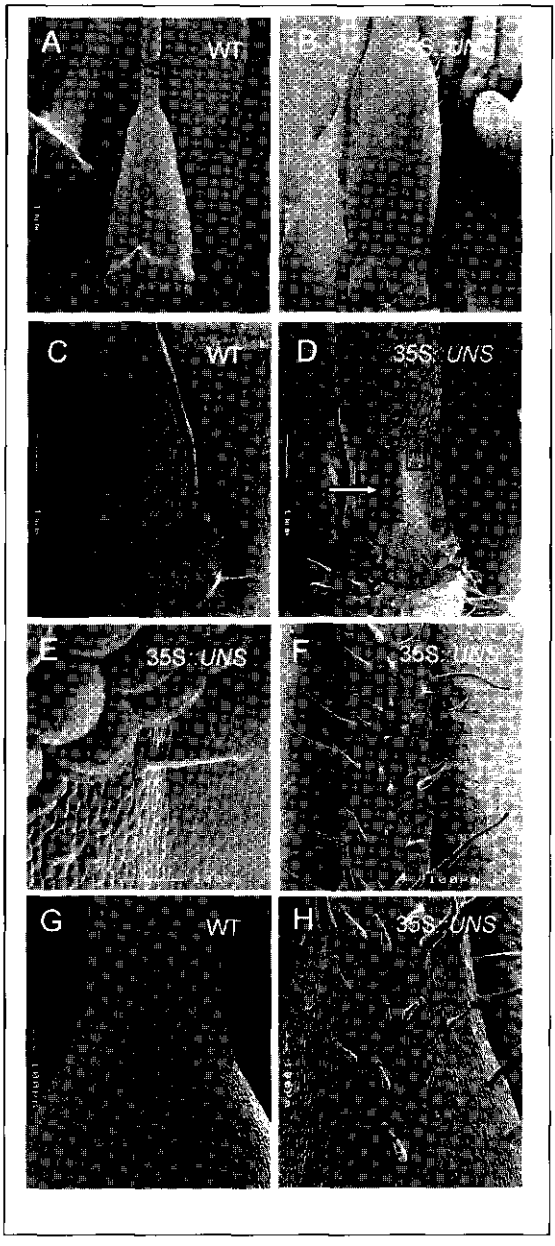

Figure 5: Scanning Electron Microscopy (SEM) of pistils of wild type (WT) and 35S.::*UNS* lines. **(A-B)** Ovaries of WT and 35S.::*UNS*. The ovary of the transgenic line is elongated compared to the WT ovary, and covered with trichomes.**(C-D)** Ovaries of WT and 35S.::*UNS* with removed ovary wall. The box in **D** is enlarged in **E** and shows a trichome which develops on the surface of the elongated septum inside the ovary (arrow in **D**).**(F-H)** Stylar tissues of WT and 35S.::*UNS* plants. Numerous trichomes develop at the stylar surface of 35S.::*UNS*, while WT pistils do not produce trichomes. Abbreviations used: *o*, ovary; *st*, style.

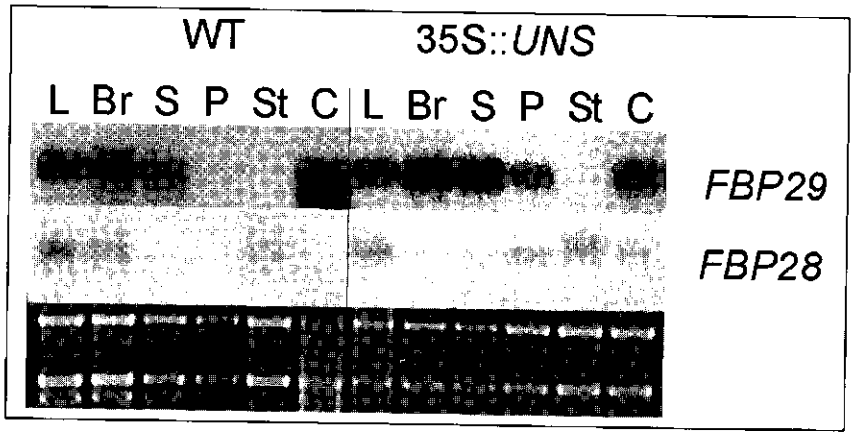

Figure 6: RNA gel blot using RNA from leaves (L), bracts (B), sepals (S), petals (P), stamens (St), and carpels (C) of wild type (left) and 35S::*UNS* (right) petunia plants The blot was hybridised with an *FBP29* (upper blot) or *FBP28* (lower blot) specific probe. Below the blot is a picture of the gel prior to blotting, stained with ethidium bromide, illustrating the amount of RNA loaded.

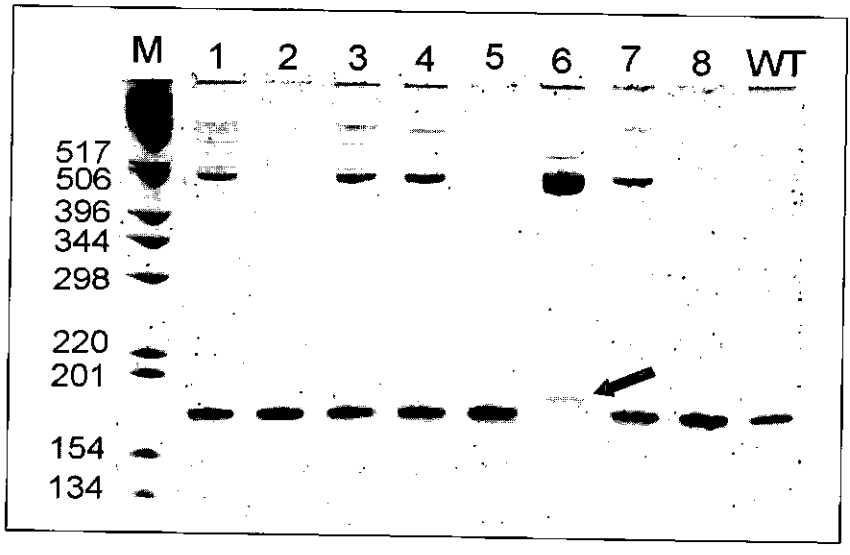

Figure 7: Analysis of individuals segregating the *dTph1* insertion in the MADS box of *UNS*. The acrylamide gel shows PCR products amplified with gene specific primers. The lower amplification product is the wild type *UNS* fragment, while the upper prominent band represents an *UNS* fragment containing the 284 base pair *dTph1* transposable element. Plants 1, 3, 4 and 7 are heterozygous for the presence of the transposon, plants 2 and 5 are wild type, and plant 6 is homozygous for the insertion. The faint band in lane 6 (indicated by the arrow) is due to somatic excision of the dTph1 element, resulting in a frame shift in the open reading frame. M: molecular marker lane. WT: wild type.

trichomes and elongated, hairy pistils are features common to all transgenic plants, while the severity of the phenotype correlated with the expression level of the transgene (results not shown).

Knock-out mutant of UNS

In order to obtain an *UNS* loss-of-function mutant, we screened a total of 5712 petunia W138 plants for transposon insertions in the open reading frame of *UNS* (Koes et al., 1995). One plant was found to be hemizygous for a *dTph1* transposable element insertion in the MADS box coding region. The small transposable element of 284 nucleotides in length (Gerats et al., 1990) was inserted 104 nucleotides downstream of the ATG start codon, in the first exon. Homozygous mutant plants were obtained upon selfing of this plant. The presence of the transposon was confirmed by PCR using genomic DNA of single plants from the same progeny. The acrylamide gel in Figure 7 shows the dTph1 insertion in some of the individuals. Several plants are hemizygous for the insertion (e.g. lane 1), whereas only plant #6 was homozygous. The faint band, slightly higher than the wild type one, is due to somatic excision of the transposon. This was confirmed by direct sequencing of the PCR product, which showed the presence of an 8bp-footprint left by the transposon after excision. Since the footprint is responsible for a frameshift in the open reading frame, no wild type UNS protein is formed in plant #6. Although carrying a *null* mutation in the *UNS* gene, the plant did not show any clear phenotypic alteration, most likely due to redundancy caused by other, closely related genes.

Co-localisation of UNS, UNSΔMI and interacting partners in petunia protoplasts

Surprisingly, both *UNS* and *UNSΔMI* resulted in similar phenotypic alterations when expressed ectopically. This suggests that the mutation is due to a dominant negative effect rather than transcriptional activation by UNS. To provide additional evidence for this hypothesis, we analysed the localisation of different MADS box fusion proteins in petunia leaf protoplasts by Confocal Laser Scanning Microscopy (CLSM). Fusion constructs containing the Yellow Fluorescent Protein (YFP) and either the full length UNS or the truncated UNSΔMI were expressed under the constitutive 35S CaMV promoter. Similarly, a fusion between the Cyan Fluorescent Protein (CFP) and the MADS box protein FBP9 was overexpressed in petunia leaf protoplasts. FBP9 is a dimerisation partner of UNS as was determined by yeast two-hybrid experiments (Table 1).

The fluorescence due to YFP expression, was localised in the cytoplasm when either UNS or UNSΔMI alone were expressed (Figures 8A-B). The

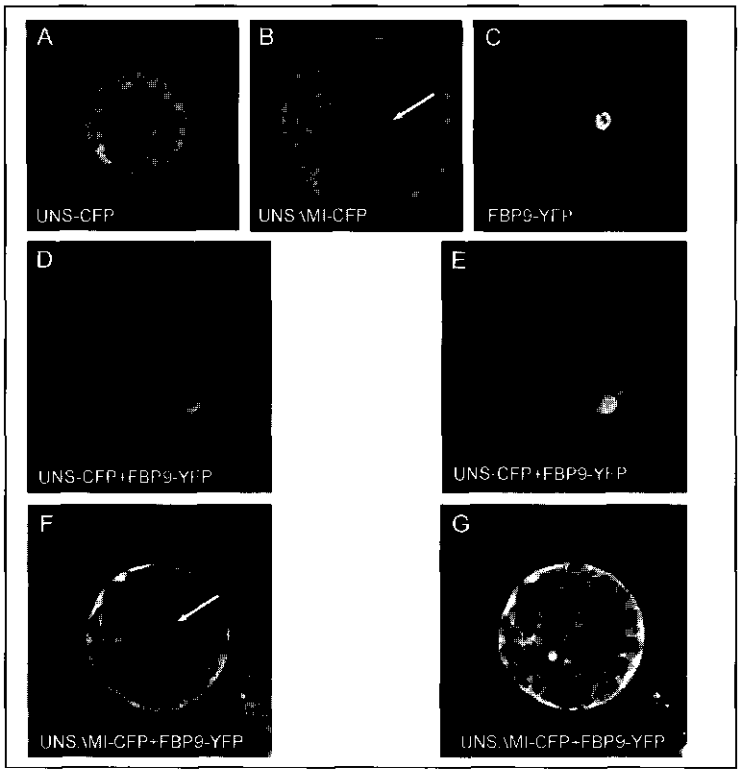

Figure 8*: Confocal Laser Scanning Microscopy (CLSM) of petunia leaf protoplasts expressing different fusion proteins. Chlorophyll auto-fluorescence is shown in red. **(A-B)** The fusion proteins between CFP and UNS full-length or UNSΔMI are localised in the cytoplasm. The arrow in B indicates the position of the nucleus. **(C)** The fusion protein FBP9-YFP is translocated to the nucleus. **(D-E)** Protoplast imaged with CFP **(D)** and YFP **(E)** filters, respectively. UNS-CFP and FBP9-YFP are colocalised in the nucleus. **(F-G)** Single protoplast expressing UNSΔMI -CFP and FBP9-YFP, imaged with CFP **(F)** and YFP **(G)** filters, respectively. Both signals are localised in the cytoplasm. Arrow indicates the position of the nucleus.

) Figure in colour in appendix, page V.

cytoplasmatic localisation of the chimeric transcription factor constructs points to the absence of a heterodimerisation partner and the inability to form homodimers (see also Table 1). In contrast to UNS, FBP9 can form homodimers (Immink et al., 2002), and the complex is transferred to the nucleus where all the yellow fluorescence is localised (Figure 8C). The use of two variants of the green fluorescent protein with different emission spectra, enables a simultaneous localisation of the two different MADS box protein constructs. The combinations tested, FBP9/UNS and FBP9/ UNSΔMI, gave rise to different fluorescence patterns. When both full-length proteins are present, the yellow and the blue

Bait – Prey	-LT	-LTA	Lac-Z
UNS – FBP9	++	++	+
UNS – AP1	++	-	-
UNSΔMI – FBP9	++	++	+
UNSΔMI – AP1	++	++	+
UNS – UNS	++	-	-

Table 1: Yeast two-hybrid assay using different protein combinations. Each interaction was tested on a selective medium lacking *Leu* and *Trp* (-LT) and on medium lacking *Leu*, *Trp* and *Ade* (-LTA). β-galactosidase activity assay was also performed (Lac-Z).

fluorescence co-localise in the nucleus (Figures 8D-E). In contrast, both FBP9 and UNSΔMI remain located in the cytoplasm, despite the ability to form heterodimers between FBP9 and UNSΔMI (Figures 8F-G). Deletion of the MADS box domain from one of the dimerising proteins, and therefore the absence of one nuclear localisation signal, thus seems to be sufficient to prevent the whole complex from being translocated into the nucleus. As a consequence, the FBP9/ UNSΔMI heterodimer can not function as a transcription factor. In this specific combination, UNSΔMI thus acts as a dominant negative form towards FBP9, preventing the complex to reach its DNA targets inside the nucleus. This suggests that the *uns* mutant phenotype is due to the elimination of an UNS dimerisation complex.

Ectopic expression of UNSΔMI phenocopies the uns mutation and generates an additional ap1-like phenotype in Arabidopsis

We transformed *Arabidopsis* plants with the 35S::*UNSΔMI* construct to monitor the effects in a heterologous system. Twenty-nine out of 44 putative primary transformants, showed variable degrees of phenotypic alterations. They all had small green petals, being almost absent in the 4 lines with the most severe aberrations (Figures 9B-G). In addition, these lines produced elongated 'unshaven' carpels (Figures 9B-C). The long stem at the basis of the mutant ovary (arrow in Figure 9F) and stellate trichomes arising from its surface, are characteristics that are very similar to the ones produced in petunia by the same construct (compare Figures 3 and 9).

Surprisingly, the *Arabidopsis* transformants exhibited extra features, not seen in the transgenic petunia lines and that closely resembled abnormalities described for *apetala1 (ap1)* mutants (Irish and Sussex, 1990; Bowman et al., 1993). As shown in Figures 9B and 9D-F, first whorl organs tend to be bract-like, being elongated and pointed and with stellate trichomes on the abaxial surface. Another

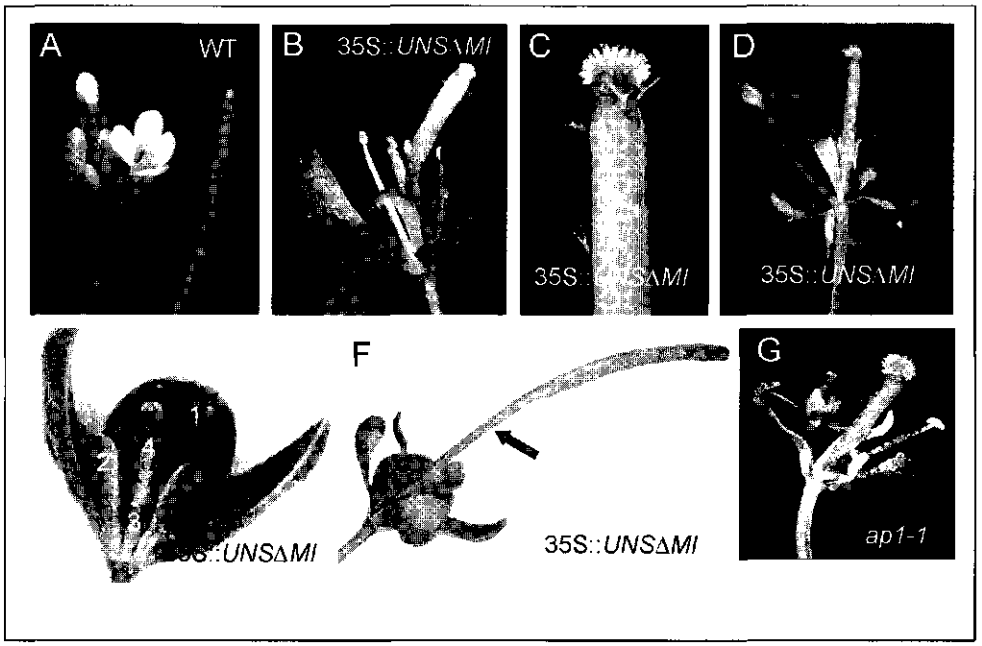

Figure 9*: Flower morphology of 35S:: *UNSΔMI* Arabidopsis lines. **(A)** Wild type Arabidopsis inflorescence (left) and silique (right). **(B-F)** Flowers of 35S::*UNSΔMI* transformants: stellate trichomes on the abaxial side of the sepals and on the pistil are visible in **B** and **C**. The arrows in **B** and **D** point to the secondary buds arising at the axil of the first whorl organs. Petals are completely absent or largely reduced in size and greenish. **(E)** A young 35S::*UNSΔMI* flower with small green organs in whorl 2. The four flower whorls are numbered. **(F)** Flower of 35S::*UNSΔMI* after fertilisation. The arrow points to the elongated stem at the basis of the silique. **(G)** *ap1-1* flower; the arrow indicates the axillary bud arising at the axil of the first whorl organs. ***) Figure in colour in appendix, page V.***

trait that was observed and that is typical for all strong *ap1* alleles, is the presence of extra flowers arising in the axils of the first whorl organs of the primary flowers (see Figure 9G). Flowers with this indeterminate, branched structure that replaces the single flower of a wild type plant, are depicted in Figures 9B and D.

RNA gel blot analysis was performed to check whether the expression of the *AP1* gene was affected in the transgenic plants overexpressing *UNSΔMI*. No reduction in the level of *AP1* mRNA was detected in any of the transgenic plants when compared to wild type plants (data not shown). This demonstrates that the *ap1-like* phenotype of the *UNSΔMI* overexpressing lines, is not caused by a transcriptional silencing of the *AP1* gene. However, in line with the mode of action in petunia, *UNSΔMI* may also act as a dominant negative factor in the translocation of a heterodimer complex to the nucleus in *Arabidopsis*.

Transgenic *Arabidopsis* plants overexpressing the full-length *UNS* gene, in contrast, did not show any morphological aberration or variations in flowering time, suggesting that the petunia full-length protein does not have the same functional interactions in *Arabidopsis* as the truncated one.

AP1 complements the ap1-like phenotype in 35S::UNSΔMI transgenic Arabidopsis plants

To analyse whether the *ap1*-like phenotype, obtained by overexpression of the *UNSΔMI* construct is caused by suppression of the *AP1* function, crosses were made between *35S::AP1* plants and *Arabidopsis* plants carrying the *UNSΔMI* construct. The progeny was analysed both at the molecular and phenotypic level. Five out of the 45 progeny plants analysed, showed partial complementation in their flowers: wild type petals were formed and the secondary buds in the axils of the sepals were absent (Figures 10D-E). Among the other plants in the progeny we observed some wild type plants and plants displaying either an *AP1* or an *UNSΔMI* overexpression phenotype (Figures 10B-C). A subset of the individuals belonging to the four phenotypic classes was checked at the molecular level by means of RNA gel blot analysis (Figure 10A). The gel blot was probed either with the *AP1* or the *UNS* gene. All wild type plants lacked ectopic expression of both transgenes, while overexpression of *AP1* or *UNSΔMI* was detected in the plants displaying the corresponding phenotype. The chimeric phenotype with both wild type and 35S::*UNSΔMI* characteristics was either due to a complementation by high expression of the *AP1* transgene (lanes 7, 8 and 9) or to a relatively low expression of *UNSΔMI* (lanes 6 and 10). This suggests that the AP1 function is affected in the 35S::*UNSΔMI* overexpressor lines.

UNS and UNSΔMI have different dimerisation partners in petunia and Arabidopsis

The *ap1-like* phenotype obtained with the truncated UNS protein in *Arabidopsis*, would imply that UNSΔMI can act as a dominant negative factor towards the *Arabidopsis* AP1 protein. To verify this hypothesis, we tested possible interactions between the petunia and *Arabidopsis* MADS box proteins in the yeast two-hybrid GAL4 system. These analyses revealed that the full-length and the truncated UNS share the majority of the interacting partners among the petunia MADS box proteins (Immink et al., in preparation). Here we confirm that differences in dimerisation specificity occur among heterologous proteins. As expected, only the petunia UNS lacking the MADS box and the I region could dimerise with the AP1 protein, while the full-length failed to interact with the *Arabidopsis* protein in any

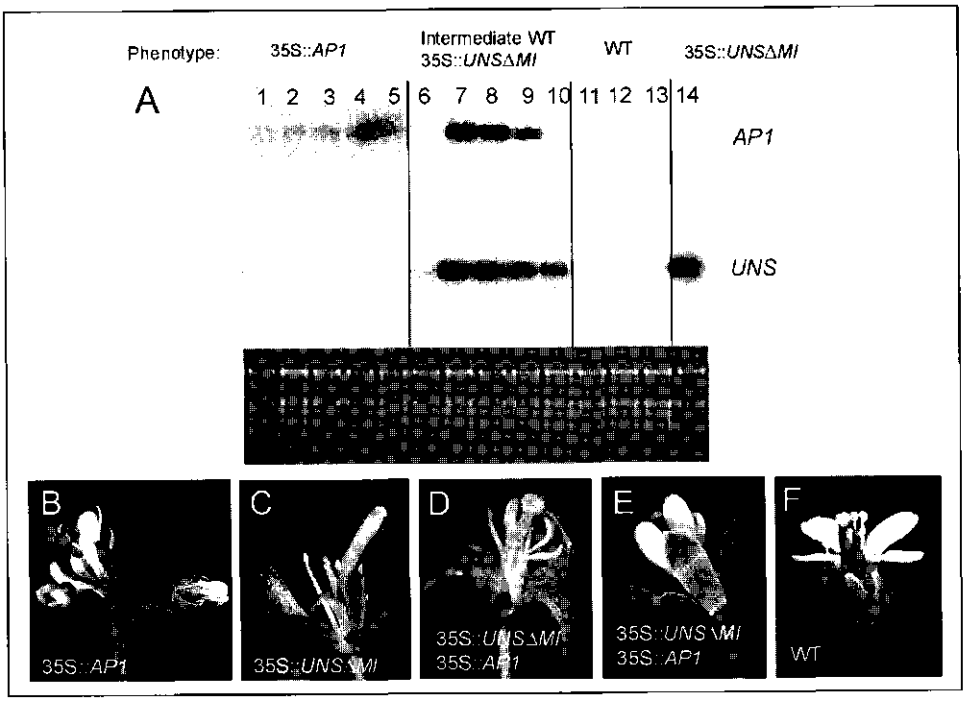

Figure 10: Analysis of progeny plants derived from a cross between 35S::*AP1* and a 35S::*UNSΔMI* line. **(A)** Expression of *AP1* and *UNS* in leaves of Arabidopsis plants derived from a cross between a 35S::*AP1* and a 35S::*UNSΔMI* line. The upper blot was hybridised with an *AP1* probe, the lower one with an *UNS* probe. The phenotype is indicated on top of the gel. Below the blots is a picture of the gel prior to blotting and stained with ethidium bromide. **(B-F)** Progeny plants from a cross between 35S::*AP1* and 35S::*UNSΔMI* segregate four classes of floral phenotypes. **(B)** Terminal flower of a 35S::*AP1* plant. **(C)** Flower of a 35S::*UNSΔMI* line. **(D-E)** Flowers from two lines carrying both 35S::*AP1* and 35S::*UNSΔMI* constructs and showing a phenotype intermediate between wild type and *UNSΔMI* overexpression. The sepals are still bract-like and petal formation is partly **(D)** or completely **(E)** restored. **(F)** Wild type Arabidopsis flower.

of the conditions tested (Table 1). This may explain the *ap1*-like phenotype as observed specifically in the UNSΔMI overexpression lines.

Discussion

MADS box genes have been recruited during plant evolution as master regulatory genes that control developmental processes such as inflorescence and flower formation. Floral homeotic functions have been the first ones to be assigned to members of this family of transcription factors. Although by now different roles have been identified for other MADS box proteins, the number of these genes that

are found to be involved in processes not related to flower development is still low. In *Arabidopsis*, SHATTERPROOF1 and SHATTERPROOF2 regulate dehiscence of siliques (Liljegren et al., 2000), FRUITFULL controls silique valve development (Gu et al., 1998) and ANR1 is involved in lateral root elongation (Zhang and Forde, 1998). Nevertheless no reports on MADS box genes are available that demonstrate a role in vegetative development.

Here we describe the identification and functional analysis of a petunia MADS box gene, *UNSHAVEN (UNS),* which is specifically expressed in vegetative and green tissues. When ectopically expressed in flowers this gene induces vegetative features suggesting a role in maintenance or specification of vegetative identity.

