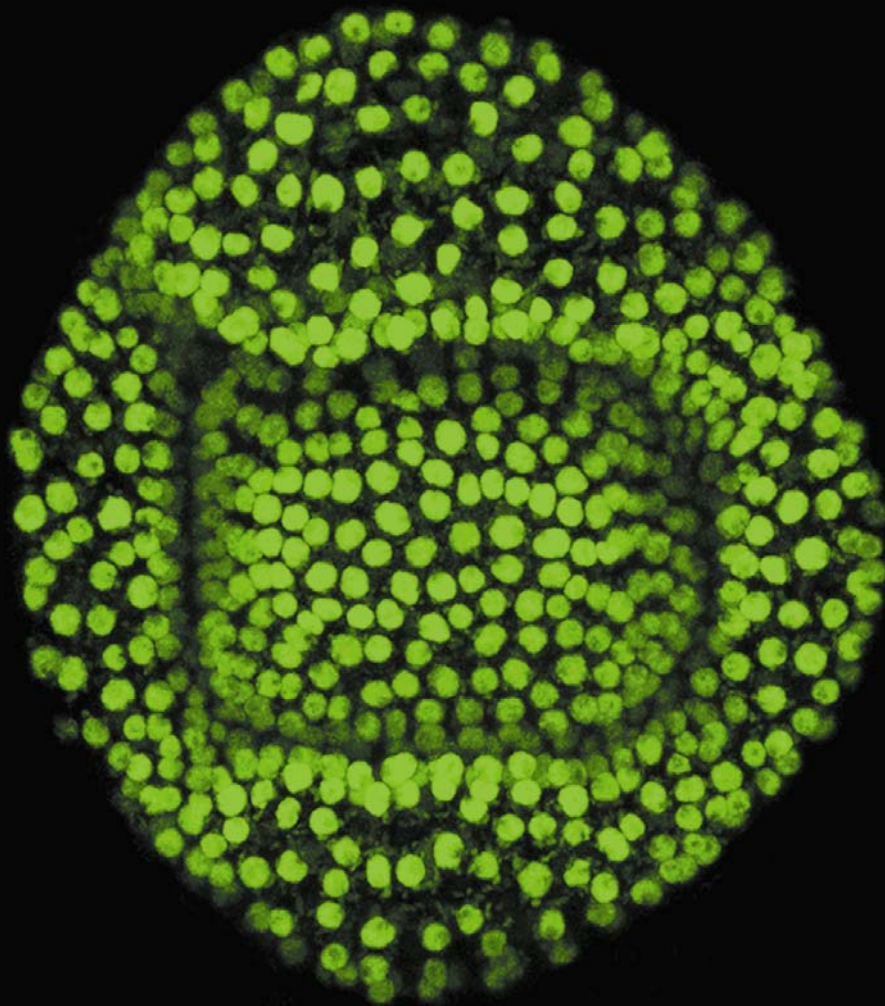


# MADS on the move

A study on MADS domain protein function and movement  
during floral development in *Arabidopsis thaliana*



Susanna Leonora Urbanus

# Propositions

1. Intercellular movement needs to be considered in the context of the specific location and developmental stage within the plant.  
[This thesis, Wu *et al.* (2003) Development]
2. The observation that some MADS domain transcription factors move and others do not, indicates that the movement has a biological relevant function.  
[This thesis]
3. In confocal laser scanning microscopy good controls are essential to avoid the mistake of regarding fluorescent artefacts as interesting results.
4. Insect-directed guiding patterns in flowers and inflorescences created by UV reflection and absorption might interfere with confocal laser scanning microscopy of these tissues.
5. Depictions of fish with air bubbles coming out of their mouths to indicate their presence in water are in most cases wrong.
6. If chewing gum improves cognitive abilities, ruminants such as cattle, goats and sheep must be philosophers.  
[Scholey *et al.* (2009) Physiology & Behavior, Smith (2009) Nutritional Neuroscience]
7. People in cars with bumper stickers have a higher tendency to show aggressive road behaviour, so watch out for cars that say "Peace".  
[Szlemko *et al.* (2008) Journal of Applied Social Psychology]
8. Growing older is the process of becoming less naive and more cynical.

Propositions belonging to the thesis, entitled

"MADS on the move: a study on MADS domain protein function and movement during floral development in *Arabidopsis thaliana*".

Susanna L. Urbanus

Wageningen, 19 May 2010

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## **Thesis Committee**

### **Thesis supervisor**

Prof. dr. ir. G.C. Angenent  
Personal chair at the Laboratory of Molecular Biology  
Wageningen University

### **Thesis co-supervisor**

Dr. ir. R.G.H. Immink  
Senior scientist, Business unit Bioscience  
Plant Research International, Wageningen

### **Other members**

Prof. dr. M.M. Kater, University of Milan, Italy  
Dr. ir. M.J. Ketelaar, Wageningen University  
Prof. dr. R.E. Koes, VU University Amsterdam  
Prof. dr. S.C. de Vries, Wageningen University

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Plant Sciences

# MADS on the move

A study on MADS domain protein function and movement  
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Susanna Leonora Urbanus

## **Thesis**

submitted in fulfilment of the requirements for the degree of doctor  
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You don't need eyes to see,

you need vision





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# Chapter 1

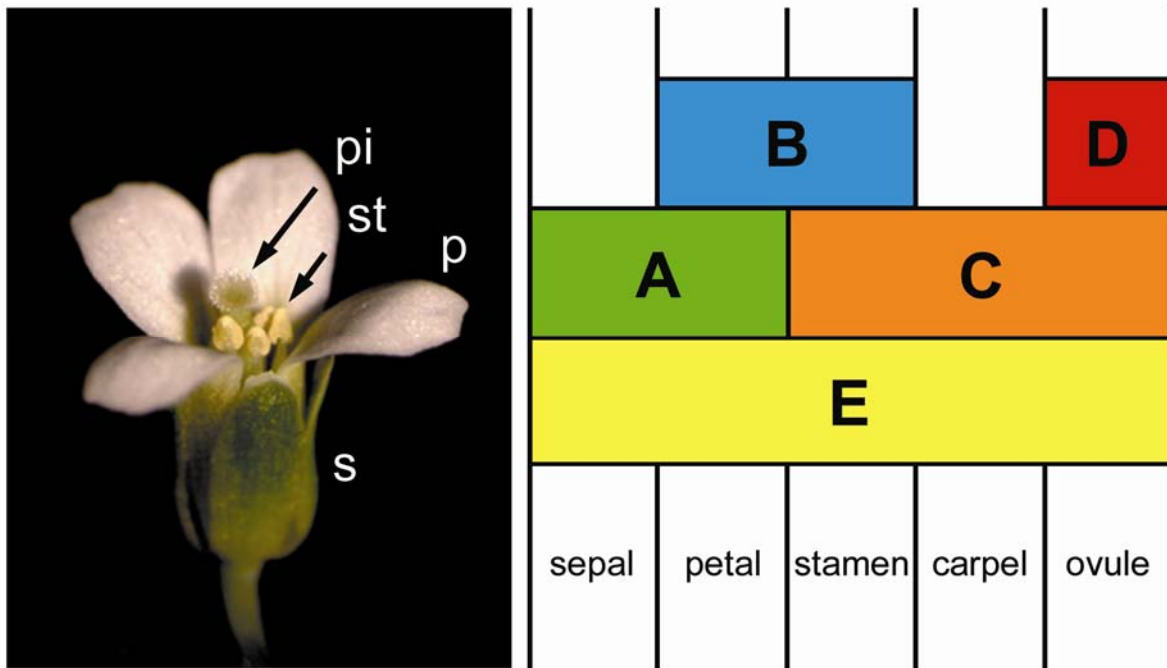
General introduction

## Flowers and MADS domain transcription factors

Evolution has graced our world with plants that bear their reproductive organs in a spectacular array of diverse and beautiful flowers. Although the flowering plant *Arabidopsis thaliana* has only small and rather humble flowers, they have been a major contributor in our understanding of floral development. Indeed, *Arabidopsis thaliana* has been in the scientific spotlight of the plant biology field ever since it was recognized that this small weed could be a good model plant under laboratory conditions [1]. This is, among others, due to its small size, rapid generation time, efficient self-pollination, prolific seed production, and the relative easy manipulation of its genetic material. *Arabidopsis thaliana* flowers consist of four sepals, four petals, six stamens, and two carpels that form the pistil (Figure 1). The stamens are the male reproductive organs and carry pollen inside the anther. The pistil, which is the female reproductive organ, holds the ovules that will develop into seeds after fertilization by the pollen. All these floral organs are developed from a source of proliferating pluripotent cells, called the floral meristem [2]. The MADS domain transcription factor family plays an important role during floral development, among others by specifying the different floral organ identities through distinct combinations of MADS domain transcription factor types. These combinations are illustrated in the extended 'ABC' model, where A+E-type MADS domain transcription factors together specify sepal identity, A+B+E-type proteins together specify petal identity, B+C+E-type proteins together specify stamen identity, C+E-type proteins together specify carpel identity, and finally C+D+E-type proteins together specify ovule identity (Figure 1) [3-9].

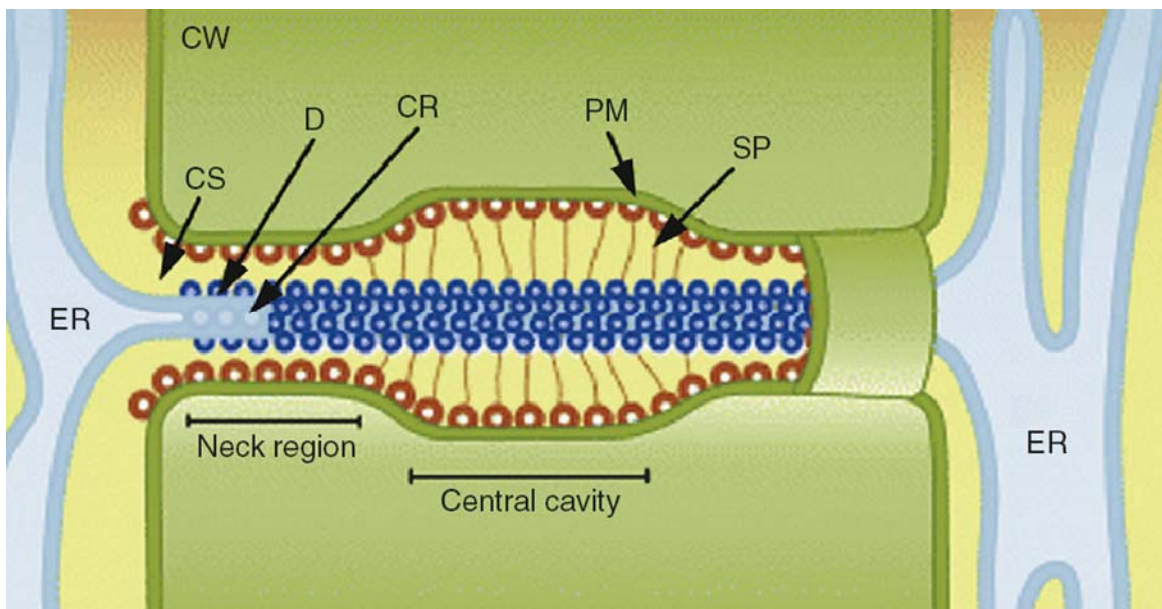
MADS domain transcription factors can initiate developmental programs through the regulation of downstream target genes [10]. Besides regulating the expression of these target genes, MADS domain transcription factors are also able to regulate their own expression via autoregulatory loops [11-14]. It is thought that MADS domain transcription factors need to be in a dimeric form, either homo- or heterodimeric, to be able to enter the nucleus where they perform their regulatory function [15-17]. The binding of MADS domain proteins to the regulatory DNA sequences of target genes is mediated by the N-terminal located conserved MADS domain, and occurs either in the form of a dimer or in a multimeric fashion, for instance in the tetrameric form that was proposed in the 'quartet' model [10, 12, 18-20]. The E-type SEPALLATA (SEP) MADS domain transcription factors that play an important role in all floral organs (Figure 1), seem to function as 'glue' proteins that facilitate the formation and functioning of these multimeric MADS domain protein complexes [5, 7, 14, 20-22].

That MADS domain transcription factors can be transported between cells was shown in *Antirrhinum majus* by the two B-type proteins DEFICIENS (DEF) and GLOBOSA (GLO) that can move outwards to the epidermal cell layer in floral meristems [23]. In contrast, the respective *Arabidopsis thaliana* B-type orthologues APETALA3 (AP3) and PISTILLATA (PI) do not appear to have the same ability for outward intercellular transport in the floral meristem, nor are these proteins able to travel inwards [24]. Also the A-type protein APETALA1 (AP1) does not travel inwards in floral meristems [25, 26]. There are however



**Figure 1. *Arabidopsis thaliana* flower and the extended 'ABC' model.**

On the left, a photograph of an *Arabidopsis thaliana* flower indicating the green sepals (s), the white petals (p), the stamens (st) that carry pollen in the yellow anthers, and the pistil (pi) that is formed from two carpels and contains ovules. On the right, a representation of the extended 'ABC' model that illustrates the distinct combinations of MADS domain transcription factor types that are needed for the specification of the sepal, petal, stamen, carpel and ovule identities.



**Figure 2. Schematic representation of a plasmodesma spanning the cell wall between adjacent cells.**

Indicated are the neck region, the central cavity, the endoplasmic reticulum (ER), the cytoplasmic sleeve (CS), the desmotubule (D), the central rod (CR), the plasma membrane (PM), and the spoke-like connections (SP) between the desmotubule and the plasma membrane that may control the aperture of the plasmodesma. Blue and red circles represent plasmodesma-specific proteins. Reprinted with permission [27]

suggestions that certain MADS domain transcription factors have non-cell-autonomous functions, i.e. functions beyond the cell in which the protein is expressed, such as the C-type protein AGAMOUS (AG) in floral meristem integrity [28, 29] and FRUITFULL (FUL) in pistil development [30].

### **Intercellular transport of transcription factors**

In plants, intercellular transport of macromolecules, such as transcription factors, is thought to be mediated by dynamic channels that connect the cytoplasm of neighbouring cells [27, 31, 32]. These symplastic connections, called plasmodesmata, consist of a plasma membrane-lined channel through the cell wall that is filled with a central desmotubule derived from the endoplasmic reticulum (ER) and a cytoplasmic sleeve (Figure 2). The cytoplasmic sleeve is the major conduit for macromolecular transport and contains components that regulate plasmodesmal structure and transport. Based on how they are created, plasmodesmata can be classified into two types [33]. Primary plasmodesmata are formed during cell divisions when cytoplasmic strands with ER tubules become enclosed in newly created cell walls. These primary plasmodesmata are initially simple channels, but they can be modified into more complex, branched structures later on. Secondary plasmodesmata, on the other hand, are actively created across existing cell walls and are often complex, branched structures.

Plasmodesmata play a crucial role in developmental processes in a plant through their control over intercellular transport of developmental signals and their ability to join cells on a supracellular level into symplastic domains [34-39]. These symplastic domains, which are partially or completely isolated from intercellular communication with surrounding tissues, make it possible that different developmental processes proceed next to each other in relative isolation. The diffusion of macromolecules through plasmodesmata is controlled by the aperture of the plasmodesmal channel. This aperture appears to reduce in size with increasing levels of tissue differentiation, allowing increasingly smaller sized macromolecules for intercellular transport. However, besides passive transport there is also the possibility of active transport through the plasmodesmata. Especially viral movement proteins are known for their ability to actively enlarge the plasmodesmal channel and thereby help the spread of viral infections throughout the plant [40]. There are also some known plant-specific proteins that have a similar ability to enlarge the plasmodesmal channel, such as the KNOTTED-LIKE HOMEODOMAIN (KNOX) transcription factors that are involved in the establishment and maintenance of plant meristems [41, 42]. Transport through plasmodesmata can also be reversibly blocked to create boundaries between symplastic domains or in response to abiotic or biotic stress [43-47]. This is achieved through callose deposition in the neck region of the plasmodesmal channel.

## Looking inside the living plant

Although genetic screens and *in vitro* and *in vivo* studies on protein-protein and protein-DNA interactions provide important information on how MADS domain transcription factors are able to function, understanding of the behaviour of MADS domain transcription factors within their own context *in planta* is still limited. Since the discovery of the GREEN FLUORESCENT PROTEIN (GFP) from the jellyfish *Aequorea Victoria* and the subsequent improvement and development of similar fluorescent markers with other spectra and physical characteristics, it has become possible to observe cellular structures and processes in living cells by fluorescence microscopy [48-53]. Especially in combination with Confocal Laser Scanning Microscopy (CLSM), where thick tissues can be optically sectioned and later reconstructed into 3-D images, the visualization of processes in intact plant tissues has become possible. In this thesis we investigated the behaviour of fluorescently-tagged MADS domain proteins during *Arabidopsis thaliana* floral development, and explored the importance of intercellular transport for MADS domain transcription factor functioning.

In Chapter 2, we describe different methods of tagging the MADS domain proteins AG, SEP3 and FUL for chromatin immunoprecipitation, chromatin affinity purification and *in planta* imaging. This research demonstrates that tagging of MADS domain proteins frequently results in loss-of-function phenotypes in the plant, especially when the MADS box genes are under the control of the constitutive *CaMV35S* promoter. Plants that express tagged MADS box genes from genomic fragments that include all or most of the regulatory elements, and therefore mimic the natural expression pattern as much as possible, show lower levels of loss-of-function phenotypes and are also more useful to investigate biologically relevant behaviour of the MADS domain proteins.

In Chapter 3, the spatio-temporal localisation patterns of GFP-tagged MADS domain proteins AG, AP1, SEP3 and FUL during floral development by CLSM are reported. These analyses show that there are several cases of MADS domain protein presence in specific tissues where no mRNA could be detected. This could indicate that there is intercellular transport of MADS domain proteins in meristematic tissues during floral development. The implications of the observed behaviour of the different MADS domain proteins for MADS domain protein functioning are discussed.

In Chapters 4 and 5 we describe the different methods that were used to investigate whether MADS domain proteins are indeed able to transport between cells during floral development. Attempts to investigate intercellular MADS domain protein transport with microinjection techniques and by using the photoconvertible fluorescent mEosFP-tag are discussed. In plants that specifically overexpress GFP-tagged MADS domain proteins AG, AP3, PI, or SEP3 in the epidermis, all tested proteins were able to move within the epidermal cell layer, while only AG could also move from the epidermis to the subepidermis. In these plants we analyzed the effects of epidermal MADS domain protein expression on the plant phenotype and also the ability of both epidermis-expressed GFP-tagged AG and AP3 to complement their corresponding mutant backgrounds.

In Chapter 6, we explore the mechanisms behind the previously observed behaviour of

GFP-tagged SEP3 during petal and stamen development (Chapter 3). Just prior to the initiation of petal and stamen primordia GFP-tagged SEP3 proteins change their subcellular localisation from predominantly nuclear to more cytoplasmic, and at later stages GFP-tagged SEP3 protein seems to disappear in the middle of the primordia without the loss of *SEP3* mRNA expression. These two processes could be regulated at a post-transcriptional level by mechanisms that are discussed. Additionally, we investigated the possible involvement of the F-box protein UNUSUAL FLORAL ORGANS (UFO) that regulates petal and stamen development.

Finally, in Chapter 7 the results of this thesis are discussed and placed into a broader context. Special attention is given to the ability of GFP-tagged AG to travel between cells and the behaviour of GFP-tagged SEP3 during petal and stamen development.



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# Chapter 2

## Tagging of MADS domain proteins for chromatin immunoprecipitation

Stefan de Folter

Susan L Urbanus

Lisette GC van Zuijlen

Kerstin Kaufmann

Gerco C Angenent

### Abstract

**Background:** Most transcription factors fulfil their role in complexes and regulate their target genes upon binding to DNA motifs located in upstream regions or introns. To date, knowledge about transcription factor target genes and their corresponding transcription factor binding sites are still very limited. Two related methods that allow *in vivo* identification of transcription factor binding sites are chromatin immunoprecipitation (ChIP) and chromatin affinity purification (ChAP). For ChAP, the protein of interest is tagged with a peptide or protein, which can be used for affinity purification of the protein-DNA complex and hence, the identification of the target gene.

**Results:** Here, we present the results of experiments aiming at the development of a generic tagging approach for the Arabidopsis MADS domain proteins AGAMOUS, SEPALLATA3, and FRUITFULL. For this, Arabidopsis wild type plants were transformed with constructs containing a MADS-box gene fused to either a double *Strep-tag*<sup>®</sup> II-FLAG-tag, a triple HA-tag, or an eGFP-tag, all under the control of the constitutive double 35S Cauliflower Mosaic Virus (CaMV) promoter. Strikingly, in all cases, the number of transformants with loss-of-function phenotypes was much larger than those with an overexpression phenotype. Using endogenous promoters instead of the 35S CaMV resulted in a dramatic reduction in the frequency of loss-of-function phenotypes. Furthermore, pleiotropic defects occasionally caused by an overexpression strategy can be overcome by using the native promoter of the gene. Finally, a ChAP result is presented using GFP antibody on plants carrying a genomic fragment of a MADS-box gene fused to GFP.

**Conclusion:** This study revealed that MADS-box proteins are very sensitive to fusions with small peptide tags and GFP tags. Furthermore, for the expression of chimeric versions of MADS-box genes it is favourable to use the entire genomic region in frame to the tag of choice. Interestingly, though unexpected, it appears that the use of chimeric versions of MADS-box genes under the control of the strong 35S CaMV promoter is a very efficient method to obtain dominant-negative mutants, either caused by cosuppression or by alteration of the activity of the recombinant protein. Finally, we were able to demonstrate AGAMOUS binding to one of its targets by ChAP.

### Background

During the last 15 years, many studies have been performed aiming at the understanding of MADS-box gene function in plants using loss- and gain-of-function approaches, which resulted in a wealth of information about their role in development [1, 2]. Far less is known about how they act at the molecular level, how they bind to DNA motifs (*cis*-elements) and activate down-stream target genes. It has been shown that MADS domain proteins are able to bind to the DNA motif CC(A/T)<sub>6</sub>GG, the so-called CArG-box (reviewed in [3]). This motif has also been found in promoter sequences of a small number of genes that have been annotated as target genes (e.g. [4-7]). Nevertheless, the exact requirements for this DNA motif to be bound by MADS-box transcription factors *in vivo* are still unknown. Therefore,

methods for the identification of DNA target sites are needed.

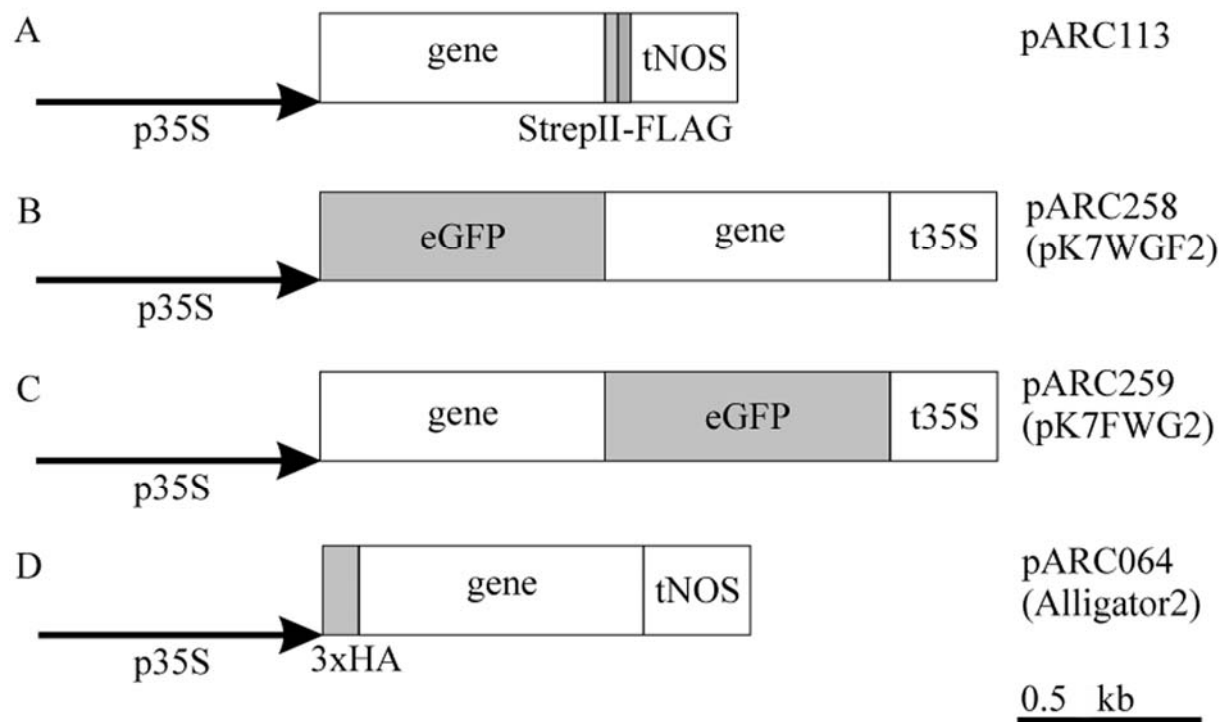
A powerful method to identify target sites is chromatin immunoprecipitation (ChIP), which allows purification of *in vivo* formed complexes of a DNA-binding protein and associated DNA (reviewed in [8]). In short, the method involves the fixation of plant tissue and the isolation of the total protein-DNA mixture, followed by an immunoprecipitation step with an antibody directed against the protein of interest. Next, the DNA can be purified, amplified, and finally identified by sequencing. Alternatively, the amplified DNA can be hybridized to micro arrays containing promoter elements or the entire genome as tiled oligonucleotides (ChIP-chip approach, [9, 10]). The identification of target genes from MADS domain proteins by ChIP has been reported recently [5, 7, 11]. A drawback of ChIP is that for each protein of interest a new specific antibody is required. To overcome this drawback, a protein tagging approach with a general tag could be followed, which we refer to as Chromatin Affinity Purification (ChAP). In this approach, a generic tag is fused to the protein of interest and subsequently used to isolate protein-DNA (or protein-protein) complexes based on affinity purification (reviewed in [12-14]).

In this study we focused on three MADS domain proteins from Arabidopsis, namely AGAMOUS (AG), SEPALLATA3 (SEP3), and FRUITFULL (FUL). AG and SEP3 are both floral organ identity proteins, and based on the ABC model [15], represent C- and E-type proteins, respectively (reviewed in [16]). AG is necessary for the formation of stamens and carpels and is expressed in the inner two floral whorls [17]. SEP3 is expressed in the inner three whorls and is essential for the formation of petals, stamens and carpels in a redundant mode of action with SEP1 and SEP2 [18-21]. FUL has a function in floral meristem identity (early function) and in fruit development (late function) [22-24], and is expressed in the inflorescence meristem, inflorescence stem, cauline leaves, and in developing ovary walls [25]. Here, we report the expression of these three MADS domain proteins in Arabidopsis fused with different tags and the analysis of the phenotypes obtained. Furthermore, the first result obtained with ChAP using a GFP antibody is presented.

## Results

### Protein tagging vectors for plant expression

Four different binary vectors were used for the tagging approach in plants (Figure 1). The first vector (Figure 1A) contains a double tag, the *Strep-tag*® II [26], followed by the FLAG-tag [27], located at the C-terminus of the protein of interest. These peptide tags are both very small, each only 8 amino acids long. Two other vectors (Figure 1B and 1C) contain the coding region for eGFP (enhanced GREEN FLUORESCENT PROTEIN, Clontech) [28, 29], which is either located at the N- or C-terminus of the protein of interest [30]. The fourth vector (Figure 1D) contains a triple HA-tag (hemagglutinin derived) [31], each encoding for a 9 amino acids long peptide. Furthermore, all vectors have a constitutive double 35S CaMV promoter [32, 33] to express the fusion products of AG, SEP3, and FUL in transgenic Arabidopsis plants.



**Figure 1. Binary tagging vectors for plant protein expression.**

(A) C-terminal fusion expression vector with the *Strep-tag*® II and the FLAG-tag. (B) N-terminal fusion expression vector with eGFP. (C) C-terminal fusion expression vector with eGFP. (D) N-terminal fusion expression vector with a triple HA-tag. All vectors contain the constitutive 35S CaMV promoter with the double enhancer for expression.

### Phenotypic and expression analyses of Arabidopsis lines expressing chimeric MADS-box versions

All constructs were introduced into Arabidopsis wild type plants, ecotype Columbia-0, and the transformants obtained were analyzed for overexpression phenotypes. The results are summarized in Table 1 and Figure 2. The expected overexpression phenotypes for *AG* are homeotic changes of floral organs, resembling an *apetala2*-like flower, curly leaves, and early flowering as described by [34]. For ectopic *SEP3* expression, curly leaves and early flowering are characteristics to be expected [35], while ectopic expression of *FUL* results in siliques that fail to shatter, because the dehiscence zone is absent [23, 24].

Overexpression phenotypes were only observed in about 10% of the plants when the eGFP protein was fused either N- or C-terminally (Figure 2B, 2C, and 2J). Surprisingly, many plants containing an eGFP fusion construct revealed a mutant phenotype (Figure 2E, 2F, 2H, 2I, and 2J). Plants with either an overexpression phenotype or a mutant phenotype, obtained with construct pARC276 and pARC277 (Table 1), were analyzed by northern blot hybridization for the expression of the introduced *AG* or *SEP3* transgenes, respectively (Figures 3A and 3B). This revealed a perfect linkage between plants with an overexpression phenotype having a high ectopic gene expression in leaves, while plants with an *ag* mutant phenotype (pARC276) showed no expression. In stead, the latter plants exhibit a smear in the Northern blot, which is often observed when a gene is cosuppressed [36, 37]. Remarkably, for plants containing the *SEP3* fusion construct (pARC277), no loss-of-function



phenotypes were observed, though, the Northern blot showed hallmarks of cosuppression, suggesting that silencing of *SEP3* may have occurred. Most likely, the paralogs and redundant genes *SEP1* and *SEP2* are not affected, which explains that no mutant phenotype was obtained. Plants carrying the *FUL* fusion construct (pARC310) were not molecularly analyzed, but mutant-like plants in a range of severity were observed, which suggest that also cosuppression had occurred. Furthermore, a few overexpression and mutant plants with the *AG*, *SEP3*, and *FUL* fused to *eGFP* were analyzed for fluorescence (Figures 2K and 2L) and confirmed the same linkage between expression and phenotype.

Plants transformed with constructs containing either the *Strep-tag*<sup>®</sup> II-FLAG-tag or the triple HA-tag displayed only a wild-type- or mutant phenotype. Transgenic plants with construct pARC117, containing the double *Strep-tag*<sup>®</sup> II-FLAG-tag, were also analyzed by Northern blot for the expression of the *FUL* fusion product (Figure 3C). Remarkably, in contrast to the *eGFP* fusion constructs, all plants with a loss-of-function phenotype revealed ectopic *FUL* expression, which was lacking in plants with a wild-type phenotype. This suggests that this mutant phenotype obtained with the double tag *Strep-tag*<sup>®</sup> II-FLAG-tag is caused by a dominant-negative effect and not by a cosuppression mechanism.

**Table 1.**

Summary of tagged MADS domain proteins in Arabidopsis plants with the observed phenotypes.