The petunia UNS gene belongs to the SOC1 clade but it does not have the same function

Close sequence similarities among proteins from different species are usually taken as an indication of similarity in function, in particular when supported by related expression patterns. The petunia *UNSHAVEN (UNS)* gene, although sharing a high degree of sequence similarity with the *Arabidopsis SOC1* gene, does not seem to fulfil the same function. As a direct target of CONSTANS, SOC1 is promoting flowering in response to a long photoperiod treatment. In fact its expression is detectable only after the plant has been exposed to long day conditions (Samach et al., 2000). The mRNA is localised in the shoot apical meristem (SAM) and in emerging leaf primordia. Furthermore, it is also present in the inflorescence meristem but it is absent from the flower meristem. *UNS* mRNA is also present in the SAM and emerging leaves, but in contrast, *UNS* mRNA appears long before the plant is committed to flowering. Therefore it is likely that the *UNS* gene does not have any direct role in the switch from vegetative to generative phase, such as *SOC1.* Although at lower levels, the *UNS* mRNA could still be detected in the inflorescence meristem and in the flower meristem till floral organ primordia start to emerge. The presence of *UNS* transcript in floral meristems and flower primordia, while *SOC1* is excluded from these domains, implies different regulatory mechanisms, also at later stages of development. For other members of the same clade, low expression levels in flowers has also been reported. Both tomato *TDR3* and tobacco *MADS1* mRNA could be detected in fully developed floral organs (Pnueli et al., 1991; Mandel et al., 1994) and we cannot exclude that a low amount of *UNS* mRNA, that could not be revealed by RNA gel blot analysis, is still present in the mature flower. Therefore, a function of the protein in floral organs cannot be ruled out completely, although we favour the hypothesis that the main role of *UNS* is in vegetative tissues.

UNS confers leaf-like features to floral organs in a dominant-negative way

Although one should be careful in drawing conclusions from ectopic expression studies, it can be particularly informative when homeotic or phase changes appear in overexpressor lines and when the gene is functionally redundant. It must be taken into account that aberrations induced by a transgene can be the result of a different mode of action of the transgenic protein, when expressed in a cellular environment, different from its original one. This is the case for UNS but it is most likely applicable to many other overexpression mutants as well. All phenotypic alterations that were induced by ectopic expression of the full-length UNS protein were also apparent in transgenic plants in which a truncated, non-functional UNS protein was expressed. The leaf-like characteristics, conferred to floral organs by both proteins, cannot be due to direct activation of a set of genes by the transcription factors, since UNS lacking the complete DNA binding domain and the I region simply cannot perform the function of the wild type protein. Similar changes in flower morphology have also been reported for tobacco plants overexpressing the potato MADS box gene *STMADS16* (Garcia-Maroto et al., 2000). The ovary was elongated, had a ridged shape and trichomes developed all over the stylar surface. These and other observations on the decreased flowering time and increased internode length in transgenic tobacco plants, led to the conclusion that overexpression of *STMADS16* can superimpose a vegetative program onto the normal floral developmental pathways. Therefore, a role for *STMADS16* in the control of vegetative growth was proposed. Given the expression patterns and overexpression phenotypes of *UNS* and *STMADS16* (exclusively expressed in vegetative organs), a similar function in promoting or maintaining the vegetative growth in petunia can also be inferred for *UNS*. However, considering the distant clades to which they belong, *UNS* is unlikely to be the petunia homologue of *STMADS16*.

As stated before, the UNS protein lacking the MADS domain cannot act as a transcription factor; evidence for this phenomenon came from the localisation experiments in petunia protoplasts. It has been shown with the *Arabidopsis* APETALA3 (AP3) and PISTILLATA (PI) MADS box proteins, that dimer formation is necessary for the translocation of both transcription factors into the nucleus; when only one of the two proteins is present, the localisation of either one is exclusively cytoplasmatic (McGonigle et al., 1996). Dimerisation as a prerequisite to target a transcription factor complex to the nucleus, appears to be a general mechanism that has been shown to occur also with the MCM complex in yeast and the Extradenticle/Homothorax homeodomain complex in *Drosophila* (Pasion and Forsburg, 1999; Abu-Shaar et al., 1999). Similarly in petunia, the UNS

protein is targeted to the nucleus only when the dimerisation partner FBP9 is present, since the UNS protein itself is not able to form a homodimer (Immink et al., 2002). Conversely, UNSΔMI still retains the ability to dimerise with FBP9 but it prevents the complex from being targeted to the nucleus, most likely because of the lack of the nuclear localisation signal. The fact that dimerisation still occurs is proven by the cytoplasmatic localisation of FBP9. This protein is able to homodimerise and when present alone in a protoplast, it is only detectable in the nucleus. The affinity for heterodimer formation with UNSΔMI is apparently higher than for homodimer formation, UNSΔMI /FBP9 complexes are assembled rather than FBP9/FBP9 homodimers. The hypothesis of a dominant-negative interaction of UNSΔMI is in line with the phenotype that the heterologous protein generated in *Arabidopsis*. Besides features similar to the one observed in petunia transformants, additional *ap1*-like characteristics were visible in the transgenic *Arabidopsis* plants. Because the phenotype is partially reminiscent of the *Arabidopsis ap1* mutant, we tested the ability of UNSΔMI to dimerise with the *Arabidopsis* AP1 protein. The positive results obtained with the yeast two-hybrid system confirmed our assumption of a dominant-negative action of the petunia protein in the floral whorls. This was also supported by the partial complementation that occurred in plants overexpressing both *AP1* and *UNSΔMI*.

The truncated UNS protein can sequester its dimerisation partners in the cytoplasm, preventing them from entering the nucleus and thus from functioning as transcriptional activators. Thus, the phenotype observed in transgenic flowers is due to a dominant-negative action of UNSΔMI towards other MADS box proteins, possibly involved in the maintenance of floral characteristics in floral organs. Due to the identical phenotypic alterations produced by the wild type UNS protein, we propose that a similar action towards the corresponding floral factors is accomplished by UNS in the flower domains. However it cannot be discriminated at this stage whether UNS is acting directly on specific target genes in a dominant-negative way, or whether it simply prevents other proteins to activate transcription by taking them away. In the first hypothesis, UNS would still bind target sites present in promoters, but without interacting with the correct transcription activator partners, it would prevent an active transcription complex to assemble at the same binding site. This competition between dominant-negative form and an active transcription factor complex has been shown to occur in mammals with the murine SRF factor (Belaguli et al., 1999). It has also been proposed as a possible mode of action for the negative regulator of floral transition SVP in *Arabidopsis* (Hartmann et al., 2000). Additional evidence should come from loss of function mutant phenotypes. Unfortunately a knock out mutant of *UNS* did not result in phenotypic alterations. Most likely *UNS* is redundant with other closely related

MADS box genes, such as *FBP21* and *FBP28*. Based on the results described in this paper we suggest a role for UNS in maintaining the vegetative program in petunia by suppressing a factor required for the induction of floral identity. A candidate for such a floral identity gene could be AP1 or another gene of the AP1 clade with a similar meristem identity function. Although a number of petunia MADS box genes belonging to this subfamily has been cloned, loss-of-function mutants of these genes are required in order to verify whether they could phenocopy the *uns* mutant phenotype.

Material and methods

Plant material

Petunia hybrida lines W115 and W138, *Arabidopsis thaliana*, ecotype Wassilevskija (Ws), and transgenic plants were grown under normal greenhouse conditions (22°C, 14/10 hr light/dark).

Screening of a cDNA library and DNA sequence analysis

The *UNSHAVEN (UNS*, accession number AF335238*)*, *FLORAL BINDING PROTEIN21 (FBP21*, accession number AF335239*)*, *FBP22* (accession number AF335240*)*, *FBP28* (accession number AF335244) and *FBP29* (accession number AF335245) cDNA clone were isolated from a young inflorescence petunia cDNA library, constructed in the Stratagene hybriZAP™ vector, according to manufacturer's instructions (Stratagene, Catalogue #235601). Approximately 100,000 plaques were screened with a mixed MADS box probe containing the 5' terminal sequence of *FBP1* (362bp) and *FBP2* (364bp) genes (Angenent et al., 1992). Hybridisation and washing of the Hybond N^+ membranes (Amersham) were done under low-stringency conditions (60°C hybridisation and wash with 2X SSC [1X SSC is 0.15M NaCl, 0.015M sodium citrate] at 60°C). 38 Clones were isolated and purified, and the pAD-GAL4 phagemids were excised in vivo according to the Stratagene protocol.

Nucleotide and amino acid sequence comparisons were performed using the ClustalW multiple sequence alignment program (Thompson et al., 1994). Amino acid alignments including the MADS box, the I region and the K box, were used to obtain the dendrogram with the Protdist and neighbour-joining (UPGMA method) programs of the PHYLIP 3.5c package (provided by Felsenstein J., Department of Genetics, University of Washington, Seattle, WA). Included in the dendrogram are also sequences of the *Arabidopsis* SOC1, AGL14, AGL19, SVP and AGL24, the tobacco MADS1, SaMADSA from *Sinapis alba* and the tomato TDR3 MADS box protein. AP3 from *Arabidopsis* was used as outgroup.

Construction of binary vectors and plant transformation

The complete open reading frame (ORF) of *UNS* was generated by PCR using a 5' primer on the ATG containing a HindIII site and a reverse primer designed after the STOP codon with a KpnI site. The PCR fragment was placed downstream the double 35S promoter in the pGD120 plasmid (Immink et al., 2002), the expression cassette was subsequently cloned into the pBIN19 binary vector (Bevan, 1984). The same cloning strategy was used to generate the *API* overexpression vector. The truncated open reading frame, *UNSΔMI*, lacking the MADS box and the I region, was also generated by PCR using the 5' primer: 5'-GGATCC**ATG**CAAACTGAAAATCAAGCTGGG-3', which introduces an ATG at bp231 of the original ORF and a BamHI recognition site, and the 3' primer: 5'-GGATCCGAAGAGGGATAGGTAGTCACC-3', containing a BamHI site.

Transformation of petunia plants was performed as described before (Colombo et al., 1995). Transformation of *Arabidopsis* plants, ecotype Ws, was performed using the floral dip method as described in Clough and Bent (1998); C58C1 is the *Agrobacterium* strain used for the transformation.

RNA gel blot analysis and in situ hybridisation

Total RNA was isolated from different tissues of petunia and *Arabidopsis* plants, according to Verwoerd et al. (1989). 10□g of the total RNAs, denaturated with 1.5M glyoxal, were fractionated on a 1.4% agarose gel and blotted onto Hybond N^+ membrane. The *UNS* gene specific fragment was used as a probe for hybridisation. The probe was labelled by random oligonucleotide priming (Feinberg and Vogelstein, 1984) and blots were hybridised as described by Angenent et al. (1992).

In situ hybridisations were performed as described by Canas et al. (1994). Digoxigenin-labelled RNA probes were synthesised by T7 polymerase-driven *in vitro* transcription, from the PCR fragment containing the full-length *UNS* ORF, according to the instructions of Boehringer Mannheim. The amplification product was obtained with the 5' primer: 5'- GTTCCTTGAAACATCTAAAAGG-3', and the 3' primer: 5'-TAATACGACTCACTATAGGGATAGGTAGTCACCAATTA-ATTC-3', containing the T7 polymerase promoter site.

Detection of UNS knock-out mutants

A modified version of the PCR-based screening described by R. Koes and collaborators (1995) was adopted to identify dTph1 insertions in the *UNS* gene. The *UNS* specific reverse primer 5'-TTCATATTGCTTGCCTCTAGGTG-3' designed at the end of the MADS box and the dTph1 inverted repeat primer with an extra EcoRI site (5'-GAATTCGCTCCGCCCCTG-3') were used in the PCR reactions

on two different populations of 3840 plants each. The PCR products, blotted onto Hybond N^+ membrane, were hybridised with the *UNS* cDNA.

Scanning electron microscopy

Samples were mounted on a stub, frozen in liquid nitrogen, coated and observed as described in Angenent et al. (1995).

Protein-protein interactions

The full length UNS and the truncated UNSΔMI were tested for dimerisation with the *Arabidopsis* APETALA1 MADS box protein, using the Stratagene hybriZAP™ two-hybrid system (Catalogue #235601). The yeast strain used in the experiments was PJ69-4A (James et al., 1996). Full-length ORF of *UNS* and *AP1* were generated by PCR using gene specific primers which introduced a 5'- *EcoRI* site before the ATG and a 3'-*SalI* after the STOP codon, for in-frame fusion in the pBD-GAL4 and pAD-GAL4 vectors. The truncated open reading frame, *UNSΔMI* was also generated by PCR using the 5' primer: 5'-GAATTC**ATG**CAAACTGAA-AATCAAGCTGGG-3', which introduces an ATG at bp231 of the original ORF and an *EcoRI* recognition site, and the same 3' primer used for the full-length clone. Yeast transformation, selection on supplemented synthetic medium lacking *Leu*, *Trp* and *Ade*, and β–galactosidase activity assay, were performed according to the manufacturer's instructions.

Construction of CFP/YFP plasmids

C-terminal in-frame fusion proteins between FBP9, UNS or UNSΔMI and CFP or YFP proteins were obtained by PCR amplification of the complete ORF of the MADS box genes and subsequent cloning in pECFP and pEYFP (Clontech catalogue #6075-1 and #6004-1, respectively). MADS box gene specific primers, introducing a *SalI* restriction site at the 5'-end and a *BamHI* site at the 3'-end, were used in the PCR reactions. The 3'- *BamHI* site replaces the endogenous stop codon. The obtained plasmids were digested with *SalI* and *XbaI* and the chimerical genes were cloned in the expression vector pGD120 (pUCAP containing the expression cassette of the binary vector previously used for plant transformation).

Protoplast transfection

Protoplasts were prepared from W115 petunia leaves and transfected as described by Denecke et al. (1989). Prior protoplasts isolation, leaves were surface sterilised by incubation in 1% bleach for 20 minutes. All measurements and imaging experiments were done after overnight incubation at 25 degrees in the dark.

Localisation studies

The localisation of the fluorescent fusion proteins was analysed by Confocal Scanning Laser Microscopy analyses (CSLM 510, Carl-Zeiss, GMBH Germany). Protoplasts were excited by a 458 and a 514 nm Ar laser lines controlled by an acousto optical tunable filter (AOTF). For CFP a HFT458 dichroic mirror and BP470-500 emission filter were used and for YFP a HFT514 dichroic mirror and BP535-590 IR emission filter. Images were obtained with a 40x oil immersion objective. Protoplasts were scanned with step size of 101 µm (X-axis) by 7.4 µm (Y-axis). The pinhole was set at 60µm, corresponding to a theoretical thickness of about 1µm. Images were analysed and adapted with Zeiss LSM510 software.

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Chapter 5

Development of a general method to obtain dominant-negative mutations for plant MADS box transcription factors

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Abstract

Mutant analysis is a frequently used and successful tool to elucidate gene functions. A way to generate mutations for a specific gene is by a dominant negative strategy. In theory, overexpression of a truncated or mutant form of a specific protein can disrupt the function of the wild-type endogenous protein. This strategy has proven to be successful for a few plant MADS box transcription factors. In this study we followed several strategies to obtain dominant-negative forms of plant MADS box transcription factors. Various dominant-negative constructs were generated for PETUNIA FLOWERING GENE (PFG) and the *Arabidopsis* protein APETALA1 (AP1). In addition, a fusion protein was made between part of the AP1 protein and a strong transcriptional repressor domain from vertebrates. Overexpression of none of these constructs resulted in a suppression phenotype, demonstrating that a general strategy to obtain a dominant-negative MADS box protein is difficult to design. Nevertheless, the work presented here gives new insights in the role that the conserved putative threonine phosphorylation site in the MADS domain plays in MADS box protein functioning. Furthermore, it shows that the presence of a farnesylation site in the C-terminus of AP1 is not essential to obtain an *AP1* overexpression phenotype.

Introduction

The identification of plant gene functions has been accelerated by the elucidation of the genomic sequence of the model species *Arabidopsis thaliana* (The Arabidopsis Genome Initiative, 2000). The function of many genes can be predicted based on sequence similarities to known genes, however, for the majority of the genes mutant analysis is still required to elucidate their function. Also for the modification of agriculturally important traits in crop plants genes have to be disrupted or overexpressed. Classical approaches to inactivate genes and thereby their functions are transposon or T-DNA insertions, antisense and cosuppression technology and more recently, RNA interference (RNAi)(Chuang and Meyerowitz, 2000).

The potential of transposons for genetic studies was recognised decades ago and targeted gene disruption by transposable elements has been often used successfully to inactivate gene functions (reviewed by Walbot, 1992). A disadvantage of this technology is the low frequency of mutagenesis accompanied by labour intensive large screening experiments. Furthermore, mutations obtained by this method are recessive and can neither be regulated nor transferred to other

species. A general drawback is also that in case of multi-copy genes or functional redundancy, each copy needs to be inactivated separately. The same disadvantages hold for gene disruption by targeted T-DNA insertion (reviewed by Krysan et al., 1999). Some of the mentioned shortcomings can be circumvented by gene silencing techniques like antisense, cosuppression and RNAi. Introduction of a single silencing construct can be sufficient to inactivate multiple copies of the same gene or various functional redundant genes, providing that they are homologous in DNA sequence. A mutation introduced by this method acts, in general, as a dominant trait. Nevertheless, due to the mode of action it is possible that genes homologous in sequence to the target gene but with a different function are silenced along with the target gene. Therefore, methods that are developed to affect gene functions in a dominant way rather than acting on DNA sequences are desirable. In nature some examples of mutations exist that behave more or less according these criteria. For instance in maize a dominant inhibitory allele has been identified for the regulatory gene *C1*, involved in anthocyanin pigmentation (Coe, 1962). More recently a study on the human MADS box gene *SRF* revealed that primary RNA transcripts of this gene are alternatively spliced, giving rise to a functional full-length protein and a C-terminal truncated protein (Belaguli et al., 1999). The truncated version appeared to compete with the full-length protein and acts as a natural dominant-negative regulator that is able to block specific SRF functions. Already in 1987 Herskowitz suggested to make use of such "dominant-negative" strategies to block specific protein functions. Proteins have multiple functional domains and by deleting a specific domain an inactive mutant protein is formed that theoretically can disrupt the activity of the endogenous wild-type protein upon ectopic expression. An additional advantage of dominant negative proteins is that tissue-specific or inducible promoters can control their expression spatially and temporally. A prerequisite for generating dominant-negative mutations is however that knowledge should be available about functional domains and the mode of action of the proteins to be inactivated.

Members of the plant MADS box transcription factor family play important roles in many developmental processes and their structural domains are well characterised (reviewed by Riechmann and Meyerowitz, 1997). The conserved N-terminally located MADS domain is involved in DNA binding and plays a role in dimerisation for at least some plant MADS box proteins. The second conserved domain, named K-box, has demonstrated to be involved in dimerisation specificity and acts together with the more variable I-region located between the MADS and K-box. The function of the C-terminal domain is not completely understood, although for some MADS box proteins it has been shown that it functions as a transcription activation domain (Cho et al., 1999; Goto et al., 2001). For some other

MADS box proteins, e.g. the class B proteins DEFICIENS and GLOBOSA from *Antirrhinum*, the C-terminus mediates ternary complex formation (Egea-Cortines et al., 1999; Honma and Goto, 2001). Because plant MADS box proteins bind DNA target sites only as dimers (Riechmann et al., 1996), overexpression of a truncated MADS box protein lacking DNA binding capacity but still able to dimerise can give rise to the formation of inactive dimers and consequently a dominant-negative effect. For the vertebrate MEF2 MADS box proteins and MCM1 of yeast, several studies describe the use of dominant-negative mutants with either mutations in the DNA binding MADS domain or in the C-terminal domain, both leaving the protein-protein interaction domains intact (Molkentin et al., 1996; Kuo et al., 1997; Ornatsky et al., 1997; Acton et al., 2000; Okamoto et al., 2000). In contrast, just a few dominant-negative mutations have been reported for plant MADS box proteins. Mizukami et al. (1996) showed for the first time a strategy to obtain dominant negative mutations for plant MADS box transcription factors. Ectopic expression of a truncated AGAMOUS (AG) protein in *Arabidopsis*, lacking the C-terminal domain resulted in an *ag* mutant phenotype. A second construct encoding AG lacking both the K-box and C-terminus appeared to have a much less severe phenotype and affected only floral determinacy. In rice overexpression of *OSMADS1* with point mutations in the MADS domain led to dominant-negative effects (Jeon et al., 2000) and more recently, Tzeng and Yang (2001) demonstrated that dominant-negative mutations can even be generated heterologously. They showed that overexpression of a N-terminally truncated LMADS1 mutant protein from lily was sufficient to block the functioning of the B-type MADS box protein APETALA3 in *Arabidopsis*.

An alternative way to obtain dominant negative mutations for transcriptional activators is by engineering fusions with transcriptional repressor domains. Margolin et al (1994) analysed fusion proteins consisting of the GAL4 transcription factor binding domain and various domains of the KOX1 zinc finger protein and revealed that the so-called Kruppel-associated box (KRAB) is essential and sufficient for transcriptional repression.

Over the last decade a few successful dominant-negative mutants were generated for plant MADS box proteins by introducing various point mutations and deletions in functional domains. However, whether the followed strategies are applicable for all plant MADS box proteins, is not known. Therefore, we analysed the phenotype obtained by overexpression of mutant forms of the *PETUNIA FLOWERING GENE* (Immink et al., 1999). Furthermore, we tested several new deletions and point mutations for the *Arabidopsis* APETALA1 (AP1) MADS box protein including a fusion of AP1 with the putative KRAB transcriptional repressor domain.

Results

Overexpression of truncated PFG proteins

To test whether a dominant negative C-terminal truncation, as described for AGAMOUS (AG) (Mizukami et al., 1996), is also applicable for other MADS box transcription factor proteins, a similar deletion was generated for PETUNIA FLOWERING GENE (PFG), resulting in the construct PFGΔC (Fig. 1A). It has been demonstrated that cosuppression of *PFG* in petunia resulted in non-flowering plants (Immink et al., 1999) and we aimed to obtain the same phenotype by a dominant negative strategy. Besides PFGΔC two more truncated PFG constructs were made, one encoding the PFG MADS and I-region (PFG-MI), comparable to the AG- MI protein (Mizukami et al., 1996), and the other one encoding PFG lacking 14 amino acid residues at its N-terminus and eight at its C-terminus (PFGΔNC) (Fig. 1A). The latter construct was designed based on comparison between the MADS domains of plant MADS box proteins and MCM1, MEF2A and SRF (Riechmann and Meyerowitz, 1997), which revealed that the N-terminal half of this domain contains conserved amino acid residues that probably play important roles in DNA binding but are not involved in dimerisation. A small deletion at the C-terminus was made, because the last few amino acid residues of this domain are the most conserved and therefore may possess a general role in the functioning of the C-terminus. All three truncated PFG forms were placed under control of the strong constitutive CaMV-35S promoter and were transformed to petunia W115 plants. Based on northern blot analyses (Fig. 1B) plants with ectopic expression of *PFGΔC* (#. 3 and 4) and plants ectopically expressing *PFGΔNC* (# 3 and 5) could be selected. Remarkably, no transgenic plants were obtained for *PFG-MI,* even not after a repeated transformation experiment. Phenotypic analysis of primary *PFGΔC* and *PFGΔNC* transformants revealed no alterations and therefore plants showing expression of the transgene were selfed. In parallel more primary transformants were generated. The progenies and new primary transformants were analysed both phenotypically and by northern blot analyses (not shown). In spite of a clear over-expression in some plants, again no phenotypic changes were apparent.

Arabidopsis APETALA1 mutations

To get more insight in the function of particular structural domains of MADS box proteins and to develop a general dominant negative strategy, more mutant forms were tested. For these purposes a switch was made to the *Arabidopsis* MADS box protein APETALA1 (AP1) because both *AP1* overexpression and knockout phenotypes have been described (Mandel et al., 1992; Mandel and Yanofsky, 1995).

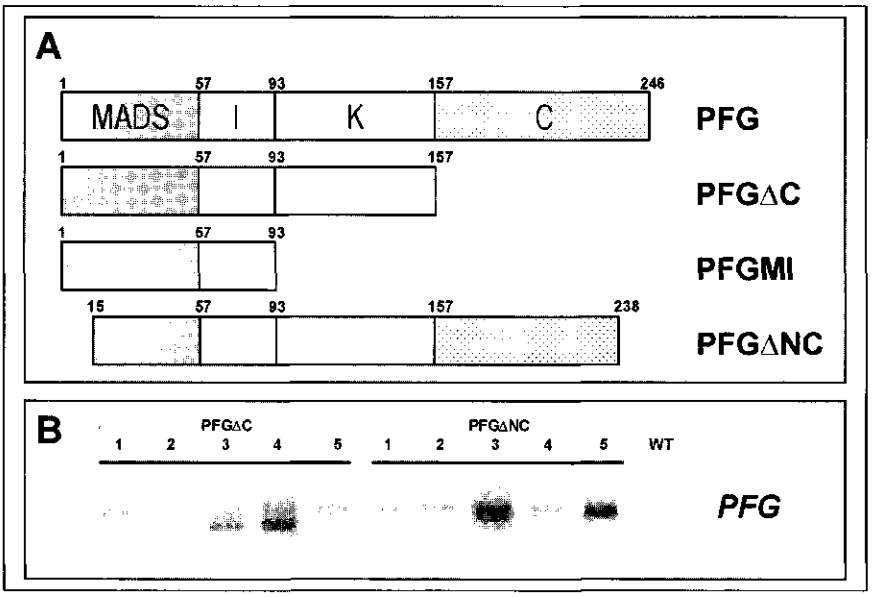

Figure 1: PETUNIA FLOWERING GENE (PFG) dominant negative mutations. A) Schematic representation of truncated PFG proteins. On top the structure of the wild-type PFG protein (246 amino acid residues). Truncations are marked with a "Δ". B) Expression of mutated *PFG* genes in leaves of primary transformants. On the right the expression of the endogenous *PFG* gene in leaves of wild-type (WT) petunia plants. N, N-terminus; M, MADS-box; I, I region; K, K-box; C, C-terminus.