Construct	Expression cassette	Plants (n)	Phenotypes (%)		
			OE	LOF	WT
pARC117	<i>35S:FUL:StrepII-FLAG:tNOS</i>	21	-	57	43
pARC118	<i>35S:AG:StrepII-FLAG:tNOS</i>	14	-	29	71
pARC276	<i>35S:AG:GFP:t35S</i>	42	12	88	-
pARC277	<i>35S:SEP3:GFP:t35S</i>	60	8	-	92
pARC308	<i>35S:GFP:AG:t35S</i>	54	7	93	-
pARC309	<i>35S:GFP:SEP3:t35S</i>	46	-	100	-
pARC310	<i>35S:GFP:FUL:t35S</i>	49	10	90	-
pARC346	<i>35S:3xHA:AG:tNOS</i>	12	-	50	50
pARC347	<i>35S:3xHA:SEP3:tNOS</i>	15	-	-	100
pARC348	<i>35S:3xHA:FUL:tNOS</i>	16	-	38	62
pARC422	<i>gAG:GFP:tNOS</i>	25	-	20	80
pARC423	<i>gSEP3:GFP:tNOS</i>	46	-	-	100
pARC424	<i>gFUL:GFP:tNOS</i>	18	-	28	72

n, number of plants; %, percentage of plants; OE, overexpression; LOF, loss-of-function phenotype; WT, wild-type

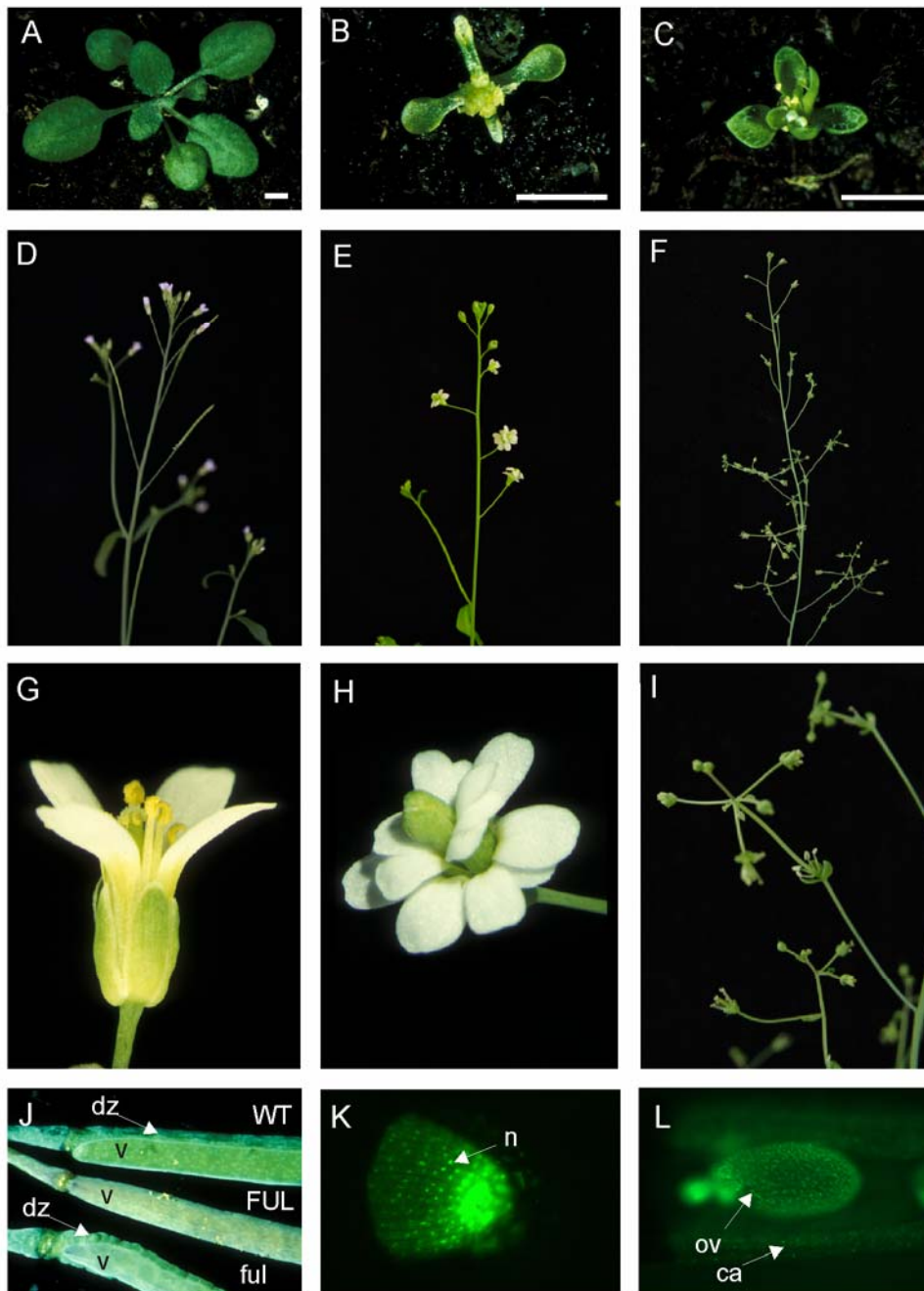
The plants with the triple HA-tag fusion constructs were analyzed by RT-PCR (data not shown). Plants with a mutant phenotype reminiscent of *ag* (pARC346) or *ful* (pARC348) mutants revealed either no expression, suggesting cosuppression, or overexpression, suggesting a dominant-negative effect, respectively.

### **Expression analysis of the *SEP3* promoter in Arabidopsis**

The constitutive and strong double 35S CaMV promoter resulted in high expression of the transgene in those plants that showed an overexpression phenotype. However, in the case of *AG* and *SEP3*, this promoter caused pleiotropic defects resulting in extremely small and early flowering plants in which only a few flowers were produced (Figures 2B and 2C). To overcome this problem, the double 35S CaMV promoter was replaced by the endogenous promoter. A 2.6 kb fragment upstream the ATG start codon of *SEP3* was fused to the  $\beta$ -glucuronidase reporter gene, encoding for GUS [38]. GUS staining in transgenic Arabidopsis plants was detected in the three inner whorls of the flower (Additional file 1), where *SEP3* is normally expressed [20]. However, GUS signal was also detected in the sepals, pedicels, and even in cauline and rosette leaves (Additional file 1), suggesting that the upstream region of *SEP3* is lacking *cis*-acting regulatory regions for correct expression.

Similar misexpression was observed for the MADS-box genes *AG* and *SEEDSTICK* (*STK*), when only the DNA region upstream the first intron or the ATG, respectively, was fused to the GUS reporter gene [39, 40]. In the case of *AG*, it appeared that the second intron, which contains various *cis*-acting regulatory elements [39, 41-43] was essential for the right spatial expression pattern, while for correct *STK* expression, the first intron should be included in the reporter constructs [40]. When the *SEP3* first intron sequence was analyzed in detail different motifs were identified that might act as *cis*-regulatory elements, including a perfect CArG-box (data not shown). To investigate the importance of the *SEP3* intron sequences, a 3.5 kb genomic fragment of *SEP3*, including upstream and intron sequences, was fused to a GFP tag (pARC423) and introduced into Arabidopsis plants. In contrast to the observed misexpression when only the *SEP3* upstream region was used, correct spatial and temporal expression was obtained when also the *SEP3* intron sequences were included (Figure 4). The *gSEP3:GFP* (pARC423) expression is predominantly visible in the nuclei of the floral meristem cells of floral buds from stage 3 onwards (comprising whorl 2, 3, and 4), while there is no or minimal expression in the rest of the inflorescence (Figure 4B). Noteworthy, the number of observed loss-of-function phenotypes with an endogenous MADS-box gene promoter (pARC422 and pARC424) is dramatically less than in the case with the 35S CaMV promoter (Table 1) or even absent in the case of *SEP3* (pARC423) (Table 1).

In summary, the reported results with *AG* and *STK* and our results with *SEP3* indicate that intron regions in MADS domain genes are important for correct spatial and temporal expression.



**Figure 2. Phenotypes of transgenic Arabidopsis plants with different tagging constructs.**

(A) Wild-type Arabidopsis at the rosette stage, (D) at the inflorescence stage, and (G) a close-up of a flower. (B) Line with *AG-eGFP* fusion construct showing an *AG* overexpression phenotype (pARC276). (C) Line with *SEP3-eGFP* fusion construct showing a *SEP3* overexpression phenotype (pARC277). Rosette stage images (A-C) were taken from plants grown under the same conditions and were of the same age (bar indicates relative size). (E,H) Line with *eGFP-AG* fusion construct showing an *ag* mutant phenotype (pARC308). (F,I) Line with *eGFP-SEP3* fusion construct showing a partial *sep*-like mutant phenotype (pARC309). (J) Siliques of lines with *GFP-FUL* fusion construct with either a *FUL* overexpression (*FUL*), *ful* mutant (*ful*) phenotype, or wild-type phenotype (WT) (pARC310). (K) Arabidopsis root tip and (L) open silique with an ovule of a line expressing *GFP-FUL* fusion construct (pARC310) observed by fluorescence microscopy. dz, dehiscence zone; v, valve; ov, ovule; n, nucleus; ca, carpel wall.

### AG protein detection and chromatin affinity purification

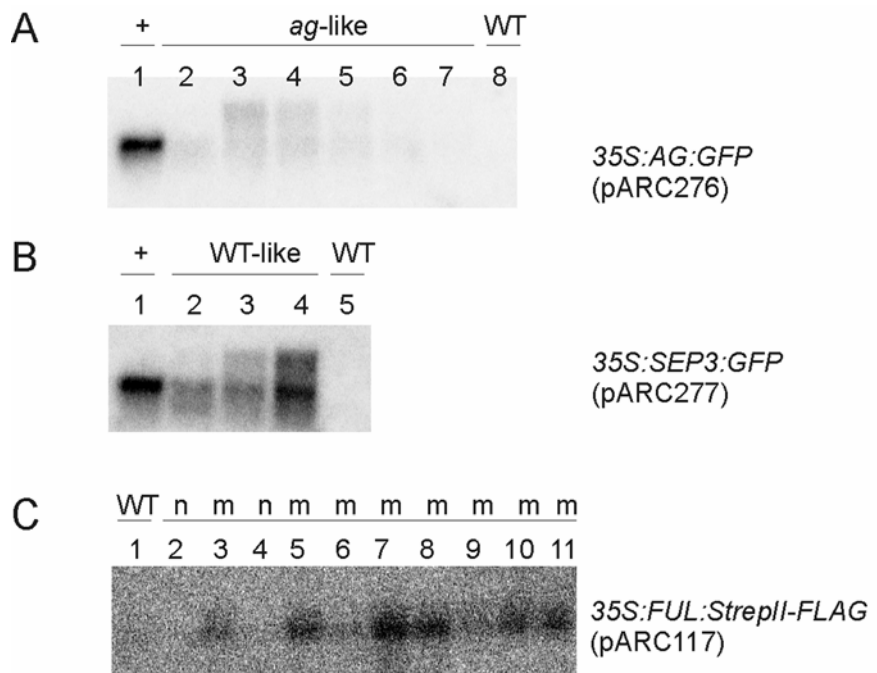
To investigate whether the ChAP procedure using tags is feasible we used transgenic Arabidopsis plants expressing *gAG:GFP* (pARC422) as example. Correct spatial and temporal AG expression was observed, predominantly in the nuclei of the floral meristem cells of floral buds from stage 3 onwards (comprising whorl 3 and 4) (Figure 4A).

First, we analyzed the *gAG:GFP* (pARC422) plants by Western blotting to see whether the chimeric AG protein is detectable with a polyclonal GFP antibody. For this, protein was isolated from nuclei extracts from wild type Arabidopsis (Col-0) plants and compared with extracts from *gAG:GFP* plants. The Western blot (Figure 4C) shows a specific band of the expected size in the *gAG:GFP* plants, which was not present in wild type plants.

Finally, a chromatin affinity purification with a GFP antibody was performed on a protein extract derived from *gAG:GFP* (pARC422) plants. As reported before, AG protein is able to bind to its own intron sequence for autoregulation [7]. This regulatory region was analyzed for enrichment by Real-time PCR, which would demonstrate that the chimeric AG protein is able to bind *in vivo* to its target sequence. The target DNA sequence (AG second intron) was 10 fold enriched after affinity purification with GFP antibody demonstrating that chimeric AG is indeed able to bind to its regulatory region (Figure 4D).

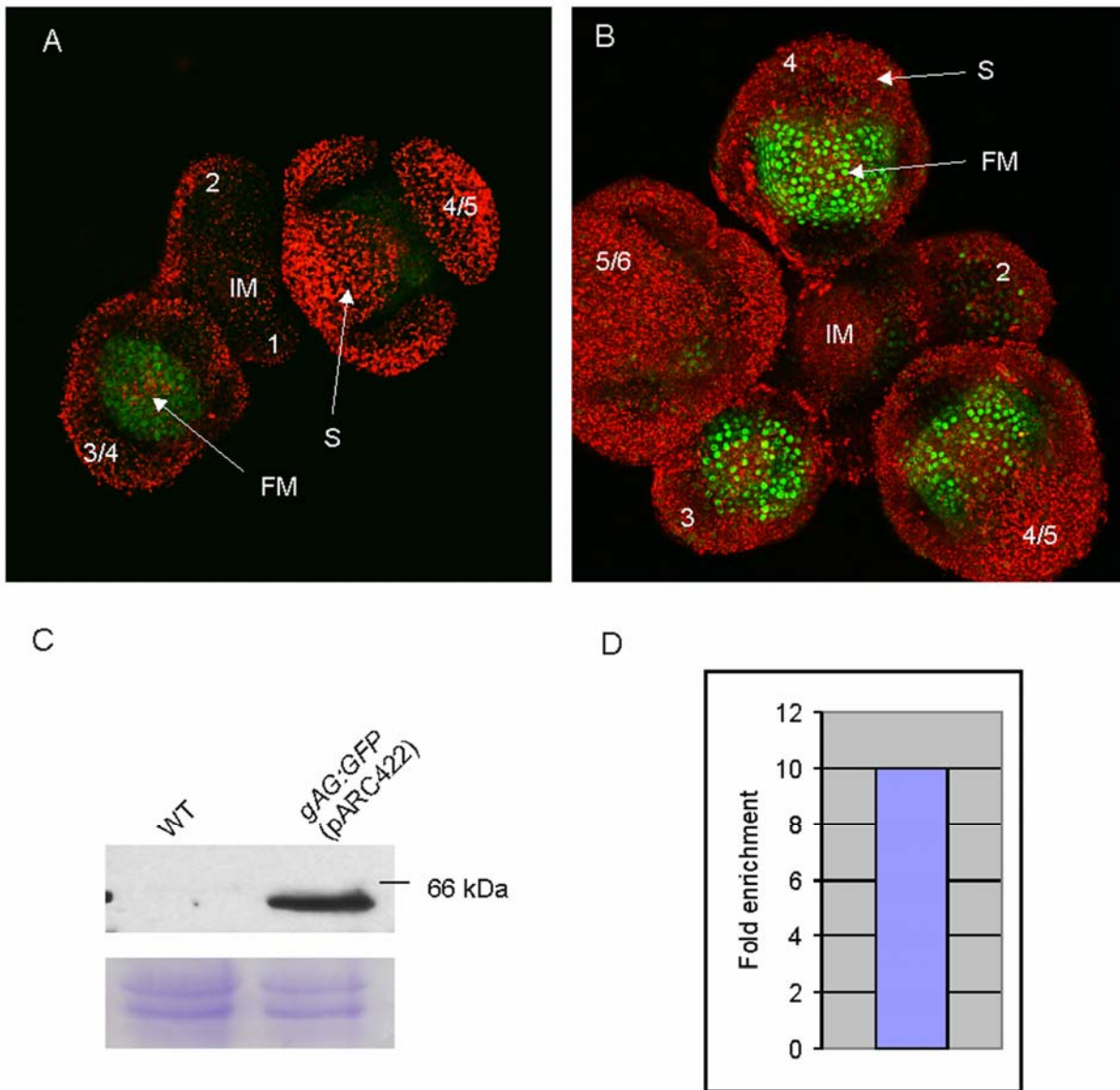
**Figure 3. Northern blot analysis of leaf tissue of different Arabidopsis lines containing various tagging constructs.**

(A) Expression analysis of *AG-eGFP* (pARC276) lines. (B) Expression analysis of *SEP3-eGFP* (pARC277) lines. (C) Expression analysis of *FUL-Strep-tag® II-FLAG-tag* (pARC117) lines, *ful*-like plants are indicated with 'm' and WT-like plants with 'n'. WT, wild-type; +, line with an overexpression phenotype.



### Discussion

The use of epitope tags can facilitate the isolation of protein-DNA or protein-protein complexes. Here, we report a first attempt of employing a generic tagging approach for the MADS domain proteins AG, SEP3, and FUL. Different tags and a combination of tags were used to produce fusion products expressed in plants. There are two important criteria before further steps can be undertaken to identify target genes by Chromatin Affinity Purification (ChAP). The first basic and most important aspect is to obtain stable expression of the fusion



**Figure 4. AG and SEP3 expression analysis and chromatin immunoprecipitation (ChIP).**

Confocal Laser Scanning Microscopical (CLSM) imaging of (A) *gAG:GFP* (pARC422) and (B) *gSEP3:GFP* (pARC423) in the inflorescence. Top view (A, B) of an inflorescence with different floral bud stages (indicated by numbers). The GFP expression (green signal) is predominantly localized in the nuclei of floral meristem cells of flower buds from stage 3 onwards (comprising whorl 3 and 4 for AG, and whorl 2, 3, and 4 for SEP3, respectively). Autofluorescence is visible as red signal. (C) Anti-GFP Western blot with material from Arabidopsis WT and *gAG:GFP* (pARC422) plants. Protein product is detectable in transgenic plants only. Bottom panel shows the Coomassie stained gel serving as loading control. (D) Enrichment of AG target DNA after ChIP with GFP antibody and compared with pre-immune. Quantification of target DNA was done by Real-time PCR using primers corresponding to sequences in the second intron of AG. FM, floral meristem, S, sepal, IM, inflorescence meristem, WT, wild-type.

protein. Secondly, an expressed fusion protein should be biologically active. Both aspects appeared not to be straight forward and appeared to be dependent on the tags used.

The expression experiments in plants using the constitutive and strong 35S CaMV promoter resulted in mutant phenotypes with all constructs, though, in many cases, not the expected overexpression phenotypes. Remarkably, the percentage of loss-of-function phenotypes obtained was very high, even up to 100% in the case of *GFP:SEP3* (pARC309). The loss-of-function phenotypes were most likely caused by two phenomena, either by cosuppression in the case of the *eGFP* fusions, or by a dominant-negative effect in the case of the *Strep-tag*® II-FLAG-tag fusions. With the triple HA-tag both phenomena could have happened. These different tags have been used in many organisms and with many different proteins (e.g. [13, 31, 44-46]), however it has never been reported that they cause these severe problems related to mRNA expression or activity of a recombinant protein. The high frequency of silencing with the *eGFP* fusions could be related to the 35S CaMV promoter, causing high expression of the transgene. Expression of MADS-box cDNAs under the control of the 35S CaMV promoter without the GFP tag (e.g. [47]) or expression of GFP tags using endogenous MADS-box gene promoters did not reveal such high percentages of cosuppression plants (Table 1), indicating that the combination of 35S CaMV promoter and the GFP tag may induce silencing. The silencing efficiencies of MADS-box gene expression using the GFP tag in combination with the 35S CaMV promoter appeared to be comparable to using an RNA interference strategy [48]. The only exception on this rule is *SEP3:GFP* (pARC277), which did not result in any plant with a loss-of-function phenotype. In contrast, all *GFP:SEP3* plants show a mutant phenotype. Although an intriguing observation, an explanation is missing. The altered biological activity of the FUL protein fused to short peptide tags, here referred to as 'dominant-negative' mode of action, could be caused by either trapping interacting proteins and forming non-functional protein complexes, steric hindrance preventing certain interactions, or altered folding of the protein. However, functionality of a fusion product with an epitope tag has to be analyzed case by case. It depends on the tag used and the effect it may have on the protein of interest. Our results indicate that the activity of MADS-box genes and their products can be dramatically affected by fusions with small peptide tags and GFP tags at both N- and C-termini. This high sensitivity to fusions, however, can also be used as an effective method to obtain high percentages of dominant loss-of-function mutants.

A drawback of an overexpression strategy could be the occurrence of unwanted pleiotropic effects, e.g. early flowering or a reduced number of flowers. Furthermore, overexpression or ectopic expression does not mimic the natural situation. The most elegant solution is to express the genes under their native promoter in a mutant background, which will directly reveal their biological activity and eliminate any competition with the untagged endogenous protein. For the isolation of the native promoter, often DNA sequences upstream the ATG start codon are cloned, although no general rules are available that can predict the promoter region (reviewed in [49]). This approach was followed for the *SEP3* promoter, however, it revealed a lack of specificity compared to previously reported *in situ* hybridization experiments [20]. As described previously for the MADS-box genes *AG* and

*STK*, intron sequences are important for correct expression [39, 40]. This appears also to be the case for *SEP3*, because fusion of *GFP* to a 3.5 kb genomic fragment of *SEP3* including upstream and intron sequences revealed correct expression patterns. Finally and most importantly, it appeared possible to perform ChAP using a GFP antibody on plants that carried a genomic *AG* fragment (including upstream and intron sequences) fused to GFP (pARC422).

## Conclusion

A powerful method to identify target genes is ChIP or related ChAP. ChAP makes use of an epitope tag fused to the protein of interest and this study revealed that the activities of MADS-box proteins are very sensitive to fusions with small peptide and GFP tags. Furthermore, for the expression of chimeric versions of MADS-box genes it is favourable to use the entire genomic region in frame with the tag of choice. Interestingly, though unexpected, it appears that the use of chimeric versions of MADS-box genes under the control of the strong 35S CaMV promoter is a very effective method to obtain loss-of-function mutants, either caused by cosuppression or by alteration of the activity of the recombinant protein. Finally, ChAP is possible with a chimeric MADS-box protein using a GFP antibody.

## Methods

### Plant growth

*Arabidopsis thaliana*, ecotype Columbia-0 (Col-0) plants were grown under normal greenhouse or growth chamber conditions (22°C, long day light regime).

### Construction of binary vectors and plant transformation

The vector with the C-terminal double tag *Strep-tag*® II (WSHPQFEK) and the FLAG-tag (DYKDDDDK) is called pARC113. The double tag is constructed with two forward and three reverse complementary primers resulting in, with *Arabidopsis* codon usage, 5'-CTCGAGTGGTCTCATCCTCAATTTGAAAAGTCTTCTGATTACAAGGATGATGATGATAAGTAAC TCGAG-3' (nucleotides coding for the tags are underlined). Between the two tags are two serine amino acid residues functioning as linker and after the FLAG-tag a stop codon is introduced. In brief, 1 µl of each primer (100 pmol/µl) were pooled together, incubated for 10 min at 96°C and slowly cooled down to room temperature to create double stranded fragments. The fragments were phosphorylated with 2 µl T4 kinase (10 U/µl) and incubated for 30 min at 37°C. Next, the 69 nucleotides double stranded fragments were isolated from a 12% polyacrylamide gel. Subsequently, the fragment was cloned into an *Xho*I digested binary pGD121 vector [50], containing a double 35S CaMV promoter (derived from pGD120; [51]). Full length open reading frames for *AG* (At4g18960; encoding 252 amino acids), *SEP3* (At1g24260; encoding 251 amino acids), and *FUL* (At5g60910; encoding 242 amino acids) were amplified with gene specific primers from the start to the stop codon, clones for C-terminal fusions lack the stop codon, and were subcloned in pGEM-T® Easy (Promega,

Madison, WI) and/or subcloned with the Gateway™ Technology (Invitrogen, Carlsbad, CA). After sequence control, all genes were cloned (in pARC113) and/or recombined (in pARC064, pARC258, and pARC259) in the appropriate vectors to make the fusion constructs.

A 2.6 kb *SEP3* region upstream of the ATG was amplified with specific primers (PRO117 5'-CACCGGCGCGCCATCCATCCATCCAAATGGGACC-3' and PRO118 5'-GAAGCTTTTTCTTTTCTTTCTCCTCTCCC-3') and recombined with the Gateway™ Technology in pENTR/D-TOPO (Invitrogen), followed by recombination in the binary vector pBGWFS7 [30], resulting in a transcriptional *eGFP-GUS* fusion construct (pARC213).

Genomic fragments for *AG* (6882 bp), *SEP3* (3489 bp), and *FUL* (5298 bp) were amplified with gene specific primers, a forward primer located in the upstream region, PRO433 *AG*-5'-CACCGATCAAAGACTACACATCAC-3', PRO407 *SEP3*-5'-CACCCATACC TTTGTGTCCATCAC-3', and PRO429 *FUL*-5'-CACCTCGATCAGAATTTGAGCTG-3', and a reverse primer in the 3'-region lacking the stop codon for each gene, PRO431 *AG*-5'-CACTAACTGGAGAGCGGTTTG-3', PRO408 *SEP3*-5'-AATAGAGTTGGTGTCATAAGG TAACC-3', and PRO430 *FUL*-5'-CTCGTTCGTAGTGGTAGGAC-3', and recombined in pENTR/D-TOPO. After sequence control, all genomic fragments were recombined in the binary vector pMDC204 [52], resulting in translational GFP6 fusion constructs (pARC422, pARC423, and pARC424, respectively).

Arabidopsis plants were transformed with *Agrobacterium tumefaciens* strain GV3101 using the floral dip method [53].

### RNA gel blot analysis

Total RNA was isolated from frozen plant tissue with the RNeasy plant RNA extraction kit (Qiagen). Five micrograms of each RNA sample was denatured by 1.5 M glyoxal, separated on a 1.2% agarose gel in 15 mM Na-phosphate buffer pH 6.5, checked for equal loading, and followed by blotting onto Hybond-N + membrane (Amersham Biosciences, Piscataway, NJ) in 25 mM Na-phosphate buffer pH 6.5. Probes were labeled with the RadPrime DNA Labeling System (Invitrogen) and blots were hybridized as described by Angenent et al. (1992) [54]. Gene specific probes were amplified by PCR with the following primers: PRO383 *AG*-5'-GGGTCAATGTCTCCCAAAGA-3' and PRO384 *AG*-5'-CTAACTGGAGAGCGGTTTG-3', PRO105 *SEP3*-5'-GTCTAGAATGGGAAGAGGGAGAG TAG-3' and PRO106 *SEP3*-5'-CGGATCCAATAGAGTTGGTGTCATAAGGTAACC-3'. The *FUL* fragment was derived from a pGEM-T® Easy (Promega) clone digested with *Xba*I-*Kpn*I.

### GUS assay

To detect  $\beta$ -glucuronidase (GUS) activity [38], plant tissue was fixed in 90% ice-cold acetone for 1 h at  $-20^{\circ}\text{C}$ , followed by three rinses with 0.1 M Na-phosphate buffer pH 7.0 containing 1 mM potassium ferrocyanide. The three rinse steps in total took 1 h and during the first rinse step vacuum was applied for ~15 min. Finally, the substrate was added to the samples, containing 50 mM Na-phosphate buffer pH 7.0, 1 mM EDTA, 0.1% (v/v) Triton X-100, 1 mM potassium ferrocyanide, and 1 mM X-Gluc (Duchefa, Haarlem, The Netherlands), and



vacuum was applied for 5 min, followed by overnight incubation at 37°C in the dark. Chlorophyll was removed by, first, 1 h incubation in 96% ethanol and then transference to 70% ethanol.

### Microscopy

Plant tissue was observed for GFP expression with a Zeiss Axioskop UV-microscope, equipped with filter set 13 (excitation BP 470/20, beamsplitter FT 493, emission BP 505-530). Images were taken with a Leica DFC320 digital camera and an exposure time of 18 seconds was used. Confocal Laser Scanning Microscopical (CLSM) imaging of plant tissue was performed with a Zeiss LSM 510 inverted confocal microscope using a 40x C-Apochromat (NA 1,2 W Korr) lens. The tissue was embedded in the wells of a Silicone Isolator (Grace Bio-Labs, Bend, OR) with 0.8% agar 0.5x MS. GFP was excited with the 488 line of an argon ion laser. The emission of GFP was filtered with a 505-530 nm bandpassfilter, while the red autofluorescence of the plant tissue was filtered with a 650 nm long-pass filter. 3-D projections of the obtained confocal z-stacks were made with the Zeiss LSM Image Browser Version 4.

### Chromatin Affinity Purification (ChAP)

The procedure was performed as previously described [7] with some modifications. Fixed (15–30 min) inflorescence tissue (~0.8 g) was used of transgenic Arabidopsis plants containing construct pARC422 that carries a genomic AG fragment fused with GFP. Chromatin was solubilized on ice with a probe sonicator (MSE, Soniprep 150) by 3 cycles of 15 sec pulses of half maximal power with 30 sec cooling time between pulses. GFP antibody was used for the affinity purification (ab290; Abcam, Cambridge, UK) and for the negative control complete rabbit serum. For pre-clearing and affinity purification Protein A-Agarose beads were used (sc-2001; Santa Cruz Biotechnology, Santa Cruz, CA). After elution of the beads, samples were treated with proteinase K, followed by precipitation. The precipitated DNA was dissolved in 100 µl water, purified with a PCR purification kit (Qiagen, Valencia, CA), and eluted with 30 µl EB (water containing 10 mM Tris, pH 8). Enrichment of the target region was determined using a real-time PCR detection system (MyiQ, Bio-Rad Laboratories, Hercules, CA) by comparing the affinity purified sample (anti-GFP) with the negative control (rabbit serum). The results between the two samples were normalized using sequences of *Heat Shock Factor1* (*HSF1*; At4g17750). The following primers were used, PRO469 AG-5'-TGGTCTGCCTTCTACGATCC-3' and PRO470 AG-5'-CAACAACCCATTAACACATTGG-3', PDS1045 *HSF1*-5'- GCTATCCACAGGTTAGATAAAGGAG-3' and PDS1046 *HSF1*-5'-GAGAAAGATTGTGTGAGAATGAAA-3'.

### Protein isolation and detection

Nuclei extraction from 0.5 g of Arabidopsis inflorescences was performed according to the protocol used for ChIP experiments [7]. The nuclei pellet was resuspended in 120 µl 2x SDS sample buffer, incubated on ice and centrifuged at 20800 x g for 10 min at 4°C. The supernatant was boiled for 5 min. Western blotting was performed essentially as described

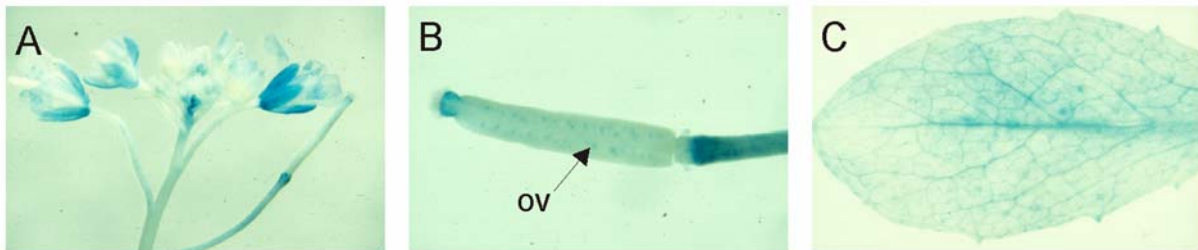
previously [55]. The GFP antibody (ab290; Abcam) was used in a 1:5000 dilution.