In figure 2A an overview is given of all *AP1* mutations that were generated. Because large truncations probably lead to instable proteins due to wrong protein folding, more subtle mutations were made. In the construct AP1ΔC only the last 11 amino acid residues of the C-terminus were deleted in stead of the complete C-domain. In a second construct the N-terminal half of the MADS domain was removed (AP1ΔM), including half of the conserved bipartite nuclear localisation signal (NLS). In addition to these truncations 4 different point mutations were generated in the MADS box domain, two in the putative conserved "RQVT" phosphorylation site, one in the conserved bipartite NLS and one in a conserved lysine residue. The Threonine (T) of the putative "RQVT" phosphorylation site had been changed into Alanine (A) and the negatively charged Aspartic acid (D). Molkentin et al. (1996) showed that either phosphorylation or the presence of a negatively charged amino acid residue at the site where normally phosphorylation occurs, enhances DNA binding activity of the MADS box transcription factor MEF2C drastically. Based on this result we hypothesised that a change of the T into an A removes the phosphorylation site and results in a dominant negative

effect. The modification into the negatively charged D may result in a hyperactive DNA binding protein.

All modified cDNAs encoding for the mutated AP1 proteins were placed under control of the CaMV-35S promoter and constructs were transformed to wild-type *Arabidopsis* plants. To be able to select primary transformants showing overexpression of the transgene, northern blot analyses were performed on leaf tissue, where the endogenous *AP1* gene is normally not expressed (Fig. 2B, not all shown). From each transformation at least five independent transformants with different ectopic expression levels were selected for further analyses. For comparison of phenotypes, figures 2C and D show a wildtype *Arabidopsis* inflorescence and a lateral inflorescence, respectively. Fig. 2E shows a flower of the *ap1-1* mutant (Koornneef and Dellaert, 1982; Mandel et al., 1992), which is characterised by leaf-like organs in whorl one with secondary flowers in their axils and a lack of petals in whorl two. Fig. 2F presents a plant with strong *AP1* overexpression phenotype, which exhibits early flowering, curled rosette leaves and an inflorescence that immediately terminates in a composite terminal flower (TFL) structure. Phenotypic analysis of the primary AP1ΔC transformants revealed an *AP1* overexpression phenotype in stead of the expected dominant negative effect. All selected primary AP1ΔC transformants had curled leaves (Fig. 2G) and their secondary inflorescences terminated in TFL structures. In high overexpressors these determinate composite flowers were also found on the primary inflorescence (Fig. 2H) resembling the phenotype of plants overexpressing the full-length *AP1* (Fig. 2F; Mandel and Yanofsky, 1995). Plants expressing *AP1ΔM*, *AP1-T20A* or *AP1-R24A*, did not show any phenotypic alterations. In contrast, *AP1-T20D* and *AP1-K30A* transgenic plants revealed a weak *AP1* overexpression phenotype, which is characterised by curled leaves and TFL structures in secondary and lateral inflorescences (Fig. 2I). Unfortunately, none of the tested constructs resulted in a clear dominant-negative effect.

To study the effect of the various AP1 constructs on flowering time, seeds from lines with high expression of the transgenes were grown under both short day (SD, 10:14h) and long day (LD, 16:8h) conditions. For all plants, the number of days till flowering was scored as well as the number of rosette leaves at the moment of bolting. The results of this analysis are summarised in Table 1. The obtained results reveal that all lines with curled leaves and TFL structures are early flowering under both short and long day conditions. Overexpression of these particular *AP1* mutants resembles overexpression of-full-length *AP1* and hence the mutated amino acids or truncated domains seem not to play an important role in AP1 functioning. Nevertheless, in all the mutants the obtained effects are less severe than those observed for the full-length *AP1* overexpressors (Fig. 2). For a

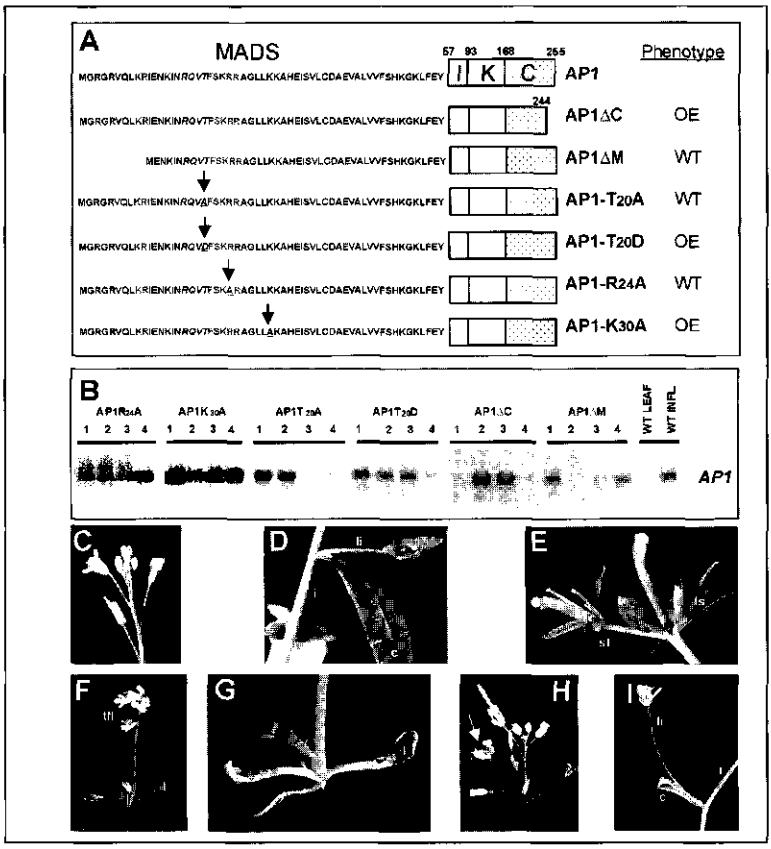

Figure 2: APETALA1 (AP1) dominant negative mutations. A) Schematic representation of generated AP1 constructs. On top the structure of the wild-type AP1 protein (255 amino acid residues). The putative threonine phosphorylation site in the MADS domain is in italics. The bipartite nuclear localisation signal is marked in red. Generated point mutations are underlined and indicated with an arrow. Truncations are marked with a "Δ", C means C-terminus and M is MADS box. On the right the phenotype of plants overexpressing various mutant genes under the control of the CaMV35S promoter is described. WT indicates similar to wild-type and OE means an *AP1* overexpression phenotype. B) Expression of various mutated *AP1* genes in leaves of primary transformants. On the right the expression of the endogenous AP1 gene in the inflorescence of wild-type (WT) *Arabidopsis* plants. No signal is obtained in wild-type leaves. C) Inflorescence of a wild-type *Arabidopsis* plant (Ws-3). D) Lateral inflorescence of a wild-type *Arabidopsis* plant. E) Flower of the *ap1-1* mutant (Ler). F) AP1 overexpression plant. G) Rosette leaves of primary AP1ΔC transformants. H) Terminal flower structures (marked with an arrow) in the primary inflorescence of AP1ΔC transformants. I) Lateral inflorescence of AP1-T20D transformant. The cauline leaf is curled and the lateral inflorescence terminates immediately with a composite floral structure. c = cauline leaf, i = primary inflorescence, l = curled rosette leaf, li = lateral inflorescence, ls = cauline-leaf-like organ, tfl = composite terminal flower structure, sf = secondary flower.

Construct	ecotype	Long Day			Short Day		
		Number of rosette leaves	Days till bolting	phenotype	Number of rosette leaves	Days till bolting	phenotype
Wildtype	Ws-3	7,3 (±1,1)	21 (± 1)	wt	10,0 (±1,3)	34 (±2)	wt
AP1ΔC	Ws-3	3,6 (±0,5)	15 (±1)	cl, TFL	4,3 (±0,7)	22 (±4)	cl, TFL
AP1ΔM	Ws-3	5,8 (±0,6)	21 (±1)	wt	8,3 (±2,1)	29 (±2)	wt
AP1-T20A	Ws-3	5,7 (±0,5)	24 (±3)	wt	8,7 (±0,5)	29 (±3)	wt
AP1-T20D	Ws-3	5,2 (±0,7)	20 (±2)	cl, TFL	7,7 (±0,7)	27 (±3)	cl, TFL
Wildtype	Col-0	12,9 (±1,1)	28 (±2)	wt	28,9 (±3,8)	59 (±5)	wt
AP1-R24A	Col-0	11 (±1,9)	24 (±2)	wt	27,6 (±7,2)	48 (±7)	wt
AP1-K30A	Col-0	9,8 (±3,5)	23 (±3)	cl, TFL	10,6 (±2,7)	28 (±3)	cl, TFL

Table 1: Analysis of flowering time under long and short day conditions in *Arabidopsis* lines overexpressing various mutant forms of *APETALA1*. The number of rosette leaves at the moment of bolting was determined as well as the number of days till bolting (n = 12). wt = wildtype, cl = curled leaves, TFL = terminal flower structures in lateral and secondary inflorescences. The standard deviation is indicated between brackets.

few of the tested constructs no aberrant phenotype was obtained. The most likely explanation for this observation is that these constructs do not produce an active AP1 protein or that the activity is insufficient to give rise to an AP1 overexpression phenotype. Another way to monitor the AP1 activity of these constructs is by analysing whether they are able to complement the *ap1* mutant. Therefore, we overexpressed one of the constructs that gave no phenotype (AP1-T20A) in the *ap1-1* mutant (Ler background). Analysis of primary transformants revealed no substantial complementation of the *ap1-1* mutation, demonstrating that this mutant form is not active (results not shown).

Analysis of plants expressing an AP1-repressor domain fusion

None of the tested deletions and point mutations in AP1 appeared to result in the desired dominant-negative effect. Because AP1 has shown to have a potential transcriptional activation domain (Cho et al., 1999), a new strategy was followed in which the AP1 C-terminal domain containing the activation domain is replaced by a putative transcriptional repressor domain. In this way we anticipated that the AP1 transcription activator is converted into a transcriptional suppresser. As repressor domain the Kruppel-associated box (KRAB A) found in C_2H_2 zinc finger proteins was used (Margolin et al., 1994). Fusions between AP1 lacking the C-terminal

transcription activation domain and the KRAB A box of KOX1 (AP1MIK-KRAB) were overexpressed in *Arabidopsis* Col-0 plants under control of the constitutive CaMV-35S promoter. Nine out of 33 primary transformants exhibited a weak *AP1* overexpression phenotype. The other plants showed no alterations.

Discussion

In this study various experiments were performed to obtain a general dominant-negative strategy to suppress plant MADS box transcription factor functions. Unfortunately, none of the tested strategies resulted in the desired dominant-negative effect. Nevertheless, the obtained results give new insights in the function and importance of conserved motifs and specific MADS box protein domains.

PFG mutations

The *PETUNIA FLOWERING GENE* (*PFG*) was chosen as a first target for generating a dominant-negative mutation, because cosuppression of this gene resulted in a non-flowering phenotype (Immink et al., 1999). In contrast to what was found in *Arabidopsis* for the C-type MADS box protein AGAMOUS (AG, Mizukami et al., 1996), overexpression of truncated PFG versions lacking the C-terminus had no phenotypic effect. A possible explanation for this failure to produce a suppression phenotype could be a wrong folding of the truncated PFG protein. However, because type II lineage MADS box proteins have a conserved domain structure (Alvarez-Buylla et al., 2000) and overexpression of AGΔC, which was made in a similar way, resulted in a dominant-negative phenotype (Mizukami et al., 1996), this explanation is not very likely. Alternatively, the C-terminus from PFG has another function than the C-terminus of AG and is not essential for PFG functioning. Comparison between C-domains of various MADS box proteins is difficult because of the variability in sequence and the lack of clear motifs in this domain and therefore no conclusions can be drawn in this direction. Taken into account the high expression level of the endogenous *PFG* gene and its broad expression pattern throughout the plant live cycle (Immink et al., 1999), it is more likely that the lack of a dominant negative effect is due to incompetence to compete out the endogenous PFG protein. This means that the overall expression level of *PFGΔC* is just too low compared to the endogenous *PFG* expression level.

APETALA1 functioning and prenylation

In the second part of the work presented here APETALA1 (AP1) from *Arabidopsis* was used as a target to obtain a general dominant negative strategy. One of the first tested constructs was a truncated AP1 protein, lacking 11 amino acid residues at its

C-terminus (AP1ΔC). The last four amino acid residues of the AP1 C-terminus are CFAA, which may present a so-called CaaX box recognition motif for farnesyltransferase (FTase, Yalovsky et al., 2000). This motif appeared to be conserved among AP1 proteins in various plant species. Overexpression of AP1 with a mutated CaaX box did not give rise to composite terminal flower structures (TFLs) and therefore Yalovsky and colleagues (2000) suggested that this motif has an important role in AP1 functioning. In contrast to their results we obtained an *AP1* overexpression phenotype for both AP1ΔC and AP1-MIK-KRAB, including the formation of TFLs in lateral shoots and secondary inflorescences. Nevertheless, TFLs were found very rarely in the primary inflorescences of these transformants despite high expression levels and therefore the last 11 amino acid residues of the AP1 protein seem to play at least a minor role in this process. Studies in the past have shown that a chimeric AP1 protein containing the AG C-terminus (Krizek and Meyerowitz, 1996 and Krizek et al., 1999) and PEAM4, the AP1 ortholog of pea, which lacks a CaaX motif (Berbel et al., 2001) are able to induce the formation of TFLs. Taken this all together, we concluded that farnesylation plays no important role in the formation of TFLs upon overexpression of *AP1* and *AP1* like MADS box genes. However, based on the experiments done we can not rule out that farnesylation is important for the functioning of the endogenous AP1 protein.

APETALA1 functioning and nuclear localisation

MADS box proteins are supposed to function as transcription factors and therefore need to be nuclear localised to become functional. The MADS domain of AP1 contains a bipartite nuclear localisation signal (NLS, Dingwall and Laskey, 1991), which is conserved among the type II lineage of plant MADS box proteins. Mc Gonigle et al (1996) showed that nuclear localisation of the MADS box proteins APETALA3 (AP3) and PISTILLATA (PI) is dependent on their simultaneous expression. Recently, we demonstrated that dimerisation is a prerequisite for MADS box proteins to become nuclear localised and that both dimerisation partners need to contain the right signals for nuclear import (Immink et al., 2002). Therefore, in theory a MADS box protein with an inactive NLS is still able to interact with its dimerisation partner, but retains this partner in the cytoplasm resulting in a dominant-negative effect. To test this hypothesis, two AP1 mutants (AP1ΔM and AP1-R24A) were generated that both affected the NLS. Overexpression of either of the two constructs failed to produce phenotypic alterations, which demonstrates that both mutations inactivate the AP1 protein in such a way that no overexpression phenotype was obtained. Furthermore, the mutations were also insufficient to provoke a dominant-negative effect.

Localisation studies with the petunia MADS box protein FBP2, which contains a similar point mutation as in AP1-R24A, have revealed that nuclear import of the homodimer is completely blocked. However, as a heterodimer it was still able to enter the nucleus although at a much lower efficiency (Immink et al., 2002). Probably, also AP1-R24A is impaired in its nuclear import, which explains the absence of an overexpression phenotype.

APETALA1 functioning and phosphorylation

All type-II lineage plant MADS box proteins contain a conserved putative threonine phosphorylation site in their MADS domain. Overexpression of AG in which the conserved Threonine (Thr) was replaced by an Alanine (Ala) resulted in a dominant-negative mutation (Ma and Mizukami, 1992). In our study however, overexpression of AP1-T20A didn't lead to phenotypic changes of *Arabidopsis* plants. Furthermore, the mutated AP1-T20A construct was not able to complement the *ap1-1* mutation. These results indicate that the AP1-T20A construct is not functional which may explain the differences observed for AG (Ma and Mizukami, 1992) and AP1 (this study). In contrast, changing the conserved Thr into a negatively charged Aspartic acid residue resulted in an AP1 protein, which gave rise to a normal AP1 overexpression phenotype. These results suggest that a negative charge at the position of the Thr, either due to phosphorylation or due to the presence of a negatively charged amino acid residue, is essential for AP1 overexpression functioning.

Overexpression of AP1 and early flowering

Overexpression of the full-length AP1 protein (Mandel and Yanofsky, 1995), as well as overexpression of many mutated AP1 proteins gave rise to early flowering under both long and short day conditions. Remarkably, some of the mutants analysed in this study with either point mutations or deletions in their MADS domain did not show a significant effect on flowering time. In contrast, deletions at the C-terminus retain the capability of AP1to induce flowering. The same effect has been observed by Jeon et al (2000), when mutant forms of the supposed rice AP1 ortholog OSMADS1 were overexpressed in rice. They suggested that the early flowering phenotype observed upon overexpression of AP1-like MADS box proteins is not a gain of function phenotype, but caused by a dominant negative effect on a MADS box protein involved in the floral transition process. The results obtained in this study with the mutant forms of AP1, which result in the AP1 "overexpression" phenotype, strengthen this hypothesis. Recently, it was demonstrated that AP1 is able to interact with the SVP MADS box protein (Pelaz et al., 2001), which is a negative regulator of floral transition (Hartmann et al.,

2000). Therefore it is tempting to speculate that the early flowering effect by overexpression of AP1 or its mutant forms is due to interaction with this floral repressor and hence inhibition of its function. However, this is not in agreement with the results obtained with the constructs showing no phenotype, which are most likely still able to interact with such a floral suppresser. More likely, the early flowering phenotype in all the *AP1* overexpression plants is the result of a normal but precocious functioning of AP1. Our hypothesis is that AP1 interacts with SVP and this complex suppresses SVP, which induces the switch to floral development. The DNA binding domain of AP1 seems to play an essential role in this process, because almost all AP1 proteins mutated in this domain are not able to induce early flowering. The antagonistic expression patterns of *SVP* (Hartmann et al., 2000) and *AP1* (Mandel et al., 1992), are in line with this hypothesis.

Generation of a general dominant negative strategy

The work presented here suggests that a general strategy to obtain a dominant-negative MADS box transcription factor is difficult to obtain. In-spite of the relative high conservation in protein structure, it seems that the function of the different domains in a MADS box protein is less conserved, which is reflected in different responses to mutations in these domains. Another aspect that has to be considered when generating dominant negative mutations is that the mutant form has to compete with the endogenous proteins. This implies that the mutated protein has to be abundantly present and that its binding capacity to dimerisation partners is not affected. Therefore, a dominant-negative strategy for a particular MADS box protein can not easily be adopted for another member of this large family of transcription factors. More knowledge about the exact functions of the various domains and the role of specific amino-acid residues is needed. Large-scale site directed mutagenesis and domain swap experiments in combination with improved computational modelling and sequence analyses will give us more insights in the folding and functioning of plant MADS box transcription factors in the near future.

Material and Methods

Plant material

The *Petunia hybrida* variety W115 was used and grown under normal greenhouse conditions. From *Arabidopsis thaliana* the ecotypes Ws-3, Col and Ler were used. The *ap1-1* mutation is in a Ler background (Koornneef and Dellaert, 1982). All *Arabidopsis* plants were grown at 20-22 °C and 16h light for long day treatment and 10h light for the short day treatment.

Construction of binary vectors

All mutated open reading frames (ORFs) were obtained by PCR with PFU proof-reading polymerase (Stratagene) and cloned in binary vector pGD121, which was generated by cloning the CaMV-35S expression cassette from pGD120 (Immink et al., 2002) as an *AscI/PacI* fragment in pBINPLUS (Engelen et al., 1995). *PFGΔC* has been obtained by PCR on the *PETUNIA FLOWERING GENE (PFG)* full-length cDNA (Immink et al., 1999) with PRAT181 (forward primer on the ATG of *PFG*) and PRAT184 (reverse primer annealing after K-box, including new TAA stop codon), yielding a fragment of 471 bp. For the amplification of *PFGMI*, the same forward primer was used in combination with PRAT183 (reverse primer annealing downstream the I-region, including new TAA stop codon), giving a fragment of 279 bp. The *PFGΔNC* fragment (664 bp) was amplified with PRAT182 (forward primer annealing 42 nt after the start codon, including a new ATG) and PRAT185 (reverse primer annealing 24 nt upstream the end of the coding region, including a new TAA stop codon). To obtain the various *APETALA1* (*AP1*) mutated ORFs, PCR reactions were performed using the full-length *Arabidopsis AP1* cDNA from the ecotype Col-0 as template. *AP1ΔM* was amplified by a forward primer annealing 36 bp downstream the native start codon and a reverse primer annealing just after the stop codon. A new ATG was introduced from the forward primer. For *AP1ΔC* a PCR was performed with a forward primer annealing just in front of the ATG start codon and a reverse primer annealing 33 nt in front of the native stop codon and introducing a new stop at that position. All point mutations were obtained by site directed mutagenesis of pGD121 containing the full-length *AP1* ORF, according the Stratagene QuickChange™ Site-Directed Mutagenesis Kit (Catalogue #200518). The Kruppel-associated box (KRAB) was generated from six partially overlapping and complementary primers (Isogen) based on the sequence of the KOX1 KRAB A-box (Margolin et al., 1994). The codon usage of the primers had been plant optimised. The six primers were mixed (8.5 ng each), heated at 95°C for 10 minutes and cooled down slowly to room temperature for optimal annealing. The obtained fragment with the sequence 5'-

AGA ACC CTT GTG ACC TTC AAG GAT GTG TTT GTG GAT TTC ACT AGG GAA GAA TGG AAG CTC CTT GAT ACT GCT CAG CAG ATT GTG TAT AGA AAT GTG ATG CTT GAG AAT TAT AAG AAT CTT GTT TCT CTT GGA TAT TGA-3' was subcloned in pGEM-T-Easy (Promega). Subsequently, the *AP1MIK* fragment (528 bp) was generated by PCR and cloned in-frame with the KRAB domain. Finally, the resulting *AP1MIK-KRAB* fragment was cloned in binary vector pGD121. All generated binary constructs were confirmed by sequencing analysis (BigDye™ sequencing kit, Applied Biosystems).

Plant transformations

Petunia W115 was transformed using the standard leaf disc transformation method (Horsch et al., 1985) and transgenic plants were regenerated as described by Van Tunen et al. (1989). All *AP1* constructs were introduced into *Arabidopsis* by the floral dip method (Clough and Bent, 1998).

Expression analysis

Total RNA was isolated from petunia leaves according to Verwoerd et al (1989) and from *Arabidopsis* leaves with the Qiagen RNeasy® Plant Mini Kit. 10 µg of total RNA was denatured by glyoxal (1.5 M) prior to electrophoresis. Equal loading of samples was verified by ethidium bromide staining of the gel. Subsequently, RNA was blotted onto Hybond N^+ membranes (Amersham). *PFG* and *AP1* cDNA fragments containing the complete coding regions were radioactive labelled by the RadPrime DNA Labelling System (Gibco BRL®). Blots were hybridised as described previously (Angenent et al., 1992).

Chapter 6

Analysis of MADS box protein-protein interactions in living plant cells

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Abstract

Over the last decade, the yeast two-hybrid system has become the tool to use for the identification of protein-protein interactions and recently, even complete interactomes were elucidated by this method. Nevertheless, it is an artificial system that is sensitive to errors resulting in the identification of false-positive and false-negative interactions. In this study plant MADS box transcription factor interactions identified by yeast two-hybrid systems where studied in living plant cells by a technique based on Fluorescence Resonance Energy Transfer (FRET). Petunia MADS box proteins were fused to either Cyan Fluorescent Protein (CFP) or Yellow Fluorescent Protein (YFP) and transiently expressed in protoplasts followed by "FRET-SPectral Imaging Microscopy (SPIM)" and "FRET-Fluorescence Lifetime Imaging Microscopy (FLIM)" to detect FRET and hence protein-protein interactions. The three petunia MADS box heterodimers identified in yeast, using FBP11 as bait, could be confirmed in protoplasts. However, in contrast to the yeast two-hybrid results, homodimerisation was demonstrated in plant cells for FBP2, FBP5 and FBP9 petunia MADS box proteins. Heterodimers were identified between the ovule-specific MADS box protein FBP11 and members of the petunia FBP2 subfamily which are also expressed in ovules suggesting that these dimers play a role in ovule development. Furthermore, the role of dimerisation in translocation of MADS box protein dimers to the nucleus is demonstrated and the nuclear localisation signal (NLS) of MADS box proteins has been mapped to the N-terminal region of the MADS domain by means of mutant analyses.

Introduction

During the last decade the complete genome of many organisms has been unravelled by large-scale sequencing projects. The challenge of this moment is to elucidate the function of the proteins encoded by the large number of predicted and unknown genes. Many proteins are functional in a combinatorial manner and interact specifically with other proteins. Determination of these interaction patterns is an essential part of functional genomics research. Even the function of unknown proteins can be predicted based on the interaction with a functionally well-characterised protein (Mayer and Hieter, 2000; Legrain et al., 2001).

Yeast two-hybrid systems have shown to be powerful techniques to identify high-throughput protein-protein interactions (Fahena et al., 2000). Both Uetz et al. (2000) and Ito et al. (2001) used the yeast two-hybrid GAL4 system

successfully to analyse the complete yeast interactome. Remarkably, the data from these two studies do not largely overlap; among other causes for this situation are limitations of the genetic yeast two-hybrid GAL4 system. Incorrect protein folding, auto-activation of selection markers and wrong post-translational processing in yeast cells are just a few of the causes leading to false positives and negatives. Therefore, interactions identified by yeast systems require confirmation by other methods in a more natural environment. Often, immuno-precipitation has been used for this purpose (Freilich et al., 1999; Shi et al., 2000), although, a general drawback of this method is that specific antibodies against the protein of interest are required. Furthermore, is not known whether the precipitated proteins interact directly with the protein of interest and in which cell compartment the specific interaction takes place.