### Authors' contributions

SdF and GA conceived and designed the experiments. SdF, SU, KK, and LvZ carried out the experiments. SdF and GA drafted the manuscript. All authors read and approved the final manuscript.

### Acknowledgements

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#### Additional file 1. *SEP3* expression analysis in transgenic *Arabidopsis* plants.

(A-C) GUS expression patterns of *SEP3* promoter *GUS* fusion (pARC213) in different tissues, (A) inflorescence, (B) silique, and (C) rosette leaf. ov, ovule.

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# Chapter 3

## In planta localisation patterns of MADS domain proteins during floral development in *Arabidopsis thaliana*

Susan L Urbanus

Stefan de Folter

Anna V Shchennikova

Kerstin Kaufmann

Richard GH Immink

Gerco C Angenent

### Abstract

**Background:** MADS domain transcription factors play important roles in various developmental processes in flowering plants. Members of this family play a prominent role in the transition to flowering and the specification of floral organ identity. Several studies reported mRNA expression patterns of the genes encoding these MADS domain proteins, however, these studies do not provide the necessary information on the temporal and spatial localisation of the proteins. We have made GREEN FLUORESCENT PROTEIN (GFP) translational fusions with the four MADS domain proteins SEPALLATA3, AGAMOUS, FRUITFULL and APETALA1 from the model plant *Arabidopsis thaliana* and analysed the protein localisation patterns in living plant tissues by confocal laser scanning microscopy (CLSM).

**Results:** We unravelled the protein localisation patterns of the four MADS domain proteins at a cellular and subcellular level in inflorescence and floral meristems, during development of the early flower bud stages, and during further differentiation of the floral organs. The protein localisation patterns revealed a few deviations from known mRNA expression patterns, suggesting a non-cell autonomous action of these factors or alternative control mechanisms. In addition, we observed a change in the subcellular localisation of SEPALLATA3 from a predominantly nuclear localisation to a more cytoplasmic localisation, occurring specifically during petal and stamen development. Furthermore, we show that the down-regulation of the homeodomain transcription factor *WUSCHEL* in ovular tissues is preceded by the occurrence of both AGAMOUS and SEPALLATA3 proteins, supporting the hypothesis that both proteins together suppress *WUSCHEL* expression in the ovule.

**Conclusions:** This approach provides a highly detailed *in situ* map of MADS domain protein presence during early and later stages of floral development. The subcellular localisation of the transcription factors in the cytoplasm, as observed at certain stages during development, points to mechanisms other than transcriptional control. Together this information is essential to understand the role of these proteins in the regulatory processes that drive floral development and leads to new hypotheses.

### Background

Major developmental steps in flowering plants, such as the transition to flowering and floral organ development are, for the most part, controlled by members of the MADS domain family of transcription factors [1]. The action of these transcription factors in defining the identity of the floral organs has been captured in a genetic model, the “ABC” model [2], which was later extended with a “D” and “E” function [3-6]. This model describes how the combinatorial activity of several classes of regulatory genes, most of which encode MIKC-type MADS domain proteins, define the identity of the five different floral organs (sepals, petals, stamen, carpels, and ovules). According to this model, the combination of the class A+E genes specifies the identity of sepals, while the A+B+E genes specify petal identity, the combination of classes B+C+E determines stamen identity, C+E genes together lead to carpel identity,



and finally the combination of classes C+D+E is responsible for ovule identity (for review see [7]). Floral organ development in *Arabidopsis thaliana* is controlled by the following genes: the A-function is represented by the genes *APETALA1* (*AP1*) and *APETALA2* (*AP2*) (not a MADS domain transcription factor); the B-function is controlled by *APETALA3* (*AP3*) and *PISTILLATA* (*PI*); *AGAMOUS* (*AG*) represents the C-function; the D-function is represented in a redundant manner by *SEEDSTICK* (*STK*), *SHATTERPROOF1* (*SHP1*) and *SHATTERPROOF2* (*SHP2*); and the E-function involves the four closely related genes *SEPALLATA1* to *SEPALLATA4* (*SEP1-4*). Furthermore, another MADS box gene *FRUITFULL* (*FUL*), which is not described in the “ABC” model, is also involved in carpel development. In addition to these functions in floral organ development, some of these genes also have other functions. For instance, both *FUL* and *AP1* are involved in the transition from inflorescence meristem to floral meristem identity, while *AG* controls the floral meristem determinacy [8-10].

The MADS domain proteins and the “ABC” model are well studied subjects for transcription factor regulation and action. Several studies have shown that at least some MADS domain proteins need to be in a dimeric form, either homo- or heterodimeric, before they can enter the nucleus [11-13]. In the nucleus, the proteins bind to DNA sequences of the target gene with the consensus CC(AT)<sub>6</sub>GG sequence, also known as the CARG box [14]. Binding to the DNA occurs either in the form of a dimer [14] or in a multimeric fashion [15, 16], for instance in a tetrameric form as proposed in the “quartet” model [17]. Our knowledge about these MADS domain protein interactions has been greatly extended by a study where interaction data obtained from a systematic Yeast Two-Hybrid experiment was combined with large scale microarray co-expression data of the corresponding genes [18]. By considering not only the capacity of proteins to interact with each other, but also the possibility for putative partners to be co-localised in the same tissues and cells, the output is narrowed down to interactions that are likely to be of biological relevance for the plant.

However, microarray studies give only a very broad view on the spatio-temporal expression pattern of genes and do not provide the necessary detail that is needed to demonstrate co-localisation of the encoded proteins. In situ mRNA hybridisation studies and promoter-reporter studies like the ones reported for *AG* [19], *SEP3* [20], *AP1* [21], and *FUL* [22] reveal the expression patterns in more detail, but these might not reflect the protein localisation patterns. In fact, it is difficult to infer the protein localisation pattern from an mRNA expression pattern for two main reasons. First of all, production and degradation rates of mRNA and proteins could be totally different, and secondly, proteins can be transported from an expressing cell to a neighbouring non-expressing cell; all resulting in protein patterns that deviate from the mRNA patterns. It is known from studies on the class B genes *DEFICIENS* and *GLOBOSA* from *Antirrhinum majus* [23] that particular MADS domain transcription factors are able to transfer from one cell to another, where they may have a non-cell-autonomous function. Therefore, to obtain information about the spatio-temporal control of these regulatory proteins, it is essential to study the localisation of the proteins themselves. Additionally, protein localisation studies can be more informative on the functioning of transcription factors by showing the specific subcellular localisation of the

proteins during development. It has been demonstrated that some MADS domain transcription factors are localised in the cytoplasm when an interaction partner is absent and only become functional when they enter the nucleus after dimerisation [11-13, 24]. Preferably, one would like to obtain a three-dimensional map of protein localisations with cellular resolution and information about the dynamics of proteins during plant development. The discovery of GREEN FLUORESCENT PROTEIN (GFP) and similar fluorescent proteins and their use as visual tags for proteins, in combination with Confocal Laser Scanning Microscopy (CLSM) has made this visualisation of fluorescently tagged proteins in living plant tissue possible [25-27].

In order to study MADS domain proteins in living tissues with CLSM during floral development, we made C-terminal GFP tagged versions of *SEP3*, *AG*, *FUL*, and *AP1*. Previously it was shown that the fusion of GFP to the C-terminus of the MADS domain protein *AP1* does not affect its function, as it is able to complement the *ap1* mutant [28]. Furthermore, it is known from studies with *AG*, *STK*, and *SEP3* that introns can contain important regulatory elements that are required for the correct expression pattern of these MADS box genes [29-31]. For this reason, we made C-terminal GFP tagged versions of *SEP3*, *AG*, *FUL*, and *AP1* using genomic fragments [29]. Here we describe the protein localisation patterns of *gSEP3:GFP*, *gAG:GFP*, *gFUL:GFP* and *gAP1:GFP* on a cellular and subcellular level in the inflorescence meristem and at various stages of floral meristem and organ formation. This detailed study reveals discrepancies between the previously reported mRNA expression patterns and the protein localisations, and sheds new light on the functioning of the MADS domain proteins in floral organ patterning and formation.

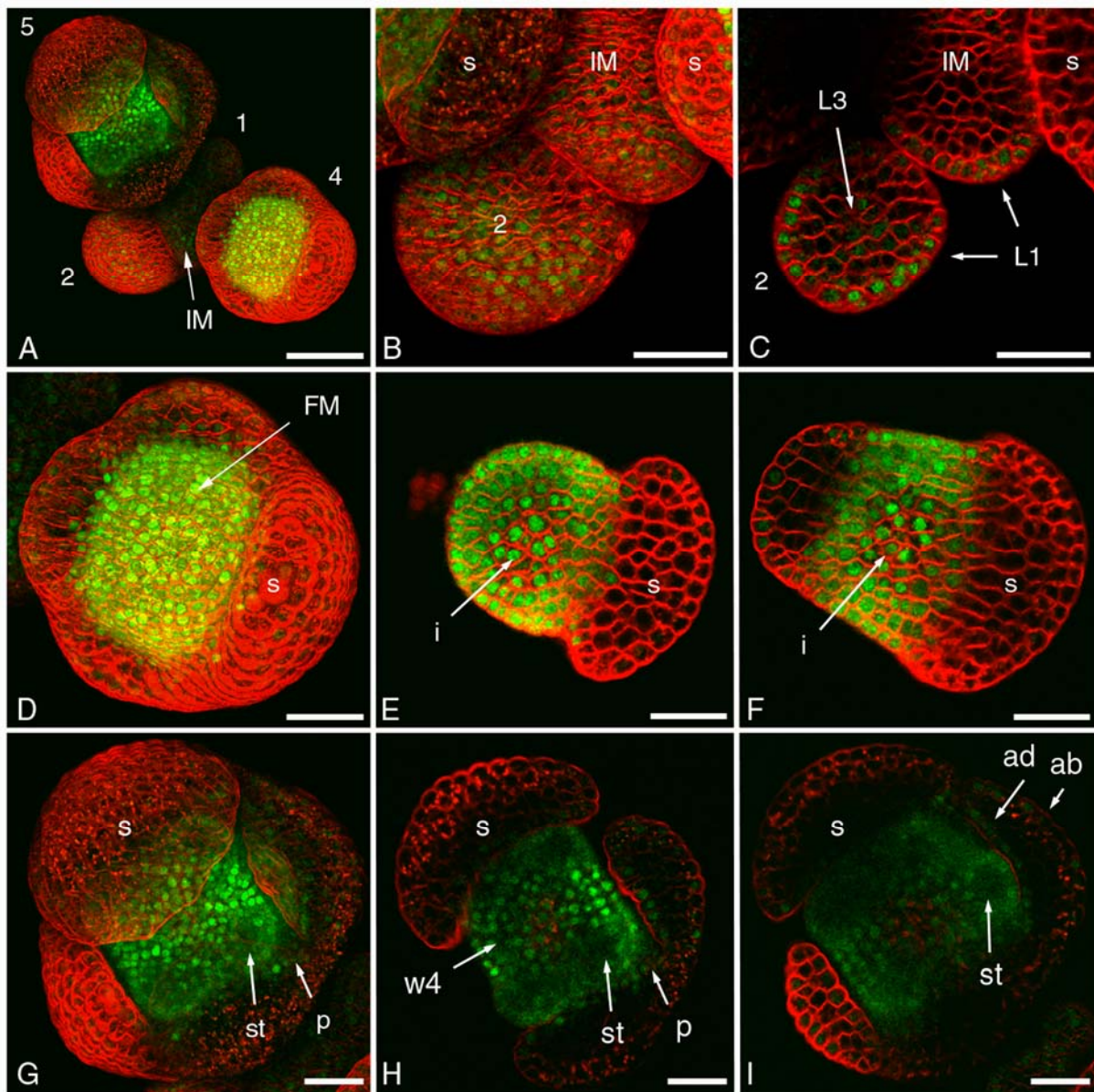
### Results and Discussion

In this study we analysed the spatio-temporal protein localisation patterns of C-terminally GFP tagged genomic clones of MADS domain proteins *SEP3*, *AG*, *FUL* and *AP1* during floral development, hereafter referred to as *SEP3:GFP*, *AG:GFP*, *FUL:GFP*, and *AP1:GFP*. The generated constructs were transformed to *Arabidopsis thaliana* wild type Col-0 plants, and at least four GFP-expressing stable primary transformants per construct were analysed for their protein localisation. These were found to be very similar in localisation patterns, although some differences in expression levels were observed. These differences in expression levels may be due to differences in transgene copy numbers, but they may also be caused by positional effects of the insertion of the transgene. Three constructs, namely *AG:GFP*, *FUL:GFP* and *AP1:GFP*, were also introduced into their respective mutant lines. These complementation experiments showed that C-terminal GFP tagged MADS domain proteins are functional, as the *AG:GFP*, *FUL:GFP* and *AP1:GFP* proteins can rescue the mutant phenotypes of *ag*, *ful* and *ap1* mutants, respectively. As the single *sep3* mutant shows very subtle phenotypic alterations due to the redundancy of *SEP3* with *SEP1* and *SEP2* [4], the *SEP3:GFP* construct was only transformed into the wild type background. The spatio-temporal protein localisation patterns described here are from representative lines homozygous for the transgene in the Col-0 wild type background.

**SEP3 localisation in inflorescence meristem and early flower bud stages**

In the inflorescence meristem, SEP3:GFP plants had a very low but definite signal in the epidermal layer that was located mostly in the nucleus (Figures 1A-C). During the initiation of the floral primordia and flower bud stages 1 and 2 this epidermal localisation pattern remained. However, stage 2 flower buds also showed a few cells in the subepidermal and inner cell layers in the centre of the floral meristem that started to have a much higher level of SEP3:GFP signal, also located mostly in the nucleus (Figure 1C). During the development towards stage 3 flower buds this signal spread out to encompass the whole dome of the floral meristem from which the second, third and fourth whorl will develop at later stages (Figure 1D). This increasing SEP3:GFP localisation fits with the reported mRNA expression pattern [20], where *SEP3* expression starts in late stage 2 flower buds and is largely confined to the three inner whorls of the flower primordium. However, the weak, but distinct epidermal presence of SEP3:GFP in the inflorescence meristem and stage 1 flower buds has not been reported before. This signal could originate from the epidermis itself, but it cannot be excluded that it is the result of epidermal transport from nearby, high expressing tissues, such as the floral meristem in stage 2/3 flower buds. We also observed signal in the epidermal layer of sepal primordia. During the development of the sepals, the SEP3:GFP signal became weaker at the abaxial side of the sepals, while the signal remained at the adaxial side (Figure 1I). In agreement with this, low level *SEP3* expression is occasionally detected on the adaxial side of sepals at later stages [20], and it was shown that *SEP1-SEP4* are involved in specifying adaxial sepal surface identity [6].

Interestingly, the subcellular localisation of the SEP3:GFP protein changed dramatically from stage 3 onwards. Just before the petal and stamen initiation, SEP3:GFP proteins in the future second and third whorl became both cytoplasmically and nuclear localised. At the same time, the proteins in the innermost part of the floral meristem clearly remained nuclear localised (Figures 1E and F). This cytoplasmic SEP3:GFP signal in the future second and third whorl could be due to higher expression levels of the gene, resulting in a temporary accumulation of SEP3:GFP proteins in the cytoplasm waiting for transportation to the nucleus. Another option could be that the appearance of new interaction partners in this region results in the cytoplasmic localisation of SEP3:GFP. For instance, it is known that the expression of *AP3*, which determines petal and stamen identity together with *PI* and *SEP3*, also starts at stage 3 and is restricted to the same area where whorl 2 and 3 will develop [32]. It would be interesting to investigate if *AP3* is also located predominantly in the cytoplasm at this point in development. Another possibility could be that higher cell division rates in the area of the future second and third whorl which cause the petal and stamen primordia to arise, result in increased unloading of previously nuclear localised proteins into the cytoplasm [33]. The smaller cell sizes in the area of the future second and third whorl compared to the innermost part of the floral meristem could indicate higher cell division rates (Figure 1E). This unloading of the nuclear localised proteins into the cytoplasm during cell division may also allow the proteins to meet new partners and form new complexes in the cytoplasm, for instance with *AP3* and *PI*. In stage 5 flower buds, the initiating petal and stamen primordia revealed an epidermal layer with mostly nuclear SEP3:GFP, while the



**Figure 1. Confocal microscopic analysis of SEP3:GFP localisation in inflorescence meristem and early flower bud stages.**

(A) Overview of an inflorescence with the inflorescence meristem and early flower bud stages 1 to 5 indicated. SEP3:GFP protein is detected as green signal and cell membranes are stained with the red dye FM4-64. (B) Detail of an inflorescence meristem and a stage 2 flower bud. (C) Section through tissue in (B) showing the SEP3:GFP signal in the epidermis and the beginning SEP3:GFP signal in the centre of the stage 2 flower bud. (D) Detail of a stage 4 flower bud showing the highest SEP3:GFP signal in the entire floral meristem and only in the epidermis of the four sepals. (E) Section through the stage 4 flower bud in (D) showing both cytoplasmic and nuclear localisation of SEP3:GFP in the future second and third whorl, and only nuclear localisation in the innermost part of the floral meristem. (F) More basal section through the stage 4 flower bud in (D) showing again both cytoplasmic and nuclear localisation of SEP3:GFP in the future second and third whorl. (G) Detail of a stage 5 flower bud showing the initiating petal and stamen primordia. (H) Section through the stage 5 flower bud in (G) illustrating that the SEP3:GFP signal in whorl 4 is mostly nuclear, while the reduced signal in the initiating petal and stamen primordia is both cytoplasmically and nuclear localised. (I) More basal section through the stage 5 flower bud in (G) showing the SEP3:GFP signal at the adaxial and abaxial sides of a sepal. (1-5) flower bud stages; (ab) abaxial; (ad) adaxial; (FM) floral meristem; (IM)

inflorescence meristem; (i) innermost part floral meristem; (L1) epidermal cell layer; (L3) inner cell layers; (p) petal; (s) sepal; (st) stamen; (w4) whorl 4. Scale bars of (A) 50  $\mu\text{m}$  and of (B-I) 25  $\mu\text{m}$ .

subepidermal and inner cell layers showed a lower SEP3:GFP signal, both cytoplasmically and nuclear localised (Figures 1G-I). Apparently, SEP3 is less needed in the inner layers of the emerging petals and stamen than in the epidermis. In the mean time the SEP3:GFP proteins in the fourth whorl remained nuclear localised (Figure 1H).

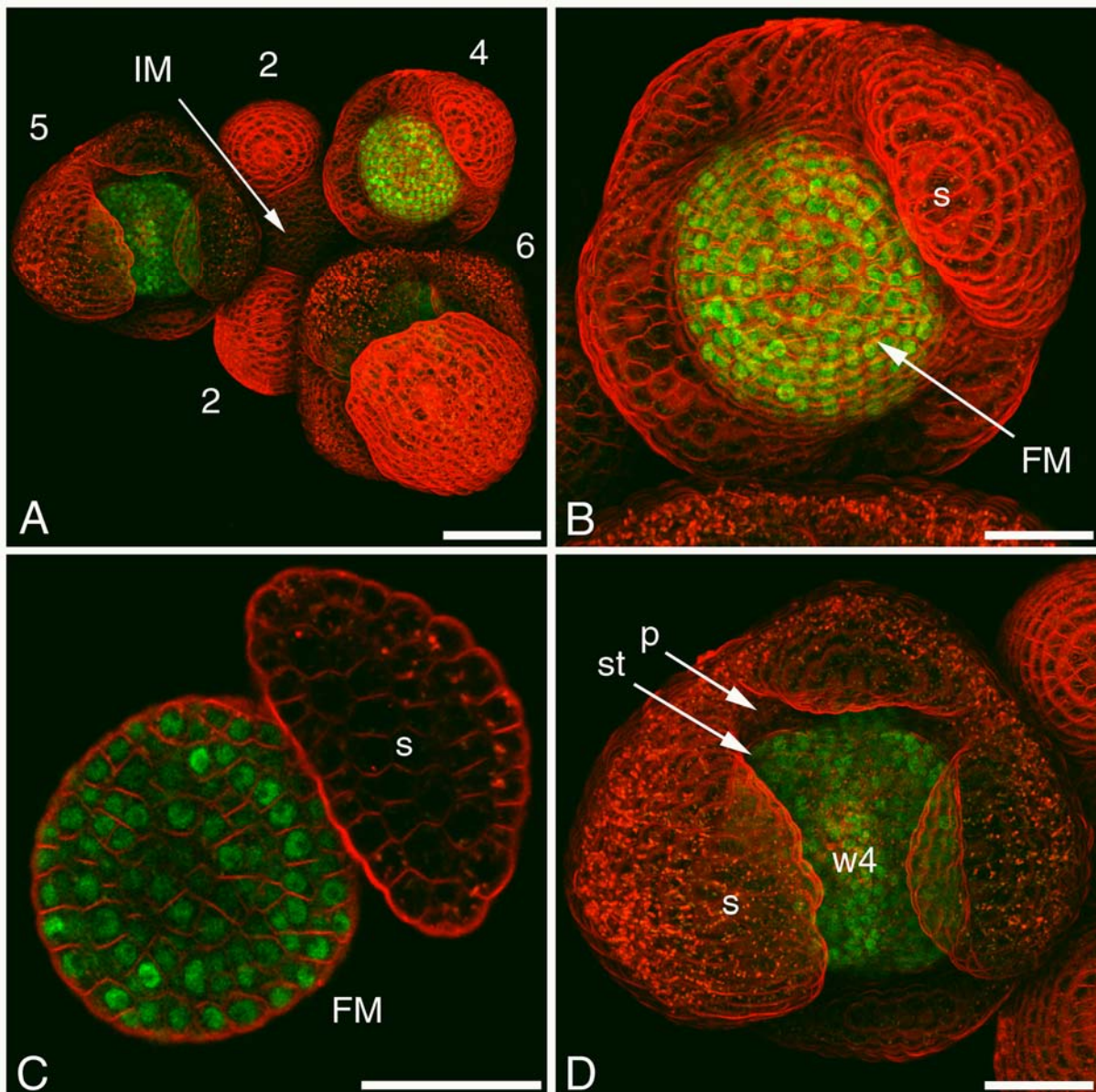
### **AG localisation in inflorescence meristem and early flower bud stages**

The AG:GFP signal appeared in a cluster of subepidermal and inner layer cells in very early stage 3 flower buds, at the time when the first sepal primordium started to arise. During the development of stage 3 flower buds the AG:GFP localisation enlarged to encompass the part of the floral meristem from which the third and fourth whorl will develop, and this pattern remained in later stages (Figures 2A, B, and D). This corresponds well to the observed AG mRNA expression pattern that starts in the floral meristem of stage 3 flower buds and continues in whorl 3 and 4 in later stages [34, 35]. During all the early stages of flower bud development the AG:GFP protein seemed to be primarily localised in the nucleus, but a substantial part of the signal was also localised in the cytoplasm (Figure 2C). In a stage 5 flower bud, AG:GFP was present throughout all cell layers of the developing stamen primordia and in the region of whorl 4 (Figure 2D).

### **FUL localisation in inflorescence meristem and early flower bud stages**

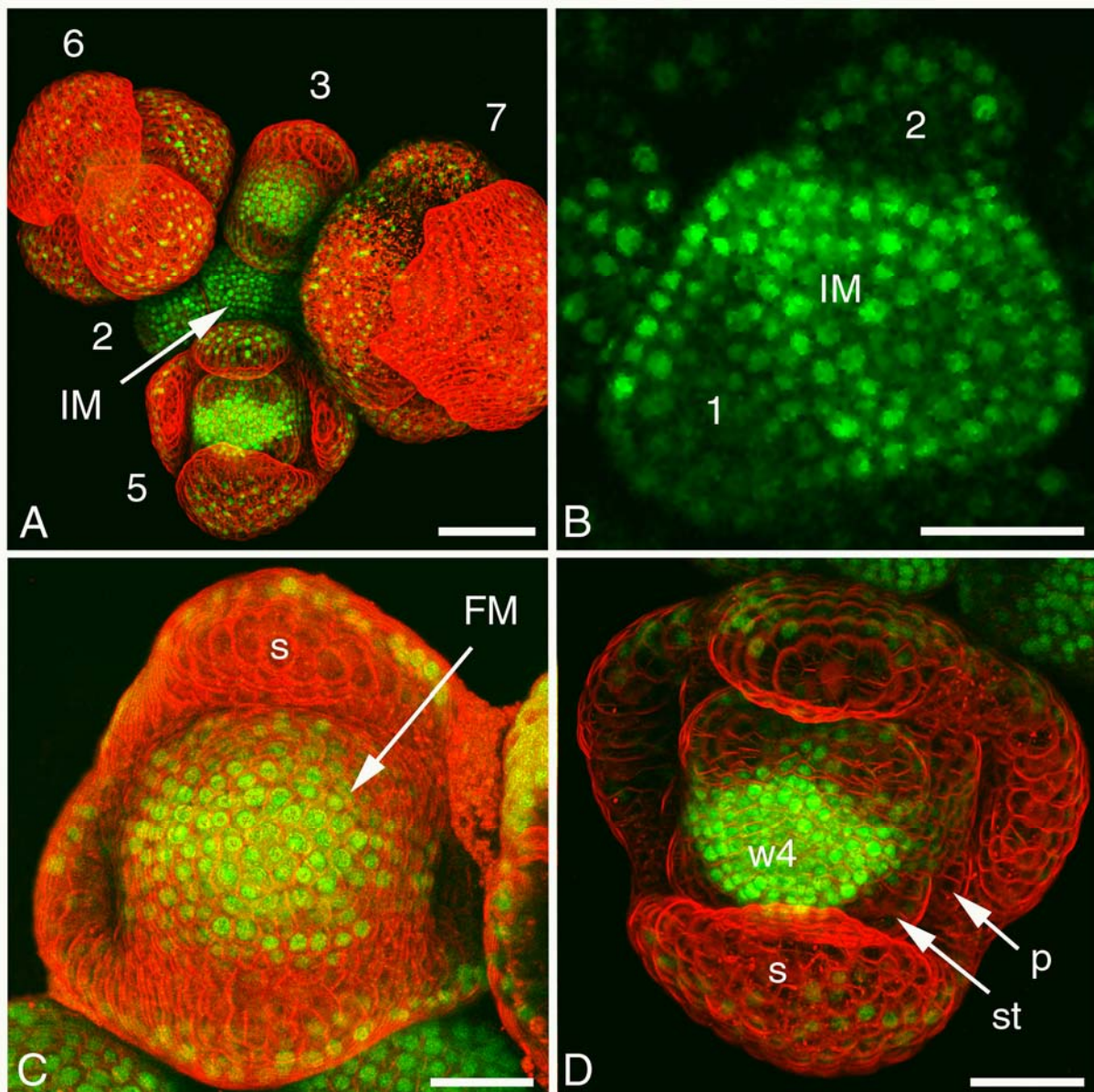
In the inflorescence meristem, FUL:GFP plants exhibited a very high fluorescence signal throughout all cell layers (Figure 3A). This signal was mostly located in the nucleus, but also in the cytoplasm (Figure 3B). As soon as the flower bud primordia were initiated the FUL:GFP signal started to reduce in the subepidermal and inner cell layers (Figure 3B), while in the centre of the emerging flower buds some cells maintained the high level of FUL:GFP signal. During the development of the sepals, the basal part of the sepal had FUL:GFP proteins in both epidermal and subepidermal layer, whereas the apical part of the sepal had only epidermal FUL:GFP. This FUL:GFP signal was maintained at the abaxial side of the sepal, whereas the adaxial side and the abaxial side at the tip showed a reduction in signal (Figures 3C and D). In stage 3, 4 and 5 flower buds the floral meristem had the highest amount of FUL:GFP protein in the innermost part of the floral meristem, while in the area of the future second and third whorl, the amount gradually reduced. However, in these initiating petal and stamen primordia the presence of FUL:GFP in the epidermis remained the longest (Figures 3C and D).

*FUL* mRNA is expressed in two distinct phases: in the inflorescence meristem and in the centre of the floral meristem from stage 3 flower bud onwards, but not in the intermediate flower bud stages 1 and 2 [22, 36]. Therefore, the FUL:GFP presence that was observed in flower bud stages 1 and 2 (Figures 3A and B) might be due to FUL:GFP protein that remained from previous stages. The renewed *FUL* gene expression in stage 3 flower buds in the centre of the floral meristem corresponds with the increased FUL:GFP protein



**Figure 2. Confocal microscopic analysis of AG:GFP localisation in inflorescence meristem and early flower bud stages.**

(A) Overview of an inflorescence with the inflorescence meristem and early flower bud stages 2 to 6 indicated. AG:GFP protein is detected as green signal and cell membranes are stained with the red dye FM4-64. (B) Detail of a stage 4 flower bud showing AG:GFP signal in the future third and fourth whorl. (C) Section through the stage 4 flower bud in (B) showing that AG:GFP is located in both the cytoplasm and the nuclei. (D) Detail of the stage 5 flower bud in (A) showing AG:GFP signal in the developing stamen and in whorl 4. (2-6) flower bud stages; (FM) floral meristem; (IM) inflorescence meristem; (p) petal; (s) sepal; (st) stamen; (w4) whorl 4. Scale bar of (A) 50  $\mu\text{m}$  and scale bars of (B – D) 25  $\mu\text{m}$ .



**Figure 3. Confocal microscopic analysis of FUL:GFP localisation in inflorescence meristem and early flower bud stages.**

(A) Overview of an inflorescence with the inflorescence meristem and early flower bud stages 2 to 7 indicated. FUL:GFP protein is detected as green signal and cell membranes are stained with the red dye FM4-64. (B) Section through an inflorescence meristem showing high FUL:GFP signal in all layers, while stage 1 and 2 flower bud primordia have reduced signal. (C) Detail of a stage 3 flower bud showing the highest FUL:GFP signal in the floral meristem and only epidermal signal in the four sepals. (D) Detail of a stage 5 flower bud showing the highest FUL:GFP signal in whorl 4 and reducing signal in the petal and stamen primordia. (2-7) flower bud stages; (FM) floral meristem; (IM) inflorescence meristem; (p) petal; (s) sepal; (st) stamen; (w4) whorl 4. Scale bar of (A) 50  $\mu\text{m}$  and scale bars of (B–D) 25  $\mu\text{m}$ .

accumulation in the centre of the floral meristem from stage 3 onwards (Figure 3C). In summary, throughout the development from inflorescence meristem to floral organs it seems that the tissues that need to remain undifferentiated, such as the inflorescence and floral meristem, have FUL:GFP protein in all cell layers. Differentiating tissues however, like the developing floral organs, seem to lose the subepidermal and inner cell layer FUL:GFP signal and only retain the signal in the epidermis.