Recently, a non-invading method based on Fluorescence Resonance Energy Transfer (Stryer, 1978; Wu and Brand, 1994) became available for monitoring protein-protein interactions in living cells (Gadella et al., 1999; Miyawaki and Tsien, 2000). FRET is the phenomenon of energy transfer between a donor fluorescent molecule and a neighbouring chromophore, the acceptor, when the two fluorescent molecules come in very close proximity (<100□). This will actually be the case when two proteins tagged with two different fluorescent dyes are physically interacting. By means of this system the specific interaction between Bcl-2 and Bax in mitochondria of single intact mammalian cells has been demonstrated (Mahajan et al., 1998). Recently, Mas et al. (2000) used the FRET technique to show specific interaction between the *Arabidopsis* photoreceptors phyB and cry2 and translocation of the formed complex to nuclear speckles in a light dependent manner.

A complicating factor in determining FRET is that the ratio of donor to acceptor fluorescence, an indicator of FRET efficiency, is dependent on microscope optics and local relative concentrations of the donor and acceptor fluorescent molecules (Gadella et al., 1999). Detecting FRET in plant cells is even more complex because of direct absorption of fluorescence by chlorophyll pigments. To avoid these problems, the occurrence of FRET can be determined by Fluorescence Lifetime Imaging Microscopy (FLIM; Gadella, 1999). FRET decreases the fluorescence lifetime of the donor fluorescent molecule independent of local chromophore concentration and absorption of donor fluorescence by chlorophyll.

We aimed to study MADS box transcription factor interactions in living plant cells. In *Arabidopsis* this large family of transcription factors comprises at least 80 members (Riechmann et al., 2000). MADS box proteins form specific homo and/or heterodimers (Davies et al., 1996; Fan et al., 1997; Pelaz et al., 2001;

Immink et al., in preparation), which direct a variety of developmental processes (Theissen et al., 2000). Recently it has been shown that even higher order ternary complexes are formed between members of this transcription factor family (Egea-Cortines et al., 1999; Honma and Goto, 2001). Although, the ability to form a particular dimer or complex has been demonstrated in yeast for several MADS box proteins, no information is available about these physical interactions in living plant cells. Furthermore, it is not clear where exactly these specific interactions occur and how MADS box proteins are translocated to the nucleus, where they are functional as transcription factors. In this work the petunia MADS box protein FLORAL BINDING PROTEIN11, which is involved in proper ovule formation, has been studied. Cosuppression of the closely related MADS box genes *FBP7* and *FBP11* has resulted in the formation of carpeloid structures in stead of ovules, which demonstrates that these genes are involved in ovule identity specification (Angenent et al., 1995). Nevertheless, ectopic expression of *FBP11* under control of the constitutive CaMV 35S promoter revealed ovule formation on the adaxial side of sepals and rarely on the petals only (Colombo et al., 1995), indicating that besides FBP11 additional factors are needed for proper ovule formation, which are most likely present in sepals and petals as well. Since MADS box proteins are functional as dimers, the specific cofactors essential for ovule development are probably represented by heterodimerisation partners of FBP11. Therefore, putative interaction partners of FBP11 were isolated by means of the yeast two-hybrid GAL4 system. Subsequently, the new FRET-FLIM technology was applied to gain insight in MADS box transcription factor interactions in living plant cells and the intra-cellular localisation of the MADS box protein monomers and dimers was determined by Confocal Scanning Laser Microscopy (CSLM). The colocalisation of the interacting partners of FBP11 and the ability to form dimers in plant cells suggest a role for these transcription complexes in petunia ovule formation. In addition, the role of a Nuclear Localisation Signal (NLS) in nuclear import of MADS box proteins and the effect of dimerisation on this process will be discussed.

Results

Yeast two-hybrid analyses

A petunia ovary specific cDNA expression library was screened with FBP11 (Angenent et al., 1995; Colombo et al., 1995) as bait to identify interacting partners for this ovule specific MADS box protein. Yeast colonies positive for all three-reporter genes were selected as true interactors and the prey vector was further investigated by sequencing analysis. Except for a small domain of a ferrodoxin

protein, which was isolated twice, all positive clones contained full-length coding sequences of either MADS box protein FBP2 (isolated 4x) (Angenent et al., 1992), or one of the novel proteins FBP5 (4x) and FBP9 (3x). For all further analyses only these full-length clones were investigated.

In a subsequent experiment, FBP11 and its isolated interacting MADS box proteins were tested for their ability to homodimerise. For FBP11, no homodimerisation could be detected in the genetic yeast GAL4 system. The three related proteins, FBP2, FBP5 and FBP9 gave auto-activation of yeast reporter genes when expressed as bait proteins. This auto-activation by MADS box proteins can be abolished by deletion of the C-terminal domain containing the transcription activation domain (Moon et al., 1999). We followed this strategy for FBP2 and revealed specific heterodimers between the truncated protein and other MADS box proteins, including the heterodimer with FBP11 (Immink et al., in preparation). Nevertheless, homodimerisation between this truncated protein and the full-length FBP2 could not be identified. Subsequently, the ability of FBP2, FBP5 and FBP9 to form homodimers was analysed in the cytoplasmic yeast two-hybrid CytoTrap™ system, in which selection for interaction is not based on transcriptional activation (Aronheim et al., 1997). As in the yeast two-hybrid GAL4 system, no homodimerisation could be identified.

Biological characteristics of FBP11 interactors.

A first prerequisite for proteins to interact in a biological context is their presence in the same tissues and at the same developmental stage. To test whether this is the case for FBP11 and its putative interactors, expression analyses were performed. Because FBP11 has demonstrated to be involved in petunia ovule development, we focussed on this tissue for the expression analyses. In-situ hybridisations demonstrate that *FBP11* and *FBP2* have overlapping expression patterns in developing ovules (Figure 1A-C). In mature ovules, both genes are highly expressed in the endothelium surrounding the embryo sac. Northern blot analyses revealed that both *FBP5* and *FBP9*, which like *FBP2* belong to the *SEPALLATA* group of MADS box genes (Fig. 1F), are also strongly expressed in ovules (not shown). Transgenic petunia plants overexpressing *FBP11* under the control of the Cauliflower Mosaic Virus (CaMV) 35S promoter resulted in the formation of ectopic ovules on sepals and rarely on the tube of the petals (Colombo et al., 1995). Expression analyses on the sepals of these *FBP11* overexpression plants, demonstrated that *FBP2* is expressed in the ectopically formed ovules and in the epidermal cell layer (Fig. 1D). In wild type petunia flowers, *FBP2* is expressed in the epidermal cell layer on the adaxial side of sepals and in the petals (Angenent et al., 1992). For comparison the expression of the *PETUNIA FLOWERING GENE*

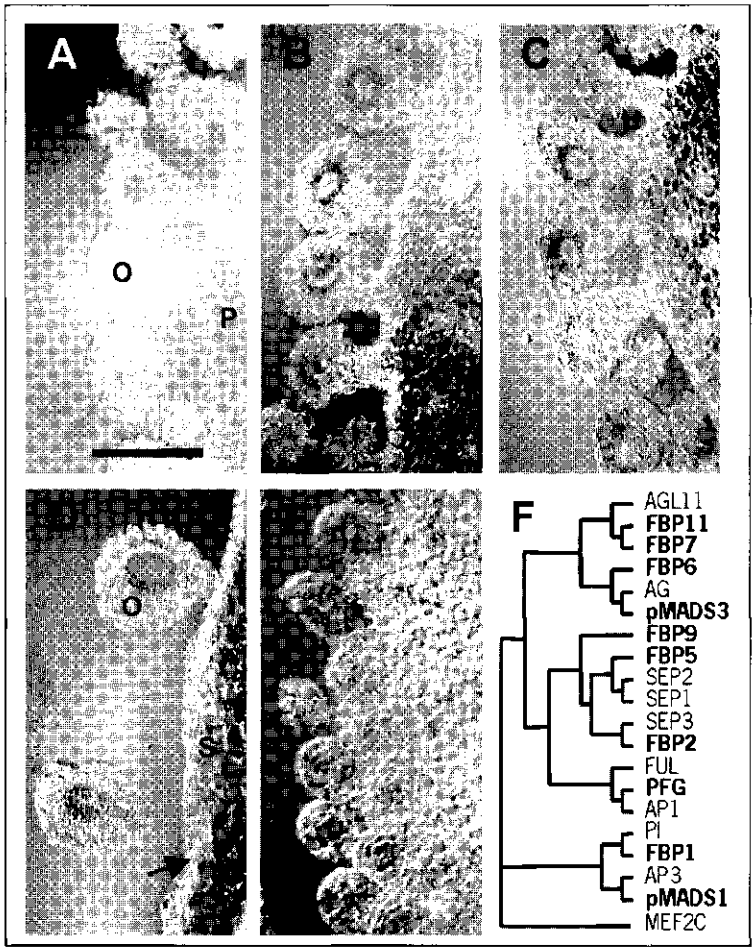

Figure 1*: Phylogenetic and expression analyses of *FBP11* and the genes encoding its interaction partners. Longitudinal sections were hybridised with digoxygenin-labelled probes (Red signal). (*A*) Almost mature W115 wildtype ovules, hybridised with a sense *FBP11* probe. (*B*) Same stage as (*A*), hybridised with *FBP11* antisense probe. (*C*) Same stage as (*A*), hybridised with *FBP2* antisense probe. (*D*) Sepal of CaMV35S::*FBP11* overexpression plant with ectopic ovule formation on adaxial side, hybridised with *FBP2* antisense probe. The signal in the epidermal cell layer is marked with an arrow. (*E*) Young developing ovules, hybridised with *PFG* antisense probe. (*F*) Phylogenetic tree of MADS box genes described in this study. As a reference *Arabidopsis* and petunia MADS box genes with a known function are included. All petunia MADS box genes are in bold. For the comparison of the proteins the MADS box, I region and K box domains were used. AP1 = *APETALA1*, AP3 = *APETALA3*, AG = *AGAMOUS*, AGL = *AGAMOUS-Like*, FBP = *FLORAL BINDING PROTEIN*, FUL = *FRUITFULL*, MEF2C = *Myocyte Enhancer Factor 2C*, O = ovule, PFG = *PETUNIA FLOWERING GENE*, PI = *PISTILLATA*, P = placenta, S = sepal (adaxial side) and SEP = *SEPALLATA*. Bar in (*A*) = 1.0 mm. ***) Figure in colour in appendix, page VI.***

(PFG) (Immink et al., 1999) in ovules of wildtype petunia plants is shown. Despite the co-expression of *PFG* and *FBP11* in ovules no interaction was detected between PFG and FBP11 by the yeast two-hybrid GAL4 system (Immink et al., in preparation).

Localisation of MADS box proteins in protoplasts

To localise FBP11 and its putative interacting partners FBP2, FBP5 and FBP9 inside a living plant cell, fusions of the MADS box proteins with Cyan Fluorescent Protein (CFP) and/or Yellow Fluorescent Protein (YFP) were made. These fusion proteins were transiently expressed in petunia leaf protoplasts and cells were imaged by Confocal Scanning Laser Microscopy (CSLM). Figure 2A shows the nuclear localisation of the FBP9-YFP fusion protein. In addition to an overall fluorescence of the nucleus, sometimes YFP signals were concentrated in small nuclear spots. Except for FBP11-CFP and FBP11-YFP, all fusion proteins tested were nuclear localised (results not shown). For the fusion proteins with FBP11, a fluorescent signal was only observed in the cytoplasm (Fig. 2B) and any nuclear import was absent. For all MADS box proteins analysed, no differences were obtained between CFP and YFP fusions.

In a subsequent experiment localisation of the supposed FBP11 heterodimers was monitored in living plant cells by transient expression of FBP11-CFP in combination with either FBP2-YFP, FBP5-YFP or FBP9-YFP. CSLM analyses revealed that all these combinations gave a fluorescent signal inside the nucleus (Fig. 2C, not all combinations shown). Surprisingly, also the FBP11-CFP signal was localised in the nucleus when co-expressed with one of its presumed interacting partners, while in contrast, the protein retained in the cytoplasm when expressed alone (Fig. 2B). For all three co-transfected combinations the CFP and YFP signals were exactly colocalised inside the nucleus, suggesting a physical interaction of the protein molecules.

Identification of MADS box protein interactions in living cells

Colocalisation of proteins, as identified for specific petunia MADS box protein combinations, is a first indication for physical interaction between the concerned proteins. However, all MADS box proteins contain the highly conserved DNA binding MADS box domain and are therefore expected to be functional as transcription factors and hence nuclear localised. To determine whether there is a physical interaction between specific MADS box proteins, both FRET-FLIM (Fluorescence Resonance Energy Transfer - Fluorescence Lifetime Imaging Microscopy) and FRET-SPIM (Fluorescence Resonance Energy Transfer - SPectral Imaging Microscopy) measurements were performed on petunia

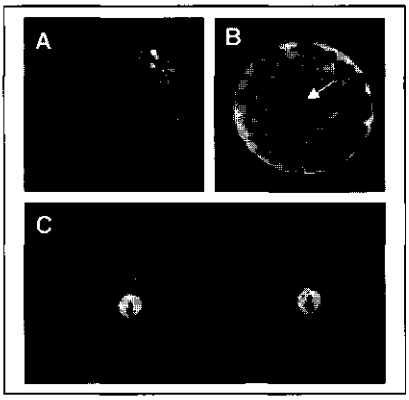

Figure 2*: Localisation of MADS box proteins in petunia leaf protoplasts imaged by Confocal Scanning Laser Microscopy (CSLM). Chlorophyll auto-fluorescence is shown in red. (*A*) Nuclear localised FBP9-YFP. (*B*) Cytoplasmic localised FBP11-CFP. The position of the nucleus is marked with an arrow. (*C*) Protoplast expressing FBP11-CFP in combination with FBP2-YFP. On the left, the nuclear localised CFP signal, on the right the YFP signal of the same protoplast. ***) Figure in colour in appendix, page VI.***

protoplasts expressing a specific MADS box protein-CFP/-YFP combination. Based on the results of the yeast two-hybrid analyses the putative heterodimers, FBP11-FBP2, FBP11-FBP5 and FBP11-FBP9, were selected. In addition, the combination FBP2-PFG (PETUNIA FLOWERING GENE) (Immink et al., 1999), for which no dimerisation was scored in yeast (Immink et al., in preparation), were evaluated by the fluorescence microspectrometry methods. Both FBP2 and PFG are nuclear localised in petunia protoplasts (not shown) and their corresponding genes are expressed in ovules (Fig. 1C and E).

In Figure 3, the results from the FRET-FLIM analyses for the combinations FBP2-CFP/FBP11-YFP (Fig. 3 A and C) and FBP2-CFP/PFG-YFP (Fig. 3 B and C) are shown. Interaction and hence FRET results in a reduction of fluorescence lifetime of the donor fluorescent molecule (CFP). FRET-FLIM measurements on petunia protoplasts expressing only a single MADS box protein-CFP fusion protein, revealed a fluorescent lifetime of about 2.3 ns for the CFP chromophore (not shown). In case of the combination FBP2-CFP/FBP11-YFP a large reduction in fluorescence lifetime from 2,3 to 1,8 ns was obtained (Fig. 3C), demonstrating FRET between CFP and YFP. In contrast, imaging of protoplasts expressing the combination FBP2-CFP/PFG-YFP yielded an average lifetime of about 2.3 ns (Fig. 3C), similarly as was observed when a single CFP construct was expressed. In a parallel experiment FRET-SPIM analyses were performed for the same two combinations. Upon excitation of CFP, interaction (FRET) leads to quenching of CFP emission (donor), while YFP (acceptor) will be sensitised, resulting in a strongly increased fluorescence at 525 nm as compared to 475 nm. This altered fluorescence emission ratio clearly occurs for the combination FBP2-CFP/FBP11-YFP (Fig. 3D), while the emission spectra of the cells expressing the combination FBP2-CFP/PFG-YFP reminisced that of a single MADS box-CFP fusion protein. The spectral change obtained for the combination FBP2-CFP/

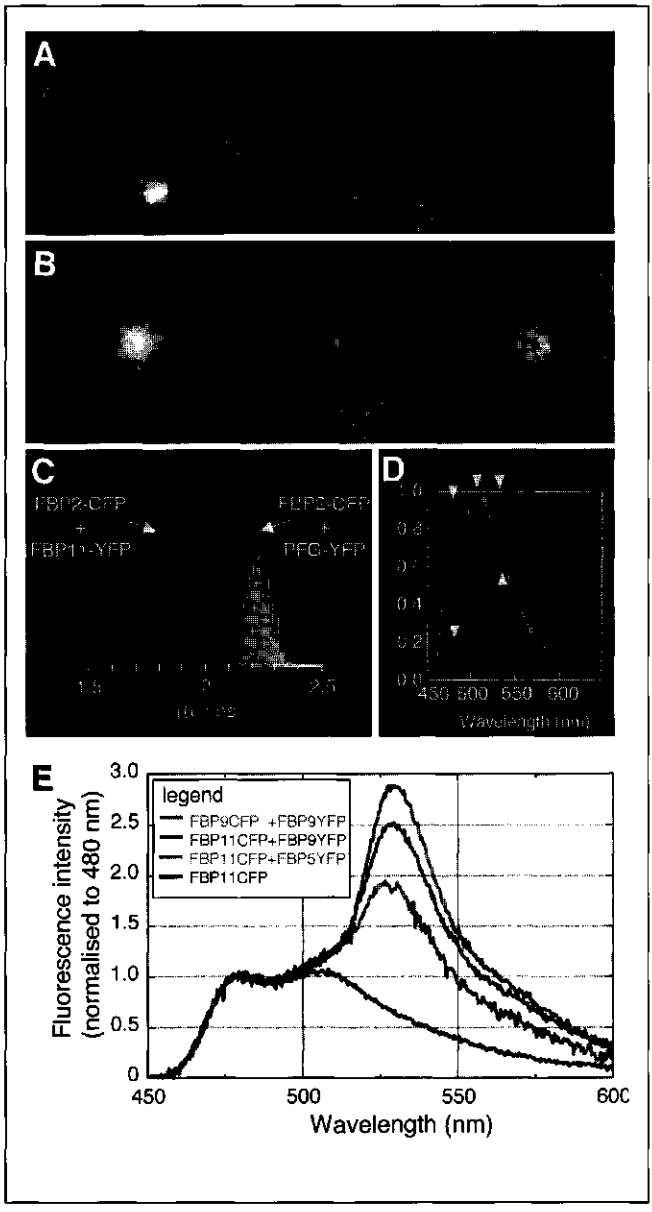

Figure 3*: FRET-FLIM and FRET-SPIM analyses of protoplasts expressing petunia MADS box proteins fused to CFP and YFP. (*A*) Protoplast expressing FBP2-CFP + FBP11-YFP. The border of the protoplast is artificially marked with a red circle. Left, Fluorescence image of a protoplast. Middle, Fluorescence intensity image (reconstructed from the FLIM-data stack). Right, *Fluorescence lifetime image*. The fluorescence lifetime at each pixel is represented in a pseudocolour index. Each micropixel represents a CFP fluorescence lifetime. Green represents a mask for pixels with low fluorescence intensity that are excluded from the lifetime analysis resulting in a lifetime image of the nucleus only. (*B*) Protoplast expressing FBP2-CFP + PFG-YFP. Left, middle, right as described for (*A*). (*C*) FRET-FLIM analysis. Temporal histogram and pseudocolour scale of the fluorescence pixel values of (*A*) and (*B*). (*D*) FRET-SPIM analysis. Spectra of protoplasts expressing FBP2-CFP + FBP11 YFP (Red line) and of protoplasts expressing FBP2-CFP + PFG-YFP (orange line). The CFP emission peaks are marked with a cyan-coloured arrowhead and the YFP emission peak with yellow arrowheads. Fluorescence intensities were normalised to one for easy comparison. (*E*) FRET-SPIM analyses of petunia protoplasts transfected with FBP11CFP, FBP11CFP + FBP5YFP, FBP11CFP + FBP9YFP and FBP9CFP +FBP9YFP. From each transfection the spectrum of one representative protoplast is shown.

) Figure in colour in appendix, page VII.

FBP11-YFP and the strong reduction in fluorescence lifetime, demonstrate the molecular interaction between these two MADS box proteins in a living plant cell. In contrast, no interaction was detectable for the MADS box proteins PFG and FBP2, despite their nuclear colocalisation, which confirms the yeast two-hybrid results. Because similar results were obtained with FRET-FLIM and FRET-SPIM analyses, only FRET-SPIM measurements were done for the two other putative FBP11 complexes. For both FBP11-FBP5 and FBP11-FBP9, a very clear spectral shift was obtained (Fig. 3E), demonstrating physical interactions between these proteins in plant protoplasts.

In order to investigate the occurrence of homodimerisation of the MADS box proteins, FBP9-CFP and FBP9-YFP were co-expressed in leaf protoplasts and subsequently a FRET-FLIM and FRET-SPIM analysis was performed. Surprisingly, in contrast to what was found by yeast two-hybrid analysis, FRET-FLIM measurements yielded a strong reduction in CFP lifetime to 1.6 ns, indicating homodimerisation of FBP9. SPIM analyses on these cells confirmed this specific interaction (Fig. 3E). Similar experiments for FBP2 and FBP5 also revealed a clear shift in spectrum towards 525nm (FRET-SPIM, not shown) and hence these proteins form homodimers in plant cells as well. For FBP11, no homodimerisation could be detected, as in the yeast two-hybrid GAL4 system.

Role of Nuclear Localisation Signal (NLS)

To predict the sequence motifs involved in nuclear import of MADS box proteins, analyses with the program PROSITE (http://www.expasy.ch/prosite/) were performed. These analyses revealed that both FBP2 and FBP11 contain a putative conserved bipartite NLS consensus sequence in the MADS domain (Fig. 4A). N-terminal truncated FBP11-CFP and FBP2-YFP constructs were generated to determine the involvement of these NLS motifs in nuclear import. The first 31 and 38 amino acids of the MADS box DNA binding domains were removed from FBP11 and FBP2, respectively. Subsequently, new start codons were introduced, yielding FBP11ΔN-CFP and FBP2ΔN-YFP (Fig. 4A). These truncated proteins were transiently expressed in petunia leaf protoplasts, resulting in a cytoplasmic fluorescent signal in both cases (Fig. 4B and C right). In parallel, double transfections were performed of the truncated fusion proteins in combination with either the full-length or N-terminal truncated interacting partner (FBP11ΔN-CFP + FBP2ΔN-YFP, FBP11ΔN-CFP + FBP2-YFP and FBP11-CFP + FBP2ΔN-YFP). All three combinations, each comprising at least one interacting partner with a truncated MADS box, appeared to result in a colocalisation of CFP and YFP signals in the cytoplasm only (Fig. 4E, not all combinations shown). In none of the

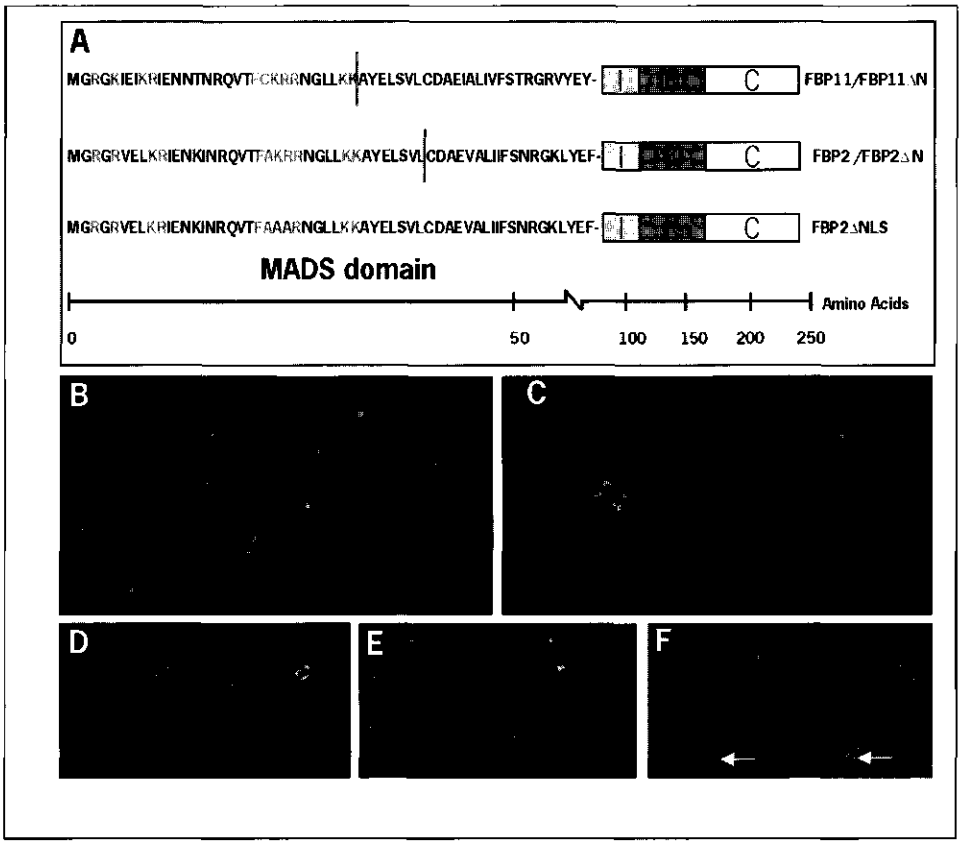

Figure 4*: Role of Nuclear localisation Signal (NLS) in MADS box protein translocation. (*A*) Representation of MADS box protein structure and the predicted position of NLSs. The positions of the truncations are indicated with a vertical red line in the protein sequence and with "Δ" in the name of the protein. The bipartite NLS localised in the N-terminal MADS domain is marked blue and conserved basic amino acids in the MADS domain are marked in green. Mutated amino acid residues in the NLS are marked in red in the protein sequence and with "ΔNLS" in the name of the protein. I = intervening region, K = K-box, C = C-terminal region. All images (*B-F*) were obtained by Confocal Scanning Laser Microscopy (CSLM). Chlorophyll auto-fluorescence is shown in red. (*B*) Localisation of FBP11-CFP (left) and FBP11ΔN-CFP (right). (*C*) Localisation of FBP2-YFP (left) and FBP2ΔN-YFP (right). (*D*) Nuclear colocalisation of FBP11-CFP and FBP2-YFP. On the left the CFP signal, on the right the YFP signal of the same protoplast. (*E*) Cytoplasmic colocalisation of FBP11ΔN-CFP and FBP2-YFP. On the left the CFP signal, on the right the YFP signal of the same protoplast. (F) Colocalisation of FBP11-CFP and FBP2ΔNLS-YFP. On the left the CFP signal, on the right the YFP signal of the same protoplast. The nucleus is marked with an arrow.

) Figure in colour in appendix, page VIII.

cells a fluorescent signal was observed in the nucleus, as has been seen for the full-length proteins (Fig. 2C and 4D). In contrast to protoplasts expressing the full-length fusion proteins, many cells accumulating the truncated fusion proteins produced fluorescent aggregates in the cytoplasm. Due to this clustering of fusion proteins, a dramatic reduction of fluorescence lifetime was observed and hence no reliable FRET-FLIM and FRET-SPIM measurements were possible for the determination of protein-protein interactions. Cells without aggregates expressed the chimeric proteins at very low levels and therefore, could not be used for microscopical measurements.

These results indicate that the NLS of MADS box proteins is positioned in the N-terminal located MADS domain and furthermore, that this domain should be present in both interacting partners for transport of the dimer to the nucleus. The consensus sequence of the bipartite NLS in this domain is "$PP(N)_{10}(P_3N_2)$", in which "P" is a positively charged amino acid and "N" represents any amino acid (Dingwall and Laskey, 1991). To test whether the predicted bipartite NLS sequence in the MADS domain is an essential signal for nuclear localisation, point mutations were generated in this motif. For this purpose two out of three positively charged amino acid residues in the NLS of FBP2 were changed in the uncharged amino acid Alanine resulting in the construct FBP2ΔNLS-YFP (Fig. 4A). Expression of this construct in leaf protoplasts revealed a cytoplasmic signal exclusively (not shown), while unmodified FBP2 is translocated to the nucleus (Fig. 4C, left). However, expression of FBP2ΔNLS-YFP in combination with FBP11-CFP resulted in a fluorescent signal in both cytoplasm and nucleus (Fig. 4F). Therefore, nuclear translocation of the FBP11-FBP2ΔNLS heterodimer seems to be impeded in these cells but not completely blocked.

Discussion

Yeast two-hybrid versus in-planta methods

In this study MADS box protein-protein interactions were analysed in living plant cells, making use of advanced fluorescent microscopy techniques. All heterodimers identified by the yeast two-hybrid GAL4 system, using FBP11 protein as bait, were confirmed in petunia protoplasts, indicating that the yeast system gives a reliable result. However in addition to the FBP11 heterodimers, homodimers of FBP2, FBP5 and FBP9 were formed in living plant cells, which could not be detected by the yeast two hybrid systems. In the yeast two-hybrid GAL4 system protein-protein interaction events are monitored at 30°C and in the CytoTrap™ system at 37°C. These relatively high non-ambient temperatures may influence the folding and hence interaction capability of some proteins.

Temperature-dependent interaction has been reported for the *Arabidopsis* class B MADS box proteins APETALA3 (AP3) and PISTILLATA (PI) (Kohalmi et al., 1996) and this may also be the case for FBP2, FBP5 and FBP9 homodimers. The lack of homodimerisation in the CytoTrap™ system seems at least not to be due to general problems related to this system, because it was used successfully to determine the interactions between 23 petunia MADS box proteins, including two homodimers (Immink et al., in preparation). Alternatively, the discrepancy between the results obtained with the yeast systems and the in planta system is due to plant specific factors or modifications that are required for homodimerisation of these three MADS box transcription factors.

Nuclear translocation of MADS box proteins

FBP11 fused to either CFP or YFP is localised in the cytoplasm of petunia leaf protoplasts in spite of the presence of the conserved bipartite Nuclear Localisation Signal (NLS). Upon expression of one of its interaction partners, FBP11 is transported to the nucleus, where a physical interaction between the two expressed proteins was demonstrated by FRET-FLIM. Inside the nucleus the fluorescent signal is not always evenly dispersed but sometimes localised in specific spots or in the nucleolus. Interestingly, the mammalian transcription factor Pit-1 also accumulates in sub-nuclear spots (Day, 1998) and Wei et al. (1999) demonstrated that transcription sites are often associated with splicing factor-rich nuclear speckles. In line with these observations plant MADS box transcription factors may accumulate in nuclear spots reflecting transcriptionally active sites.

In contrast to FBP11, the MADS box proteins FBP2, FBP5 and FBP9 are nuclear localised when individually expressed. This nuclear transport can be driven by a specific interaction with an endogenous MADS box protein present in the leaf protoplasts or, alternatively, the nuclear localisation of FBP2, FBP5 and FBP9 in the living plant cell is driven by their homodimerisation. The presence of FBP11 in the cytoplasm, a MADS box protein which is not able to homodimerise, is in line with the latter option. Based on the results we hypothesised that dimerisation of MADS box proteins occurs in the cytoplasm and is essential for nuclear localisation. More evidence for this hypothesis came from experiments with truncated proteins. Expression of FBP11-CFP, lacking 31 amino acids of the MADS domain, in combination with FBP2-YFP resulted in a colocalised CFP/YFP signal in the cytoplasm. The fact that FBP2 is retained in the cytoplasm implies that there is interaction in the cytoplasm between FBP2 and the truncated FBP11, lacking the Nuclear Localisation Signal (NLS). Due to the lack of a proper nuclear localisation signal this heterodimer remains in the cytoplasm. Because no FBP2-YFP signal was observed in the nucleus, homodimerisation of FBP2

proteins seems to be less efficient than the formation of heterodimers between FBP2 and the truncated FBP11. Dimerisation as a prerequisite for nuclear localisation of MADS box proteins has been suggested before by Mc Gonigle et al. (1996), who did similar localisation studies with the *Arabidopsis* B-type MADS box proteins APETALA3 (AP3) and PISTILLATA (PI). However, they were not able to show a direct interaction between these two proteins in living plant cells as is demonstrated here for the petunia MADS box proteins. The necessity for dimerisation to obtain nuclear localisation has been reported for other transcription factors as well (Spit et al., 1998; Chida et al., 1999; Nagoshi and Yoneda, 2001) and seems to be a common fine-tuning mechanism for transcriptional regulation.

Which domains or signals are exactly involved in nuclear translocation of MADS box proteins is unknown. McGonigle et al. (1996) mapped the NLS to the first 69 amino acids of AP3 and to the first 105 amino acids of PI. We were able to fine map the NLS to the first 40 amino acids of FBP2 and FBP11 and nuclear localisation was even abolished when this N-terminal domain was missing from either one of the two interacting partners, demonstrating once again that the presence of one complete bipartite NLS is not sufficient. In order to map the nuclear localisation signal more exactly a point mutation was generated in the conserved bipartite NLS of FBP2. This mutation appeared to be sufficient to abolish nuclear localisation of the FBP2 homodimer and to drastically impair the translocation of the heterodimer with the full-length FBP11. This result suggests that the complete bipartite NLS in both protein partners is required for efficient nuclear localisation. However, it can not be ruled out that other signals within the MADS box domain play a minor role in nuclear targeting. NLSs are in general rich in basic amino acids (Dingwall and Laskey, 1991) and comparison of the first 40 amino acids of 23 petunia MADS box proteins revealed that there are 4 more conserved basic amino acids close to the conserved bipartite NLS (see Fig 4A). A whole set of point mutations are required to determine the exact role of these amino acid residues in nuclear import of MADS box dimers.

Biological function of identified MADS box protein dimers

In this study it is demonstrated that FBP11 interacts specifically with the three very closely related SEPALLATA like petunia MADS box proteins FBP2, FBP5 and FBP9. Expression analyses showed that all corresponding genes are highly expressed in ovules. FBP11 is responsible for the determination of ovule identity as has been demonstrated by suppression and overexpression mutants (Angenent et al., 1995; Colombo et al., 1995). In-situ hybridisation on sepals of the *FBP11* overexpression plants revealed the presence of *FBP2* mRNA in the ectopically formed ovules. These results together, suggest a role for the identified FBP11

heterodimers in the development of ovules and probably the very closely related proteins FBP2, FBP5 and FBP9 play a redundant role in this process. *FBP2* and *FBP11* are also co-expressed at early developmental stages during wild type ovule initiation and in the epidermal cells on the adaxial side of sepals of the *FBP11* overexpressor, which suggests a role for the FBP2-FBP11 heterodimer in ovule initiation. Nevertheless, *FBP2* is also expressed in other floral organs (Angenent et al., 1992), while no ectopic ovules were identified on these organs in the *FBP11* overexpression plant (Colombo et al., 1995). Therefore, additional factors are needed or alternatively another complex is involved in the initiation of ovule formation. The FBP2, FBP5 or FBP9 homodimers seem not to be these ovule initiation factors, because ovule formation was completely blocked in the *fbp11* cosuppression mutant (Angenent et al., 1995), despite the expression of *FBP2*, *FBP5* and *FBP9*. Previously it has been demonstrated that FBP2 functions as an identity mediating (IM) MADS box protein involved in the development of the inner three floral whorls (Angenent et al., 1994), like the *Arabidopsis* SEPALLATA proteins (Pelaz et al., 2000). The results obtained in this study suggest an additional function for FBP2 like proteins in ovule development and probably these two different functions are effected by heterodimerisation of FBP2 with different MADS box protein partners.

Future prospects

The yeast two-hybrid GAL4 system has proven to be a robust system for the fast identification of protein-protein interactions. However, this study showed clearly some limitations of this yeast system and hence validation of obtained results is needed in a more natural background. The FRET system described here is a very powerful tool for these *in-vivo* analyses. Another possibility is the sophisticated Protein Complementation Assay (PCA) technology, published very recently by Subramaniam et al. (2001). Protein interaction in this system results in a reconstitution of enzymatic activity, which can be monitored by the specific binding of a labelled product. A drawback of PCA is that a fluorescent dye needs to be added as substrate and that the interacting protein can not be followed individually. In contrast, CFP/YFP fusion proteins can be followed individually in time and space. Nevertheless, the FRET-FLIM system is labour intensive and not suitable for large-scale screenings at this moment. Furthermore it has to be emphasised that it is a transient assay, like PCA, with relatively high expression levels for the fusion proteins, compared to endogenous proteins. These high expression levels may result in clustering of proteins, as has been shown here for the truncated MADS box proteins and we can not exclude that it induces small changes in protein characteristics. Therefore, it will be better to generate stable

transformants in the future, in which expression of the fusion proteins is driven by the promoter of the corresponding genes rather than by the strong CaMV 35S promoter. In general, it needs to be notified that FRET is not a direct screening for dimerisation and that a FRET signal demonstrates the presence of both CFP and YFP labelled proteins in a complex with a mutual distance of 8nm or less. Recently, it has been shown that MADS box proteins are able to form ternary complexes (Egea-Cortines et al., 1999; Honma and Goto, 2001) and therefore, we can not rule out that the FRET signals obtained in this study are the result of higher order complex formation. The same comments can be made to yeast two-hybrid analyses that neither give information about the stoichiometry of formed complexes. Nevertheless, the FRET results obtained in this study demonstrate indisputably specific MADS box protein complex formation in living plant cells and the importance of this complex formation for MADS box transcription factor functioning. The results are a first step towards the complete understanding of MADS box transcription factor action at the intracellular level. A next step will be the analysis of ternary MADS box protein complexes in vivo and hopefully, the properties of the green fluorescent protein (GFP) and its derivatives in combination with the fast developments in the microscopy field will make this possible in the near future.

Materials and Methods

Yeast two-hybrid GAL4 system

The ovary specific cDNA expression library was constructed from poly(A)$^+$ RNA from young petunia ovaries, according the manufacturers instructions (Stratagene, Catalogue # 235601). In parallel, the FBP11 encoding open reading frame was generated by polymerase chain reaction (PCR) and cloned in-frame in the bait vector (pBD-GAL4).

Instead of the YRG-2 yeast host strain supplied by Stratagene, the PJ69-4A yeast strain has been used (James et al., 1996). The bait plasmid was transformed to yeast according the CLONTECH Yeast Protocols Handbook (Protocol # PT3024-1) and transformants were checked for auto-activation of reporter genes, which appeared not to occur. Subsequently, yeast cells containing the bait construct were transformed with "ovary" phagemid library DNA, according the CLONTECH protocol. In total $1{,}5.10^6$ individual yeast transformants were generated. Screening for protein-protein interaction events was performed according the Stratagene manual (Catalogue # 235601). Clones positive for all three-yeast reporter genes were sequenced (BigDye™ sequencing kit, Applied Biosystems), to identify the cDNA-inserts.

Yeast two-hybrid CytoTrap™ system

The CytoTrap™ Vector Kit from Stratagene was used (Catalogue #217438). Instead of the enclosed pSOS vector, pSOS-NES was used, containing a Nuclear Export Signal (NES) fused in-frame between the SOS coding region and the Multiple Cloning Site (MCS). This vector and the yeast cdc25H mating type "a" strain were obtained from Stratagene US. FBP2, FBP5 and FBP9 were cloned in the pMYR and pSOS-NES vectors as described above for the yeast two-hybrid GAL4 system bait vector construction. All constructs were confirmed by sequencing (BigDye™ sequencing kit, Applied Biosystems).

The pMYR-petunia-MADS-box-gene constructs were transferred to yeast strain cdc25H mating type "a" and the pSOS-NES-petunia-MADS-box-gene constructs to cdc25H mating type "□" according to the manual. Double transformants were obtained by mating and screened for protein-protein interaction events as described by the manufacturer.

In-situ hybridisations

Plant materials were fixed and embedded in paraffin and sections were made as described by Canas et al. (1994). Digoxygenin-labelled probes were generated from pSPT18/pSPT19 vectors by in vitro-transcription (Boehringer Mannheim). All probes consist of specific cDNA fragments, lacking the conserved 5' MADS-domain encoding sequence. Hybridisation and immunological detection were performed as described before (Canas et al., 1994).

Construction of CFP/YFP plasmids

Complete ORFs of MADS box genes *FLORAL BINDING PROTEIN 2(FBP2), FBP5* (AF335235), *FBP9* (AF335236), *FBP11* and *PETUNIA FLOWERING GENE (PFG)* were amplified with gene specific primers (Isogen), to remove the stop codon and for C-terminal in-frame fusion with the coding region of *CFP* and *YFP* (from *pECFP* and *pEYFP*, Clontech catalogue #6075-1 and #6004-1, respectively). To obtain FBP2ΔN and FBP11ΔN, lacking 38 and 31 AAs at the N-terminus respectively, a second set of gene specific forward primers were designed for these two genes annealing 114 and 93 nt downstream the start codon, respectively, and introducing an in-frame new ATG start codon. All PCR reactions were performed with PFU proof-reading polymerase (Stratagene). Subsequently, "MADS box gene-CFP/YFP" products were cloned in the expression vector pGD120 (pUCAP containing the expression cassette: CaMV 35SDE-AMV leader sequence-MCS-NOS terminator). The FBP2ΔNLS-YFP plasmid with the point mutation in the NLS was generated by site directed mutagenesis on pGD120-FBP2YFP with primers PRI690 (5'-CAATAGACAAGTTACCTTTGCTGCGGCAAGAAATGG

ACTATTGAAAAAAGC-3') and PRI691 (5'-GCTTTTTTCAATAGTCCATTT CTTGCCGCAGCAAAGGTAACTTGTCTATTG-3'), according the Stratagene QuickChange™ Site-Directed Mutagenesis Kit manual (Catalogue #200518). All final expression constructs were confirmed by sequencing (BigDye™ sequencing kit, Applied Biosystems).

Protoplast transfection

The generated MADS box CFP/YFP fusion proteins were transiently expressed in living petunia leaf protoplasts. Protoplasts were prepared from fresh full-grown leaves of W115 petunia plants grown in the greenhouse. Leaves were surface sterilised by incubation in 4% bleach for 20 minutes. Protoplasts were isolated from these leaves and transfected (PEG method) as described by Denecke et al. (1989). All measurements and imaging experiments were done after overnight incubation at 25 °C in the dark.

Localisation studies

Localisation of MADS box-CFP/YFP fusion proteins in petunia protoplasts was determined by Confocal Scanning Laser Microscopy analyses (CSLM 510, Carl-Zeiss, GMBH Germany). Excitation was provided by the 458 and 514 nm Ar laser lines controlled by an acousto optical tunable filter (AOTF). Three dichroic beam splitters were used to separate excitation from emission and to divide the fluorescence emission into the CFP, YFP and chlorophyll channels. The HFT 458/514 dual dichroic beam splitter was used as a primary dichroic mirror reflecting excitation and transmitting fluorescence emission, an NFT 635 dichroic mirror was used as a secondary splitter and an NFT 515 was used as tertiary dichroic splitter. Fluorescence reflected by both the NFT 635 and NFT 515 splitters was filtered through a BP470-500 nm filter yielding the CFP signal. Fluorescence reflected by the NFT 635 but transmitted by the NFT 515 splitter was filtered through a BP535-590 nm filter yielding the YFP channel. Fluorescence transmitted by both the NFT 515 and 635 splitters, was additionally filtered by an LP650 filter to yield the chlorophyll image. Cross-talk free CFP and YFP images were acquired by operating the microscope in the multi-tracking mode, in which the 514 nm excitation was coupled to activation of the YFP detection channel, and the 458 nm excitation was coupled to activation of the CFP and chlorophyll detection channels. Images were obtained with a 40x oil immersion objective (N.A. 1.3). The pinhole was set at an approximately 1 airy disk unit, corresponding to a theoretical thickness of about 1µm in the object plane. Images were analysed and adapted with Zeiss LSM510 software.

FRET-SPIM

For SPIM, excitation was provided by a 100 W Hg Arc lamp of which the 435 nm line was selected by inserting an Omega (Brattleboro, VT, USA) 435DF10 bandpass filter in the excitation light path. The excitation light was reflected onto the sample by an Omega 430DCLP dichroic mirror. A Leica 20x HR-fluotar air objective (NA = 0.5) was employed. Residual excitation light was rejected using a Schott (Mainz, Germany) GG455 longpass filter. In the spectrograph (Chromex 250 is (Chromex Inc., Albuquerque, NM, USA)) a 150 grooves/mm grating was used with a central wavelength of 500 nm. For further details of the set-up see Goedhart and Gadella (27). Single protoplasts expressing specific MADS box protein-CFP and MADS box protein-YFP fusion proteins were positioned by aligning them across the entrance slit of the spectrograph (set at 200 µm width corresponding with a line of 10 µm width in the object plane). Acquisition time was 1-2 s. Regions of the image spectrum corresponding to the nucleus or cytosol of labelled cells were distance averaged (typically 5-10rows of pixels) and the resulting fluorescence spectra were corrected for background fluorescence and camera bias by background subtraction using an extracellular region just next to the plasma membrane region from the same spectral image. The resulting spectra were not corrected for the spectral instrument response yielding a slightly underestimated intensity in the blue edge of the spectra. For each specific MADS box protein combination at least 5 independent protoplasts were imaged.

FRET-FLIM

FRET-FLIM measurements were performed on individual protoplasts expressing a specific MADS box protein-CFP and MADS box protein-YFP fusion protein. For each MADS box protein combination measurements were repeated at least 3 times with independent protoplasts. FLIM was performed on a wide-field frequency-domain instrument described in detail elsewhere (Gadella, 1999). The cells were excited with the 457 nm argon-ion laser line modulated at 60.116 MHz and the CFP fluorescence was selectively imaged using an Omega 470 DCLP dichroic mirror and an Omega 487RDF42 band-pass emission filter. 20 phase images (1-3 s each) were taken (10 with increasing and 10 with decreasing the phase allowing correction for photobleaching (which was less than 10% in all cases)). Reference phase settings and modulation were calibrated approximately ever 30′ by measuring a glass microcuvette filled with an erythro-shine-B solution in water (single component fluorescence decay with a lifetime of 0.08 ns). The microscope set-up, lifetime image calculation and image processing are described in detail elsewhere (Gadella, 1999). CFP fluorescence was imaged using a 470-500 nm bandpass emission filter after excitation with a 457 nm argon-ion laser line

modulated at 40 MHz. Used microscope set-up, calculations and image processing for frequency domain FLIM, were described before (Gadella, 1999; Gadella et al., 1999; Goedhart and Gadella, 2000; Gadella et al., 1994).

Chapter 7

General Discussion: Identification and exploitation of MADS box protein-protein interactions

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Shortened version submitted for publication

The completion of the *Arabidopsis* genome sequencing project in 2000 was a major milestone in plant genomics (The Arabidopsis Genome Initiative, 2000). Based on sequence comparison and common motifs in the encoded proteins about 69% of the 25.000 genes present in the 125 Mbp genome could be functionally classified. Despite of this information our knowledge about the role of these proteins in the life cycle of the plant is very limited. The challenge for the future is to find the functions for all predicted unknown genes in a systematic high-throughput manner.

The current functional genomics toolbox consists of several techniques to reach this objective, such as gene knockouts, transcriptome analysis by micro arrays, proteomics tools and metabolic profiling. Among these tools, forward and reverse genetics are powerful and well-established methods for the functional analysis of genes. Screening for gene knock outs by transposons or T-DNA insertions are performed systematically in *Arabidopsis* and recently, various large-scale knock-out facilities became available like the T-DNA knock-out service from the university of Wisconsin (Krysan et al., 1999; Bouche and Bouchez, 2001). Although, it is a very powerful technology, screening for knock-outs is labour intensive and often no phenotypes are obtained due to functional redundancy or because aberrant phenotypes appear only under very specific conditions. Transcriptome analysis by micro-array analysis is a more high-throughput functional genomics tool. By means of this approach clusters of genes expressed in the same tissues or upon the same stimuli can be easily identified (Kuhn, 2001; Aharoni and Vorst, 2002). Nevertheless, no knowledge is obtained about post-transcriptional regulation or the function of the proteins encoded by the transcripts.

Most proteins are functional in complexes and depend on protein interactions for their functioning. Therefore, mapping of protein-protein interactions can be a next step to elucidate protein functions. To analyse protein-protein interactions in a high-throughput manner, yeast two-hybrid systems have proven to be very powerful (Reviewed by Fashena et al., 2000). A well-known example is the study of the yeast interactome, in which interaction patterns were determined for 6000 predicted yeast proteins (Uetz et al., 2000; Ito et al., 2001). Based on the designed interaction maps functions could be predicted for many unknown proteins.

In this general discussion, various yeast and in-planta methods for the identification of protein-protein interactions will be discussed and compared. Based on several examples we will show how protein interactions can be used in genomic research. Interaction patterns give not only clues about protein functions but provide also insight in the presence of functional redundancy. Furthermore, examples will be given how protein-protein interaction mapping can be exploited

to identify orthologues from non-related species, which appear to be a novel approach to transfer knowledge from model species to crop plants.

The MADS box transcription factor family has been chosen as subject of study, because it is one of the most important families of regulatory proteins in plants and numerous members of this family have been studied in detail (reviewed by Riechmann and Meyerowitz, 1997; Theissen et al., 2000). In *Arabidopsis* this large family of transcription factors consist of at least 100 members (Riechmann et al., 2000; The Arabidopsis Genome Initiative, 2000). A complicating factor for functional analysis of this family is the existence of many paralogues that causes functional redundancy among the MADS box transcription factors (reviewed by Smyth, 2000). MADS box proteins are an ideal subject to demonstrate the power of protein-protein interaction analysis as a functional genomics tool, because members of this large family form specific homo- and heterodimers and even ternary complexes (Egea-Cortines et al., 1999; Honma and Goto, 2001).