### **AP1 localisation in inflorescence meristem and early flower bud stages**

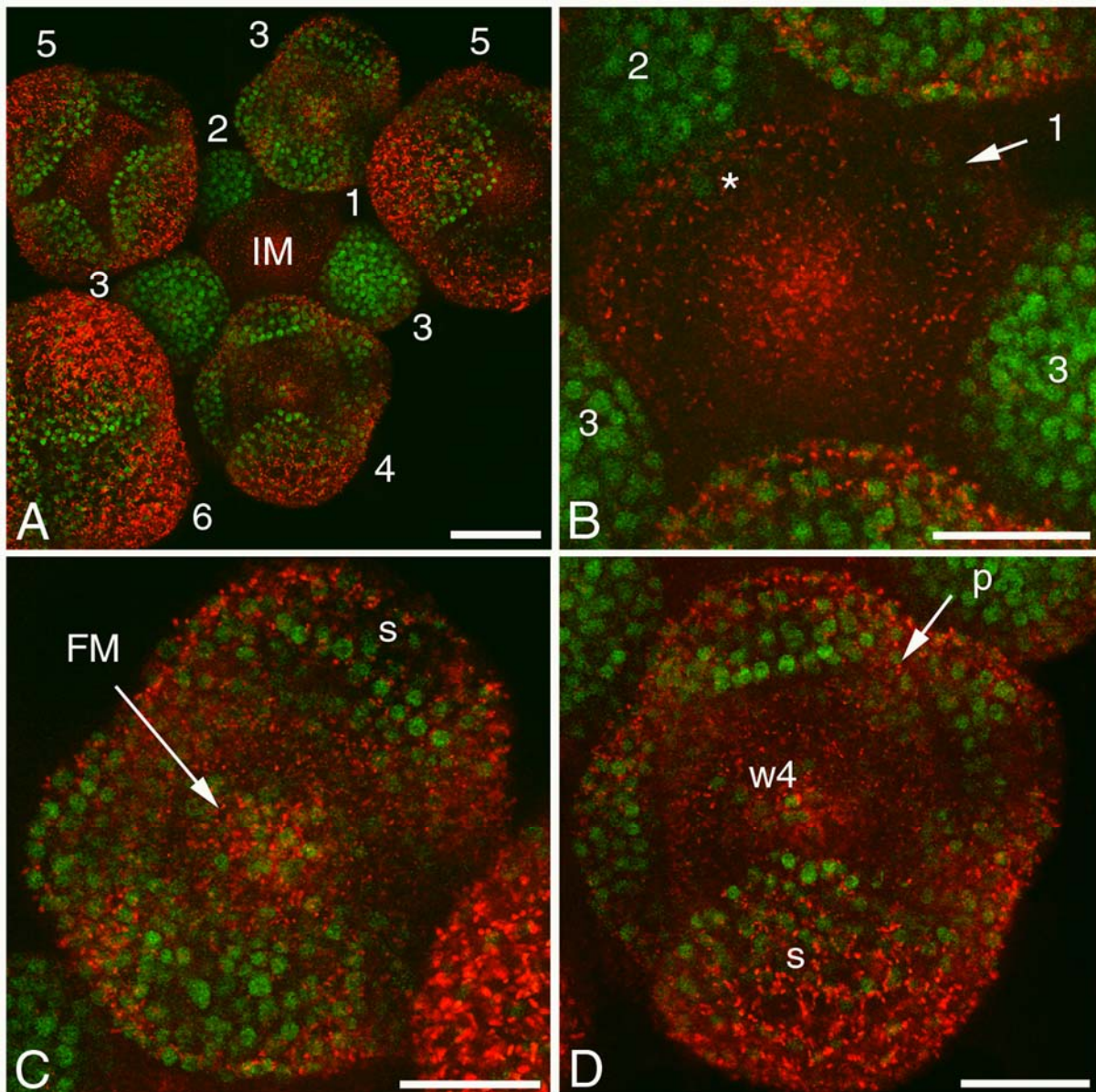
The AP1:GFP signal was first detected in a few cells of the epidermal and internal cell layers of the emerging flower bud primordium (Figures 4A and B). From flower bud stages 1 to 3 the AP1 fusion protein was found throughout all cell layers and was located predominantly in the nucleus (Figure 4C). In flower bud stages 4 and 5, the AP1:GFP protein in the sepals was most abundant in the apical tips (Figures 4A and D). The signal diminished in the third and fourth whorl at this stage, while the signal remained in the second whorl where the petal primordia would emerge. However, a few epidermal cells in the centre of the fourth whorl still had a low AP1:GFP signal, perhaps representing the last meristematic cells in the differentiating floral meristem (Figure 4D). This localisation pattern corresponds with the reported *AP1* mRNA expression [21, 37], where *AP1* expression starts in stage 1 flower bud primordia and increases during the development of the flower bud until stage 3. At the end of stage 3, the *AP1* expression starts to reduce in the centre of the floral meristem as a result of the negative regulation of AG protein present there [37] (Figures 2A and B). However, as our AG:GFP and AP1:GFP localisation studies revealed, there is a clear time-lag between the termination of *AP1* mRNA expression in early stage 3 flower buds, and the reduction in time of the AP1:GFP protein starting in late stage 3 flower buds (Figure 4C). The same time-lag is seen for the reduction of the FUL:GFP signal starting in stage 1 flower buds (Figure 3B), where *FUL* mRNA expression is suppressed due to the presence of AP1 protein [36]. This negative regulation of AP1 on *FUL* expression is probably also apparent in the developing sepals, where eventually the FUL:GFP protein presence is highest in the basal parts of the sepals (Figure 3D), while AP1:GFP protein is more abundant in the apical tips of the sepals (Figure 4D).

### **SEP3, FUL and AP1 localisation during petal development**

The function of the MADS domain proteins in the floral transition and in the determination of floral organ identities is well established, however, less is known about putative later functions of MADS domain proteins in differentiating floral organs. Recently, a report showed that *AG* has a late function in stamen development [38], and it was already known that *FUL* has a late function in pistil and fruit development [22, 39]. In view of this and the fact that MADS domain proteins were still present in differentiating floral organs, we studied the SEP3:GFP, FUL:GFP and AP1:GFP localisations during the development of the petal in more detail.

At stage 5, we observed that SEP3:GFP signal was predominantly located in the epidermis of the emerging petal primordium (Figures 1G and 1I), while AP1:GFP signal was





**Figure 4. Confocal microscopic analysis of AP1:GFP localisation in inflorescence meristem and early flower bud stages.**

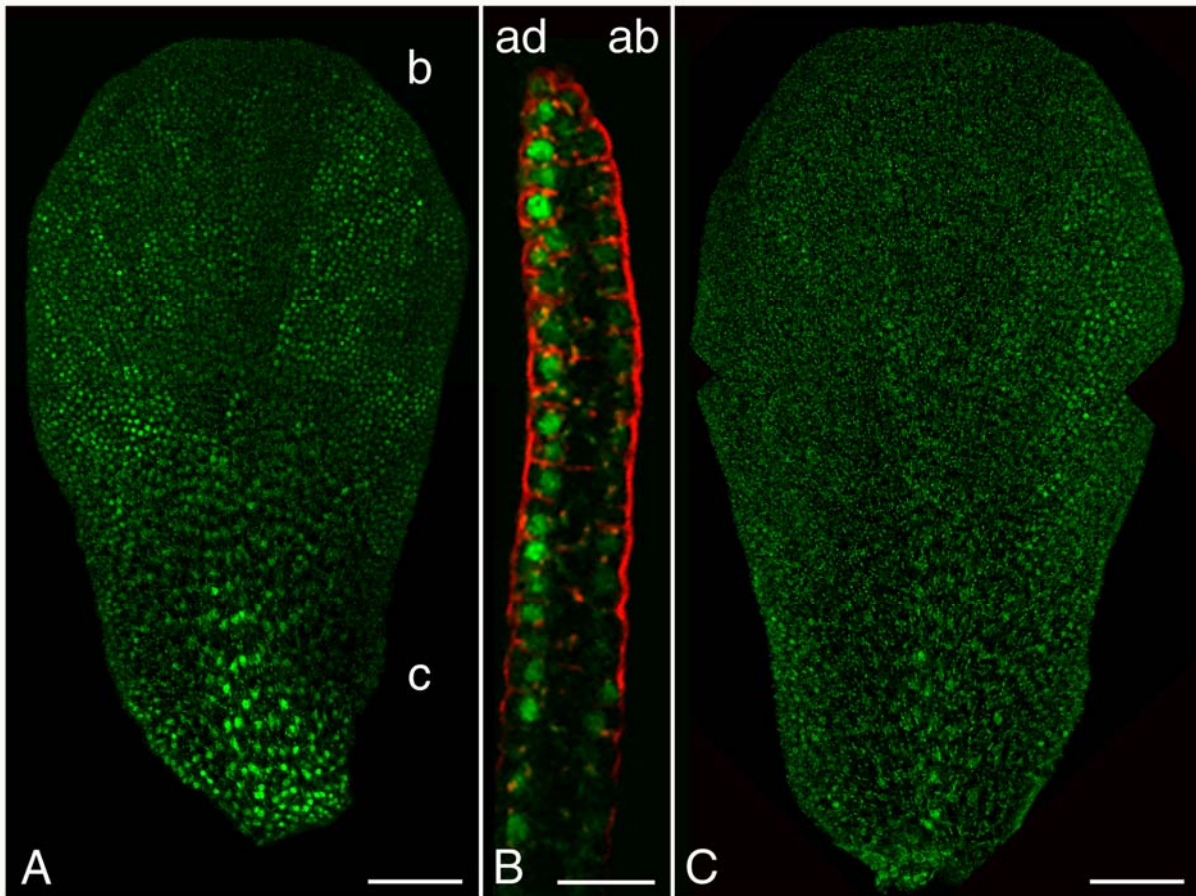
(A) Overview of an inflorescence with the inflorescence meristem and early flower bud stages 1 to 6 indicated. AP1:GFP protein is detected as green signal in a red autofluorescent background. (B) Detail of the inflorescence meristem in (A) showing that the AP1:GFP signal starts in a few cells in stage 1 flower buds and spreads to all cell layers in flower bud stage 2 and 3. The asterisk denotes AP1:GFP signal in nuclei of the adjacent stage 2 flower bud projecting through the overlying inflorescence meristem tissue. (C) Detail of a late stage 3 flower bud showing AP1:GFP signal in all cell layers of the four sepals and the floral meristem. (D) Detail of a stage 4 flower bud showing the highest AP1:GFP signal in the tips of the four sepals and lower signal at positions where the petals will emerge. The signal in whorl 3 and whorl 4 is reducing, although the signal does remain in whorl 4. (1-6) flower bud stages; (FM) floral meristem; (IM) inflorescence meristem; (p) petal; (s) sepal; (w4) whorl 4. Scale bar of (A) 50  $\mu\text{m}$  and scale bars of (B–D) 25  $\mu\text{m}$ .

present throughout all cell layers (Figure 4D). FUL:GFP protein was hardly present at this stage of petal development (Figure 3D). Around stage 9, when petals start to increase rapidly in size [40], high AP1:GFP signal was detected throughout all cell layers. This signal slowly reduced in time and was almost abolished in a stage 12 petal (data not shown). This is in agreement with the *AP1* mRNA expression pattern reported for petals [21, 37]. Remarkably, FUL:GFP protein was observed in the centre of the claw around stage 10 (data not shown), where it might be involved in the vascular development of the petal [22]. The SEP3:GFP signal in a stage 9 petal was higher in the adaxial epidermis of the petal than the abaxial epidermis (Figure 5B). This difference in adaxial and abaxial patterning of SEP3:GFP fusion protein remained until stage 12 petals, although the signal gradually reduced. In a stage 11 petal the SEP3:GFP signal on the adaxial side was strongest in the blade and in the middle of the claw (Figure 5A), while at the abaxial side the edges of the blade and the middle of the claw had the strongest SEP3:GFP signal (Figure 5C). The asymmetric accumulation of SEP3:GFP protein in the petal epidermis is in contrast with the uniform *SEP3* mRNA expression reported for petals [20], but it does resemble the epidermal SEP3:GFP localisation pattern that we observed in sepals (Figure 11), suggesting that SEP3 could play a role in the adaxial/abaxial patterning of both organs.

### **FUL, SEP3 and AG localisation during pistil development**

It is well-known that *FUL* has a late function in valve differentiation in the developing pistil and in fruit elongation [22, 39]. In contrast, much less is known about putative late functions of *SEP3* and *AG* during pistil development. Therefore, we studied the localisation patterns of FUL:GFP, SEP3:GFP and AG:GFP in the developing pistil until flower bud stage 12 (stage after [40]), when style, valves, valve margins, and replum are being formed. Note that in stage 12 pistil tissue the confocal microscope laser could not reliably penetrate beyond five cell layers.

We observed that FUL:GFP is predominantly located in the two valves and the replum and, to a much lesser extent, in the valve margins and the basal half of the style (Figure 6A). In the valves FUL:GFP was present in the first five cell layers, whereas we detected signal in the replum and the style only in the first two layers, and in the valve margins only in the first layer (Figure 6F). In a younger gynoecium (stage 10) this FUL:GFP localisation pattern was already visible, although with lower intensity, but in this stage the future replum had only relatively high signal in the basal part (data not shown). The presence of FUL in the epidermal and subepidermal layers of the replum and valve margins has not been reported before in either developing pistils or fruits [9, 22, 36]. Nevertheless, *ful-1* mutant fruits do not only have defects in the valve tissue but also fail to dehisce, indicating defects in the replum and valve margins [9, 22]. Also, the presence of the FUL:GFP protein in the replum does not transform the cells into valve cells, whereas this was reported happening when constitutively expressing *FUL* in the entire gynoecium [41]. This suggests that either the replum identity is already established prior to or around stage 10 before the FUL:GFP expression is basal-apically up-regulated in the replum, or that for the conversion of replum to valve cells FUL protein needs to be present in all cell layers of the replum. The suggestion that FUL could be

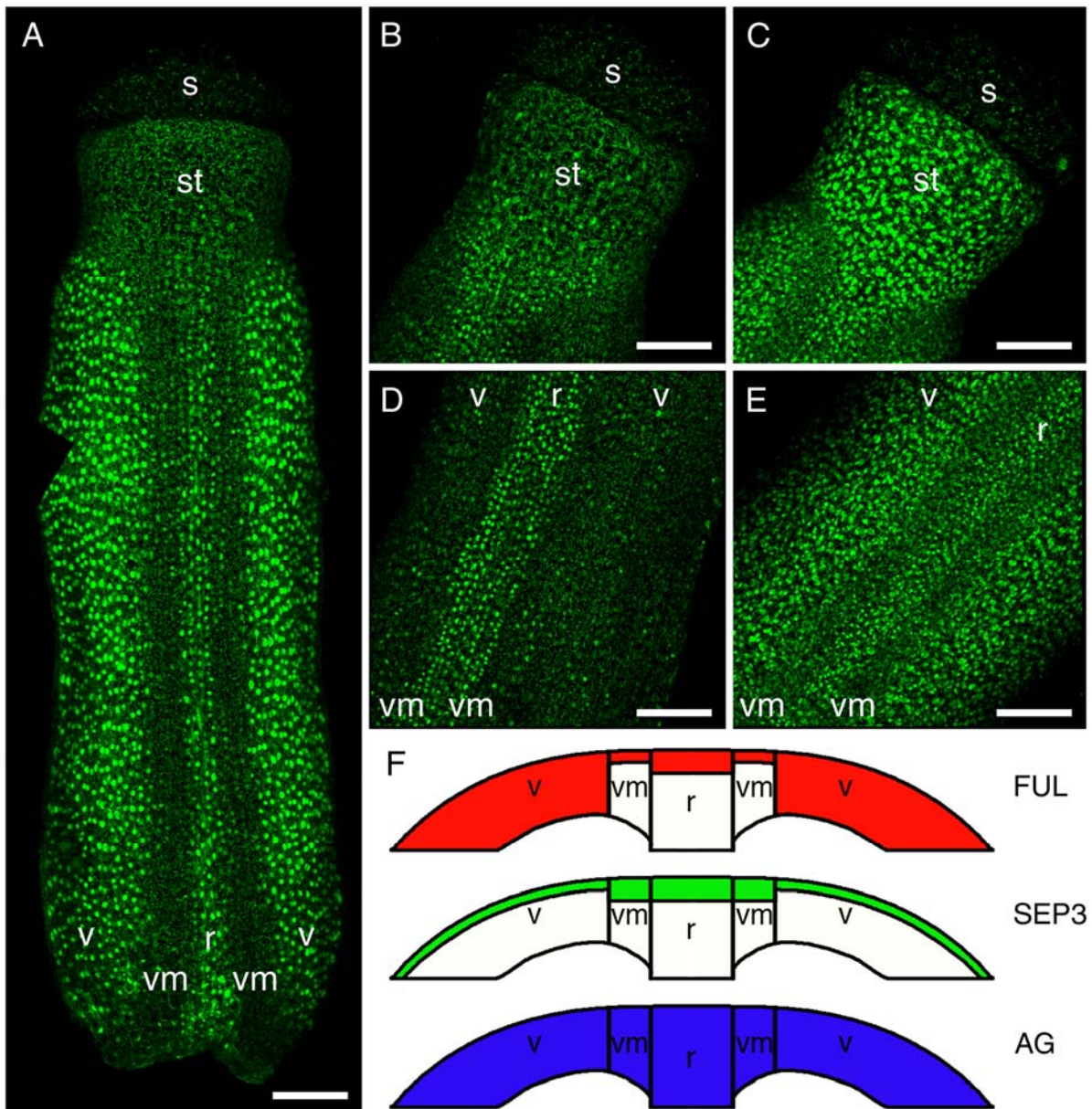


**Figure 5. Confocal microscopic analysis of SEP3:GFP during petal development.**

(A) SEP3:GFP signal at the adaxial side of a stage 11 petal. Nuclei with SEP3:GFP proteins are visible as bright green spots against the green autofluorescent background of the petal. (B) Cross section through a petal of approximately stage 10 showing higher SEP3:GFP signal in the adaxial epidermis than the abaxial epidermis. Cell membranes are stained with the red dye FM4-64. (C) SEP3:GFP signal at the abaxial side of a stage 11 petal. The images in (A) and (B) are each composed of two separate, overlapping projections. (ab) abaxial side; (ad) adaxial side; (b) blade of petal; (c) claw of petal. Scale bars of (A) and (C) 50  $\mu\text{m}$  and scale bar of (B) 15  $\mu\text{m}$ .

intercellularly transported from the valve tissue to the replum [22] is supported by our localisation data. On the other hand, most studies on *FUL* expression focused more specifically on fruit development (after stage 12), it could therefore be that *FUL* expression in the epidermal and subepidermal layers of the replum and valve margins in the developing pistil (until stage 12) has been overlooked.

SEP3:GFP fusion protein is most abundant in the replum and the valve margins, while the valves and the style showed a much lower signal (Figures 6B and D). In the replum and the valve margins SEP3:GFP was located in the first two cell layers, whereas the style and the valves had only signal in the epidermal layer at this stage of pistil development (Figure 6F). However, in a younger gynoecium (stage 10) the same localisation pattern with lower intensity was found, but at least three cell layers deep in the future style and two cell layers deep in the future valve margins, the future replum, and the future valves (data not shown). Therefore, it seems that during the development of the gynoecium SEP3:GFP becomes restricted to the epidermal and subepidermal layers.



**Figure 6. Confocal microscopic analysis of FUL:GFP, SEP3:GFP and AG:GFP localisation in stage 12 pistils.**

(A) FUL:GFP localisation in a stage 12 pistil, with the highest signal in the valves and the replum, and lower signal in the valve margins and the basal half of the style. The image is composed of three separate, overlapping projections. Nuclei with FUL:GFP proteins are visible as bright green spots against the green autofluorescent background of the pistil. (B) SEP3:GFP localisation in the apical part of a stage 12 pistil with the highest signal in the valve margins and replum, and lower signal in the style and valves. (C) AG:GFP signal in all cell layers of the apical part of a stage 12 pistil. (D) SEP3:GFP localisation in the middle part of a stage 12 pistil with the highest signal in the valve margins and replum, and lower signal in the valves. (E) AG:GFP signal in all cell layers of the middle part of a stage 12 pistil. (F) Schematic cross sections of the FUL:GFP, SEP3:GFP and AG:GFP localisations in the ovary wall. (r) replum; (s) stigma; (st) style; (v) valve; (vm) valve margin. All scale bars 50  $\mu$ m.

We observed that AG:GFP is present throughout all cell layers of the whole pistil including the style and stigma (Figures 6C, E and F). In a younger gynoecium (stage 10) the same pattern is already present (data not shown). It was shown that AG mRNA expression at early gynoecium stages is throughout the whole tissue, but in contrast to our protein localisation data, this expression becomes restricted over time to the stigma and no or hardly any expression exists in the ovary walls in a stage 12 pistil [19]. The high uniform signal of AG:GFP that we observed throughout stage 10 and stage 12 pistils could be explained by a low turnover of the protein molecules in those tissues.

### **SEP3 and AG localisation correlated with *WUS* expression during ovule development**

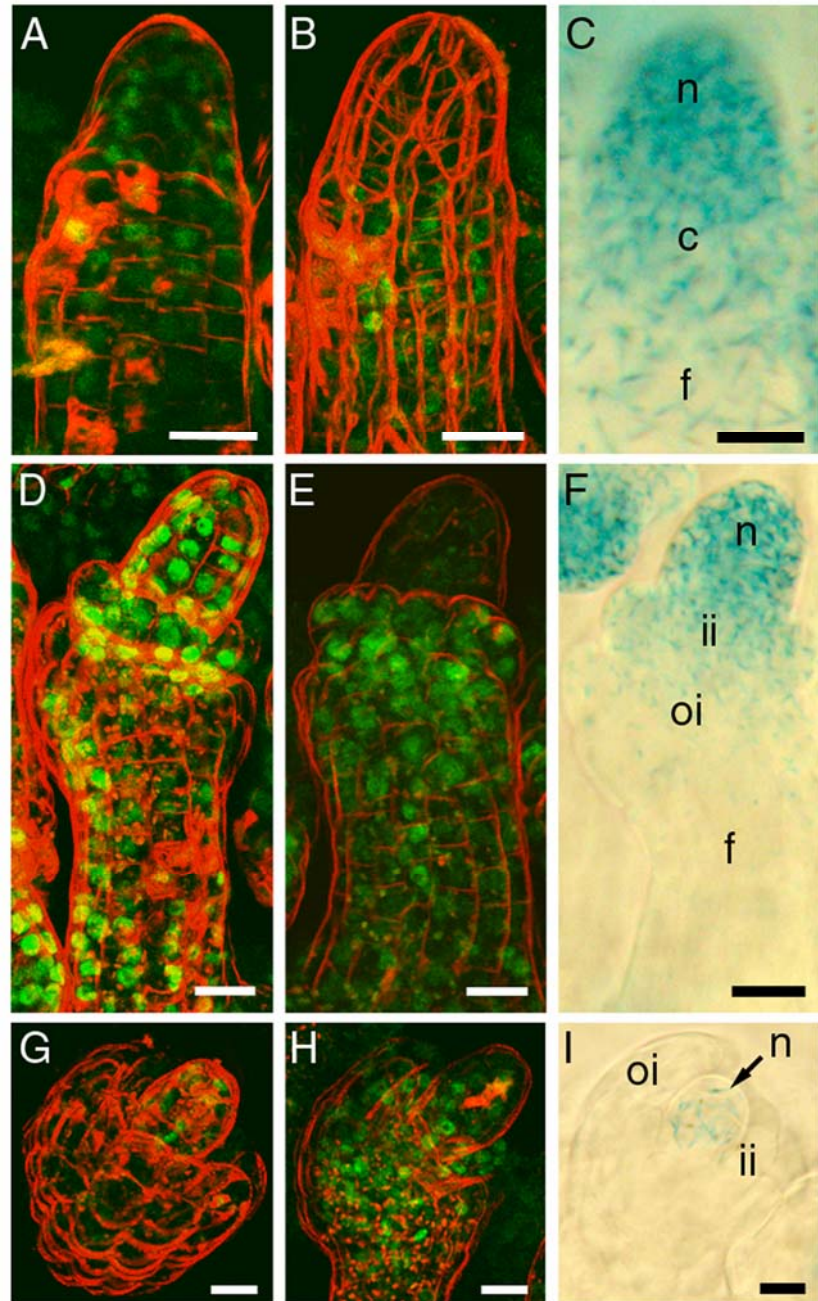
We studied the spatio-temporal localisation pattern of SEP3:GFP and AG:GFP during ovule development in relation to the expression pattern of *WUSCHEL* (*WUS*). *WUS* is a homeodomain transcription factor involved in meristem cell identity maintenance in shoots and an important regulator of ovule development [42, 43]. It is expressed in the centre of the shoot apical meristem, inflorescence and floral meristem and later in the developing ovule [43-45]. The down-regulation of *WUS* in the terminating floral meristem is thought to be regulated by AG [46, 47]. Additionally, D- and E-type MADS domain proteins like STK and SEP3, might be involved in the suppression of *WUS* and the termination of the floral meristem [48] (RI and GA, unpublished results). In ovules, *WUS* is thought to be down-regulated by the SEP3-AG dimer combined with homeodomain transcription factor BELL1 [49]. For this reason, the localisation pattern of SEP3:GFP and AG:GFP was correlated with the expression pattern of *WUS* in ovules, which may reveal whether this interaction between these MADS domain proteins and the *WUS* gene is tightly correlated.

Both SEP3:GFP and AG:GFP were present from the protrusion stage of ovule development stage 1 onwards (stages after [50]). At the beginning of stage 2 SEP3:GFP was present in the whole protrusion, while AG:GFP seemed to be limited to the funiculus and the chalaza with hardly any fluorescence in the nucellus (Figures 7A and B). During the initiation of the inner and the outer integuments, stages 2-II to 2-III, the amount of AG:GFP protein in the nucellus increased. At the same time, the initiating inner and outer integuments showed the highest AG:GFP levels (Figure 7E). Also SEP3:GFP signal peaked during the stages 2-II to 2-III, with the highest signal in the initiating inner integument and the nucellus (Figure 7D). After stage 2-III, both AG:GFP and SEP3:GFP were clearly present in the nucellus (Figures 7G and H). These spatio-temporal patterns of AG:GFP and SEP:GFP fit with the reported AG mRNA expression [51] and the *SEP3* mRNA expression [20]. However, no AG expression was seen in the nucellus [51], whereas we did see increasing AG:GFP signal in the nucellus (Figures 7E and H). This could be the result of AG:GFP transport from the developing integuments towards the nucellus. The transcriptional *pWUS:GUS* reporter line [43] showed that *WUS* expression gradually becomes restricted to the integument primordia and the nucellus in the stages 2-II to 2-III (Figures 7C and F), until it was only present in the nucellus after stage 2-III (Figure 7I). *WUS* expression peaked in the nucellus during the stages 2-II to 2-III and afterwards diminished. Therefore it seems that this proximal-distal down-regulation of *WUS* expression in the different ovular tissues is preceded by increasing

proximal-distal AG:GFP and SEP3:GFP signals and only occurs in the tissues where both SEP3:GFP and AG:GFP were present, supporting the hypothesis that SEP3 and AG are together involved in the repression of *WUS* expression [49].

**Figure 7. Localisation of SEP3:GFP, AG:GFP and expression of *pWUS:GUS* during ovule development.**

Confocal microscopic analysis of SEP3:GFP localisation (**A**, **D**, and **G**) and AG:GFP localisation (**B**, **E**, and **H**) during ovule development. GFP tagged MADS domain proteins are detected as green signal and cell membranes are stained with the red dye FM4-64. DIC microscopy of *pWUS:GUS* expression in developing ovules (**C**, **F**, and **I**) where GUS activity is detected as a blue colour. (**A**) SEP3:GFP signal in the nucellus, chalaza, and funiculus of an ovule at stage 2-II. (**B**) AG:GFP signal in the chalaza and funiculus of an ovule at stage 2-II. (**C**) *pWUS:GUS* expression in an ovule at stage 2-II in an increasing basal-to-apical gradient. (**D**) High SEP3:GFP signal specifically in the nucellus, the inner integument, and the funiculus in an ovule at stage 2-III. (**E**) AG:GFP signal in an ovule at stage 2-III, with beginning signal in the nucellus and the highest signal in the developing inner and outer integuments. (**F**) *pWUS:GUS* expression in the nucellus and the two integuments of an ovule at stage 2-III in an increasing basal-to-apical gradient. (**G**) SEP3:GFP signal in an ovule at stage 2-IV with the highest signal in the nucellus. (**H**) AG:GFP signal in the nucellus, the two integuments, and the funiculus of an ovule at stage 2-IV. (**I**) *pWUS:GUS* expression only in the nucellus in an ovule at stage 2-IV. (c) chalaza; (f) funiculus; (ii) inner integument; (oi) outer integument; (n) nucellus. All scale bars 10  $\mu$ m.



## Conclusions

The results described here show that in some cases a discrepancy exists between the previously reported mRNA expression patterns and our protein localisations. Therefore, mRNA expression patterns alone are not sufficient to form hypotheses about gene function and they need to be supported by protein localisation data. For instance, in a recent paper [52] it was suggested that *FUL*, unlike *AP1*, cannot be directly involved in the regulation of flowering time genes (e.g. *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1*), simply because it is not expressed in the early flower bud stages. Our localisation data, however, shows that *FUL:GFP* is present in the early flower bud stages and therefore could have the same function as *AP1* in regulating these flowering time genes. This is also in agreement with the hypothesis that both *AP1* and *FUL* act as hubs between the flower induction protein network and the floral organ protein network [18], and previous genetic studies that showed redundancy between *AP1* and *FUL* during the floral transition [9]. Other examples, such as the asymmetric localisation of *SEP3* in the epidermis of both sepals and petals and its possible role in the adaxial/abaxial patterning of these organs, the presence of *FUL* protein in the replum of developing pistils, or the presence of *AG* in the nucellus of the developing ovule during the down-regulation of *WUS* were also not apparent from the reported mRNA patterns. These cases demonstrate the importance of studying protein patterns and protein levels for a better understanding of transcription factor functioning. Some caution is needed however, as we analysed the behaviour of tagged proteins, and we cannot exclude that the localisation patterns of these fusion proteins differ from those of the native proteins. It is possible that the increased size of the fusion protein could have an effect on the transport abilities of the protein, or that the presence of the tag could interfere with the ability to form (multimeric) protein complexes. Furthermore, the presence of the tag could change the stability of the protein, which could lead to a different localisation pattern. We also have to realise that the transgenes are inserted on other positions in the genome than the endogenous genes, which may cause some positional effects on expression pattern and level. Nevertheless, because of the capacity of the *AG*, *FUL* and *AP1* fusion proteins to complement their respective mutants and the lack of ectopic expression phenotypes in the transgenic lines during floral development, it is very likely that the localisations of the fusion proteins mimic the patterns of the endogenous proteins.

Discrepancies between mRNA and protein patterns may suggest non-cell autonomous action of the proteins by intercellular transport. This has been shown for a number of transcription factors, such as *KNOTTED-1* in maize [53], and *SHORTROOT* [54] and *CAPRICE* [55] in *Arabidopsis*. Also MADS domain proteins may move from one cell to another, as has been reported for the *Antirrhinum majus* MADS domain proteins *DEFICIENS* and *GLOBOSA* [23]. In our study there are a few examples where the presence of MADS domain proteins could be due to intercellular transport. For instance, the low level, but distinct presence of *SEP3* in the epidermis of the inflorescence meristem and stage 1 flower buds could be due to epidermal transport from high expressing stage 2/3 flower buds. Another example is the increasing *AG* presence in the nucellus in the developing ovule,

where the high expressing integuments might be the source of the AG proteins in the nucellus. Also, as previously suggested [22], the FUL proteins in the replum in developing pistils could originate from the high expressing valve cells.