Identification of protein-protein interactions by yeast systems

Over the last decade, yeast two-hybrid systems have become the general approach to identify protein-protein interactions (Fashena et al., 2000; Legrain et al., 2001). The original yeast two-hybrid GAL4 system (Fields and Song, 1989) is the most commonly used and is based on the modular nature of the DNA binding and activation domain of the GAL4 transcription activator (Fig. 1A). By means of this genetic system, specific dimerisation has been shown for plant MADS box proteins from different species (Chapter 2; Chapter 6; Davies et al., 1996; Fan et al., 1997; Davies et al., 1999; Moon et al., 1999a,b; Pelaz et al., 2001). A relatively large number of MADS box proteins studied by the GAL4 system appeared to contain intrinsic transcriptional activation domains resulting in auto-activation of the reporter genes. Studies with other plant transcription factors in yeast demonstrated transcriptional activity for members of various families (Solano et al., 1995; Stockinger et al., 1997; Meijer et al., 2000). A strategy to circumvent the auto-activation problem is to map the transcriptional activation domain in the protein studied and remove it from the bait. This strategy has been used successfully by Moon et al. (1999b) for the rice MADS box protein OsMADS6 and Pelaz et al (2001) for the *Arabidopsis* MADS box protein APETALA1 (AP1). However, differences in interaction patterns between full-length and truncated proteins were identified for some petunia MADS box proteins. The petunia MADS box protein FLORAL BINDING PROTEIN2 (FBP2) lacking the C terminus has shown to have less interaction partners than the full-length FBP2 protein (Chapter2). In contrast, the UNSHAVEN (UNS) lacking 77 amino acid residues at

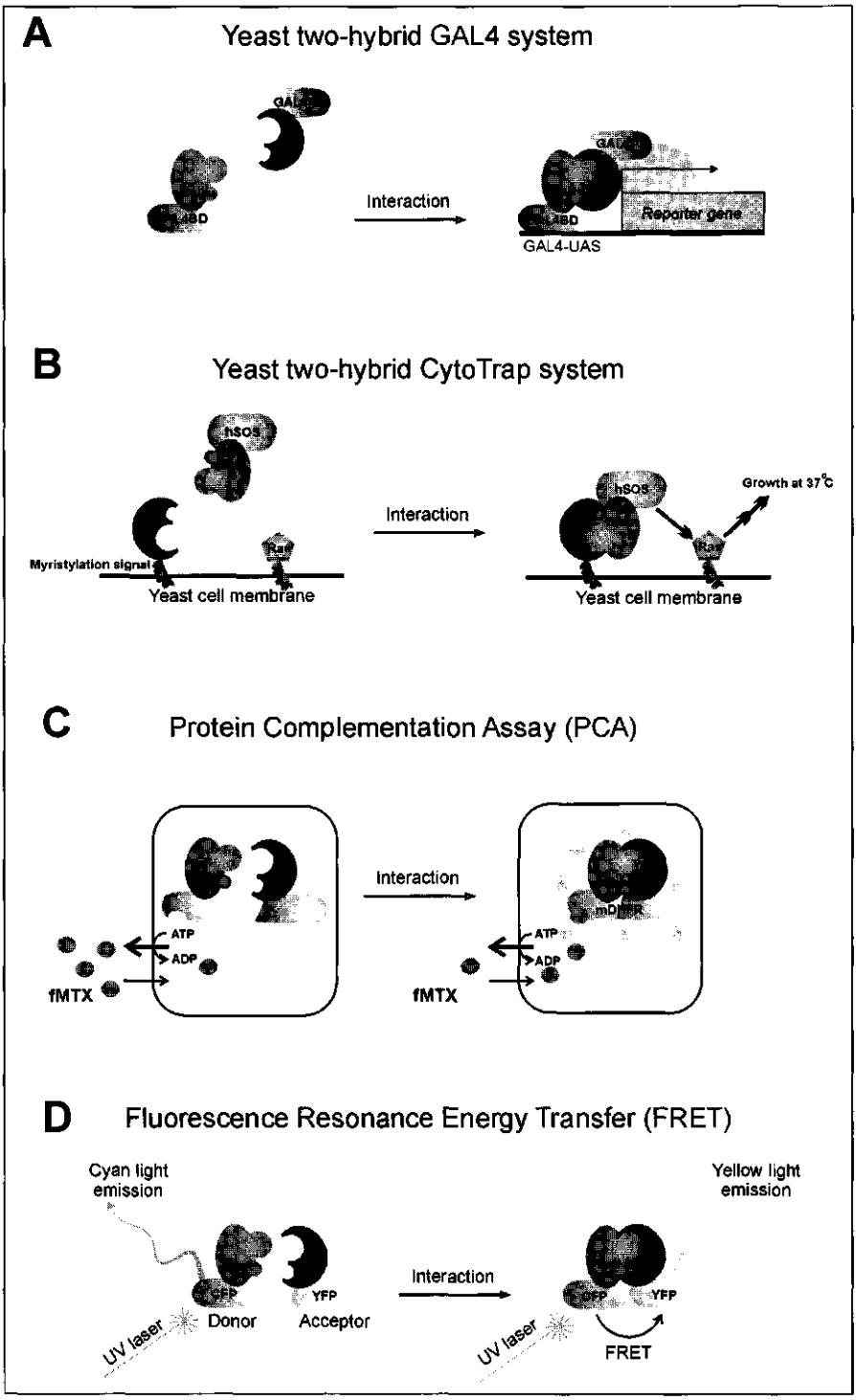
A
Yeast two-hybrid GAL4 system
GAL
DBD
Interaction
GAL
DBD
Reporter gene
GAL4-UAS
B
Yeast two-hybrid CytoTrap system
hSOS
Myristylation signal
Yeast cell membrane
Interaction
hSOS
Growth at 37°C
Yeast cell membrane
C
Protein Complementation Assay (PCA)
ATP
ADP
fMTX
Interaction
ATP
ADP
fMTX
D
Fluorescence Resonance Energy Transfer (FRET)
Cyan light emission
Yellow light emission
CFP
YFP
Donor
Acceptor
UV laser
Interaction
CFP
YFP
UV laser
FRET

Figure 1: Yeast and in planta systems for the identification of protein-protein interactions. A) Yeast two-hybrid GAL4 system. In this genetic system the GAL4 transcription factor is split into an inactive DNA binding domain (GAL4BD) and transcription activation domain (GAL4AD) and various reporter genes are placed under control of the GAL4 up-stream activating sequence (GAL4-UAS). The bait protein is fused to the GAL4BD domain and the prey protein to the GAL4AD domain. Upon interaction between bait and prey proteins the activity of the GAL4 transcription factor is restored, resulting in the activation of the reporter genes. B) Yeast two-hybrid CytoTrap system. Cytoplasmic system based on the Ras signal transduction cascade. The bait protein is fused to the human SOS protein (hSOS) and the prey protein is fused to a myristylation signal peptide, which targets the fusion protein to the membrane. Upon interaction between bait and prey proteins the hSOS protein is anchored to the membrane of the yeast cells, which subsequently activates the Ras signal transduction cascade enabling the yeast cells to grow at 37°C. C) In planta Protein Complementation Assay (PCA). The murine dihydrofolate reductase (mDHFR) enzyme is split into two inactive complementary domains and these domains are fused to bait and prey proteins. Interaction between bait and prey will restore the capability of mDHFR to bind fluorescein-conjugated methotrexate (fMTX). Free fMTX is actively transported out of plant cells, while mDHFR-fMTX complexes are retained inside the cell and can be visualised by spectroscopy methods. D) In planta fluorescence Resonance Energy Transfer (FRET) assay. FRET occurs only when two fluorescent molecules with overlapping emission and excitation spectra come in very close proximity (<100Å). In this system the bait protein is fused to Cyan Fluorescent Protein (CFP) and the prey protein to Yellow Fluorescent Protein (YFP). Interaction between bait and prey brings CFP and YFP close together and hence FRET will occur between the two chromophores upon excitation of CFP, which can be monitored by various spectroscopic methods.

its N-terminus appeared to interact less specifically and has more interaction partners than the full-length UNS protein (Chapter 2; Chapter 4). For transcription factors belonging to other families it is even more difficult to follow this strategy, because the exact position of the transcription activation domain is unknown or because multiple activation domains are present. In conclusion, engineering truncated bait proteins without transcriptional activation capacity is not easy and data obtained from studies with truncated proteins need to be interpreted carefully and should be confirmed with full-length proteins.

A few yeast systems have been described, in which the identification of protein-protein interactions is not based on transcriptional activation and therefore are suitable for proteins with transcriptional activity. An example is the so-called repressed transactivator system (RTA), that is based on the general yeast transcriptional repressor TUP1 (Hirst et al., 2001). In this system the bait protein is fused to the GAL4 binding domain and the prey protein to the strong TUP1 repressor. Upon a protein-protein interaction event TUP1 represses the transcriptional activity of the bait protein and by that the expression of the *URA3* reporter gene, which results in resistance to 5-FOA. Other systems that have

proven to be useful are the "split-ubiquitin" method (Stagljar et al., 1998) and SOS recruitment system. The commercially available CytoTrap system is an example of the latter mentioned technology. This cytoplasmic two-hybrid system is based on the recruitment of the human SOS protein (hSOS) to the membrane of the yeast cells upon a protein-protein interaction (Fig. 1B). Subsequently, the hSOS-bait fusion protein activates the RAS signal transduction cascade enabling the temperature-sensitive cdc25H yeast strain to grow at high temperatures (Aronheim et al., 1997). The dimerisation pattern of 23 petunia MADS box proteins has been determined successfully by means of this system (Chapter 2). Nevertheless, also this yeast system has some drawbacks. One of the major problems is reversion of the mutant cdc25H yeast strain, which enables the yeast to grow at 37°C independent of a protein-protein interaction. This appeared to be the case for about 3 % of the clones tested in the screening with the petunia MADS box proteins (Immink and Angenent, unpublished results). For screening of cDNA expression libraries with specific bait proteins, this percentage of revertants will give rise to unacceptable high numbers of false positives. Furthermore, the relatively high non-ambient temperature of 37°C at which the screening is performed, may influence the folding and hence interaction capability of some proteins. Temperature-dependent interaction has been reported for the *Arabidopsis* B-type MADS box proteins APETALA3 and PISTILLATA (Kohalmi et al., 1996) and seems to occur for some petunia MADS box proteins as well (Immink and Angenent, unpublished results).

The identification of several MADS box protein-protein interactions from different plant species by yeast two-hybrid systems, clearly demonstrates the power of the yeast systems. Nevertheless, some combinations of interacting proteins are missed and others are scored as interaction partners based on doubtful results due to problems with auto-activation, contradictions between full-length and truncated proteins, temperature sensitivity of specific interactions or wrong post-translational processing of the proteins in yeast cells. Furthermore, some proteins may be able to interact in yeast cells but are never colocalised in the same compartments of the plant cell and therefore, will never interact in their natural environment. In conclusion, yeast two-hybrid systems are powerful and robust systems to get a first idea about possible interaction patterns, although identified interactions require confirmation by other means, preferably in a more natural environment.

Identification of protein-protein interactions by in-planta systems

Two methods have been described in literature, which can be used to monitor protein-protein interactions in living plant cells. One of the two technologies is a Protein Complementation Assay (PCA), which is based on reconstitution of murine dihydrofolate reductase (mDHFR) upon a protein-protein interaction event followed by binding of a fluorescent probe (Subramaniam et al., 2001). For this purpose the mDHFR protein is split into two complementary fragments, which are fused to the proteins of interest (Fig 1C). The fusion proteins are expressed in living plant cells and in case of a protein-protein interaction the two mDHFR fragments are reassembled into an active enzyme, which is able to bind fluorescein-conjugated methotrexate (fMTX). Free fMTX is actively transported out of cells, while fMTX bound to the reconstituted mDHFR retains inside the plant cell and can be monitored by fluorescence microscopy or fluorescence-activated cell sorting (FACS). A disadvantage of this method is that first a fluorescent dye (fMTX) needs to be added before measurements can be performed. Furthermore, no information is obtained about the localisation of the individual fusion proteins.

The other available technique to identify protein-protein interactions in-planta is based on the Fluorescence Resonance Energy Transfer (FRET) principle. FRET is the process of energy transfer from one fluorescent molecule, the donor, to another fluorescent molecule, the acceptor. Energy transfer occurs only when the two chromophores are separated by a distance of less then 100 angstrom and when the emission and absorbtion spectra of the donor and acceptor chromophores respectively, are overlapping (Gadella et al., 1999). Cyan Fluorescent Protein (CFP) and Yellow Fluorescent Protein (YFP), two derivatives of Green Fluorescent Protein (GFP) from the jellyfish, have demonstrated to be a good FRET donor-acceptor combination. For the identification of protein-protein interactions, fusions between CFP or YFP and the proteins of interest are expressed in living plant cells (Fig. 1D). Subsequently, the donor molecule is excited, which in case of a protein-protein interaction between the proteins of interest will lead to FRET. The occurrence of FRET can be determined by both spectral imaging microscopy (FRET-SPIM) and Fluorescence Lifetime Imaging Microscopy (FRET-FLIM). For plant cells, which have a high absorption and filtering of fluorescence by chlorophyll pigments, the latter method is preferred (Gadella et al., 1999). FLIM is based on the lifetime of the excited donor, which is reduced when energy can be transferred to the acceptor. Recently, the usefulness of FRET for the identification of MADS box transcription factor interactions in living plant cells has been demonstrated (Chapter 6). An advantage of FRET compared to PCA is that the

individual fusion proteins can be localised and followed in time. A disadvantage is that the method is labour intensive and relies on sophisticated fluorescence microscopy set-ups. For both in-planta methods holds that fusion proteins are used for the identification of protein-protein interaction events, which can interfere with a correct folding of the proteins studied. Based on all discussed items a comparison has been made between the two in planta methods and the yeast systems (Table 1).

	Yeast systems		In-planta systems	
	GAL4	CytoTrap	PCA	FRET
High-throughput	++	+	+/-	-
Quantitative	+	-	+	+
Reliability	+/-	+/-	++	++
Biological relevance	+/-	+/-	++	++

Table 1: Comparison between yeast and in-planta systems for the identification of protein-protein interactions.

Prediction of protein functions based on interaction patterns

Protein-protein interactions appeared to be conserved among different species and therefore comparison of interaction maps enables to address specific functions to unknown proteins. In addition knowledge will be gained about the occurrence of functional redundancy and about evolutionary aspects of protein functioning. Figure 2 presents the interaction map of petunia (Chapter 2), *Antirrhinum* (Davies et al., 1996; Davies et al., 1999) and *Arabidopsis* (Kohalmi et al., 1996; Fan et al., 1997) B- and C-type MADS box proteins. Comparison of the interaction maps from these not directly related species reveals the conservation of interaction patterns among the studied MADS box proteins. It appeared that in contrast to *Arabidopsis* petunia contains at least two B-type MADS box proteins in the PISTILATA lineage, FBP1 and pMADS2 (Angenent et al., 1993; Kush et al., 1993), which based on interaction patterns are most likely functionally redundant. From an evolutionary point of view, the two petunia B-type genes are probably derived from a single ancestor B-type gene by a gene duplication event. The same holds for the two known petunia C-type proteins FBP6 and pMADS3 (Kater et al., 1998), although small differences were observed in their interaction patterns, suggesting that they evolve further. In general the existence of identical interaction patterns for two proteins provide evidence for the presence of functional redundancy.

An example of a MADS box protein with unknown function to which a function can be addressed based on its interaction pattern is the petunia MADS box

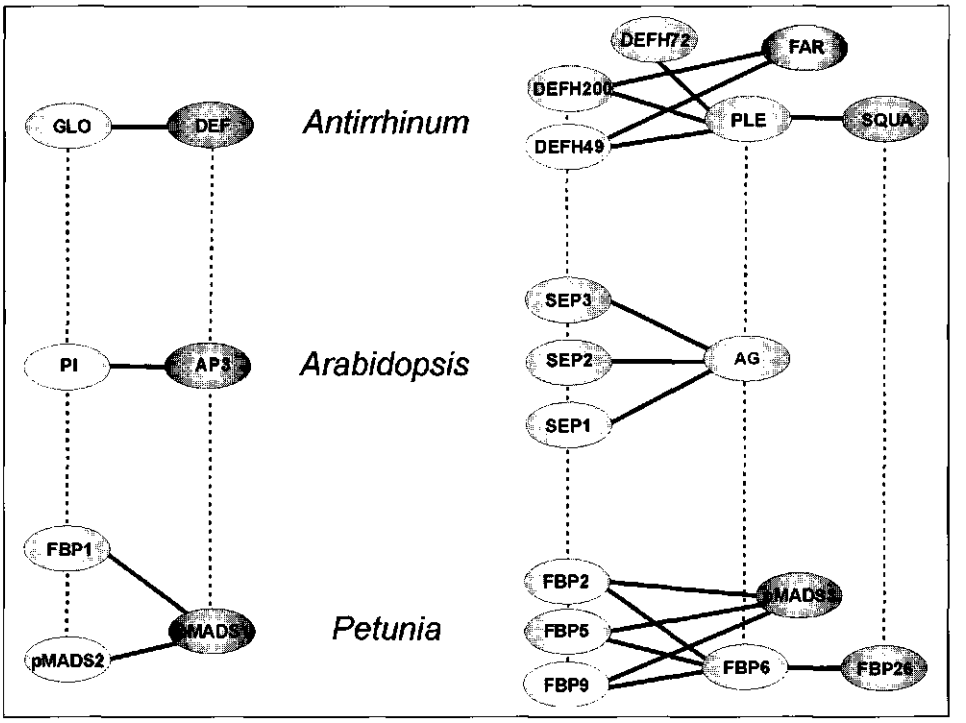

Figure 2: Identification of paralogues and orthologues based on protein-protein interaction patterns. Comparison of the interaction maps of petunia, *Arabidopsis* and *Antirrhinum* B, C and E-type of MADS box proteins. Interacting proteins are connected by a solid line and supposed paralogues and orthologues by dotted lines. AG, AGAMOUS; AP3, APETALA3; DEF, DEFICIENS; DEFH, DEFICIENS Homologue; FAR, FARINELLI; FBP, FLORAL BINDING PROTEIN; GLO, GLOBOSA; PI, PISTILLATA; PLE, PLENA; pMADS, petunia MADS box protein; SEP, SEPALLATA; SQUA, SQUAMOSA.

protein FBP26. Its interaction with FBP6 in combination with a high percentage of sequence homology with SQUA from *Antirrhinum* and the specific expression pattern of the *FBP26* gene (Chapter 3), suggests a function similar to SQUA for FBP26.

In addition to the interaction scheme of a given protein as shown above, the interaction with a well-characterised protein may give clues about its function. The petunia MADS box protein FBP2 has been classified as an E-type MADS box protein acting in a combinatorial way with B and C class proteins to establish floral organ identity (Theißen, 2001). This conclusion was based on expression pattern, phylogenetic analysis and the phenotype of the *fbp2* cosuppression plant (Angenent et al., 1994). However, it has been demonstrated that FBP2 also interacts with the D-type MADS box protein FBP11 (Chapter 6). Because FBP11 is involved

in proper ovule development (Angenent et al., 1995; Colombo et al., 1995), the formation of the FBP11-FBP2 dimer suggests an additional role for FBP2, besides its E-type function, in the formation of ovules (Chapter 6). More evidence for this hypothesis comes from the fact that in transgenic plants overexpressing *FBP11* the formation of ectopic ovules is accompanied by expression of the *FBP2* gene (Colombo et al., 1995; Chapter 6).

Heterologous interaction screenings for the identification of functional homologues

Comparison of interaction patterns determined within a species (see Fig. 2) provides a first clue on the conservation of protein functions. A new screen has been developed to proof that proteins from different species are indistinguishable in their ability to form heterodimer transcription factors. For this, heterologous 2-hybrid screenings were performed between the *Arabidopsis* SEPALLATA3 (SEP3) protein and the 23 known petunia MADS box proteins (Fig. 3A). The results revealed that SEP3 interacts specifically with the same MADS box proteins as FBP2 and FBP5 from petunia, which indicates that SEP3 can be classified as the functional homologue (orthologue) of FBP2. This was also demonstrated by reverse genetics experiments in Petunia (Angenent et al. 1994) and *Arabidopisis* (Pelaz et al., 2000), although these experiments are much more labour intensive as the 2-hybrid screens. For other species, these mutant analysis approaches are even not possible or hardly doable, while for the heterologous protein interaction screens virtually no limitations are present.

In a similar way, Moon et al. (1999a) demonstrated that the putative rice AP3 homologue OsMADS16 is able to interact with PI from *Arabidopsis*. However, the supposed rice PI homologue OsMADS4 was not able to interact with AP3 from *Arabidopsis*. In addition, transcription activation ability was shown for OsMADS16 in yeast, which is not present in AP3. This latter observation suggest a slightly different regulation of the B-function in the monocot rice compared to the dicot *Arabidopsis*, which is also reflected in small differences in heterologous interactions.

Because some MADS box proteins appear to be functional in ternary complexes, we have also determined whether these ternary interactions can occur heterologously. Honma and Goto (2001) demonstrated a ternary interaction between a heterodimer of the *Arabidopsis* B-type proteins PI-AP3 and the floral meristem identity protein AP1. In a yeast three-hybrid experiment the interaction between the petunia B-type heterodimer FBP1-pMADS1 and AP1 from *Arabidopsis* was tested (Fig. 3B). As was the case for dimerisation, also ternary

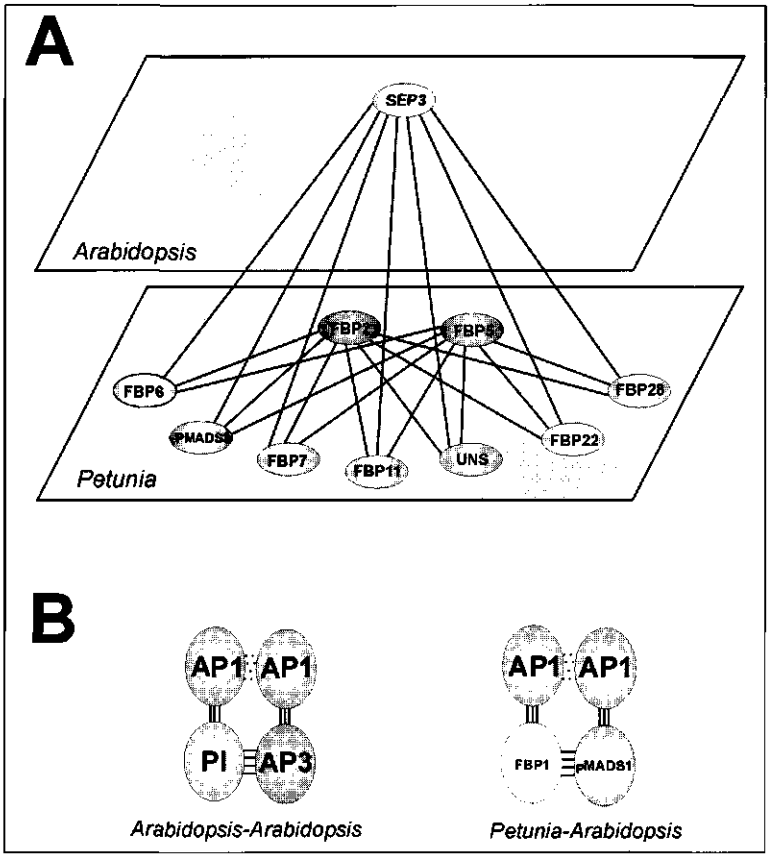

Figure3: Identification of orthologues by heterologous protein-protein interaction screens. A) Heterologous interactions between SEPALLATA3 (SEP3) from *Arabidopsis* and petunia MADS box proteins identified by the yeast two-hybrid CytoTrap system. SEP3 appeared to have identical interaction partners as the paralogues FBP2 and FBP5 from petunia, confirming their identical functions. B) Heterologous ternary complex formation identified by the yeast three-hybrid GAL4 system. The supposed *Arabidopsis* APETALA1 homodimer is able to interact with the petunia FBP1-pMADS1 heterodimer (B-type proteins) in a similar way as it interacts with the *Arabidopsis* PISTILATA(PI)-APETALA3(AP3) heterodimer.

complex formation appeared to occur between MADS box proteins from different species. This result shows once again that protein-protein interactions are conserved among higher plant species and therefore can be used to identify orthologues of proteins in other species. In the future cDNA expression libraries of crop plants can be screened with well-characterised proteins from model species to identify specific proteins of interest. The big advantage of this technology is that no sequence or expression information is needed and that it allows a reliable and high throughput identification of protein functions.

Prospects

In this discussion, we have shown that protein interaction mapping is a very useful functional genomics tool to get insight in the function of MADS box transcription factor complexes. Based on the overall conservation of protein-protein interaction patterns, this functional genomics strategy can be extended most likely to complete plant proteomes as well. Furthermore, it appears that interactome analysis represents a powerful tool in *comparitive genomics*, from which knowledge can be extracted to be used for the transfer of genomics data from model species to crop plants. The potential of these types of genomics analyses will expand in the near future due to developments in high throughput interaction screens. To ensure the reliability of the outcome of these analyses, several large-scale interaction screenings have to be compared and integrated, as has been demonstrated by Tong et al. (2002). The emerging protein-array technology will be a perfect additional method for this purpose (MacBeath and Schreiber, 2000; Walter et al., 2000).

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Summary

Homeotic genes specify the identity of different plant tissues and organs. Mutations in these genes result in replacement of a specific organ or tissue by another plant body part or tissue, which is normally formed at a different position. An important group of homeotic genes in plants is represented by members of the MADS box family of transcription factors. The first members isolated from this large family were involved in specifying the identity of floral organs. Based on the genetic and molecular analysis of these genes the "ABC(DE)" model of floral development was established. Meanwhile, MADS box transcription factors with other functions were identified, like the regulation of flowering time.