Subcellular localisations of proteins can provide clues on the regulation and functioning of transcription factors. The subcellular SEP3:GFP localisation in the cytoplasm in the area of the floral meristem that will become whorl 2 and 3 is an example of a localisation that is not expected based on the nature of transcription factors. The cytoplasmic localisation could indicate that the SEP3:GFP protein in the cytoplasm is not in a dimeric form, and therefore cannot be transported to the nucleus [11-13, 24]. Alternatively, it could also be that post-translational modifications cause the cytoplasmic retention of the SEP3:GFP protein, possibly facilitating intercellular transport [56] or breakdown of the protein [57]. Recently, it has also been shown that the MADS domain protein MPF2 from *Physalis floridana* is located in the cytoplasm and imported into the nucleus upon hormone treatment with cytokinin [58]. This shows that non-nuclear localisation is an intriguing mechanism for the regulation of transcription factor functioning.

Summarising, our analysis with GFP tagged proteins expressed under the control of the endogenous promoter revealed the spatio-temporal dynamics of the MADS domain proteins in various tissues of the living plant, leading to a deeper understanding of the behaviour of these MADS domain proteins and allowing the formation of new hypotheses about their function and regulation during early and later stages of floral development.

## Methods

### ***Arabidopsis thaliana* plants**

All plants were grown at 22 °C in growth chambers under a long-day light regime (16 h light/8 h dark).

The construction of transgenic lines expressing gAG:GFP, gFUL:GFP and gSEP3:GFP was previously described [29]. The AG genomic clone has a promoter region of approximately 2.6 kb upstream from the translational start, the FUL genomic clone has a promoter region of approximately 2 kb and the SEP3 clone has a promoter region of approximately 1.5 kb. To make the translational gAP1:GFP fusion construct, a genomic clone fragment of AP1 (6616 bp) was amplified with the following two gene specific primers: the forward primer PDS298 (5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTGTTTAACATCCAAGATTTGTTTTACATAATCGTTAC-3') located 2992 bp upstream from the translational start, and the reverse primer PDS297 (5'-GGGGACCACTTTGTACAAGAAAGCTGGGTCTGCGGCGAAGCAGCCAAGGTT-3') lacking the stop codon at the 3' end of the coding sequence. The amplified product was inserted into the pENTR/D-TOPO vector (Invitrogen) and, after sequence controls, recombined into the binary vector pMDC107 [59]. *Arabidopsis* plants were transformed with *Agrobacterium tumefaciens* strain GV3101 using the floral dip method [60]. Transformed seeds were selected on MS agar plates with 10 µg/ml hygromycin, and stable transgenic lines were maintained afterwards. Furthermore, a transcriptional pWUSCHEL:GUS line [43] was used to analyse the expression pattern of WUSCHEL in



developing ovules.

### Complementation experiments

Stable transgenic plant lines expressing *gAG:GFP* and *gFUL:GFP* in wild type Col-0 background were crossed with the SALK\_014999 *ag* T-DNA insertion mutant line and the *ful-1* mutant [22] respectively, while the *gAP1:GFP* construct was directly transformed into the SALK\_056708 *ap1* T-DNA insertion mutant line. In the progeny, the presence of the wild type allele, the mutant allele, and the GFP tagged MADS box gene construct was determined by PCR and CLSM. For *AG* the following primer pairs were used: *AG* wild type allele, forward primer PRO182 (5'-GGATCCATGGCGTACCAATCGGAGCT-3') annealing immediately after the START codon and reverse primer PDS1985 (5'-CATTTCCTTCAGCCTATATTACC-3') located in the 3' UTR 18 bp downstream of the STOP codon; *ag* T-DNA mutant allele, forward primer PRO433 (5'-CACCGATCAAAGACTACACATCAC-3') located in the 5' UTR 2634 bp upstream of the START codon and reverse primer PDS404 (5'-TGGTTCACGTAGTGGGCCATCG-3') located on the left border of the T-DNA. The presence of *gAG:GFP* was determined by CLSM in the inflorescences. For *FUL*, the following primer pairs were used: *FUL* wild type allele, forward primer PDS1024 (5'-CTTACGTCAGTGTAGACTCAGC-3') located in the 5' UTR 201 bp upstream of the START codon and reverse primer PDS1023 (5'-AAAGAGTGAGATAGTTCTACTCG-3') in the 3' UTR 16 bp downstream of the STOP codon; *ful-1* mutant allele, forward primer PDS1025 (5'-TTCATCCCTTTTTCAGGGTTGTC-3') corresponding with the inserted DsE element and reverse primer PDS1023; and *gFUL:GFP*, forward primer PDS920 (5'-ATCACTTACGTCAGTGTAGACTCAGC-3') in the 5' UTR 204 bp upstream and reverse primer PDS914 (5'-CATCATGTTTGTATAGTTCATCCATGCC-3') 5 bp upstream of the STOP codon of *mGFP6*. For *AP1*, the following primer pairs were used: *AP1* wild type allele, forward primer PDS912 (5'-AAAACCTTTAGGGCCGTAGTGAAGTGAAC-3') 385 bp downstream of the START codon and reverse primer PDS1105 (5'-ATTGGATGAAAAGAGCCTAGCCAC-3') in the 3' UTR 89 bp downstream of the STOP codon (which will give no product for the *ap1* T-DNA mutant allele); *gAP1:GFP*, forward primer PDS912 and reverse primer PDS915 (5'-GACCAGGGTTGGCCATGGAACAGG-3') 183 bp downstream of the START codon of *mGFP6*.

### Confocal laser scanning microscopy

To observe the localisation of the GFP tagged proteins in living plant tissue, inflorescence material was dissected until the relevant meristems and flower buds became visible. After stage 5 flower buds, when sepals started to enclose the floral meristem, it became difficult to visualise the underlying developing floral organs without dissecting the flower buds. The tissues were embedded as previously described [29]. The dye FM4-64 (Molecular Probes, Leiden, The Netherlands) was used as a red counter stain for cell membranes and added at a concentration of 5  $\mu$ M to the embedding mixture of 0.8% agar, 0.5x MS. The incubation time of the sample in the embedding mixture with FM4-64 was at least 20 minutes. Confocal Laser Scanning Microscopy (CLSM) of the living plant tissue was performed with a Zeiss

LSM 510 inverted confocal microscope using a 40 x C-Apochromat (NA 1.2 W korr) lens. Both GFP and the FM4-64 dye were excited with the 488 nm line of an Argon ion laser. The GFP emission was filtered with a 505-530 nm band pass filter, while the FM4-64 dye emission and red autofluorescence was filtered with a 650 nm long pass filter. The optical slices in the confocal z-stacks were made as a sum of 4 scans and were median filtered afterwards. Three-dimensional projections of the obtained confocal z-stacks were made with the Zeiss LSM Image Browser version 4 and adjusted with Adobe Photoshop version 5.0.

### **GUS assay**

To analyse the expression pattern of *WUSCHEL* in developing ovules, inflorescences of the *pWUS:GUS* line were fixed and a  $\beta$ -glucuronidase (GUS) assay was performed overnight at 37 °C as previously described [61] (modified from [62]). After GUS detection and chlorophyll removal, the inflorescences were kept in Hoyer's solution (7.5 g Arabic gum, 100 g chloral hydrate, 5 ml glycerol, and 60 ml water). Whole siliques of one inflorescence were put under a cover slip and observed with a Nikon Optiphot microscope. Bright field images of the ovules were taken with a Leica DFC320 digital camera using a 40x Plan DIC objective.

### **Authors' contributions**

For the MADS domain protein localisations SdF, AV and KK created the GFP-tagged MADS box constructs, and SU selected the transgenic plant lines and analysed the protein localisation patterns with CLSM. For the *WUS* expression pattern in ovules RI performed the GUS assay on inflorescences of *pWUS:GUS* plants and SU imaged the ovules. SU wrote the manuscript and RI and GA critically revised it. All authors read and approved the final manuscript.

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# Chapter 4

## Investigating intercellular protein transport

Susan L Urbanus

Jan W Vos

Martijn A Fiers

QD (Peter) Dinh

Richard GH Immink

Gerco C Angenent

### Abstract

Comparisons between mRNA patterns of MADS box genes and their protein localisations indicate that during floral development in *Arabidopsis thaliana* intercellular transport of MADS domain proteins probably occurs. To support this hypothesis we investigated the intercellular trafficking ability of MADS domain proteins with more direct methods. We attempted to set up microinjection systems in *Tradescantia virginiana* stamen hairs and *Arabidopsis thaliana* inflorescences in which transiently expressed MADS domain proteins with a fluorescent tag could be tested for their ability to actively transport between cells. In a different line of investigation we created two different sets of stable transgenic *Arabidopsis thaliana* plants that express fluorescently-tagged MADS domain proteins in inflorescence tissue. In one set of transgenic plants expressing mEosFP-tagged MADS domain proteins under the control of their own promoter, we attempted to follow the intercellular transport of these proteins in their native environment by using a photoconversion technique. In another set of transgenic plants expressing GFP-tagged MADS domain proteins specifically in the epidermal cell layer, we successfully demonstrated both active and passive intercellular transport of MADS domain proteins by combining confocal laser scanning microscopy with a photobleaching technique.

### Introduction

In the previous chapter the localisations of selected GFP-tagged *Arabidopsis thaliana* MADS domain proteins are described and compared to their known mRNA expression patterns. This comparison shows that there are several instances during floral development where MADS domain proteins may be transported between cells. We tried to test with more direct methods if MADS domain proteins indeed have the ability to traffic between cells. Generally it is thought that intercellular transport of proteins is mediated through dynamic channels, called plasmodesmata, which connect the cytoplasm of neighbouring cells [1]. The connectivity between cells depends on the size exclusion limit (SEL) of the plasmodesmata, which is a measure for the maximum size of macromolecules that can freely diffuse through the plasmodesmata. Previously it was shown that less differentiated tissues, such as meristems, have a higher SEL than more differentiated tissues [2-4]. Besides a passive flow of macromolecules between cells, there is also the possibility of active transport of large macromolecules by temporarily enlarging the SEL of plasmodesmata to allow passage [5, 6]. With the methods described in this chapter we tried to determine if MADS domain proteins have any ability for active or passive intercellular transport in inflorescences during floral development.

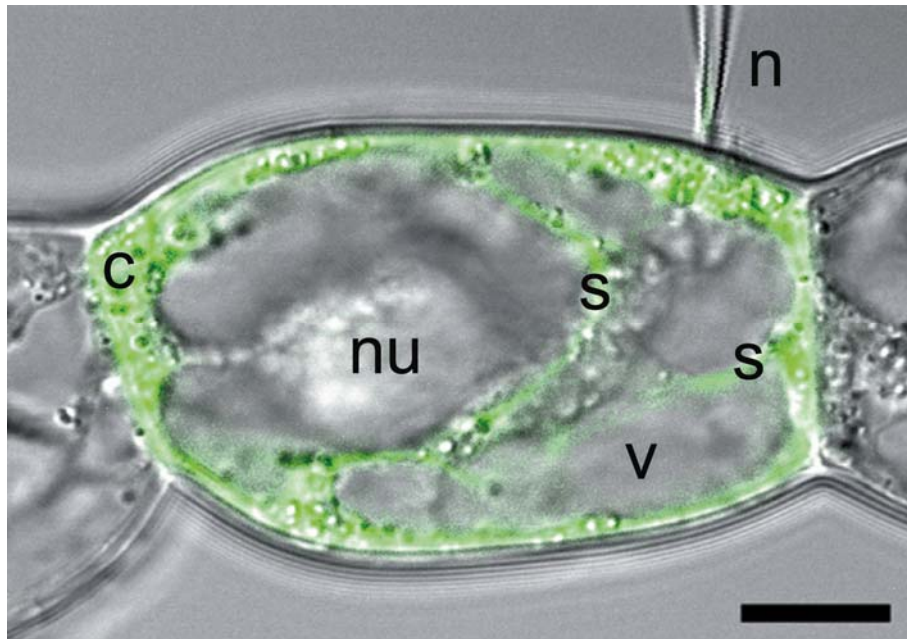


## Microinjection experiments

Two different types of microinjection techniques were used to transiently express fluorescently-tagged MADS domain proteins and fluorescent dyes in plant cells, and to subsequently follow their possible transport to non-injected cells with confocal laser scanning microscopy (CLSM). For our first microinjection experiments we made use of a pressure-microinjection system with living stamen hairs from *Tradescantia virginiana*, used frequently in cell division research [7]. Microinjection experiments in stamen hairs from *Tradescantia virginiana* and *Setcreasea purpurea* have been used before in research on plasmodesmata, because the spread of fluorescently-tagged macromolecules is easily followed in the single file of large stamen hair cells [8, 9]. We attempted to introduce binary vectors into the stamen hair cells, containing sequences that allow the constitutive expression of fluorescently-tagged MADS domain proteins from *Arabidopsis thaliana* or *Petunia hybrida* involved in stamen development. Control dyes, such as fluorescein isothiocyanate (FITC)-dextran, Rhodamine 123 and Lucifer Yellow, were used to visualize if the microinjections in the cytoplasm were successful (Figure 1). The aim of the experiment was to compare the possible transport of the fluorescently-tagged MADS domain proteins to the passive transport of fluorescent dyes of different molecular weights, and in that way determine if MADS domain proteins are able to actively transport through the plasmodesmata of the stamen hair cells. However, since we were not able to detect fluorescently-tagged MADS domain proteins in the injected stamen hair cells or in adjacent cells within two or three days after the injection, we discontinued this line of investigation. We also tried to perform microinjections on the relatively small *Arabidopsis thaliana* inflorescence meristem and floral meristems. Since *Arabidopsis thaliana* meristems consist of very compact cells, pressurized microinjections would probably be too destructive for the cells. Instead we used an iontophoretic microinjection technique where the injection load is introduced into the cell by a small current. To our knowledge there are no reports on microinjections in *Arabidopsis thaliana* inflorescences, although there are reports on iontophoretic microinjections in the bigger shoot apical meristems of *Betula pubescens* and *Sinapis alba* [10, 11]. The aim of the experiment was similar to the previous microinjections, but with the difference that binary vectors would be injected into cells that normally express the endogenous untagged genes. We were able to perform successful injections with fluorescent dyes in both inflorescence and floral meristems, which demonstrated that there are open connections between the meristematic cells (Figure 2). Unfortunately, we did not perform injections with binary vectors in *Arabidopsis thaliana* inflorescences due to time limitations.

## Specific ectopic expression in transgenic plants

Another method of introducing proteins into specific tissues is by stably expressing them in a plant under the control of a tissue-specific promoter. We created transgenic *Arabidopsis thaliana* plants that express selected GFP-tagged MADS box genes under the control of an epidermis-specific promoter, and determined the presence of the GFP-tagged proteins in



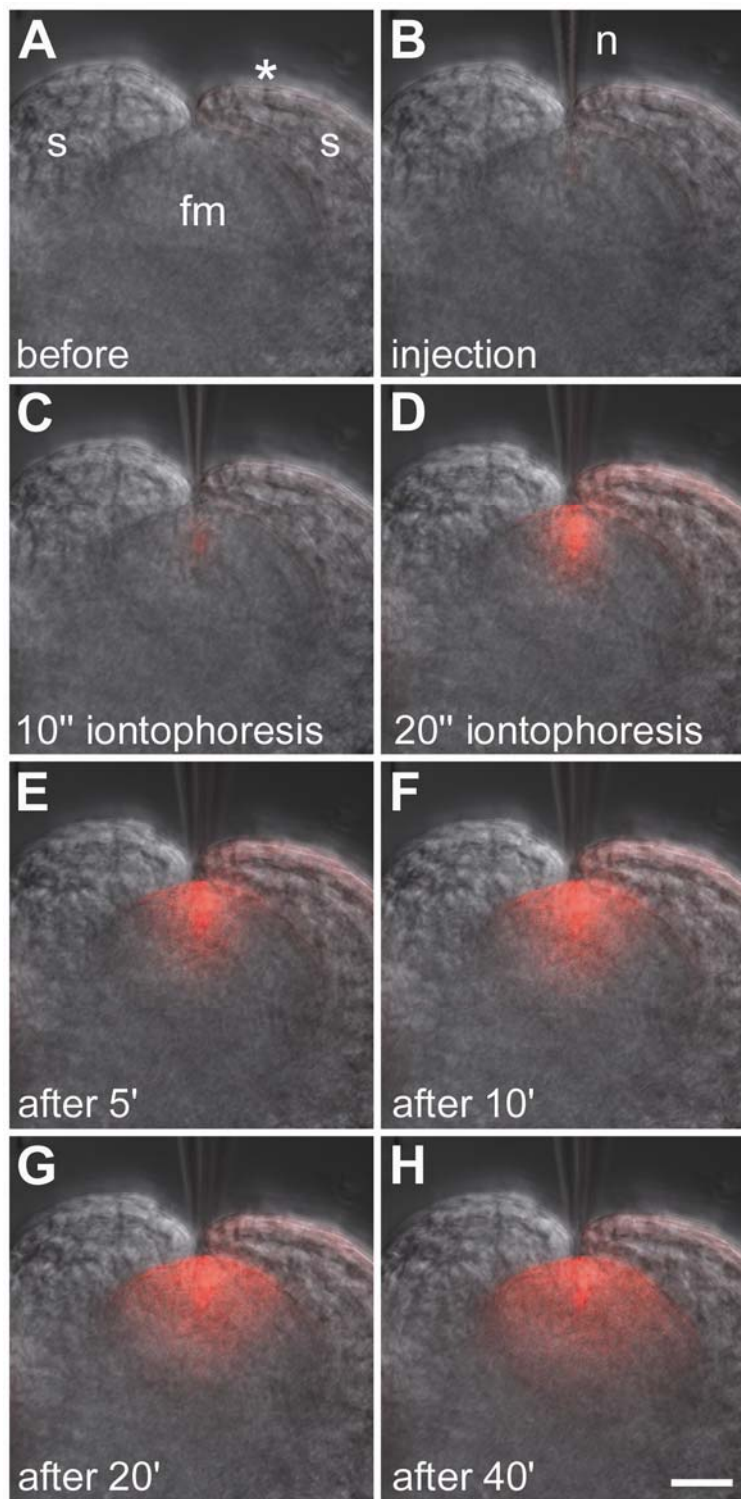
**Figure 1. FITC-dextran microinjection into a *Tradescantia virginiana* stamen hair cell.**

Example of a pressure microinjection with fluorescein isothiocyanate (FITC)-labeled dextran (10 kDa) in a *Tradescantia virginiana* stamen hair cell. The green fluorescent dye is visible in the tip of the needle (n), the cytoplasm (c) and the cytoplasmic strands (s), but not in the nucleus (nu), the vacuole (v), or the neighbouring cells (left and right). Scale bar is 10  $\mu\text{m}$ .

both epidermal and non-epidermal tissues by CLSM. Although this is not a method that directly visualizes protein transport, it can demonstrate that proteins have moved out from the cell layers where they were expressed, if leaky expression and intercellular transport of mRNA can be excluded. With this transgenic approach we demonstrated that one particular MADS domain protein, AGAMOUS, can move inwards from the epidermal cell layer to the subepidermal cell layer in a controlled manner in floral meristems. This result and others are further described in Chapter 5.

### Photobleaching experiments

To be able to directly follow the movement of proteins between cells, we made use of the phenomenon that fluorophores can irreversibly lose their fluorescent ability when they are photochemically altered by high intensity laser light. This photobleaching allows the tracking of fluorescently-tagged protein movement by monitoring the recovery of fluorescence in a photobleached area, commonly called Fluorescence Recovery After Photobleaching (FRAP). Alternatively, photobleaching can be used to track the movement of photobleached fluorescently-tagged proteins by monitoring the decrease in fluorescence in non-bleached areas, known as Fluorescence Loss In Photobleaching (FLIP) [12, 13]. These FRAP and FLIP methods are generally used to quantitatively investigate intracellular movement of molecules and to show continuity or transport between subcellular compartments in the cell.



**Figure 2. Rhodamine-6G microinjection into an *Arabidopsis thaliana* floral meristem.**

Confocal microscopy time series of an iontophoretic microinjection with the red fluorescent dye Rhodamine-6G (0.48 kDa) in an *Arabidopsis thaliana* floral meristem stage 5. The floral meristem (fm), the two visible sepals (s) and the needle (n) are indicated. The sepal on the right side (\*) already contains some Rhodamine-6G dye from a previous microinjection in the epidermis. (A) The floral bud before microinjection. (B) The floral bud after insertion of the needle in the center of the floral meristem in the subepidermal layer, (C) after 10 seconds of iontophoresis and (D) after 20 seconds of iontophoresis. (E-H) Spread of the Rhodamine-6G dye in the floral meristem after 5 minutes (E), 10 minutes (F), 20 minutes (G), and 40 minutes (H). Scale bar is 20  $\mu\text{m}$ .

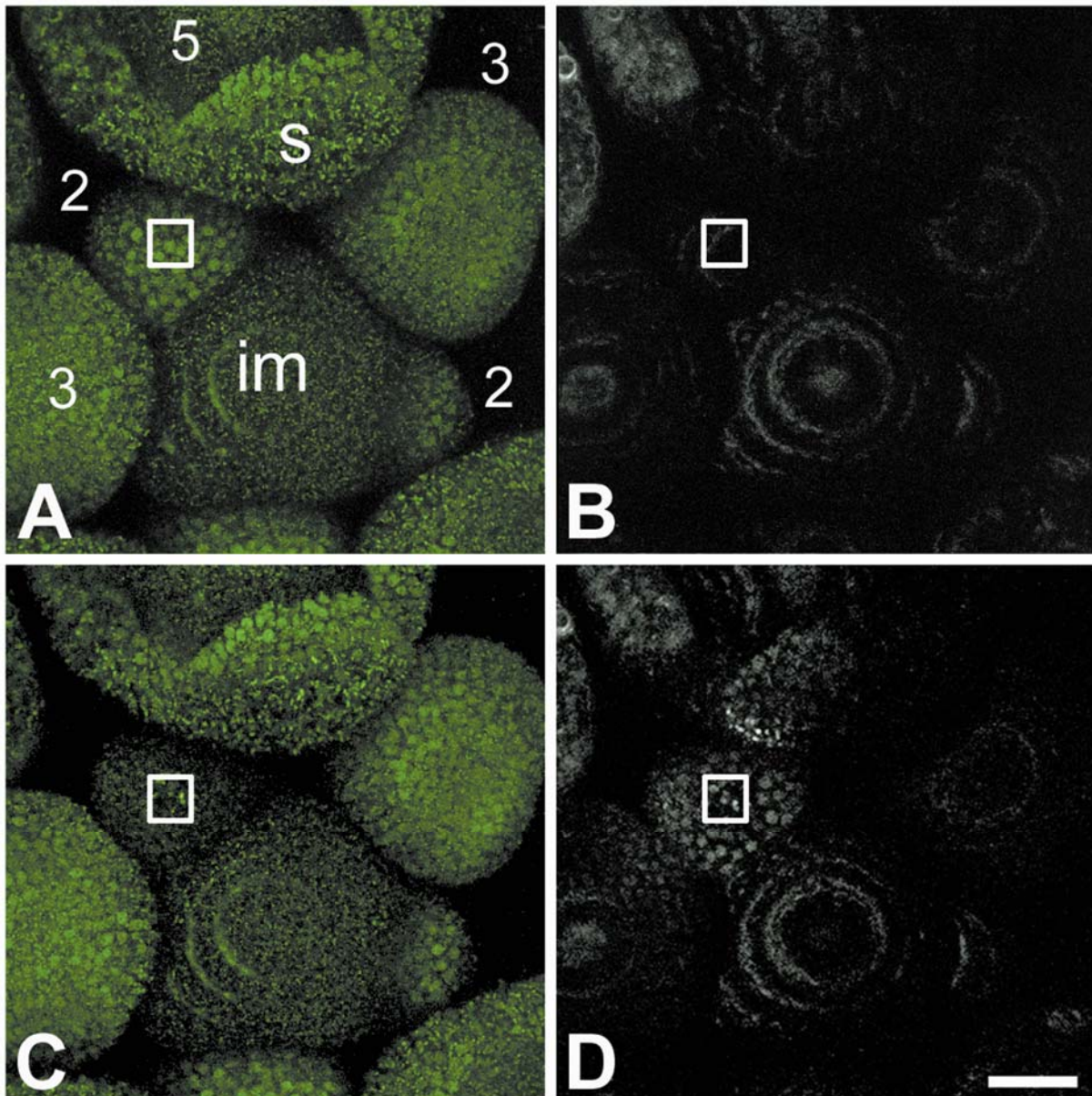
However, we used photobleaching to investigate the intercellular movement of proteins by removing the fluorescence from a few selected epidermal cells in floral meristems of transgenic *Arabidopsis thaliana* plants that specifically express GFP-tagged MADS domain proteins in the epidermis. The two-dimensional aspect of the fluorescent epidermal cell layer of the floral meristem greatly simplified the monitoring of the fluorescent recovery in the bleached area by CLSM. With this photobleaching technique we demonstrated that several MADS domain proteins can diffuse through the epidermal cell layer, presumably in a passive manner. This technique and the results are further described in Chapter 5.

### Photoconversion experiments

With the fairly new photoconvertible fluorescent tags it is possible to visualize the behaviour of a selected subset of tagged proteins within a total pool of tagged proteins, simply by changing the fluorescent colour of the tag [14]. The photoconvertible tag monomeric EosFP (mEosFP) can be changed from green to red fluorescence by irreversibly changing the structure of the fluorophore with laser light of around 400 nm [15]. To study the intercellular movement of MADS domain proteins in their own environment, we attempted to create transgenic *Arabidopsis thaliana* plants that express selected mEosFP-tagged MADS box genes under control of their own promoter, similar to the DNA constructs used in Chapter 2. Unfortunately we encountered two separate problems in this endeavour. First of all, it was very difficult to obtain enough stable primary transformants without mutant phenotypes and with good amounts of mEosFP fluorescence. It seemed that there was a high rate of transgene silencing in these plants, however transformation in a mutant background for gene silencing (*sgs2-1*) did not improve the number of stable transformants (Table 1). Another, more prominent problem was the inadvertent photoconversion of mEosFP-tagged proteins in non-selected regions in the inflorescences of stable transgenic plants. There seemed to be extensive scattering of the photoconverting 405 nm laser light far away from the selected photoconversion area, since we observed unwanted photoconversion of the mEosFP-tagged proteins in remote areas (Figures 3 and 4). Changing the embedding medium for the samples from agar to water did not change this scattering phenomenon. It is known that cells and their subcellular components can extensively scatter laser light [16], and it is therefore very likely that the easily diffracted short wavelength laser light is scattered by the curved and dense inflorescence tissue. As a result of this inadvertent photoconversion of non-selected areas, reliable investigation of intercellular movement of mEosFP-tagged proteins in *Arabidopsis thaliana* inflorescences was not possible.

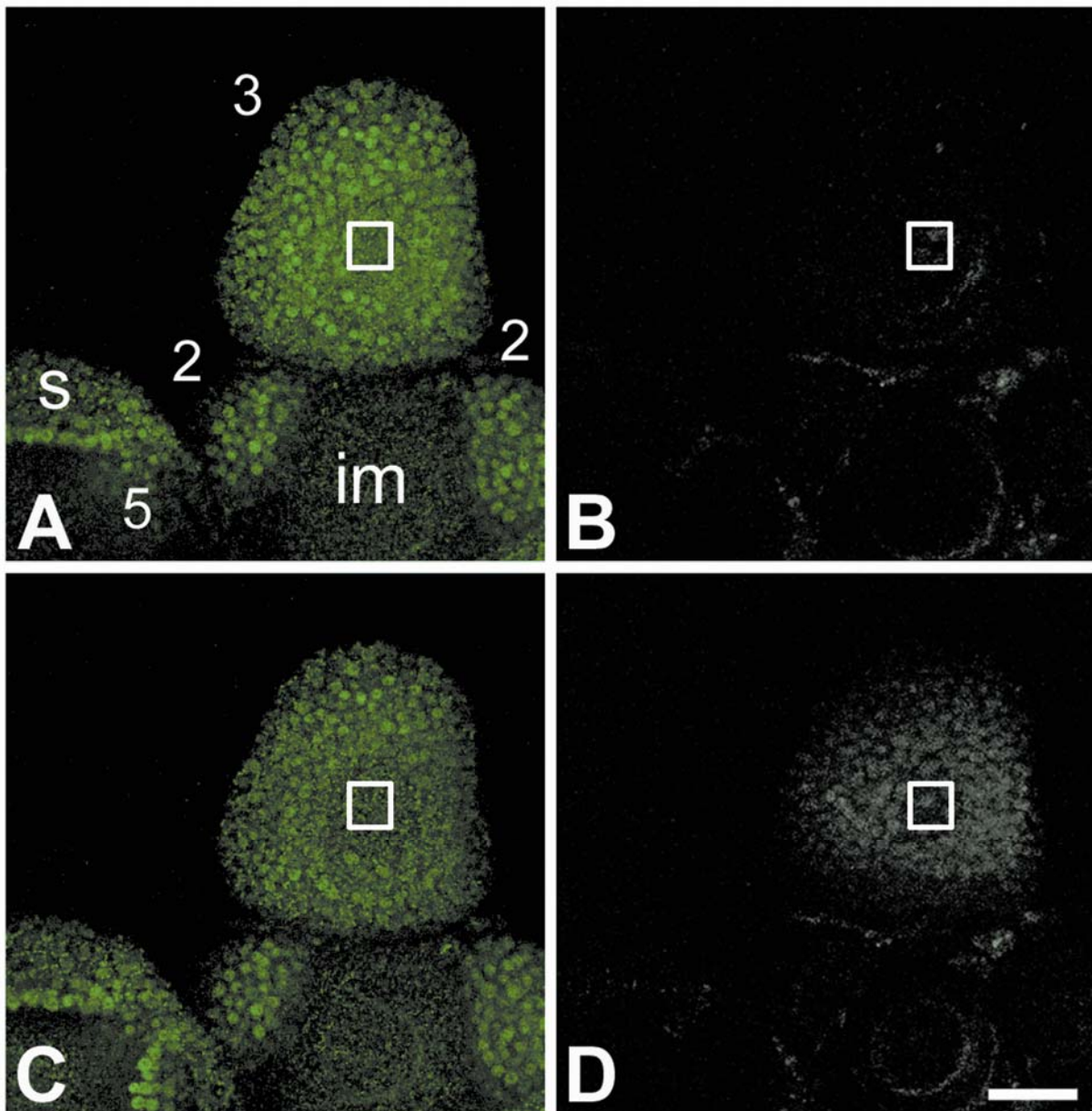
### Discussion

Comparisons of mRNA patterns and correlated protein localisations in plant tissues can reveal discrepancies between mRNA presence and protein presence in cells. These discrepancies can indicate intercellular transport of the protein if the protein is present in cells where the mRNA is not. However, to proof this intercellular protein transport other methods are needed, preferable methods that can show both intercellular transport of proteins from expressing cells to non-expressing cells and intercellular transport between expressing cells. The proposed microinjection experiments in wild type *Arabidopsis thaliana* inflorescence tissue would have been a relatively quick, transient method to test the transport abilities of fluorescently-tagged MADS domain proteins in their native environment. However, where other studies report protein expression after introducing constructs into tissues by way of microinjection [17, 18] or microbombardment [19, 20], we were not able to detect fluorescently-tagged proteins after injecting binary constructs into *Tradescantia virginiana* stamen hair cells in different phases of cell division. Other researchers circumvented this



**Figure 3. Photoconversion of AP1:mEosFP with 405 nm laser light at 100% power.**

Confocal microscopy of mEosFP-tagged MADS domain protein APETALA1 (AP1) in a wild type *Arabidopsis thaliana* inflorescence before and after photoconversion of the mEosFP tag. The nuclei filled with the unchanged AP1:mEosFP are visible as bright green dots, while the green autofluorescence is visible as small green dots. Nuclei filled with the photoconverted red version of AP1:mEosFP are visible as bright white dots, while the preconversion scans show the presence of red autofluorescence in white. AP1 proteins are not present in the inflorescence meristem (im) and start to appear from floral bud stage 1 onwards. In a stage 5 floral bud especially the tips of the sepals (s) show high levels of AP1:mEosFP. Photoconversion was performed in a maximal zoomed-in target area (indicated) in the stage 2 floral bud by scanning one z-section 30 times with 405 nm laser light at 100% laser power. Localisations of green mEosFP-tagged AP1 proteins before (A) and after photoconversion (C); showing overall reduced green fluorescence in the targeted floral bud after photoconversion. Localisations of red mEosFP-tagged AP1 proteins before (B) and after photoconversion (D); demonstrating that not only the targeted floral bud was photoconverted, but also the adjacent floral buds. Scale bar is 25  $\mu\text{m}$ .



**Figure 4. Photoconversion of AP1:mEosFP with 405 nm laser light at 25% power.**

Confocal microscopy of mEosFP-tagged MADS domain protein APETALA1 (AP1) in a wild type *Arabidopsis thaliana* inflorescence before and after photoconversion of the mEosFP tag. The nuclei filled with the unchanged AP1:mEosFP are visible as bright green dots, while the green autofluorescence is visible as small green dots. Nuclei filled with the photoconverted red version of AP1:mEosFP are visible as bright white dots, while the preconversion scans show the presence of red autofluorescence in white. AP1 proteins are not present in the inflorescence meristem (im) and start to appear from floral bud stage 1 onwards. In a stage 5 floral bud especially the tips of the sepals (s) show high levels of AP1:mEosFP. Photoconversion was performed in a maximal zoomed-in target area (indicated) in the stage 3 floral bud by scanning one z-section 30 times with 405 nm laser light at 25% laser power. Localisations of green mEosFP-tagged AP1 proteins before (A) and after photoconversion (C); showing reduced green fluorescence in the middle of the targeted floral bud. Localisations of red mEosFP-tagged AP1 proteins before (B) and after photoconversion (D); demonstrating that almost the whole targeted floral bud contains photoconverted AP1:mEosFP proteins. Scale bar is 25  $\mu\text{m}$ .

problem by injecting purified proteins generated in *Escherichia coli* cultures [6, 21]. The difficulty of trying to express fluorescently-tagged proteins from injected constructs in combination with time limitations, made us decide to discontinue this line of investigation. An important remark to make on the use of microinjections in protein transport research is that it was shown that microinjections can affect the function of the plasmodesmata, usually by decreasing the SEL of the plasmodesmata [9, 22, 23]. This decrease in SEL is caused by callose deposition in and around the plasmodesmata and is probably a general defensive mechanism against wounding and stress [24]. It is therefore very important that the investigated tissue is manipulated as little as possible. In our experiments with inflorescence tissue from stable transgenic plants it was relatively simple to avoid direct damage to the investigated inflorescence and floral meristems, since only the older flower buds had to be removed from the cut-off inflorescence stem. Although it is a longer process to create transgenic plants, in the end we learned the most about intercellular transport of MADS domain proteins from transgenic plants that stably express fluorescently-tagged versions of these proteins in their native environment. We successfully used a set of transgenic plants that constitutively express fluorescently-tagged MADS domain proteins specifically in the epidermal cell layer, to demonstrate both passive and active transport of these proteins in floral meristems. Unfortunately the plants that promised to be the most informative on protein behaviour, namely the set of transgenic plants that express MADS box genes with the photoconvertible tag *mEosFP* under the control of their own promoters, were not useful for studying intercellular protein transport. The system for photoconversion of the fluorescence of a subset of proteins in a targeted area lacked the much needed precision to properly study intercellular protein transport over short and medium distances in inflorescence tissue. This precision could be improved upon by using another confocal microscope set-up where it is possible to target areas smaller than a few meristematic cells, since the *mEosFP* tag and other photoconvertible tags have successfully been used before to study the dynamics of subcellular components [14, 25, 26]. However, the lack of precision could also be a problem inherent to the combination of the short wavelength photoconversion laser light and the curved and dense *Arabidopsis thaliana* inflorescence tissue.

## Material and methods

### Microinjection experiments

Microinjection experiments in combination with confocal laser scanning microscopy were performed on *Tradescantia virginiana* stamen hair cells and *Arabidopsis thaliana* inflorescences with a Zeiss LSM5 PASCAL Axiovert 200M inverted microscope equipped with a Narishige micromanipulation system. All images were adjusted with Adobe Photoshop version 5.0. The *Tradescantia virginiana* stamen hair cells were prepared and microinjected with pressure as described previously [7], and imaged with a Plan-Apochromat 63x/ 1.4 Oil DIC lens. We attempted to introduce binary vectors containing *GFP*- or *YFP*-tagged MADS box genes under the control of the *CaMV35S* promoter in a single stamen hair cell. We used the following MADS box genes: *Arabidopsis thaliana* *AGAMOUS* (*AG*) and *SEPALLATA3*

(*SEP3*), and *Petunia hybrida* *SEPALLATA*-like *FLORAL BINDING PROTEIN 5* (*FBP5*). Control dyes, such as fluorescein isothiocyanate (FITC)-dextran, Rhodamine 123 and Lucifer Yellow, were used to visualize whether the microinjections were successful. *Arabidopsis thaliana* inflorescences were dissected and prepared in a similar manner as the *Tradescantia virginiana* stamen hair cells, microinjected with the red dye Rhodamine-6G by way of iontophoresis with a current of 15 nA and imaged with a LD Plan-Neofluar 40x/0.6 Korr lens.

### **Construction of binary vectors for mEosFP-tagging and plant transformation**

From the pcDNA3 EosFP T158H/V123T vector [15], we amplified the 681 bp *monomeric EosFP* (*mEosFP*) fragment by proofreading PCR with the two primers PDS1224 (5'-ATAGGATCCAGTACTATGAGTGCGATTAAGCCAG-3') and PDS1225 (5'-ATAGGATCC TTATCGTCTGGCATTGTCAG-3'), which introduced a N-terminal BamHI and Scal restriction sites and also a C-terminal BamHI restriction site. Subsequently this fragment was introduced into the pGD120 vector [27] by BamHI restriction and ligation, creating the vector CZN319. A Gateway box (GW) with reading frame B (Invitrogen) was introduced upstream of the *mEosFP* fragment by Scal restriction and ligation, creating the vector CZN320. The expression cassette containing the *CaMV35S promoter\_GW\_mEosFP\_NOS terminator* from CZN320 was then introduced into the binary vector pGD121 [28] by double digestion with AscI/PacI and ligation, creating the destination vector CZN321. Subsequently, the *CaMV35S* promoter element was cut out from CZN321 by double digestion with AscI/XbaI followed by self-ligation, creating another destination vector CZN351. This destination vector was used to make the final expression clones by LR reaction with the previously made entry vectors containing the genomic clones of the MADS box genes *AGAMOUS* (*AG*), *APETALA1* (*AP1*), *FRUITFULL* (*FUL*), and *SEPALLATA3* (*SEP3*) [29, 30]. The expression clones *gAG:mEosFP* (CZN386), *gFUL:mEosFP* (CZN387), *gSEP3:mEosFP* (CZN388), and *gAP1:mEosFP* (CZN389) were then transformed to wild type *Arabidopsis thaliana* Columbia-0 (Col-0) plants via *Agrobacterium tumefaciens* strain GV3101 using the floral dip method [31]. Primary transformants were selected on 0.8% agar 0.5x MS plates with 25 µg/ml kanamycin, and later scored for their level of mEosFP fluorescence. Since most transformants in wild type Col-0 background from two different transformations had severe problems with silencing of the transgene (see Table1), we also used the *sgs2-1* (also known as *sde1* and *rdr6*) mutant line for transformation. *Sgs2-1* mutant plants are defective in gene silencing, but have no obvious defects in flower development [32, 33]. Also in this mutant background only a few stable transformants with detectable expression levels were obtained (see Table1).

### **Photoconvertible mEosFP Confocal Laser Scanning Microscopy**

To observe the localisation of the mEosFP-tagged proteins in living inflorescences, the inflorescence material was dissected and embedded as previously described [30]. Confocal laser scanning microscopy of the living plant tissue was performed with a Leica SPE DM5500 upright microscope with a 63x ACS APO (NA 1.15 CORR) lens, using the LAS AF 1.8.2 software. The green version of the photoconvertible mEosFP tag was excited with the 488 nm solid state laser and scanned at a bandwidth of 505-550 nm, while the



photoconverted red version of the mEosFP tag was excited with the 532 nm solid state laser and scanned at a bandwidth of 575-620 nm. The obtained confocal z-stacks were median filtered, converted to 3-D maximum projections and adjusted with Adobe Photoshop version 5.0. Photoconversion of the mEosFP fluorophore was performed on selected areas by using the maximal square zoom function (approximately 5 to 7 meristematic cells) and repeatedly scanning this zoomed-in area in one z-section with 405 nm laser light. Several laser intensities and photoconversion times were tested in order to obtain optimal photoconversion of the mEosFP-tagged MADS domain proteins.

## Acknowledgements

We are grateful to Jörg Wiedenmann for providing the *mEosFP* vector.

**Table 1.** Number of primary transformants after plate selection.

		CZN386 <i>gAG:mEosFP</i>	CZN387 <i>gFUL:mEosFP</i>	CZN388 <i>gSEP3:mEosFP</i>	CZN389 <i>gAP1:mEosFP</i>
	total	2	14	8	21
WT Col-0 background	stable	0 (0%)	5 (36%)	0 (0%)	16 (76%)
	mEosFP		2 (+/-)		2 (+/-)
			2 (+)		4 (+)
			1 (++)		8 (++)
				2 (+++)	
	total	4	26	8	13
<i>sgs2-1</i> background	stable	0 (0%)	12 (46%)	2 (25%)	9 (70%)
	mEosFP		6 (+)	1 (+/-)	1 (+)
			6 (++)	1 (+)	8 (++)

+/- very weak mEosFP fluorescence; + weak mEosFP fluorescence; ++ mEosFP fluorescence;  
+++ good mEosFP fluorescence

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# Chapter 5

## Intercellular transport of epidermis-expressed MADS domain transcription factors and their effect on plant morphology and floral transition

Susan L Urbanus  
Adriana P Martinelli  
QD (Peter) Dinh  
Lilian CB Aizza  
Marcelo C Dornelas  
Gerco C Angenent  
Richard GH Immink

### Summary

During the lifetime of an angiosperm plant various important processes, such as floral transition, floral organ identity specification and floral determinacy, are controlled by members of the MADS domain transcription factor family. To investigate the possible non-cell-autonomous function of MADS domain proteins, we expressed GFP-tagged clones of *AGAMOUS* (*AG*), *APETALA3* (*AP3*), *PISTILLATA* (*PI*) and *SEPALLATA3* (*SEP3*) under the control of the *MERISTEM LAYER 1* promoter in *Arabidopsis thaliana* plants. Morphological analyses revealed that epidermal overexpression was sufficient for homeotic changes in floral organs, but that it did not result in early flowering or terminal flower phenotypes that are associated with constitutive overexpression of these proteins. Localisations of the tagged proteins in these plants were analyzed with confocal laser scanning microscopy in leaf tissue, inflorescence meristems and floral meristems. We demonstrated that only *AG* is able to move via secondary plasmodesmata from the epidermal cell layer to the subepidermal cell layer in the floral meristem and to a lesser extent in the inflorescence meristem. To study the homeotic effects in more detail, the capacity of trafficking *AG* to complement the *ag* mutant phenotype was compared to the capacity of the non-inwards-moving *AP3* protein to complement the *ap3* mutant phenotype. While epidermal expression of *AG* gave full complementation, *AP3* appeared not to be able to drive all homeotic functions from the epidermis, perhaps reflecting the difference in mobility of these proteins.

### Introduction

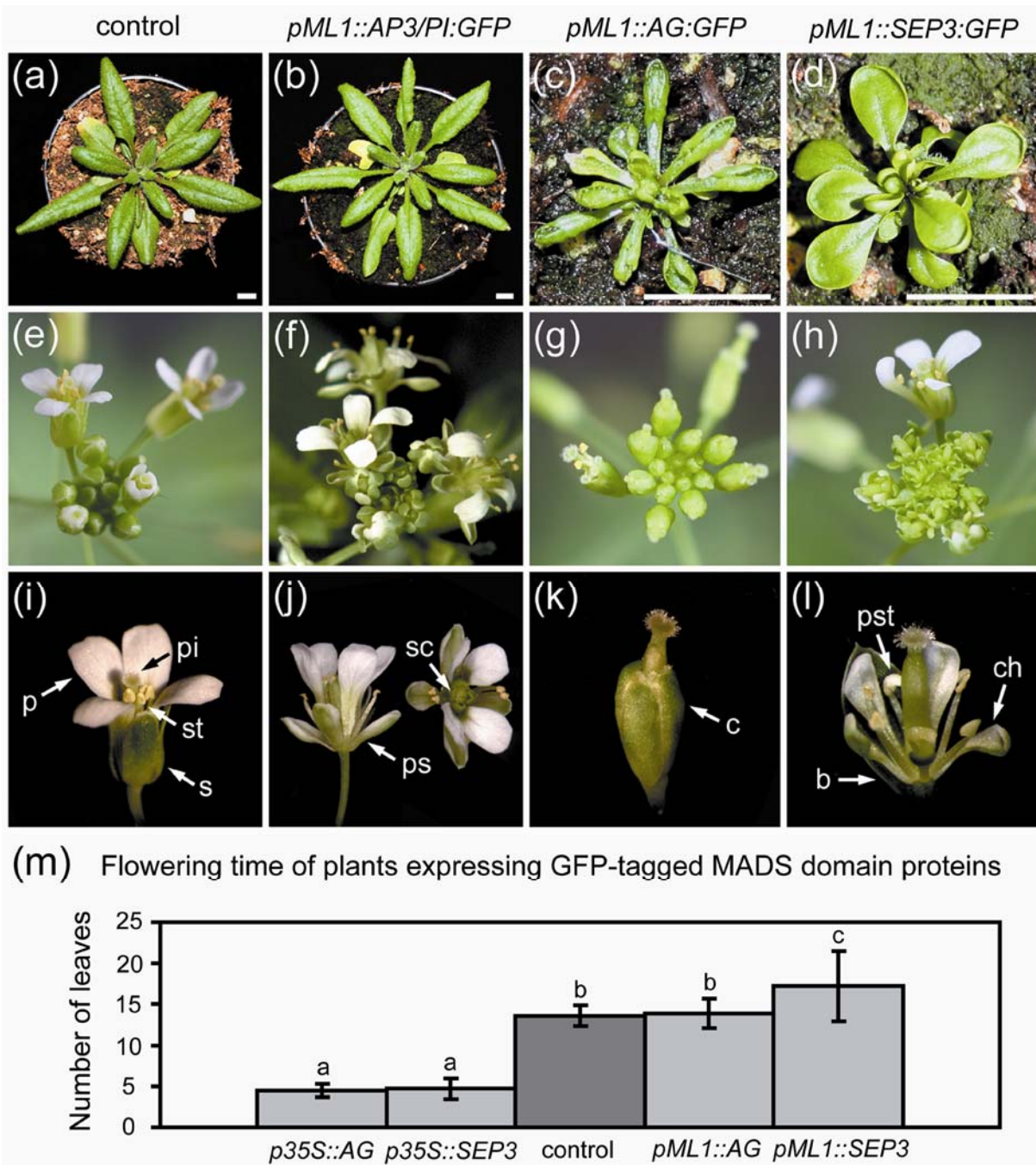
For the indefinite growth of above-ground parts, plants depend on a group of proliferating pluripotent cells in the central zone of the shoot apical meristem (SAM) (for reviews see [1-3]). In the surrounding peripheral zone the cells that are displaced from the central zone are recruited for organogenesis and below these zones lies the rib meristem that contributes to stem growth. Additionally, the SAM can be divided into clonal cell layers and as such *Arabidopsis thaliana* has two tunica cell layers and a corpus. The two tunica layers, the epidermis (L1) and subepidermis (L2), are two sheets of cells with only anticlinal cell divisions that cover the internal corpus cells (L3), which do not have clearly oriented cell divisions. Both the central and peripheral zones consist of cells from all three clonal layers. The *A. thaliana* SAM produces rosette leaves during the vegetative phase of the plants life cycle, followed by the production of cauline leaves and floral structures after the transition to the reproductive phase. Flowers develop from floral meristems (FM) that arise in the peripheral zone of the reproductive SAM, also called the inflorescence meristem (IM), and from axillary IMs. The structural organization of the FM is similar to that of the SAM, with the difference that FM growth is determinate.

The cells in the SAM and FMs are symplastically connected to each other by intercellular dynamic channels, called plasmodesmata (PD) that allow cell-to-cell transport of macromolecules, such as nutrients, proteins, mRNAs and gene silencing signals [4]. The sister cells in the two tunica layers are mainly connected to each other by primary PD that

are created during cytokinesis, whereas the secondary PD that connect the different clonal cell layers to each other are actively created across the cell wall [5]. The passive diffusion of macromolecules between cells is controlled by the size exclusion limit (SEL) of the PD. In less differentiated tissues the SEL is larger than in more differentiated tissues, therefore the connectivity between cells is greater in meristems than in differentiated tissues [6-8]. However, transport of proteins larger than the SEL is possible by active enlargement of the symplastic channels. This was first recognized in plant virology research where the spread of viruses in the plant is facilitated by viral movement proteins that actively increase the SEL of PD [9]. The first identified endogenous plant protein that moves between cells by actively increasing the SEL, was the transcription factor KNOTTED1 (KN1) in *Zea mays* [10]. Nowadays it is known that several transcription factors are able to traffic between cells in the SAM and have non-cell-autonomous functions, such as two other KNOTTED-LIKE HOMEODOMAIN (KNOX) proteins KNOTTED-LIKE FROM ARABIDOPSIS THALIANA (KNAT1/BP) and SHOOT MERISTEMLESS (STM) [11], the FM identity protein LEAFY (LFY) [12], and the B-type MADS domain proteins GLOBOSA (GLO) and DEFICIENS (DEF) [13].

The family of MADS domain transcription factors plays an important role in floral transition, floral organ identity specification, and floral determinacy. The four different floral organs that are developed from the FM are specified by combinations of different types of MADS domain proteins; A+E-type together specify sepals, A+B+E-type petals, B+C+E-type stamens, C+E-type the carpels that make up the pistil, and the C+D+E-type specify the identity of the ovules inside the pistil [14-19]. The C-type protein AGAMOUS (AG) also plays an important role in floral determinacy by eventually terminating the expression of the homeodomain protein WUSCHEL (WUS) that regulates the proliferation of the pluripotent cells in the central zone [20-23]. In the FM *WUS* is expressed in the central zone in a few cells located underneath the subepidermis [24], whereas AG protein is present in the centre of the FM in both the tunica layers and the internal cells [25].

As mentioned before, the *Antirrhinum majus* B-type proteins DEF and GLO are able to traffic between cell layers, but in contrast the respective *A. thaliana* orthologues APETALA3 (AP3) and PISTILLATA (PI) do not seem to have this capacity [26]. Functional A-type APETALA1 (AP1) proteins also do not seem to have the ability to move from the epidermal layer inwards in the FM [12, 27]. However, it has been suggested that the AG protein might have a non-cell-autonomous role in FM integrity [28-30]. To further investigate the possible non-cell-autonomous abilities of *A. thaliana* MADS domain proteins, we expressed GFP-tagged clones of AG, AP3, PI and the E-type SEPALLATA3 (SEP3) under the control of the *MERISTEM LAYER 1* (ML1) promoter in *A. thaliana* plants. As a control for passive diffusion we used 2xGFP, which has approximately the same molecular weight as a GFP-tagged MADS domain protein. The epidermal overexpression of MADS domain proteins was sufficient to induce homeotic changes in the flowers and morphological changes in the leaves, but it did not affect flowering time or SAM indeterminacy. Subsequently, we analyzed the behaviour of these epidermis-expressed proteins with Confocal Laser Scanning Microscopy (CLSM) in leaf tissue, in IMs, and in FMs. This showed that in the FM all investigated MADS domain proteins were able to move between epidermal cells, but only AG



**Figure 1. Effects of epidermal MADS domain protein expression on plant morphology and flowering.**

(a, e, i) Morphology of a non-transgenic control line showing a rosette (a), an inflorescence (e), and a flower with the sepals, petals, stamens and pistil indicated (i). (b, f, j) Morphology of the double transgenic line *pML1::AP3:GFP/pML1::PI:GFP*, which is a combination of the two single lines, showing a rosette (b), an inflorescence (f), and side view and top view of a flower with the petaloid sepals and staminoid carpels indicated (j). (c, g, k) Morphology of the transgenic *pML1::AG:GFP* line showing a rosette with small curled-up leaves (c), an inflorescence (g), and a flower with the first-whorl carpels indicated (k). (d, h, l) Morphology of the transgenic *pML1::SEP3:GFP* line showing a rosette with small leaves that are curled up at the tip (d), an inflorescence with an increasing level of disorganization in the flowers (h), and a flower with bract, petaloid stamens and chimeric organs indicated (l). (m) Graph showing the flowering time of the transgenic lines *pCaMV35S::AG:GFP*, *pCaMV35S::SEP3:GFP*, *pML1::AG:GFP*, and *pML1::SEP3:GFP* compared to the non-transgenic control line, as measured by



the number of rosette leaves. Standard deviation (bars) and significance (a,b,c:  $p < 0.001$ ) are indicated. b, bract; c, carpel; ch, chimeric organ; p, petal; pi, pistil; ps, petaloid sepal; pst, petaloid stamen; s, sepal; sc, staminoid carpel; st, stamen. All scale bars are 0.5 cm.

was also able to move inwards from the epidermis to the subepidermis. To demonstrate the functionality of these subepidermal AG proteins we did a complementation assay with the epidermis-expressed AG:GFP in an *ag* mutant background, which showed that epidermal and subepidermal AG:GFP complements fully. In comparison, complementation of the *ap3* mutant with AP3:GFP that was detected only in the epidermal layer showed only partial rescue of floral organ identity. The possible involvement of the non-cell-autonomous activity of AG during floral development is discussed.

## Results

### Effect of epidermal overexpression on plant morphology and floral transition

To explore the possible non-cell-autonomous functions of MADS domain proteins during floral transition and floral organ identity specification, we constitutively expressed four selected GFP-tagged MADS domain proteins in the epidermal cell layer of *A. thaliana* plants throughout development. The expression of AP3:GFP and PI:GFP in the epidermis of *pML1::AP3:GFP*, *pML1::PI:GFP* plants had no obvious effects on the morphology of the vegetative phase compared to the control line (Figure 1a,b). The double expression of AP3:GFP and PI:GFP in the epidermis of *pML1::AP3:GFP/pML1::PI:GFP* plants also had no obvious morphological effects (Figure 1b), whereas it was reported previously that AP3/PI overexpression results in early flowering, small plants with curled-up leaves [31]. In the case of *pML1::AG:GFP* and *pML1::SEP3:GFP* plants, the morphology of the vegetative phase was affected by the expression of epidermal AG:GFP and SEP3:GFP proteins. The rosettes of these plants could be up to six times smaller than the rosettes of the control line and had smaller leaves with curled-up edges (Figure 1c,d), resembling the leaf phenotype of plants constitutively overexpressing AG or SEP3 [32, 33]. As the *pML1::AG:GFP* and *pML1::SEP3:GFP* plants had an altered vegetative morphology and it is known that constitutive overexpression of AG or SEP3 results in early floral transition [33, 34], we paid special attention to the flowering time of these two lines. Compared to the control line the epidermal overexpressing lines did not flower earlier, while two lines constitutively overexpressing AG:GFP or SEP3:GFP did flower considerably earlier (Figure 1m). The *pML1::SEP3:GFP* line did on average have a slightly increased flowering time compared to the control line, but also showed a larger variation in flowering time.

In the reproductive phase, the constitutive overexpression of AG or SEP3 is associated with a terminal flower phenotype where the IM is converted into a terminal FM after the production of only a few flowers [32, 33]. In contrast, specific epidermal expression of AG:GFP, SEP3:GFP, AP3:GFP, PI:GFP, or AP3:GFP/PI:GFP did not result in such a terminal flower phenotype (Figure 1e-h). All lines, however, did show homeotic changes in the flowers. In *pML1::PI:GFP* flowers the first whorl organs changed from green sepals into petaloid sepals with white edges (Figure 1j and Figure S1). These petaloid sepals extended

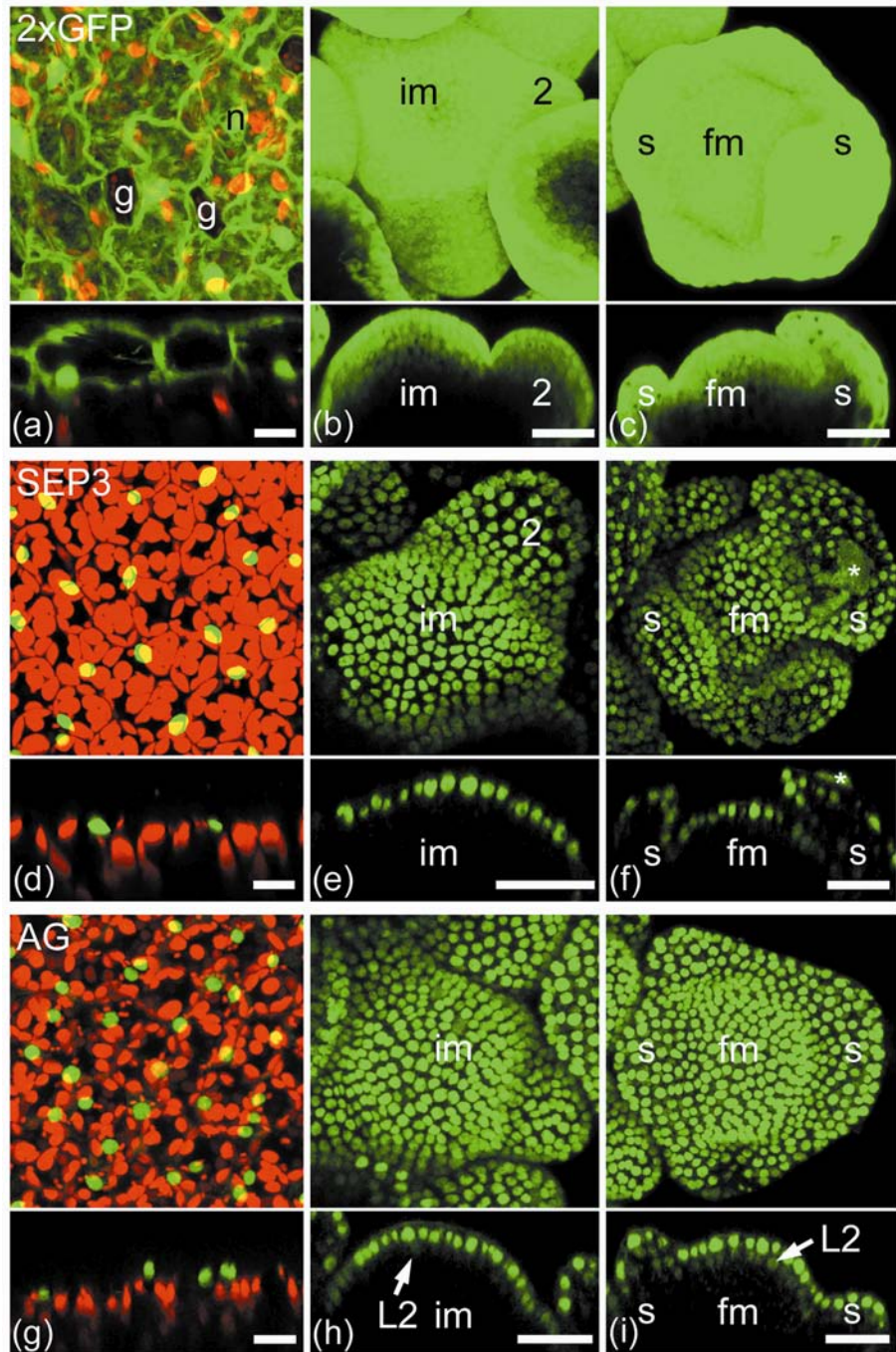
away from the floral axis at a greater angle than sepals in wild type flowers. These changes are similar to the ones reported for constitutive PI overexpression [31]. In *pML1::AP3:GFP* flowers the fourth whorl organs changed from two carpels with stigmatic papillae and ovules into more than two staminoid carpels, usually without stigmatic papillae and ovules (Figure 1j and Figure S1). This is only a mild homeotic change compared to the complete homeotic transformation of the fourth whorl carpels into stamens that is seen in constitutive AP3 overexpressing plants [35]. The double transgenic *pML1::AP3:GFP/pML1::PI:GFP* plants merely combined the mild homeotic changes from the single transgenic plants (Figure 1j), whereas constitutive overexpression of the AP3/PI combination results in flowers with only petals and stamens [31]. In *pML1::AG:GFP* flowers the first whorl organs changed from sepals into carpels with stigmatic papillae and ovules, while the second whorl petals were either not present or staminoid in nature (Figure 1k), which is similar to the changes reported for constitutive AG overexpression [32]. In *pML1::SEP3:GFP* inflorescences the first few flowers were normal, although they had an additional bract subtending the flower that varied from a rudimentary organ to a fully developed bract. Later flowers, though, became increasingly more disorganized, both in the specification of the floral organs and in the placement of these organs (Figure 1l). This is very similar to the floral phenotypes reported for the triple mutant of the flowering time genes *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1* (*SOC1*), *SHORT VEGETATIVE PHASE* (*SVP*) and *AGAMOUS-LIKE 24* (*AGL24*) that normally repress ectopic *SEP3* expression in a redundant manner to prevent premature floral differentiation [36].