To gain insight in the molecular mechanisms underlying MADS box transcription factor functioning, the MADS box family of the model species *Petunia hybrida* has been analysed. MADS box proteins are active as homo- or heterodimers and even higher-order ternary and quaternary complex formation have been shown recently. Therefore, this study focuses mainly on the characterisation of protein-protein interactions. Initially, some new members of the petunia MADS box family were isolated from inflorescence and ovary cDNA-libraries, bringing the total number of identified petunia MADS box genes up-to 23. Members of this family are either named *FLORAL BINDING PROTEINs* (*FBPs*) or *PETUNIA MADS* (*pMADS*). The 23 members were all characterised by expression pattern analysis, phylogenetic comparison and yeast two-hybrid screenings for the identification of protein-protein interactions. The obtained results were compared with data from MADS box transcription factor families of other species, like *Antirrhinum majus* and *Arabidopsis thaliana*. This comparison demonstrated that MADS box genes and their functions are largely conserved among flowering plants and therefore, functions could be predicted for some unknown petunia and *Arabidopsis* MADS box proteins. Furthermore, some of the characterised 23 genes appeared to have one or more close relatives that share similar expression patterns and protein-protein interactions. Most likely these closely related genes are paralogues and functionally redundant. This phenomenon is more common among plant MADS box transcription factors and complicates their functional analysis because all redundant genes need to be suppressed in order to obtain a mutant phenotype. Cosuppression has proven to be a way to suppress multiple genes with a high percentage of sequence similarity and therefore, it is a suitable method to identify functions for redundant genes. This technology has been applied successfully to determine the function of the petunia MADS box transcription factor FBP10. Petunia transformants repressing this gene revealed to be unable to flower and therefore *FBP10* was renamed to *PETUNIA*

FLOWERING GENE (PFG). Molecular analysis of these non-flowering plants demonstrated that besides *PFG* expression also the expression of *FBP26* was repressed. However, most likely this repression is indirect and PFG and FBP26 are not functionally redundant.

Overexpression of a gene of interest is another way to analyse its function and this technology has been used to gain insight in the function of FBP20, which is a MADS box gene expressed during the vegetative phase of development. Plants overexpressing this gene had leaf-like petals and ectopic trichome formation on floral organs. Based on these phenotypic alterations the gene was renamed to *UNSHAVEN* (*UNS*). Surprisingly, overexpression of a truncated UNS protein lacking the MADS domain resulted in the same phenotypic changes in both petunia and *Arabidopsis*. Molecular analysis of the mutated plants in combination with yeast two-hybrid analyses revealed that the observed phenotype is most likely caused by a dominant-negative effect on another protein involved in maintenance of the floral identity instead of a gain of function of UNS. As a consequence, the native function of UNS is still unclear. Nevertheless, the obtained results show that conclusions concerning gene function drawn from overexpression studies should be handled with care.

To determine whether dominant-negative mutations can be used more generally to obtain MADS box transcription factor mutants, several mutated forms of PFG were overexpressed in petunia. In addition mutations for the MADS box transcription factor APETALA1 (AP1) from *Arabidopsis* were generated and tested for their ability to repress *AP1* gene function in a dominant-negative manner. Unfortunately, overexpression of none of the generated constructs resulted in a dominant-negative effect, demonstrating that it is difficult to generate a universal dominant-negative strategy. Nevertheless, this study has elucidated the importance of some motifs and domains in the AP1 protein for its functioning. From these experiments it was also clear that more knowledge about the function of specific MADS box protein domains and their interactions is essential to predict which domain needs to be modified to obtain a dominant-negative version. To obtain information about the interactions between MADS box transcription factors, yeast two hybrid-experiments can be performed, because these systems have proven to be powerful methods to determine protein-protein interactions. However, no knowledge is obtained about the existence of these interactions in living plant cells and in which cell compartments the interactions take place. To get insight in the biological relevance of the identified interactions, in-planta analyses need to be performed. For this reason a few of the identified petunia MADS box protein dimers were analysed in leaf protoplasts by means of spectroscopy techniques based on Fluorescence Resonance Energy Transfer (FRET). All the in yeast

identified heterodimers for the ovule specific MADS box protein FBP11 could be confirmed in living cells and, in addition, homodimerisation was observed for the MADS box proteins FBP2, FBP5 and FBP9. This homodimerisation could not be demonstrated with the yeast systems. Subsequently, localisation studies were conducted with full-length and truncated proteins lacking the supposed bipartite Nuclear Localisation Signal (NLS), making use of Confocal Laser Scanning Microscopy (CLSM). The obtained results demonstrated that both partners of a MADS box protein dimer need to contain the NLS signal for nuclear localisation and furthermore, dimerisation appeared to be essential for this translocation from cytoplasm to nucleus. All these observations demonstrate again the importance of protein-protein interactions for MADS box protein functioning. The knowledge about these interactions does not only teach us about the molecular mechanisms underlying MADS box transcription factor functioning but gives also information about the presence of functional redundancy among members of this large family of transcription factors. Furthermore, a pilot experiment has shown that protein-protein interaction screens can be exploited for the identification of functional homologues (orthologues) of specific MADS box proteins from species that are genetically not very well characterised. These findings demonstrate that interaction mapping provide an additional tool for functional genomics.

Samenvatting

Homeotische genen bepalen de identiteit van de verschillende plantenweefsels en organen. Mutaties in deze genen leiden tot de vorming van organen of weefsels op plaatsen waar ze normaal niet gevormd worden. Leden van de MADS box familie van transcriptiefactoren vertegenwoordigen een belangrijke groep van homeotische genen. De eerste leden die geïsoleerd werden van deze grote familie van transcriptiefactoren, waren allen betrokken bij de ontwikkeling van bloemorganen. Genetische en moleculaire analyses van deze genen resulteerden in het bekende "ABC(DE)" model voor bloemontwikkeling. Tegelijkertijd werden MADS box transcriptiefactoren gekarakteriseerd, die betrokken waren bij ander functies, zoals de regulatie van de bloei.

Om inzicht te verkrijgen in de moleculaire regulatie, die ten grondslag ligt aan het functioneren van MADS box transcriptiefactoren, is de MADS box familie van de modelplant *Petunia hybrida* bestudeerd. MADS box eiwitten zijn actief als homo- of heterodimeren en recentelijk zijn zelfs ternaire en quarternaire MADS box eiwitcomplexen aangetoond. Om deze reden staat in deze studie de karakterisatie van MADS box eiwit-eiwit interacties centraal. In eerste instantie zijn enige nieuwe leden van de petunia MADS box familie geïsoleerd uit een bloeiwijzemeristeem- en zaadknopspecifieke cDNA bank. Genen behorend tot de petunia MADS box familie worden *FLORAL BINDING PROTEINs* (*FBPs*) of *PETUNIA MADS* (*pMADS*) genoemd en in totaal zijn er nu 23 bekend. Deze 23 genen zijn gekarakteriseerd middels sequentie analyse om te kijken naar fylogenetische verwantschap en met behulp van northern blot analyses om de expressie patronen te bepalen. Daarnaast zijn gist two-hybrid systemen gebruikt om de interacties van de eiwitten, die gecodeerd worden door de 23 MADS box genen, te bepalen. De verkregen resultaten zijn vergeleken met gegevens van andere MADS box families in de literatuur, o.a. van *Antirrhinum majus* en *Arabidopsis thaliana*. Hieruit is gebleken dat MADS box genen en hun functies in zijn algemeenheid geconserveerd zijn bij bloeiende planten. Gebruik makend van deze kennis konden functies van een aantal onbekende petunia en *Arabidopsis* MADS box transcriptiefactoren worden voorspeld. Daarnaast bleek dat een aantal van de 23 geanalyseerde transcriptiefactoren nauw verwant zijn aan andere leden van de familie en vergelijkbare expressie patronen en eiwit-eiwit interacties bezitten. Zeer waarschijnlijk zijn deze verwante genen paralogen en functioneel redundant. Dit blijkt een algemeen voorkomend fenomeen te zijn onder planten MADS box transcriptiefactoren en bemoeilijkt hun functionele analyse. In het geval van functionele redundantie moeten namelijk alle verwante genen worden

uitgeschakeld om een mutant fenotype te verkrijgen en zodoende inzicht in de functie van de genen.

Co-suppressie heeft bewezen een methode te zijn om meerdere, op sequentie niveau nauw verwante genen, uit te schakelen. Het is daarom een geschikte methode om de functies van redundante genen te bepalen. In deze studie is deze technologie succesvol toegepast om de functie van de petunia MADS box transcriptiefactor FBP10 te achterhalen. Petunia transformanten met repressie van dit gen waren niet in staat om te bloeien en derhalve is *FBP10* hernoemd tot *PETUNIA FLOWERING GENE (PFG).* Moleculaire analyse van deze niet bloeiende transformanten heeft aangetoond, dat er naast repressie van *PFG* ook repressie van *FBP26* was. Deze gecombineerde repressie is echter waarschijnlijk indirect en daarom zijn PFG en FBP26 niet functioneel redundant.

Overexpressie van een gen is een andere manier om inzicht te krijgen in de functie van het betreffende gen en deze technologie is toegepast om het MADS box gen *FBP20* te analyseren, die in een wildtype plant tot expressie komt gedurende de vegetatieve fase van ontwikkeling. Overexpressie van dit gen in transgene planten resulteerde in bladachtige kroonbladeren en ectopische trichoomvorming op bloemorganen. Gebaseerd op dit fenotype is het gen hernoemd tot *UNSHAVEN (UNS).* Opmerkelijk was dat overexpressie van een UNS eiwit zonder het MADS domein in zowel petunia als *Arabidopsis* hetzelfde effect gaf. Op basis van moleculaire analyses en gist two-hybrid experimenten werd geconstateerd dat het fenotype waarschijnlijk niet direct iets te maken heeft met de normale functie van UNS maar het gevolg is van een dominant-negatief effect op een andere factor, die betrokken is bij handhaving van de bloemidentiteit. Hieruit blijkt duidelijk dat we voorzichtig moeten zijn met het trekken van conclusies omtrent genfuncties uitsluitend op basis van overexpressie studies.

Om te bepalen of dergelijke dominant-negatieve mutaties meer algemeen toegepast kunnen worden om MADS box transcriptiefactor mutanten te genereren, zijn verschillende gemodificeerde vormen van PFG tot overexpressie gebracht in petunia. Daarnaast zijn mutaties gemaakt in de MADS box transcriptiefactor APETALA1 (AP1) van *Arabidopsis* en uitgetest in transgene planten. Helaas gaf geen van de geteste constructen het gewenste dominant-negatieve effect. Dit resultaat geeft duidelijk aan dat het niet eenvoudig is om een universele dominant-negatieve strategie te ontwikkelen voor MADS box transcriptiefactoren van planten. Desalniettemin heeft deze studie inzicht gegeven in het belang van bepaalde motieven en domeinen van het AP1 eiwit voor zijn functioneren. Verder werd ook duidelijk dat meer kennis vereist is over de rol van verschillende domeinen in het functioneren van MADS box transcriptiefactoren. Een manier om hier inzicht in te krijgen is door eiwit-eiwit interacties te bestuderen. Een methode

om eiwit-ewit interacties te bepalen is met behulp van gist two-hybrid experimenten zoals hiervoor al is beschreven voor de petunia MADS box familie. Deze gistsystemen zijn echter artificieel en geven beperkte informatie over de biologische relevantie van de gevonden interacties. Het is bijvoorbeeld mogelijk dat bepaalde interacties in een levende plantencel nooit optreden, omdat de twee eiwitten eenvoudig weg elkaar niet tegen komen. Daarom is het nodig om interacties, die aangetoond zijn met behulp van gist systemen, te bevestigen in levende plantencellen. In deze studie is dit uitgevoerd voor heterodimeren van het zaadknopspecifieke MADS box eiwit FBP11, die met behulp van het gist two-hybrid systeem waren aangetoond. De betreffende dimeren zijn geanalyseerd in bladprotoplasten met behulp van spectroscopische technieken gebaseerd op "Fluorescence Resonance Energy Transfer" (FRET). Middels deze technologie konden alle in gist geïdentificeerde FBP11 heterodimeren worden bevestigd in levende plantencellen. Bovendien werd homodimerisatie aangetoond voor de MADS box eiwitten FBP2, FBP5 and FBP9, die niet aangetoond kon worden met behulp van de gistsystemen. Vervolgens zijn lokalisatiestudies uitgevoerd voor complete MADS box eiwitten en MADS box eiwitten zonder het veronderstelde kern lokalisatie signaal (NLS), gebruik makend van Confocal Laser Scanning Microscopie (CLSM). Hieruit werd duidelijk dat beide partners van een MADS box eiwit het NLS signaal moeten bezitten voor transport van cytoplasma naar kern. Daarnaast bleek dimerisatie essentieel te zijn voor transport van de MADS box transcriptiefactoren naar de kern. Hieruit blijkt opnieuw het belang van eiwit-eiwit interacties voor het functioneren van MADS box transcriptiefactoren. Informatie over eiwit-eiwit interacties geeft namelijk informatie over het moleculaire werkingsmechanisme van MADS box eiwitten maar ook over functionele redundantie binnen deze grote familie van transcriptiefactoren. Aan de hand van een pilot experiment is zelfs gebleken dat technieken voor het aantonen van eiwit-eiwit interacties geëxploiteerd kunnen worden voor het identificeren van functionele homologen (orthologen) van specifieke MADS box eiwitten in belangrijke gewassen, die genetisch niet goed gekarakteriseerd zijn. Deze nieuwe methode voor het identificeren van orthologen op basis van interacties lijkt uitermate geschikt te zijn als nieuw gereedschap in de functionele genomica.

Nawoord

Vier jaar, 5 maanden, 30 dagen, ongeveer 200 vrijdagmiddagborrels, 4 labuitjes en meer dan 100 pagina's, zijn we al verwijderd van het startpunt van mijn promotie. En eindelijk is dan het moment aangebroken om de laatste regels te vullen en er een punt achter te zetten. De mens is gemaakt om vooruit te komen, dus de hoogste tijd om aan wat nieuws te beginnen! Toch kijk ik met erg veel plezier terug op de afgelopen 4 jaar en zal ik het "AIO-schap", dat door sommige mensen ook wel wordt gezien als een verlenging van het eeuwige studenten leven, zeker gaan missen. Naast schaatsen, concerten en festivals bezoeken, reizen, tuinieren en een avondje stappen is het doen van wetenschappelijk onderzoek een van mijn grootste hobby's geworden.

Vele mensen van de afdeling "Ontwikkelingsbiologie" (CPRO-DLO), die recentelijk is opgegaan in de business unit "Plant Ontwikkeling en Reproductie" (Plant Research International) hebben een steentje bijgedragen aan het onderzoek beschreven in dit proefschrift. Dit geldt zeker voor mijn directe collega's binnen de cluster "Plant Architectuur". Zonder hun kennis en inzet was het onmogelijk geweest om in 4 jaar tijd het geheel af te ronden. Silvia Ferrario (Mad about MADS), John Franken (King of Petunia), Jeroen Peters ("J"), Marco Busscher (*in-situ* forever), Stefan de Folter, Jacqueline Busscher, Mark Aarts (*Arabidopsis* rules), Diana Rigola, Gaby Tichtinsky, Anna Schchennikova, Olga Shulga, Esther van Tiel, Antonio Chalfun, Jurriaan Mes, Maurice Konings, Tineke Creemers, Yaxin Ge, allemaal bedankt voor de hulp op het lab! Ook wat betreft het denkwerk zijn er meerdere brains in serie geschakeld. Vele uurtjes heb ik samen met mijn kamergenotes Silvia, Olga en Anna zitten broeden op moeilijke biologische vraagstukken en dit was zeker niet zonder succes. *Last but not least*, wil ik onze clusterleider en tevens mijn copromotor Gerco Angenent bedanken. Het moet voor hem haast gevoeld hebben alsof hij zelf voor de tweede keer ging promoveren. Iedere keer als hij dacht aan een ontspannend weekend te beginnen kwam ik op de late vrijdagmiddag nog weer een manuscript bij hem inleveren. Middels de goede discussies die we samen gehad hebben en zijn kritische blik op mijn schrijfstijl, heeft hij een grote bijdrage geleverd aan dit promotieonderzoek.

Naast de mensen van Plant Research International zou ik ook graag mijn promotor Prof. Linus van der Plas willen bedanken. De discussies met hem en Sander van der Krol waren zeer nuttig om het overzicht te behouden en om op de juiste momenten de knopen door te hakken. Ook de mensen van het "MicroSpectroscopie centrum Wageningen", met name Dorus Gadella, wil ik bij deze graag bedanken voor de hulp bij de FRET-FLIM en FRET-SPIM metingen.

Daarnaast hebben de studenten Maarten Kooiker, Peter Zwiers en Erwin Brosens een bijdrage geleverd aan dit proefschrift gedurende het afstuderen. Ze dachten onderzoek aan planten te gaan doen in Wageningen, maar in de praktijk hebben ze nauwelijks planten gezien en werd het gist, gist en nog eens gist. Alle drie bedankt voor jullie inzet.

Tot slot wil ik nog graag een aantal mensen buiten het laboratorium noemen. Allereerst, mijn ouders, broer en zusje, die altijd enthousiast waren over dit werk. Bedankt, een betere stimulans kun je niet krijgen. Hetzelfde geldt voor Berlinda en Mark. En dan blijft er nog een iemand over: Dianne!!!! Vier jaar lang heb je mij op alle mogelijke manieren gesteund. Echt te gek!

Curriculum vitae

Richard Geerhard Herman Immink zag het eerste daglicht op 13 maart 1973 in Lemele en bracht zijn jeugd door in dit prachtige dorpje. In 1989 behaalde hij het MAVO diploma, gevolgd door het HAVO diploma twee jaar later. Vervolgens ging hij plantenveredeling studeren aan de Christelijke Agrarische Hogeschool (CAH) te Dronten. Al snel werd hier zijn interesse gewekt voor de moleculaire kant van dit vakgebied en daarom werd voor de specialisatie "Plantenbiotechnologie en Veredeling" gekozen. De stage in het derde jaar werd uitgevoerd bij het biotechnologiebedrijf MOGEN International NV, waar hij gewerkt heeft aan de optimalisatie van koolzaadtransformatie middels *Agrobacterium tumefaciens*. In 1995 werd de studie afgerond met het behalen van de Ingenieurs titel (Ing.). Vervolgens werd het doorstroomprogramma voor HBO studenten gevolgd aan de Universiteit van Wageningen. Voor zijn afstudeervak deed hij onderzoek in de groep van Prof. dr. ir. P.J.M. de Wit naar de rol die transcriptiefactoren spelen bij de afweer van de tomaat tegen de schimmel *Cladosporium fulvum*. Voor dit onderzoek kreeg hij in 1998 de "Moleculaire Fytopatholgie Prijs" uitgereikt. Het doctoraalexamen van de universitaire studie met als richting "Plantenveredeling en Gewasbescherming" en specialisatie "Moleculaire Biologie" werd in augustus 1997 afgerond. De bijbehorende Ingenieurs (Ir) titel werd verkregen met lof. Aansluitend aan deze studie werd hem de mogelijkheid geboden om als assistent in opleiding in dienst te treden bij de vakgroep Plantenfysiologie van Wageningen Universiteit. Van hieruit werd hij gedetacheerd bij Plant Research International (voorheen CPRO-DLO) in de groep van Dr. Gerco Angenent om eiwit-eiwit interacties van MADS box transcriptiefactoren uit planten te bestuderen. Deze studie heeft in ruim 4 jaar tijd geresulteerd in dit proefschrift. Sinds 1 februari 2002 heeft hij een aanstelling bij Plant Research International in dezelfde groep, om een betrouwbaar *high-throughput* systeem op te zetten voor het aantonen van eiwit-eiwit interacties en om de moleculaire achtergrond van het functioneren van MADS box transcriptiefactoren verder te analyseren.

Cover design: Monique Immink
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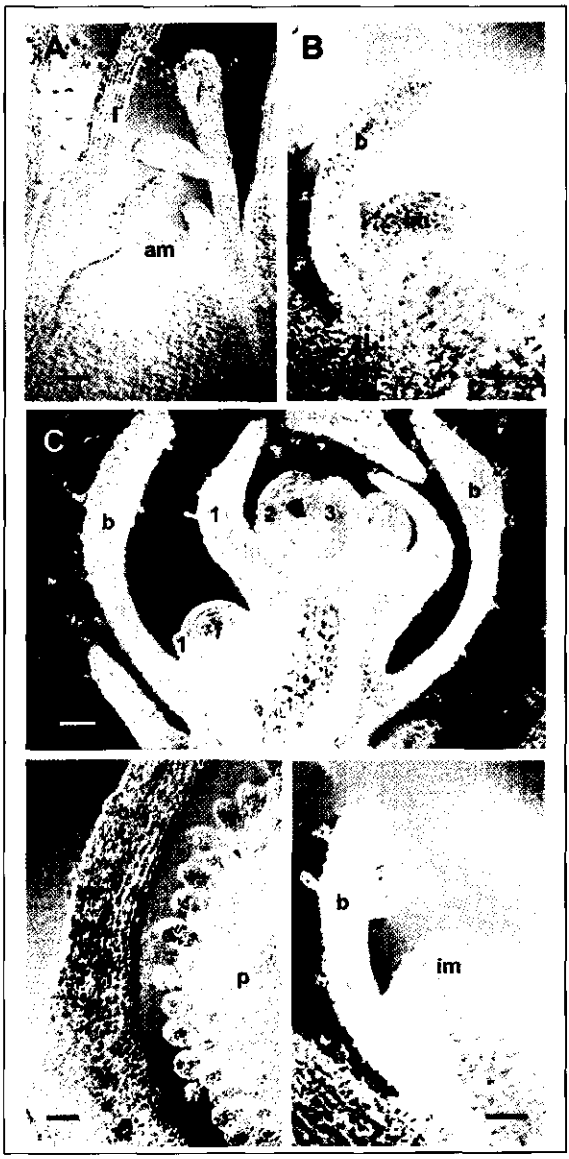
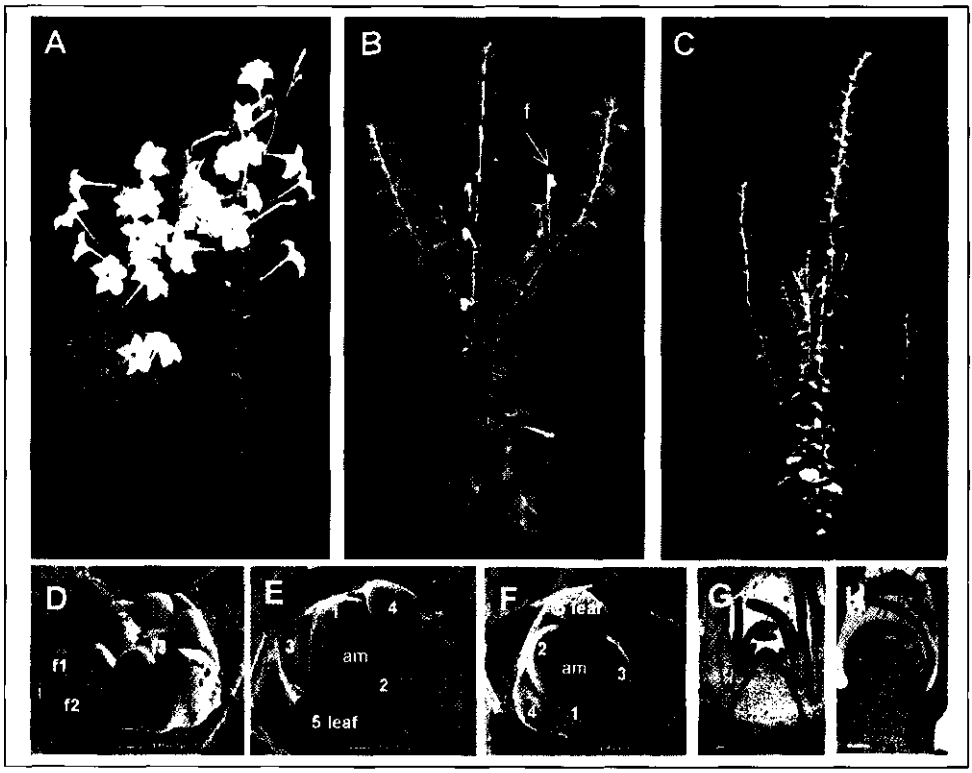
Appendix

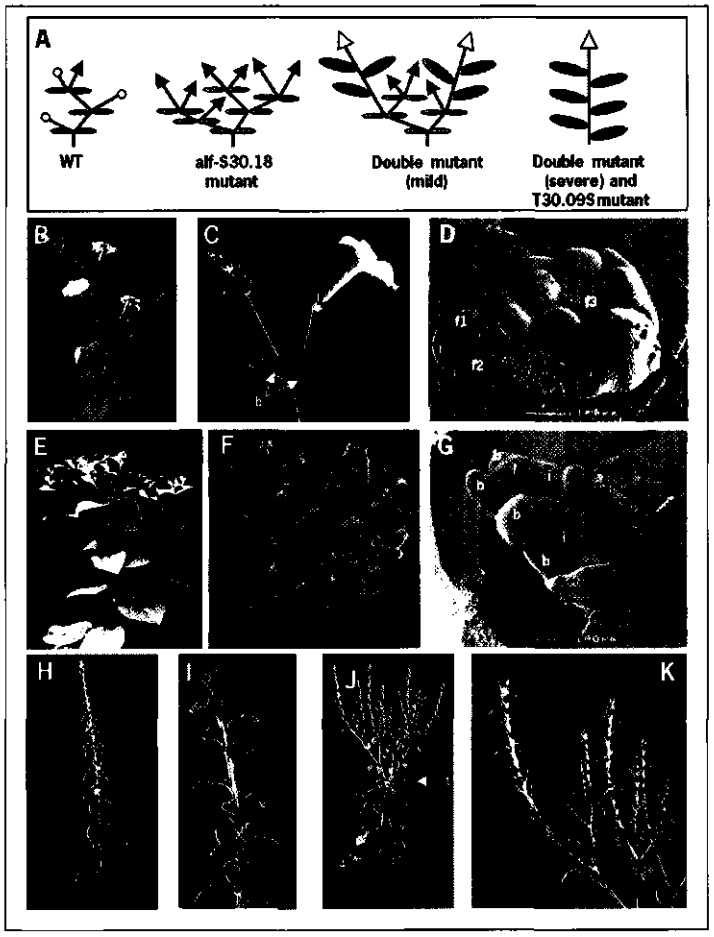
Colour figures



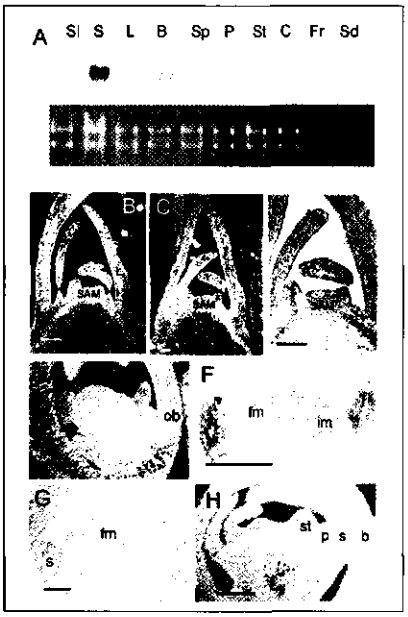
Chapter 3, Figure 3: *Localization of PFG mRNA in vegetative and inflorescence meristems, floral buds and carpels of* wild-type petunia plants (W115). Longitudinal sections were hybridized with an antisense digoxygenin-labeled *PFG* RNA-probe (A) to (D) or to a sense digoxygenin-labeled *PFG* RNA-probe as a negative control (E). (A) Vegetative shoot apex with emerging leaves on its flanks. (B) and (E) Inflorescence meristem in the axil of a bract. (C) Longitudinal section through an inflorescence with a floral bud and floral meristem. Sepal primordia start to emerge from the floral meristem. The floral whorls are indicated: sepal (1), petal (2), and stamen(3). (D) Part of the bottom part of an ovary showing hybridization signals in ovules and ovary walls. am, vegetative apical meristem; b, bract; ow, ovary wall; f, floral meristem; im, inflorescence meristem; l, leaf; o, ovule; p, placenta. Bars = 100 μm.