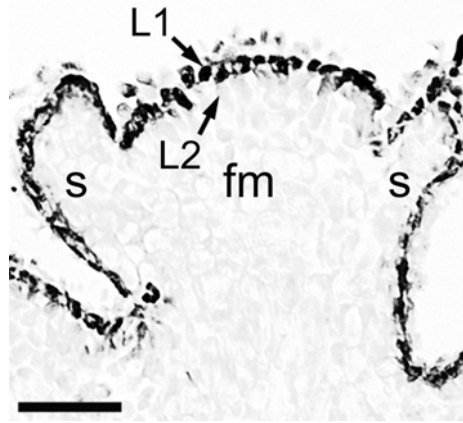
### **Intercellular transport of MADS domain proteins**

To correlate the effect of epidermis-expressed GFP-tagged MADS domain proteins with the presence of these proteins in the different tissues and cell layers we analyzed the localisations of the GFP-tagged proteins in rosette leaves, IMs and early stage FMs by CLSM. The control line *pML1::GFP:GFP* showed both nuclear and cytoplasmic localized 2xGFP protein in all three tissues. However, in the rosette leaf tissue the 2xGFP presence was restricted to the epidermis, while the IM and FM showed an inward decreasing gradient of GFP signal over several cell layers (Figure 2a-c). This demonstrates the ability of the 2xGFP molecule to move between different clonal cell layers in meristematic tissues, as shown before [8, 27]. In *pML1::SEP3:GFP* plants all tissues showed only epidermal nuclear localized *SEP3:GFP* protein (Figure 2d-f). In rosette leaf tissue of *pML1::AG:GFP* plants, the *AG:GFP* protein is only localized in the nuclei of the epidermal cells (Figure 2g). However, in the IM and FM *AG:GFP* was visible in both the epidermis and the subepidermis and was predominantly nuclear localized (Figure 2h,i). When the subepidermal GFP signal was calculated as a percentage of the epidermal GFP signal, the FM showed a subepidermal GFP signal of  $24.9\% \pm 5.2$  (SD) and the IM only  $8.3\% \pm 2.1$  (SD). In contrast, *AG:GFP* mRNA is expressed only in the epidermis and is not detectable in the subepidermis (Figure 3). This demonstrates the ability of the *AG:GFP* protein to move from the epidermal cell layer to the subepidermal cell layer via the secondary PD in the FM and to a lesser extent in the IM. Since *AG* is normally not present in IMs [25], the ability of *AG* to move from the epidermis

**Figure 2. Protein localisations of 2xGFP, SEP3:GFP and AG:GFP.**

Confocal microscopy of GFP-tagged proteins in rosette leaf tissue, IM and early flower buds. GFP fluorescence can be seen as bright green signal, while autofluorescence of plastids in the IM and in early flower buds can be seen as small green dots. Autofluorescence of chloroplasts in the leaf tissue is shown in red. Upper panels show the 3-D projections of confocal z-stacks, while the lower panels show a cross section through the confocal z-stack. (a) Cytoplasmic and nuclear localisation of 2xGFP in epidermal cells in rosette leaf tissue in the control line *pML1::GFP:GFP*. (b, c) Cytoplasmic and nuclear localisation of 2xGFP in an IM (b) and in an early flower bud (c), both with an inward decreasing gradient. (d) Nuclear localisation of SEP3:GFP in epidermal cells in rosette leaf tissue in the *pML::SEP3:GFP* line. (e, f) Predominant nuclear localisation of SEP3:GFP in epidermal cells in an IM (e) and in an early flower bud (f). (g) Nuclear localisation of AG:GFP in epidermal cells in rosette leaf tissue in the *pML::AG:GFP* line. (h, i) Predominant nuclear localisation of AG:GFP in both epidermal and subepidermal cells in an IM (h) and in an early flower bud (i). \*, speck of dust; 2, stage 2 flower bud; fm, floral meristem; g, guard cell; im, inflorescence meristem; n, nucleus; L2, subepidermal cell layer; s, sepal. Scale bars of (a), (d), and (g) are 10 µm, all other scale bars are 25 µm.





**Figure 3. Epidermal expression of AG:GFP mRNA.**

*In situ* hybridization on fixed *pML1::AG:GFP* inflorescence tissue, showing clear expression of AG:GFP mRNA in the epidermis (L1) of the floral bud and not in the subepidermis (L2). The floral meristem (fm) and two sepal primordia (s) are indicated. Scale bar is 25  $\mu$ m.

to the subepidermis in the IM is probably not of biological relevance. The nuclear presence of SEP3:GFP and AG:GFP in all three tissue types is in agreement with the earlier mentioned changes in the *pML1::SEP3:GFP* and *pML1::AG:GFP* plant morphology.

Rosette leaf tissue and IMs of *pML1::AP3:GFP* plants (Figure 4a,b) and *pML1::PI:GFP* plants (Figure 4d,e) showed no or only faint cytoplasmic GFP signal in the epidermis. In contrast, *pML1::AP3:GFP* FMs had a strong GFP signal in the epidermis of the inner three whorls, predominantly nuclear localized, while the first whorl organs had no or very faint AP3:GFP signal (Figure 4c). In the case of *pML1::PI:GFP* FMs GFP signal was visible in the epidermis of the second and third whorl, predominantly nuclear localized, while the first and fourth whorl had no or very faint PI:GFP signal (Figure 4f). These localisations show that AP3:GFP and PI:GFP are only clearly visible in the tissues where the obligate native heterodimerization partner is present, namely PI in the inner three whorls of the FM and AP3 in the second and third whorl and in a few cells at the base of the first whorl organs, respectively [37-39]. In contrast, when both AP3:GFP and PI:GFP are expressed in the double *pML1::AP3:GFP/pML1::PI:GFP* plants, all three analyzed tissues have (predominantly) nuclear GFP fluorescence in the epidermis (Figure 4g-i). These results demonstrate that only the AP3/PI heterodimeric form is able to enter the nucleus, as shown earlier [40, 41]. However, the fact that both AP3:GFP and PI:GFP were hardly detectable in the tissues where the partner was not natively present or introduced by us, strongly suggests that the non-heterodimeric forms are not only less stable, but are also rapidly degraded. This could point to a specific mechanism whereby non-heterodimeric AP3 and PI are actively degraded, and by this means leaving only the heterodimeric forms to be transported into the nucleus. The nuclear presence of AP3:GFP or PI:GFP in only parts of the FM (Figure 4c,f) correlates well with the fact that only some floral homeotic changes were found in the *pML1::AP3:GFP* and *pML1::PI:GFP* plants. Curiously, the double presence of both AP3:GFP and PI:GFP in the epidermis in *pML1::AP3:GFP/pML1::PI:GFP* plants did not lead to extra morphological changes, such as early flowering, curled-up leaves or flowers consisting of only petals and stamens [31]. This could indicate that only epidermal presence of AP3/PI is not sufficient to bring about these morphological changes. Alternatively it could indicate that, at least for the B-type proteins, the presence of two GFP tags in one heterodimer prevents normal functioning of this particular heterodimer, although it does not seem to affect the heterodimerization or the nuclear transport of this heterodimer.

**Figure 4. Protein localisations of AP3:GFP, PI:GFP and AP3:GFP/PI:GFP.**

Confocal microscopy of rosette leaf tissue, IM and early flower buds.

GFP fluorescence can be seen as bright green signal, while autofluorescence of plastids in the IM and in early flower buds can be seen as small green dots.

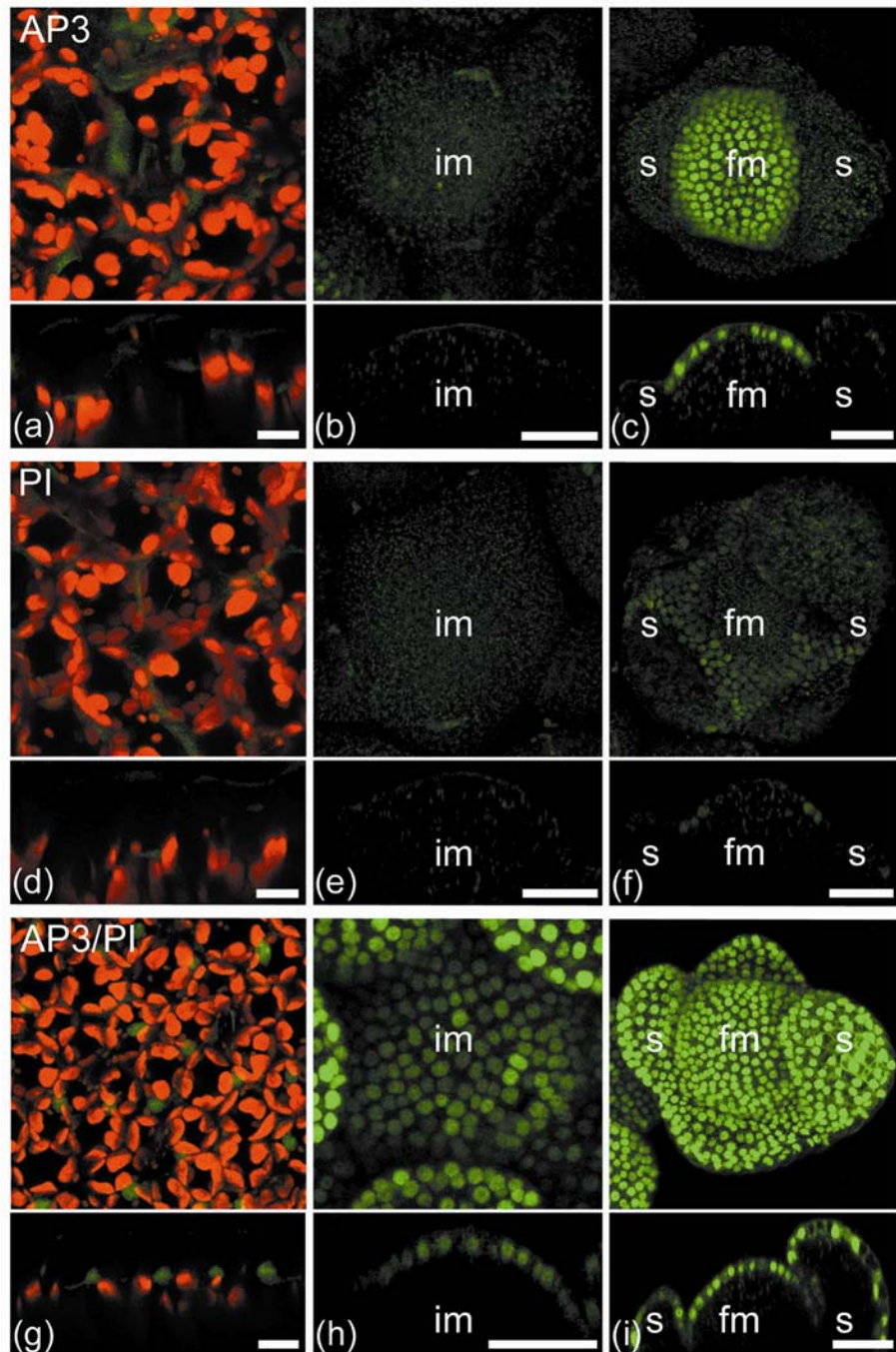
Autofluorescence of chloroplasts in the leaf tissue is shown in red.

Upper panels show the 3-D projections of confocal z-stacks, while the lower panels show a cross section through the confocal z-stack.

(a) Faint cytoplasmic AP3:GFP fluorescence in epidermal cells in rosette leaf tissue in the *pML1::AP3:GFP* line.

(b) No or very faint cytoplasmic AP3:GFP fluorescence in epidermal cells in an

IM. (c) Predominant nuclear localisation of AP3:GFP only in those epidermal cells of an early flower bud where its native partner PI is present. (d) Faint cytoplasmic PI:GFP fluorescence in epidermal cells in rosette leaf tissue in the *pML1::PI:GFP* line. (e) No or very faint cytoplasmic PI:GFP fluorescence in epidermal cells in an IM. (f) Nuclear localisation of PI:GFP only in those epidermal cells of an early flower bud where its native partner AP3 is present. (g) Nuclear localisation of GFP signal in epidermal cells in rosette leaf tissue in double line *pML1::AP3:GFP/pML1::PI GFP*. (h, i) Predominant nuclear localisation of GFP in epidermal cells in an IM (h) and in epidermal cells in an early flower bud (i). fm, floral meristem; im, inflorescence meristem; s, sepal. Scale bars of (a), (d), and (g) are 10  $\mu\text{m}$ , all other scale bars are 25  $\mu\text{m}$ .

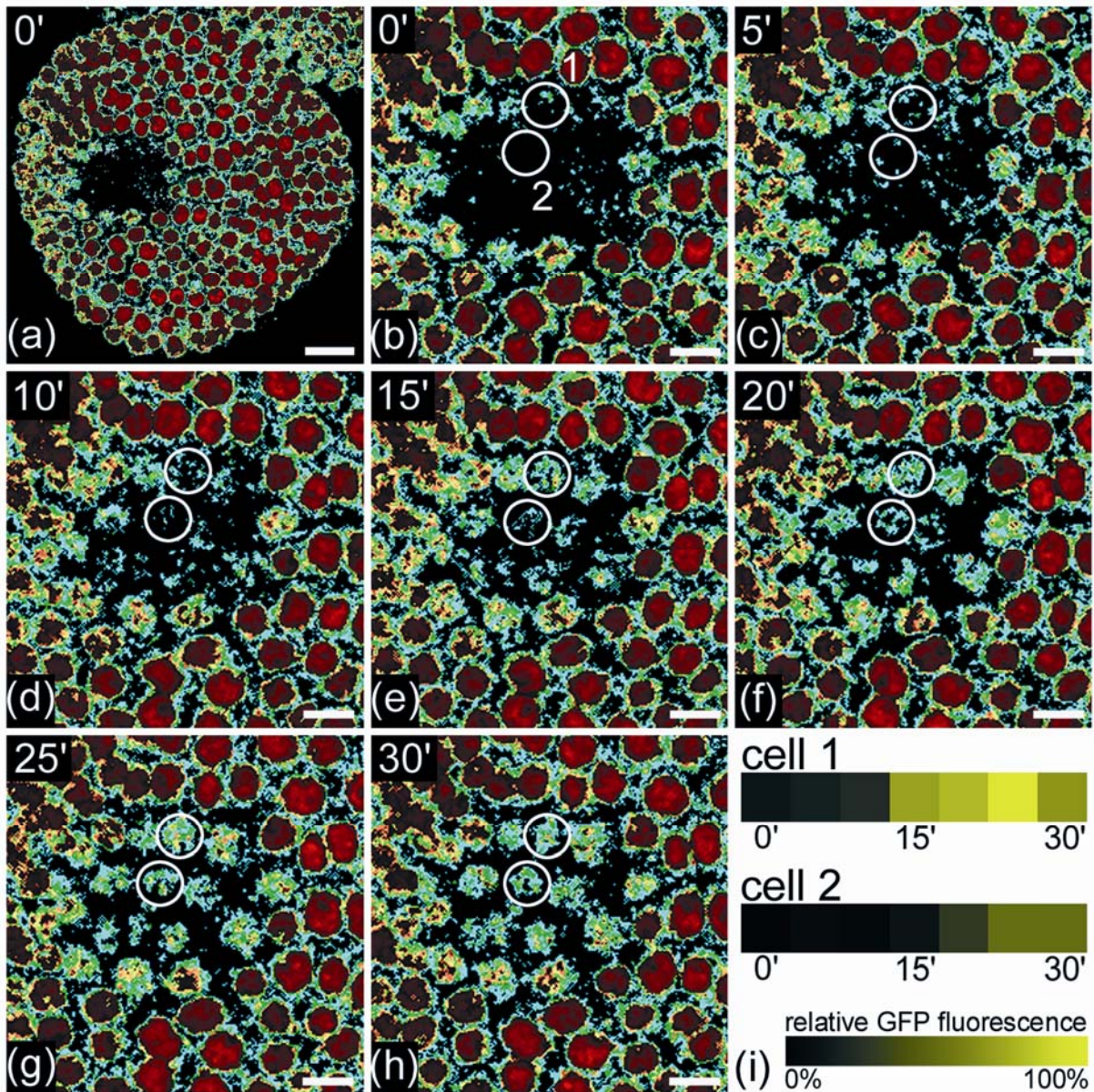


Additionally we assessed the ability of MADS domain proteins to move between cells within the epidermis via primary PD by performing a photobleaching assay on sections of early stage FMs (Figure 5 and Figure S2). The speed and diffusive inward-moving direction of fluorescence recovery in the photobleached area indicates that this fluorescence recovery is largely due to epidermal intercellular transport of the GFP-tagged MADS domain proteins, since *de novo* protein synthesis is expected to occur simultaneously in all epidermal cells. This demonstrates the ability of MADS domain proteins to traffic between epidermal cells in FM tissue.

### **Complementation assay with epidermis-expressed AG and AP3**

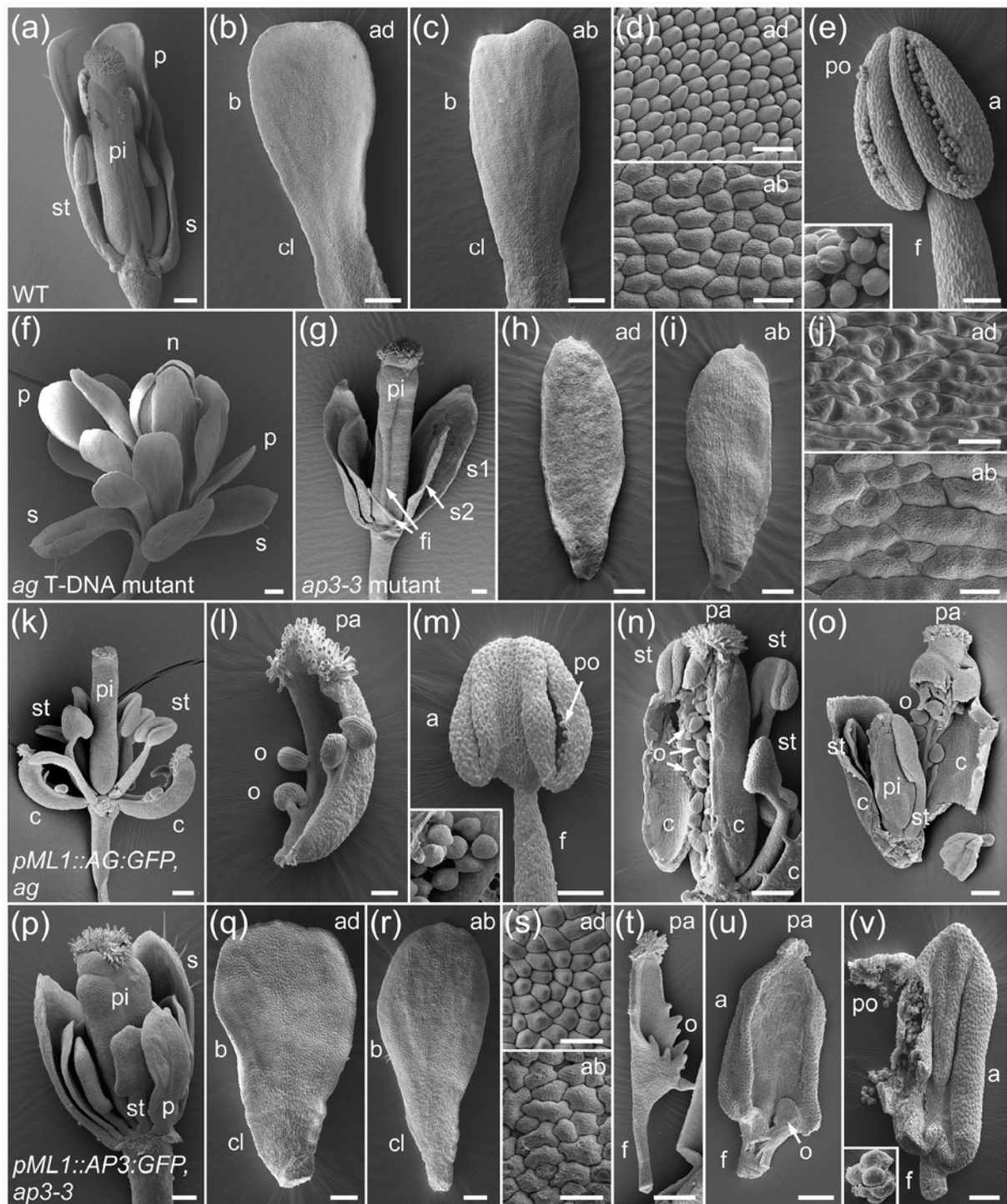
To investigate the functional relevance of AG:GFP transport from the epidermis to the subepidermis in the FM, we performed a complementation assay with *pML1::AG:GFP* in a SALK\_014999 *ag* T-DNA insertion mutant background. We compared this with an assay with *pML1::AP3:GFP* in an *ap3-3* mutant background, where we were not able to detect AP3:GFP transport to subepidermal layers. Since AP3 is a B-type protein involved in petal and stamen formation and AG a C-type protein involved in stamen and pistil formation, the focus of the comparison was on the degree of complementation of the stamens in the third floral whorl.

The control *sgs2-1* line flowers were identical to Col-0 wild type flowers (Figure 1a and Figure 6a), whereas the SALK\_014999 *ag* T-DNA insertion mutant flowers developed petals instead of stamens in the third whorl and had an indeterminate FM with repeating whorls of sepals and petals (Figure 6f). The *pML1::AG:GFP, ag* flower had completely restored stamen and pistil function (Figure 6k) and in addition, the first whorl sepals and second whorl petals of the *pML1::AG:GFP, ag* flower were changed into carpels and staminoid organs due to ectopic AG overexpression (Figure 1k and Figure 6k,l). The complemented third whorl stamens, which are similar in shape and size to stamens of the control line, consisted of a green filament and a yellow or yellowish anther with two thecae (Figure 6e,m). Of the 16 *pML1::AG:GFP, ag* plants from the total 59 checked F2 progeny plants, at least two plants produced mature pollen that was released after dehiscence (Figure 6m). Some of this pollen was used to fertilize an emasculated Col-0 wild type plant that successfully produced progeny with the *pML1::AG:GFP* construct. The complemented pistil, similar in shape and size to pistils of the control line (Figure 6a), consisted of two valves separated by a dehiscent zone and had stigmatic papillae on top of the style (Figure 6n). Inside these complemented pistils normal ovules were present. Younger plants sometimes had flowers with shorter, plumper complemented pistils in which a new flower consisting of staminoid organs and a pistil was formed, demonstrating that there still was some floral indeterminacy in these particular flowers (Figure 6o). Occasional floral indeterminacy also occurs when *ag* mutant plants are complemented with a wild type AG construct expressed in all three cell layers of the FM [42]. This complementation assay therefore demonstrates that the epidermis-expressed AG:GFP that moves to the subepidermis is sufficient for organ identity specification and outgrowth, which corresponds well with earlier observations that AG needs to be active in the subepidermis for staminoid and carpelloid tissue identity [29]. It also demonstrates that the presence of AG:GFP in both the epidermis and the subepidermis is



**Figure 5. Epidermal intercellular transport of AG:GFP.**

Recovery of GFP fluorescence after photobleaching as a result of intercellular transport of AG:GFP in the epidermis of an early flower bud. The 3-D projection of the flower bud is shown in artificial heat map colouring, which presents the minimum GFP fluorescence value in light blue and the maximum value in bright red. (a) A *pML1::AG:GFP* stage 3 flower bud immediately after photobleaching. (b-h) Close-ups of bleached area immediately after photobleaching (b), after 5 minutes (c), after 10 minutes (d), after 15 minutes (e), after 20 minutes (f), after 25 minutes (g), and after 30 minutes (h). A cell in the outer ring of recovery (1) and a cell in the inner region of recovery (2) are indicated with white circles. (i) Colour bars illustrate the GFP fluorescence recovery of the indicated cell 1 and cell 2, as measured by the normalized pixel sum per  $\mu\text{m}^2$ . Note that the fluorescence recovers first in the outer ring of the bleached area and slightly later in the inner region, demonstrating the inward movement of fluorescent AG:GFP molecules into the bleached area. Scale bar of (a) is 10  $\mu\text{m}$ , all other scale bars are 5  $\mu\text{m}$ .



**Figure 6. Complementation assay with epidermis-expressed AG and AP3.**

Scanning electron micrographs of flowers and floral organs. (a) Overview of a flower from the control line *sgs2-1* with a few organs removed to show first whorl sepals, second whorl petals, third whorl stamens, and fourth whorl pistil. (b) Adaxial side and (c) abaxial side of *sgs2-1* petals. (d) Close-ups of conical adaxial epidermal cells of (b) and less conical abaxial epidermal cells of (c). (e) *Sgs2-1* stamen with detail of pollen in inset. (f) Overview of a SALK\_014999 *ag* T-DNA insertion mutant flower, which shows the floral indeterminacy of the mutant flower that consists of only sepals and petals. (g) Overview of an *ap3-3* mutant flower with a few floral organs removed to show first whorl sepals, smaller second whorl sepals, filamentous third whorl organs, and fourth whorl pistil. (h) Adaxial side and (i) abaxial side of *ap3-3* second whorl sepals. (j) Close-ups of epidermal cells of (h) and (i), both



showing irregular shaped, elongated cells interspersed with guard cells. **(k)** Overview of a *pML1::AG:GFP*, *ag* flower with a few organs removed to show first whorl carpels, second whorl staminoid organs, third whorl stamens, and fourth whorl pistil. **(l)** *pML1::AG:GFP*, *ag* first whorl carpel with ovules at basal part of the carpel and papillae at the top. **(m)** *pML1::AG:GFP*, *ag* third whorl stamen with detail of pollen in inset. **(n)** Opened up *pML1::AG:GFP*, *ag* pistil with ovules inside. **(o)** Opened up *pML1::AG:GFP*, *ag* pistil with ovules and new flower with pistil and staminoid organs indicated. **(p)** Overview of a *pML1::AP3:GFP*, *ap3-3* flower with a few organs removed to show first whorl sepals, second whorl petaloid organs, third whorl staminoid organs, and fourth whorl pistil. **(q)** Adaxial side and **(r)** abaxial side of *pML1::AP3:GFP*, *ap3-3* second whorl petaloid organs. **(s)** Close-up of conical adaxial epidermal cells of **(q)** and close-up of less conical abaxial epidermal cells interspersed with guard cells of **(r)**. **(t)** *pML1::AP3:GFP*, *ap3-3* third whorl carpelloid organ on filament with ovular outgrowths at the base and papillae at the top. **(u)** *pML1::AP3:GFP*, *ap3-3* third whorl carpelloid organ on filament with only minor ovular outgrowth at the base and a few papillae at the top. **(v)** *pML1::AP3:GFP*, *ap3-3* third whorl stamen with immature pollen locked inside. Detail of immature pollen in inset. a, anther; ab, abaxial; ad, adaxial; b, blade; c, carpel; cl, claw; f, filament; fi, filamentous organ; n, new flower; o, ovule/ovular outgrowth; p, petal/petaloid organ; pa, papillae; pi, pistil; po, pollen; s, sepal; s1, first whorl sepal; s2, second whorl sepal; st, stamen/staminoid organ. Scale bars of **(a-c)**, **(f-i)**, **(k)**, **(n-p)**, **(t)** are 200  $\mu\text{m}$ , scale bars of **(e)**, **(l)**, **(m)**, **(q)**, **(r)**, **(u)**, **(v)** are 100  $\mu\text{m}$ , and scale bars of **(d)**, **(j)**, **(s)** are 20  $\mu\text{m}$ .

sufficient for floral determinacy, although it was shown before that AG needs to be active in both the subepidermis and the internal cell layers for floral determinacy [29].

In comparison, the complementation assay with *pML1::AP3:GFP* in an *ap3-3* mutant background showed a strikingly lower degree of complementation. Where the *ap3-3* mutant flower had small green sepals in the second whorl and filamentous third whorl organs that were sometimes fused to the fourth whorl pistil (Figure 6g), the *pML1::AP3:GFP*, *ap3-3* flower had second whorl whitish-green petaloid organs, and third whorl carpelloid or staminoid organs that were sometimes fused to the normal pistil (Figure 6p). Compared to petals from the control line (Figure 6b,c), the *pML1::AP3:GFP*, *ap3-3* petaloid organs had a similar shape (Figure 6q,r), but they were smaller in size like *ap3-3* second whorl sepals (Figure 6h,i). The epidermal cell identity of *pML1::AP3:GFP*, *ap3-3* petaloid organs was restored, although the abaxial epidermal cells were still interspersed with guard cells as seen in the *ap3-3* second whorl sepals (Figure 6d,j,s). Also, the mesophyll cells of the petals still contained chloroplasts as in the *ap3-3* second whorl sepals, giving the petaloid organs their whitish-green appearance. The third whorl organs showed a large range of carpelloid/staminoid phenotypes, both between flowers from different plants and between flowers from the same plant (Figure 6t-v). Of the 17 *pML1::AP3:GFP*, *ap3-3* plants from the total 85 checked F2 progeny plants of two independent lines, only three plants showed stamen-like organs consisting of a green filament and a yellowish anther, occasionally with minor ovular outgrowths at the base of the anther (Figure 6p,v). In the thecae of these anthers immature pollen developed (Figure 6v). The other 14 partially complemented plants had either filamentous organs with or without papillae at the top, carpelloid organs on a filament with papillae at the top and ovular outgrowths at the base (Figure 6t), or more anther-like organs on a filament with minor papillar growth at the top and minor ovular

outgrowth at the base (Figure 6u). It therefore seems that epidermal AP3:GFP presence is sufficient only for certain aspects of petal and stamen identity and function, like epidermal cell identity and to some extent the shape of the organs. However, the inner cell layer identity and the outgrowth of the organs are not sufficiently influenced by only epidermal AP3:GFP. This corresponds well with earlier observations that the presence of AP3 in the epidermis of second whorl organs has a strong influence on organ shape, while AP3 presence in the subepidermis influences organ size [26]. Also, it was noted before that in *ap3-3* flowers epidermally complemented with either AP3 or the *A. majus* orthologue DEF, the maturation of pollen, suppression of chlorophyll formation, and elongation of petals and stamen filaments were incomplete [43].

### Discussion

The family of MADS domain transcription factors controls various important processes in the life of a flowering plant, such as floral transition and floral organ identity specification. To investigate the possibility of non-cell-autonomous function of these transcription factors, we made *A. thaliana* plants that specifically express GFP-tagged versions of AG, SEP3, AP3, PI, or the combination of AP3 and PI in the epidermal cell layer and analyzed the behaviour of these proteins. Next, the developmental effect of epidermis-expressed MADS domain proteins on plant morphology and flowering time were investigated and related to the localisations and behaviour of these proteins, which lead to insights into the functioning of these MADS domain transcription factors.