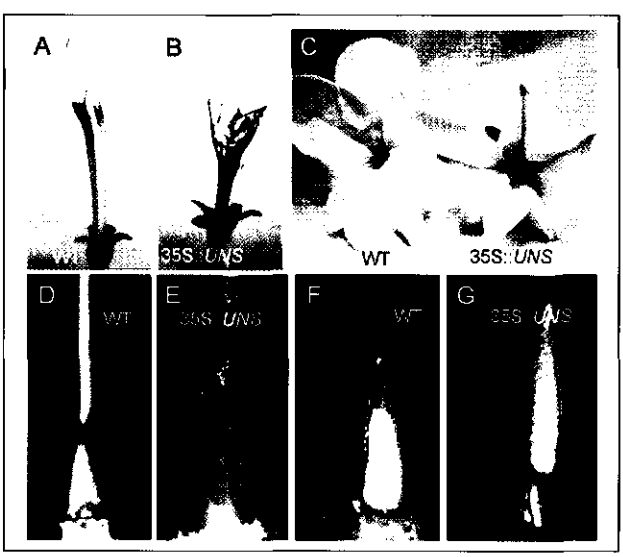
Chapter 3, Figure 4: Comparison of morphology and development of petunia wild-type plants (W115) and *pfg* cosuppression plants. (A) Petunia wild-type plant. After flower induction flowers are continuously produced from the apical inflorescence meristem. (B) Primary *pfg* cosuppression transformant in which the switch to generative development is initially made and some flowers (f) are formed, whereafter it reverts to vegetative growth. (C) Severe *pfg* cosuppression plant completely blocked in the switch from vegetative to generative development. (D) Scanning Electron Microscopy (SEM) image of a wild-type inflorescence. The inflorescence meristem (i) has generated three floral meristems (f1 to f3), from which the oldest one (f3) has initiated five stamen primordia (third whorl) and five petal primordia (second whorl). The sepals that enclose the flower partly at this stage were removed except for one. (E) SEM image of shoot apex from a vegetative wild type plant. Leaves are generated in a spiral phyllotaxy, characteristic for vegetative development. Leaves are numbered from 1 (youngest) to 5 (oldest) in order they are generated from the apical meristem (am). (F) SEM micrograph of shoot apex from an elongated shoot of a severe *pfg* cosuppression plant at a stage, when wild-type plants are already flowering. Leaves are numbered as described for (E). (G) Light micrograph of longitudinal section of a vegetative wild-type shoot apex. (H) Light micrograph of longitudinal section of an elongated shoot apex of a severe *pfg* cosuppression plant. Bars = 100 μm.



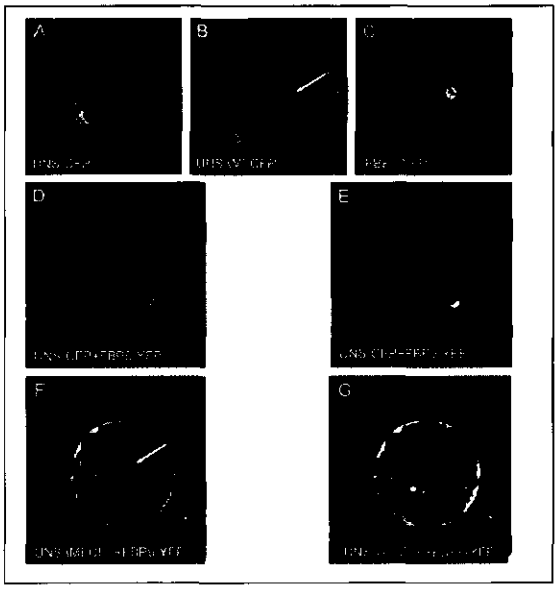
Chapter3, Figure 6: Comparison of phenotypes and development of wild-type petunia plants (W115), *alf-S3018* mutant plants and *pfg alf* double mutant plants. (A) Schematic representation of inflorescence structures of a wild-type plant, *alf-S3018* mutant and mild and severe *pfg alf* double mutants. Bracts and leaves are indicated by small light green and big dark green ovals, respectively. Vegetative meristems and inflorescence meristems are shown by open and closed triangles, respectively. Open circles are flowers. (B) Wild-type petunia plant (W115). (C) Structure of a wild-type petunia inflorescence. An indeterminate inflorescence meristem and a flower meristem develop in the axils of two bracts (b) which are arranged in an opposite position. (D) Scanning Electron Microscopic (SEM) image of a wild-type inflorescence apex. The inflorescence has generated three floral meristems (f1 to f3), from which the oldest one (f3) has initiated five stamen primordia (third whorl) and five petal primordia (second whorl). The sepals that enclose the flower partly at this stage were removed except for one. (E) Side view of an *alf-S3018* inflorescence in a W115/W138 background. Bifurcation of the *alf* inflorescence meristem is similar to that in wild-type inflorescences. However, both meristems behave as inflorescence meristems and continue to develop bracts on their flanks and divide again to form new inflorescence meristems, finally giving rise to a highly branched structure. (F) Top view of an *alf-S3018* inflorescence. (G) SEM image of *alf-S3018* inflorescence. i, inflorescence meristem; b, bract. (H) Severe *pfg alf* double mutant, in which the switch from vegetative to generative development is abolished. This double mutant phenotype is indistinguishable from that of a *pfg* single mutant. (I) Elongated shoot of a severe *pfg alf* double mutant, with leaves arranged in a spiral phyllotaxy. (J) Mild *pfg alf* double mutant. The switch from vegetative to generative development (t) is initially made and the *alf* mutant phenotype becomes apparent. After a few bifurcations the inflorescence reverts to vegetative growth with spirally arranged leaves like in the *pfg* single mutant. (K) Detail of (J).



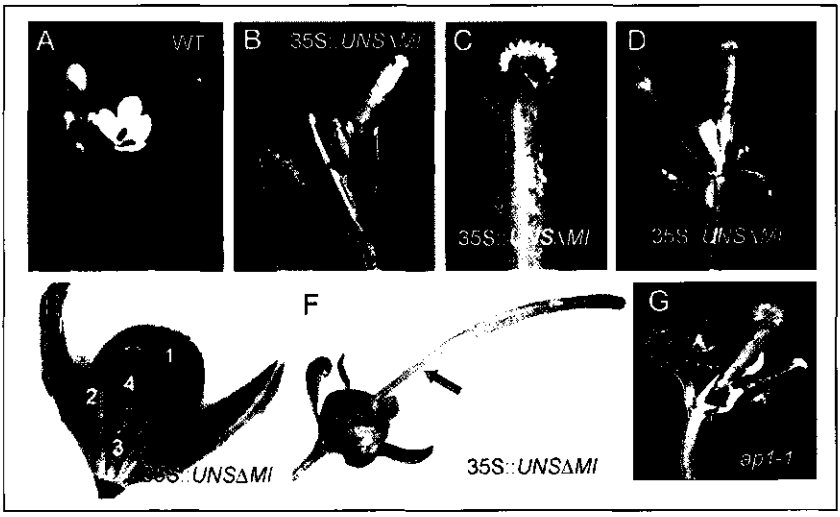
Chapter 4, Figure 2: Expression of *UNS* in wild type tissues of petunia. **(A)** RNA gel blot using RNA from seedlings (Sl), stems (S), leaves (L), bracts (B), sepals (Sp), petals (P), stamens (St), carpels (C), fruits (Fr) and seeds (Sd) of wild type petunia hybridised with an *UNS* specific probe. Below the blot is a picture of the gel prior to blotting, stained with ethidium bromide, illustrating equal loading of the samples. **(B-H)** RNA *in situ* localisation of *UNS* in shoot apical meristems and developing petunia inflorescences. Longitudinal sections were hybridised with a digoxygenin-labelled sense *UNS* probe **(B)** or antisense UNS RNA **(C-H).** Abbreviations used are: L, leaf; SAM, shoot apical meristem; ob, old bract; b, bract; fm, floral meristem; im, inflorescence meristem; s, sepal; p, petal; st, stamen. **(B-D)** Shoot apical meristems with emerging leaves on their flanks. The *UNS* mRNA is equally distributed throughout the apex and the emerging young leaves. **D** shows a higher magnification of **C**. **(E-F)** Inflorescence apex with developing floral meristem. **F** is a higher magnification of **E**. The expression is strong in young bracts, but weak hybridisation signals are also present in the floral and inflorescence meristems. **(G)** Floral meristem with developing sepals. **(H)** Young flower bud with petal and stamen primordia. Bars represent 200um in **B-F** and 500um in **G** and **H.**



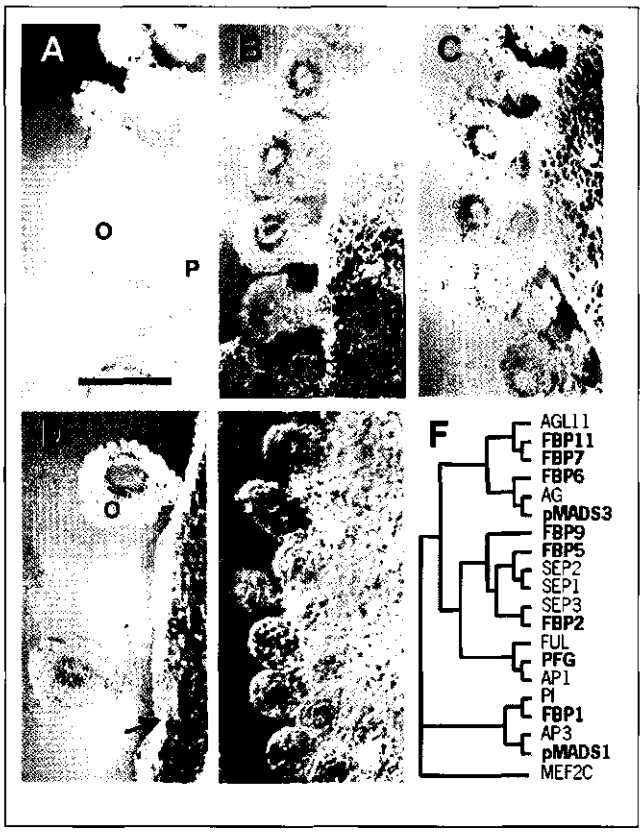
Chapter 4, Figure 3: Flowers and ovaries of wild type and 35S::*UNS* petunia plants. **(A-B)** wild type (WT) and transgenic (35S::*UNS*) petunia buds at the same stage of development. The transgenic bud is significantly shorter and greener than a bud of WT plants. **(C)** WT and 35S::*UNS* mature flowers. The reduced dimensions are maintained till full maturity of the transgenic flower, which does not open completely. **(D-G)** Ovaries of WT **(D, F)** and 35S::*UNS* **(E, G)** plants. The transgenic ovaries are significantly longer than those from WT. The outer epidermis is covered with trichomes and a stem-like structure develops at the basis of the septum (arrow in **G**). One carpel is removed from the ovaries in **F** and **G**.



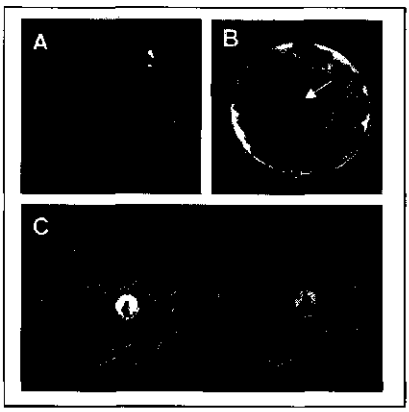
Chapter 4, Figure 8: Confocal Laser Scanning Microscopy (CLSM) of petunia leaf protoplasts expressing different fusion proteins. Chlorophyll auto-fluorescence is shown in red. **(A-B)** The fusion proteins between CFP and UNS full-length or UNSΔMI are localised in the cytoplasm. The arrow in B indicates the position of the nucleus. **(C)** The fusion protein FBP9-YFP is translocated to the nucleus. **(D-E)** Protoplast imaged with CFP **(D)** and YFP **(E)** filters, respectively. UNS-CFP and FBP9-YFP are colocalised in the nucleus. **(F-G)** Single protoplast expressing UNSΔMI -CFP and FBP9-YFP, imaged with CFP **(F)** and YFP **(G)** filters, respectively. Both signals are localised in the cytoplasm. Arrow indicates the position of the nucleus.



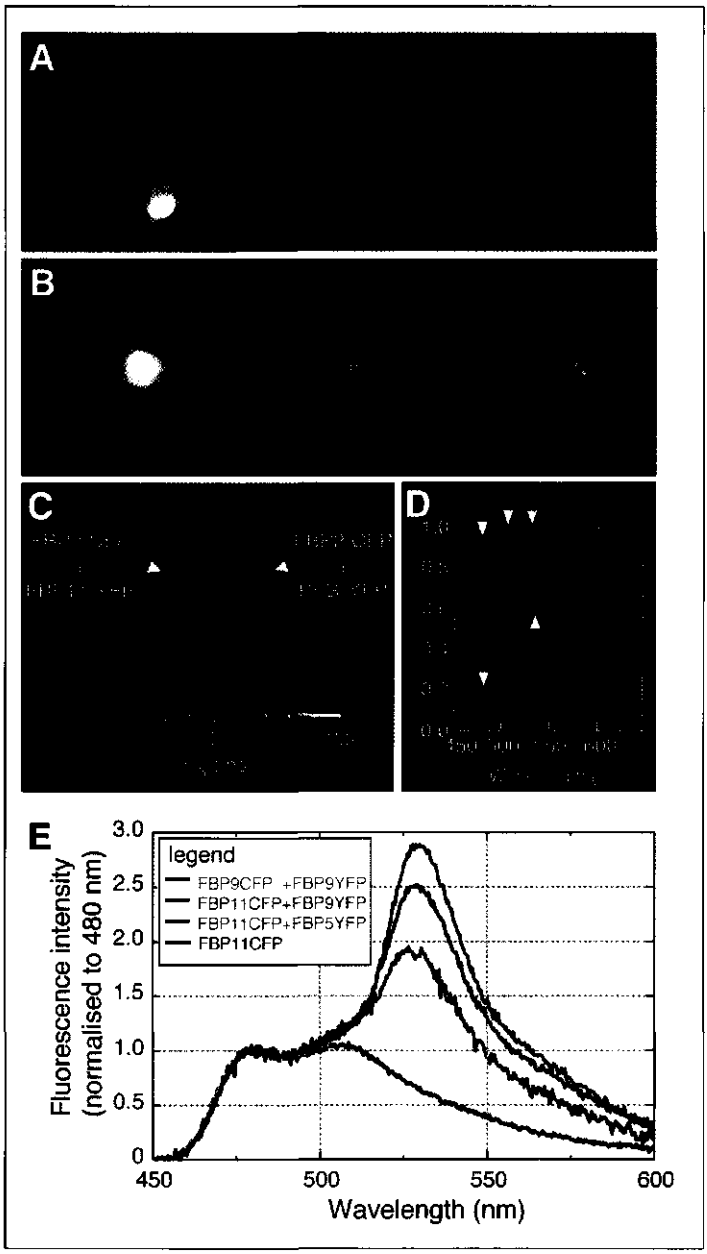
Chapter4, Figure 9: Flower morphology of 35S:: *UNSΔMI* Arabidopsis lines. **(A)** Wild type Arabidopsis inflorescence (left) and silique (right). **(B-F)** Flowers of 35S::*UNSΔMI* transformants: stellate trichomes on the abaxial side of the sepals and on the pistil are visible in **B** and **C**. The arrows in **B** and **D** point to the secondary buds arising at the axil of the first whorl organs. Petals are completely absent or largely reduced in size and greenish. **(E)** A young 35S::*UNSΔMI* flower with small green organs in whorl 2. The four flower whorls are numbered. **(F)** Flower of 35S::*UNSΔMI* after fertilisation. The arrow points to the elongated stem at the basis of the silique. **(G)** *ap1-1* flower; the arrow indicates the axillary bud arising at the axil of the first whorl organs.



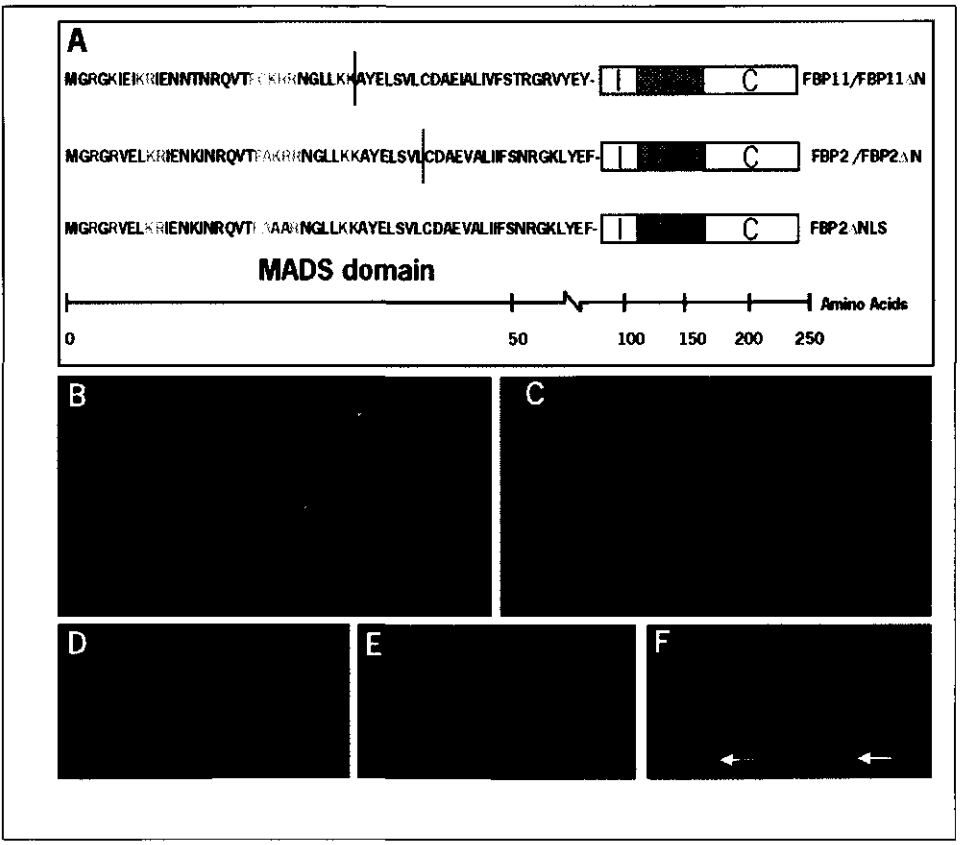
Chapter 6, Figure 1: Phylogenetic and expression analyses of *FBP11* and the genes encoding its interaction partners. Longitudinal sections were hybridised with digoxygenin-labelled probes (Red signal). (*A*) Almost mature W115 wildtype ovules, hybridised with a sense *FBP11* probe. (*B*) Same stage as (*A*), hybridised with *FBP11* antisense probe. (*C*) Same stage as (*A*), hybridised with *FBP2* antisense probe. (*D*) Sepal of CaMV35S::*FBP11* overexpression plant with ectopic ovule formation on adaxial side, hybridised with *FBP2* antisense probe. The signal in the epidermal cell layer is marked with an arrow. (*E*) Young developing ovules, hybridised with *PFG* antisense probe. (*F*) Phylogenetic tree of MADS box genes described in this study. As a reference *Arabidopsis* and petunia MADS box genes with a known function are included. All petunia MADS box genes are in bold. For the comparison of the proteins the MADS box, I region and K box domains were used. AP1 = *APETALA1*, AP3 = *APETALA3*, AG = *AGAMOUS*, AGL = *AGAMOUS-Like*, FBP = *FLORAL BINDING PROTEIN*, FUL = *FRUITFULL*, MEF2C = *Myocyte Enhancer Factor 2C*, O = ovule, PFG = *PETUNIA FLOWERING GENE*, PI = *PISTILLATA*, P = placenta, S = sepal (adaxial side) and SEP = *SEPALLATA*. Bar in (*A*) = 1.0 mm.



Chapter 6, Figure 2: Localisation of MADS box proteins in petunia leaf protoplasts imaged by Confocal Scanning Laser Microscopy (CSLM). Chlorophyll auto-fluorescence is shown in red. (*A*) Nuclear localised FBP9-YFP. (*B*) Cytoplasmic localised FBP11-CFP. The position of the nucleus is marked with an arrow. (*C*) Protoplast expressing FBP11-CFP in combination with FBP2-YFP. On the left, the nuclear localised CFP signal, on the right the YFP signal of the same protoplast.



Chapter 6, Figure 3: FRET-FLIM and FRET-SPIM analyses of protoplasts expressing petunia MADS box proteins fused to CFP and YFP. (*A*) Protoplast expressing FBP2-CFP + FBP11-YFP. The border of the protoplast is artificially marked with a red circle. Left, Fluorescence image of a protoplast. Middle, Fluorescence intensity image (reconstructed from the FLIM-data stack). Right, Fluorescence lifetime image. The fluorescence lifetime at each pixel is represented in a pseudocolour index. Each micropixel represents a CFP fluorescence lifetime. Green represents a mask for pixels with low fluorescence intensity that are excluded from the lifetime analysis resulting in a lifetime image of the nucleus only. (*B*) Protoplast expressing FBP2-CFP + PFG-YFP. Left, middle, right as described for (*A*). (*C*) FRET-FLIM analysis. Temporal histogram and pseudocolour scale of the fluorescence pixel values of (*A*) and (*B*). (*D*) FRET-SPIM analysis. Spectra of protoplasts expressing FBP2-CFP + FBP11 YFP (Red line) and of protoplasts expressing FBP2-CFP + PFG-YFP (orange line). The CFP emission peaks are marked with a cyan-coloured arrowhead and the YFP emission peak with yellow arrowheads. Fluorescence intensities were normalised to one for easy comparison. (*E*) FRET-SPIM analyses of petunia protoplasts transfected with FBP11CFP, spectrum of one representative protoplast is shown.



Chapter6, Figure 4: Role of Nuclear localisation Signal (NLS) in MADS box protein translocation. (*A*) Representation of MADS box protein structure and the predicted position of NLSs. The positions of the truncations are indicated with a vertical red line in the protein sequence and with "Δ" in the name of the protein. The bipartite NLS localised in the N-terminal MADS domain is marked blue and conserved basic amino acids in the MADS domain are marked in green. Mutated amino acid residues in the NLS are marked in red in the protein sequence and with "ΔNLS" in the name of the protein. I = intervening region, K = K-box, C = C-terminal region. All images (*B-F*) were obtained by Confocal Scanning Laser Microscopy (CSLM). Chlorophyll auto-fluorescence is shown in red. (*B*) Localisation of FBP11-CFP (left) and FBP11ΔN-CFP (right). (*C*) Localisation of FBP2-YFP (left) and FBP2ΔN-YFP (right). (*D*) Nuclear colocalisation of FBP11-CFP and FBP2-YFP. On the left the CFP signal, on the right the YFP signal of the same protoplast. (*E*) Cytoplasmic colocalisation of FBP11ΔN-CFP and FBP2-YFP. On the left the CFP signal, on the right the YFP signal of the same protoplast. (F) Colocalisation of FBP11-CFP and FBP2ΔNLS-YFP. On the left the CFP signal, on the right the YFP signal of the same protoplast. The nucleus is marked with an arrow.