### Intercellular transport

The photobleaching experiments we performed demonstrated that all tested epidermis-expressed GFP-tagged MADS domain proteins were able to diffuse through the epidermal cell layer through the primary PD. Whether the intercellular epidermal transport of MADS domain proteins in FMs is more than short-distance cannot be concluded from these experiments. Nevertheless, this intercellular transport of MADS domain proteins within the epidermis could explain previous observations of MADS domain protein localisation in epidermal tissues where their mRNA presence is not reported [25]. Additionally, the observed intercellular transport of AG in the epidermis supports previous suggestions that AG signal can extend laterally in the FM [28, 30]. We also demonstrated that from all the tested epidermis-expressed GFP-tagged MADS domain proteins only AG:GFP was also present in the subepidermal cell layer in FMs and the IM, indicating that AG:GFP proteins are able to move from the epidermis to the subepidermis. Especially in the FM, where AG normally occurs [25, 44, 45], the subepidermal AG:GFP protein amount was a substantial one-fourth of the epidermal protein amount. AG is not the only transcription factor that has the ability to traffic in the SAM, other examples are the MADS domain proteins GLO and DEF [13], the KNOX proteins KN1, KNAT1/BP and STM [10, 11] and the FM identity protein LFY [12]. The transport of AG:GFP via the secondary PD that connect the epidermis and the subepidermis [5] does not seem to be a passive process, as the other GFP-tagged MADS

domain proteins of similar molecular weight were not present in the subepidermis of the FM or the IM. It is possible that the inward transport of AG is an active process that is controlled by intrinsic properties of AG in combination with the type of secondary PD found in the FM and IM, since there is no inward transport of AG through secondary PD in leaf tissue. Alternatively, the process of AG transport from the epidermis to the subepidermis could be facilitated by a factor or chaperone that is present in the FM and IM and not in leaf tissue [46]. A possible transport facilitator that is indeed specifically expressed in the areas where AG:GFP is able to move between cell layers, is the trafficking KNOX protein STM that accumulates in punctae in the cell wall that are presumably PD [11, 47]. Interestingly, STM is thought to be involved in AG upregulation in the third and fourth whorl and also in carpel development [48, 49]. The AG upregulation would be mediated by heterodimers of STM and the two BELL1-like homeobox (BLH) proteins PENNYWISE (PNY) and POUND-FOOLISH (PNF), which have similar expression domains as STM in the centre of the inflorescence and FM [48, 50-52]. It has been shown before that MADS domain transcription factors can interact with BLH proteins, as BELL1 interacts with AG/SEP3 heterodimers to promote ovule development [53]. Perhaps STM, possibly together with PNY and PNF, interacts with AG to facilitate both AG intercellular transport and AG upregulation in the FM centre.

#### **Floral transition and SAM indeterminacy**

Both the epidermal overexpression experiments with AG:GFP, SEP3:GFP, AP3:GFP, PI:GFP and the complementation assays with epidermis-expressed AG:GFP and AP3:GFP showed that the GFP-tagged versions of these transcription factors are active. Furthermore, the fact that full complementation was obtained upon expression of C-terminally GFP-tagged version of the MADS domain proteins AG, AP1 and FRUITFULL (FUL) in their respective mutants, provides evidence that GFP does not interfere with the functioning of these MADS domain proteins [25]. Nevertheless, we cannot exclude the possibility that the fluorescent tag might hinder the functioning of particular MADS protein complexes, as might be the case for the double GFP-tagged AP3/PI heterodimer. The floral homeotic changes observed in plants with epidermis-expressed AG:GFP, SEP3:GFP, AP3:GFP and PI:GFP are similar to the changes seen in plants that constitutively overexpress these proteins [31, 32, 35, 36]. Also, the altered leaf morphology of plants overexpressing epidermal AG:GFP and SEP3:GFP is similar to that of constitutive overexpressing plants [32, 33]. Unlike constitutive overexpression, specific epidermal expression of AG:GFP, SEP3:GFP, AP3:GFP, or PI:GFP does not lead to early flowering plants or terminal flower phenotypes [32, 33]. This demonstrates that epidermal presence of MADS domain proteins, or in the case of AG:GFP both epidermal and subepidermal presence, does not affect floral transition.

#### **Does AG have a non-cell-autonomous function?**

Is it possible that AG requires non-cell-autonomous abilities to fulfil its two functions of floral organ identity specification and floral determinacy? The results from this study demonstrated that in the FM AG is able to move between epidermal cells and is also able to move inwards from the epidermal layer to the subepidermal layer. In addition, the complementation assay

with epidermis-expressed AG:GFP demonstrated that this construct can fully complement the *ag* mutant, suggesting that the trafficking AG proteins are functional in both floral organ identity specification and floral determinacy. Earlier it was proposed that AG might be able to move outward from the subepidermis to the epidermis during floral development [29] and we demonstrated that AG is able to move inwards from the epidermis to the subepidermis, so perhaps AG protein can freely move in both directions between the epidermis and the subepidermis. It might be that the ability of AG to traffic between cells and between cell layers is essential for the spread of the AG expression domain over all the three clonal layers in the FM [25, 44]. AG expression is thought to be initiated by the combination of WUS, LFY and possibly also SEP3 in a few inner layer cells in the central zone, called the organizing centre [21, 24, 36]. As soon as AG expression has been initiated, it can become a self-perpetuating signal due to the AG positive feed-back loop [54]. Protein localisations demonstrated that AG presence is first visible in a few scattered cells in the subepidermal and inner cell layers in the central zone in early stage 3 flower buds, while later stage 3 flower buds show AG presence in all the three cell layers in the presumptive third and fourth whorl [25]. It is therefore conceivable that the AG expression domain spreads outward from the organizing centre to the subepidermal and epidermal layers through the action of a few AG proteins that traffic a short distance across cell layers and subsequently initiate AG expression in those cell layers. In line with this model, enhancement of AG expression in the centre of the floral meristem by expression of this gene from the *CLAVATA3 (CLV3)* promoter results in lateral expansion of AG expression [30]. Our results strongly suggest that the so called principle of 'regulation by tuning' for C-type gene expression [30, 55] is mediated by an AG autoregulatory loop in *A. thaliana* involving short distance AG protein transport. Additionally, the ability of AG to traffic inwards in the FM could be required for the AG-induced termination of the *WUS* expression in the organizing centre of the FM, which results in the consumption of the last meristematic cells for the proper development of the pistil and ovules [2, 21, 23]. Especially for the floral determinacy function of AG it seems that a high amount of AG proteins needs to be present in the subepidermis and the internal cells to be able to eventually repress *WUS* expression in the organizing centre underneath the subepidermis [20, 21, 29]. The ability of AG proteins to travel inwards in the FM could provide a failsafe mechanism, on top of the transcriptional regulation, to ensure that enough AG molecules are eventually present in the organizing centre to repress *WUS* expression and to develop a fully functional flower.

## Experimental procedures

### Plant materials and growth conditions

All *A. thaliana* plant lines were grown at 22°C in growth chambers under a long-day light regime (16 h light/8 h dark). For the transformation of all constructs the *sgs2-1* mutant line in Columbia-0 (Col-0) background was used. The *sgs2-1* (also known as *sde1* and *rdr6*) mutant line is defective in gene silencing, but has no obvious defects in flower development [56, 57]. This mutant background was chosen because of problems with transgene silencing in the

Col-0 wildtype background [58]. A double transgenic line expressing both *pML1::AP3:GFP* and *pML1::PI:GFP* constructs was obtained by crossing the single transgenic lines. For the complementation experiments the *pML1::AG:GFP* and *pML1::AP3:GFP* transgenic lines were crossed into the SALK\_014999 *ag* T-DNA insertion mutant line and the *ap3-3* mutant line [38], respectively.

### Construction of binary vectors and plant transformation

A 3388 bp promoter fragment from the *MERISTEM LAYER 1 (ML1)* gene was generated from Col-0 gDNA by proofreading PCR with primers PDS1209 and PDS1210 (see Table S1 in supplementary material for sequences). The same fragment was shown to be sufficient to give specific epidermal expression in both vegetative and reproductive tissues [59]. Next, the pK7FWG2 gateway destination vector [60] was modified by removing the *CaMV35S* promoter element with a HindIII/SpeI double digestion and replacing this fragment with the amplified *ML1* promoter fragment. Gateway entry clones containing cDNA clones of *AG*, *AP3*, *GFP*, *PI*, *SEP3* lacking their STOP codon, were recombined into the new destination vector. The resulting six expression clones *pML1::AG:GFP* (CZN301), *pML1::AP3:GFP* (CZN303), *pML1::GFP:GFP* (CZN304), *pML1::PI:GFP* (CZN305) and *pML1::SEP3:GFP* (CZN306) were introduced into *Agrobacterium tumefaciens* strain GV3101 and transformed to *sgs2-1* mutant plants by floral dip method [61]. Additionally, two constructs *pCaMV35S::AG:GFP* (pARC276) and *pCaMV35S::SEP3:GFP* (pARC277) previously made [58], were also transformed into the *sgs2-1* mutant line.

### Confocal Laser Scanning Microscopy

The presence of the GFP-tagged proteins was determined in three different living tissues, namely rosette leaves, IMs and stage 3-5 floral buds (stages after [62]). In order to observe the localisation in rosette leaves, tips of young leaves were put between glass slides in 0.5 x MS and scanned. To observe the localisation of the GFP-tagged proteins in inflorescences, the inflorescence material was dissected and embedded as previously described [58]. Confocal Laser Scanning Microscopy (CLSM) of the living plant tissue was performed with a Leica SPE DM5500 upright microscope with a 63x ACS APO (NA 1.15 CORR) lens, using the LAS AF 1.8.2 software (Leica, <http://www.leica-microsystems.com>). Further details about the imaging and photobleaching experiments are provided in the supporting information.

### *In situ* hybridization

Inflorescences from *pML1::AG:GFP* plants were fixed and sections were hybridized with sense and antisense probes for the *GFP* gene. A detailed protocol is provided in the supporting information.

### Plant morphology and microscopy

The plant morphology of all the transgenic lines was analyzed, with specific attention to the flower phenotypes. Bright field images of the mutant and rescued flower phenotypes were taken with a Leica DFC320 digital camera mounted on a Zeiss Stemi SV8 binocular (Carl

Zeiss, <http://www.zeiss.com>) with IM500 software. For scanning electron microscopy (SEM), inflorescences were collected and fixed overnight at 4°C in aqueous 4% paraformaldehyde. Samples were dehydrated on an ethanol series to 70% ethanol and stored at 4°C until needed. Samples were further dehydrated through absolute ethanol and critical point dried through CO<sub>2</sub> in a Balzers CPD 020 (Baltec, <http://www.bal-tec.com>). Dried samples were dissected under a dissecting microscope before being mounted on metallic stubs with adhesive tape, and were further dissected when essential. Samples were then sputter-coated with colloidal gold and observed at 10-20 kV under a LEO 435 VP scanning electron microscope (Carl Zeiss) and digital micrographs were obtained.

### Flowering time measurement

Plants of *pML1::AG:GFP* and *pML1::SEP3:GFP* transgenic lines were grown together with *sgs2-1* control plants on soil, while *pCaMV35S::AG:GFP* and *pCaMV35S::SEP3:GFP* primary transformants were grown on 0.5 x MS 0.8% agar plates containing 25 µg/ml kanamycin. As a measure of flowering time the number of rosette leaves was counted at the time of bolting. The presence of the constructs in the plants was determined by examining the flower phenotype and the presence of GFP fluorescence in the leaves. In total 25 *pML1::AG:GFP* plants, 23 *pML1::SEP3:GFP* plants, 46 *sgs2-1* control plants, 10 *pCaMV35S::AG:GFP* plants and 7 *pCaMV35S::SEP3:GFP* plants were analyzed. This data was explored with a one-way ANOVA and Tukey HSD post hoc test in the statistical program SPSS version 11.5 (SPSS, <http://www.spss.com>).

### Complementation analysis

The presence of the wild type allele and the mutant allele was determined by PCR in the F2 progeny of the two crosses, *pML1::AG:GFP* x SALK\_014999 *ag* T-DNA insertion mutant line and *pML1::AP3:GFP* x *ap3-3* mutant line, while the presence of the GFP-tagged MADS box gene construct was determined by CLSM. For the *AG* wild type allele primers PRO182 and PDS1985 were used and for the *ag* T-DNA mutant allele primers PRO433 and PDS404 (see Table S1). For the *AP3* wild type allele primers PDS2027 and PDS2029 were used and for the *ap3-3* mutant allele primers PDS2028 and PDS2029.

### Acknowledgements

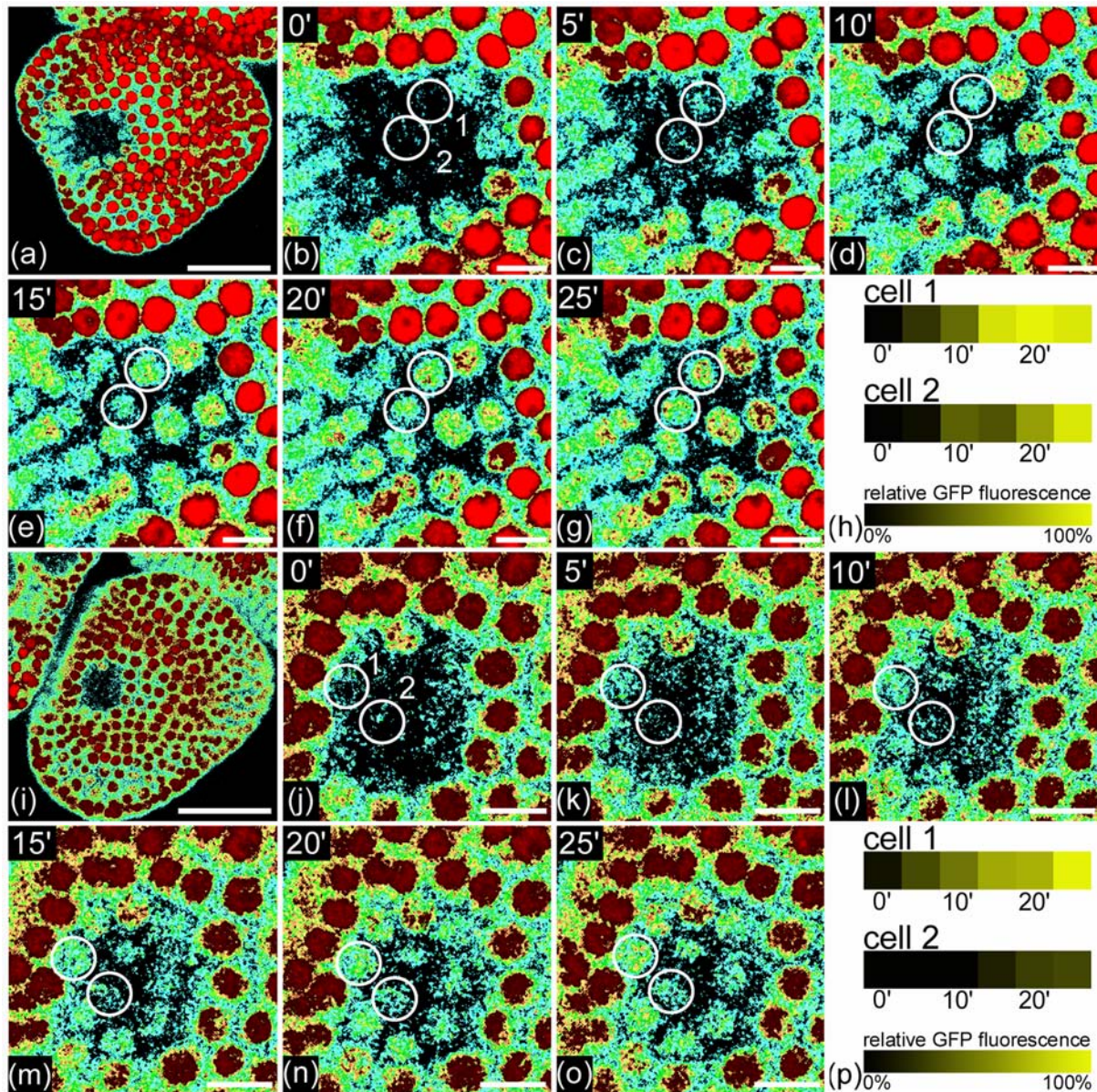
APM, LCBA and MCD are financed by CAPES and CNPq, Brazil. GA and RI are financed by CBSG. We acknowledge NAP/MEPA-ESALQ, University of São Paulo, for use of the electron microscopy facilities.

Supporting information



**Figure S1. Floral phenotypes of *pML1::AP3:GFP* and *pML1::PI:GFP* plants.**

(a) A *pML1::AP3:GFP* flower showing an unfused pistil with more than two staminoid carpels. (b) A *pML1::PI:GFP* flower showing petaloid sepals that extend at a great angle from the floral axis.



**Figure S2. Epidermal intercellular transport of *SEP3:GFP* and *AP3:GFP/PI:GFP*.**

Recovery of GFP fluorescence after photobleaching as a result of intercellular transport of GFP-tagged MADS domain proteins in the epidermis of early flower buds. The 3-D projections of the flower buds are shown in artificial heat map colouring, which presents the minimum GFP fluorescence value in light blue and the maximum value in red. (a) A *pML1::SEP3:GFP* stage 3 flower bud immediately after photobleaching. (b-g) Close-ups of bleached area immediately after photobleaching (b), after 5

minutes (c), after 10 minutes (d), after 15 minutes (e), after 20 minutes (f), and after 25 minutes (g). A cell in the outer ring of recovery (1) and a cell in the inner region of recovery (2) are indicated with white circles. (h) Colour bars illustrate the GFP fluorescence recovery of the indicated cell 1 and cell 2, as measured by the normalized pixel sum per  $\mu\text{m}^2$ . Note that the fluorescence recovers first in the outer ring of the bleached area and slightly later in the inner region, demonstrating the inward movement of fluorescent SEP3:GFP molecules into the bleached area. (i) A *pML1::AP3:GFP/pML1::PI:GFP* stage 3 flower bud immediately after photobleaching. (j-o) Close-ups of bleached area immediately after photobleaching (j), after 5 minutes (k), after 10 minutes (l), after 15 minutes (m), after 20 minutes (n), and after 25 minutes (o). (p) Colour bars illustrate the GFP fluorescence recovery of the indicated cell 1 and cell 2, as measured by the normalized pixel sum per  $\mu\text{m}^2$ . Note that the fluorescence recovers first in the outer ring of the bleached area and slightly later in the inner region, demonstrating the inward movement of fluorescent AP3/PI molecules into the bleached area. Scale bars of (a) and (i) are 30  $\mu\text{m}$ , all other scale bars are 5  $\mu\text{m}$ .

### Appendix S1. Detailed experimental procedures for confocal laser scanning microscopy and photobleaching assay

GFP was excited with the 488 nm solid state laser. The GFP emission was scanned at a bandwidth of 505-530 nm, while the red autofluorescence of the chloroplasts in leaf tissue was detected at a bandwidth of 600-700 nm. All scans for the leaf tissue and also all scans for the IMs and FMs were made with the same confocal settings, which sometimes resulted in some oversaturated images like the scans for the 2x GFP control plants. The obtained confocal z-stacks were median-filtered and converted to 3-D maximum projections or cross section images. All images were adjusted with Adobe Photoshop version 5.0 (Adobe, <http://www.adobe.com>).

To quantify the GFP signal ratio between the epidermal and subepidermal cell layers in the centre of the IM and FM in cross sections, the pixel sum per  $\mu\text{m}^2$  in the GFP channel was determined in two regions of interest (ROI) created around the epidermal nuclei and the subepidermal nuclei. For both the IM and the FM, the ratios were calculated in two perpendicular cross sections through the centre of the meristem in four different samples. The cross sections used for this quantification had a minimal amount of saturated pixels (<3%). Oversaturated scans were occasionally made to verify the absence of GFP fluorescence in the subepidermal and inner cell layers.

Photobleaching experiments were performed on early flower buds by using the maximal square zoom function and repeatedly photobleaching this zoomed-in area in one z-section with 488 nm laser light at 100% power for five minutes. The fluorescence recovery in the photobleached area was monitored by scanning the whole flower bud immediately after the photobleaching and every five minutes afterwards, until a maximum of thirty minutes. The obtained confocal z-stacks were median-filtered and converted to 3-D maximum projections and adjusted with Adobe Photoshop version 5.0. An artificial heat map colouring was used for easy visualization of signal intensity changes over time. For two cells in each experiment the recovery of the GFP fluorescence in time was quantified in two circular ROIs in the 3-D projection as the normalized pixel sum per  $\mu\text{m}^2$ , and visualized in a colour-coded bar.



**Appendix S2. Detailed experimental procedure for *in situ* hybridization**

Inflorescences from *pML1::AG:GFP* plants were collected and fixed in aqueous 4% paraformaldehyde with 0.25% glutaraldehyde for two hours at room temperature. After fixation the samples were dehydrated through an ethanol/xylene series and finally embedded in Paraplast Plus (Sigma-Aldrich, <http://www.sigmaaldrich.com>). Sections of 5-6  $\mu\text{m}$  were obtained with a rotary microtome, placed on 3-aminopropyltriethoxysilane-covered slides (Pierce, <http://www.piercenet.com>) and baked for 16 hours at 50°C. Paraplast was removed from the sections by three consecutive five minute washes in xylene. A pre-hybridization treatment with proteinase-K (1  $\mu\text{g}/\text{ml}$  in 0.05 M Tris-HCl, pH7.5) for five minutes at 37°C was followed by three washes with diethyl pyrocarbonate (DEPC)-treated water. The sections were hybridized with sense and antisense probes for the *GFP* gene. The digoxigenin (DIG-UTP)-labelled probes were made according to the instructions of the manufacturer (Roche, <http://www.roche.com>), with the use of a 372 bp PCR product template made from the *pML1::AG:GFP* construct with primers GFF1 and GFR1 (see Table S1). After overnight hybridization at 42°C, the sections were washed four times to remove excess probe. The hybridization signal was detected with an anti-DIG antibody diluted 1:1000 in 0.01 M Tris-HCl, 0.15 M NaCl, 1% Blocking Reagent (Roche) and visualized after reaction with a commercial solution of nitro-blue tetrazolium chloride (NBT) and 5-bromo-4-chloro-3'-indolyphosphate p-toluidine (BCIP) plus 1 mM levamisole (Pierce). Bright field images were taken with a Zeiss Axioscope (Carl Zeiss, <http://www.zeiss.com>), and adjusted with Adobe Photoshop version 5.0.

**Table S1.** Sequences of primers used in experiments.

Primer	Sequence 5'- 3'	Annealing site
GFF1	TGAATTAGGTTGACACTATAAGTCTCAGGTAGTGGTTGTCTG SP6 promoter	157 bp downstream of START codon of <i>GFP</i>
GFR1	GCTCTAATACGACTCACTATAGGGCACATGAAGCAGCACGACTT T7 promoter	529 bp downstream of START codon of <i>GFP</i>
PRO182	GGATCCATGGCGTACCAATCGGAGCT	Immediately after START codon of <i>AGAMOUS</i>
PRO433	CACCGATCAAAGACTACACATCAC	2634 bp upstream of START codon of <i>AGAMOUS</i>
PDS404	TGGTTCACGTAGTGGGCCATCG	On left border of T-DNA
PDS1209	TTAAAGCTTATCAAAGAAAAACAAGAAC HindIII	4983 bp upstream of START codon of <i>MERISTEM LAYER1</i>
PDS1210	AAAAGTAGTTAACCGGTGGATTCAGGGAGTTTCTTTA SpeI	1596 bp upstream of START codon of <i>MERISTEM LAYER1</i>
PDS1985	CATTTCCCTCAGCCTATATTACC	In 3' UTR of <i>AGAMOUS</i> 18 bp downstream of STOP codon
PDS2027	AGAACCAGACAAACAGACAAG	In first exon of <i>APETALA3</i> 32 bp downstream of START codon
PDS2028	AGAACCAGACAAACAGATAAG	In the first exon of <i>apetala3</i> 32 bp downstream of START codon
PDS2029	GCACCAGCAAACCTTTTAGC	In the 3' UTR of <i>APETALA3</i> 6 bp downstream of STOP codon

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# Chapter 6

About SEP<sub>3</sub>, black holes and UFO

Susan L Urbanus

QD (Peter) Dinh

Zijing (Tina) Liu

Gerco C Angenent

Richard GH Immink

### Abstract

In *Arabidopsis thaliana*, the E-type SEPALLATA MADS domain transcription factors play an important role in the development of the four floral organs, sepals, petals, stamens and carpels. The spatio-temporal localisation pattern of GFP-tagged SEPALLATA3 (SEP3) shows that prior to the initiation of the petal and stamen primordia in the second and third whorl, the SEP3:GFP protein presence suddenly changes from predominantly nuclear localized to more cytoplasmically localized. During further development of the petal and stamen primordia the SEP3:GFP presence in subepidermal and inner cell layers reduces greatly, while the SEP3 presence in the epidermal cell layer remains. We named this last phenomenon SEP3:GFP 'black holes'. The *SEP3* mRNA expression in the second and third whorl tissues during these flower bud stages has previously been described as uniform, which suggests that the SEP3:GFP black holes are caused by post-transcriptional processes. We tried to determine if the SEP3:GFP black holes are due to protein degradation, but could not proof the involvement of the 26s proteasome. We also investigated whether the second and third whorl expressed F-box protein UNUSUAL FLORAL ORGANS (UFO), thought to be a subunit of an SCF E3 ubiquitin ligase complex, is involved in the sudden change in SEP3:GFP subcellular localisation and the formation of SEP3:GFP black holes. However, SEP3:GFP localisations in different genetic backgrounds did not reveal an involvement of UFO in these two processes.

### Introduction

In *Arabidopsis thaliana* flowers the E-type MADS domain transcription factors play an important role in the development of the four different floral organ types from the floral meristem [1-3]. SEPALLATA3 (SEP3) and the other SEPALLATA proteins seem to function as 'glue' proteins that facilitate the formation and functioning of the different multimeric MADS domain protein complexes that are essential for the identity specification of the first-whorl sepals, the second-whorl petals, the third-whorl stamens and the fourth-whorl carpels [4-8]. The spatio-temporal localisation pattern of a GFP-tagged genomic *SEP3* clone shows that prior to the initiation of the petal and stamen primordia in the second and third whorl, SEP3:GFP protein presence suddenly changes from predominantly nuclear localized to more cytoplasmically localized. During further development of the petal and stamen primordia the SEP3:GFP presence in subepidermal and inner cell layers reduces greatly, while the SEP3 presence in the epidermal cell layer remains [9]. Because of the lack of SEP3:GFP presence in the centre of these developing organs, we named the last phenomenon SEP3:GFP 'black holes'. Interestingly, the *SEP3* mRNA expression is described as uniform during the early development of petals and stamens [1], which could indicate that these SEP3:GFP black holes are the result of post-transcriptional processes.

Simultaneously with the change to a more cytoplasmic SEP3:GFP localisation in specific regions of stage 3 flower buds [10], the overlapping expression of the two B-type MADS domain transcription factor genes *APETALA3* (*AP3*) and *PISTILLATA* (*PI*) starts in these



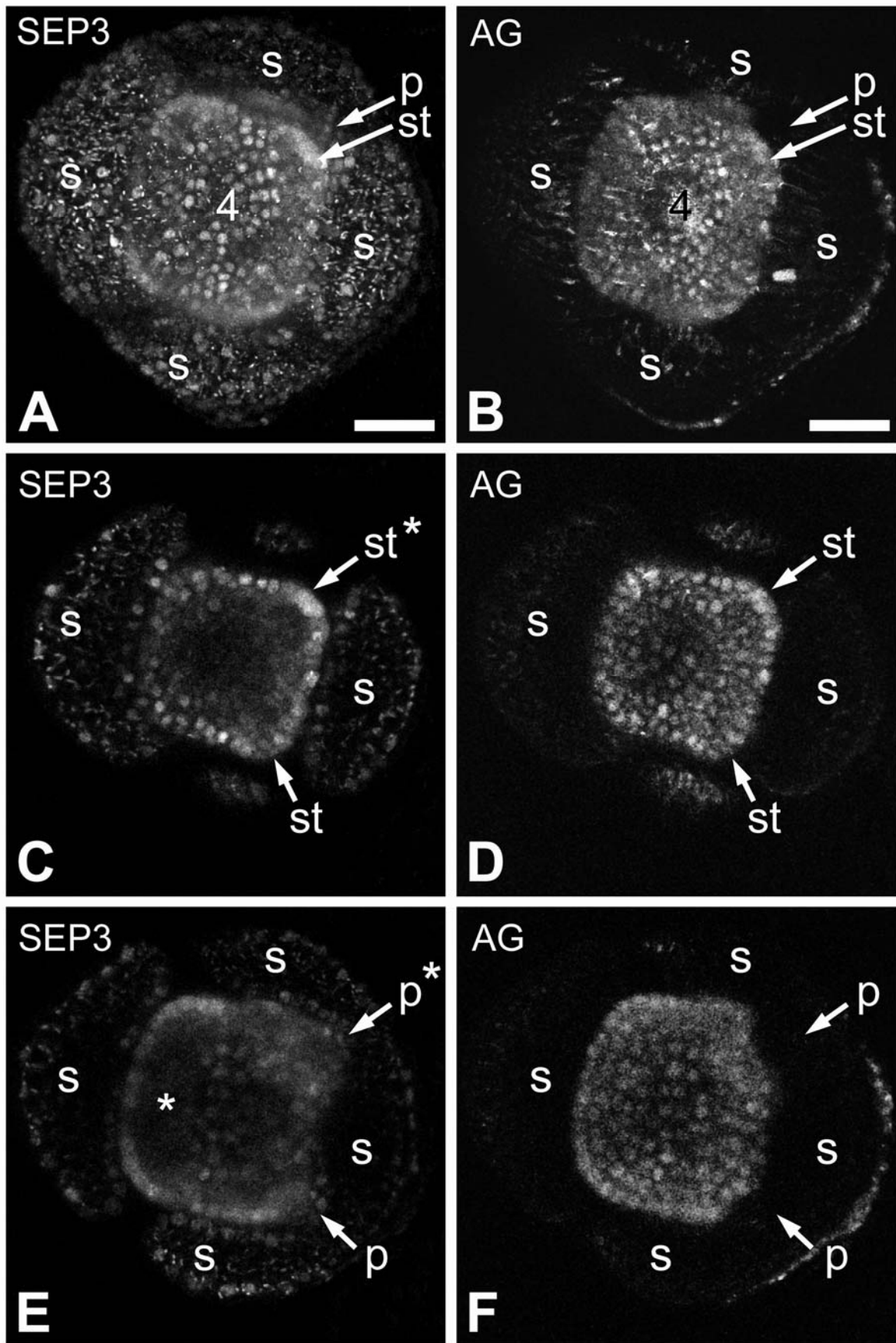
same regions [11-13]. According to the 'ABC' model the heterodimeric complex of these two B-type proteins in combination with an E-type protein and an A- or C-type protein is required for petal and stamen development, respectively [3, 7, 14, 15]. It is known that *AP3* expression is regulated by UNUSUAL FLORAL ORGANS (UFO), which is also expressed in the presumptive second and third whorl [16-19]. UFO is an F-box protein that is thought to be a subunit of an SCF E3 ubiquitin ligase complex that modifies target proteins by ubiquitination [20-23]. In these SCF complexes it is the F-box protein that provides the specificity for the target proteins. Since poly-ubiquitination commonly marks proteins for degradation by the 26s proteasome [24], the attractive theory was formed that UFO could be involved in the degradation of a repressor of LEAFY (LFY), the transcriptional activator of *AP3* [21]. Later it was confirmed that proteasome activity is indeed required for LFY activity, but it was also shown that UFO physically interacts with LFY and may function as a transcriptional co-factor [19, 25]. Additionally, mono-ubiquitination is thought to affect the function of target proteins without proteasome degradation, for instance by modifying the activity or the subcellular localisation [26].

In this chapter we describe the different experiments that were performed to discover whether the sudden change in subcellular SEP3:GFP localisation and the formation of SEP3:GFP black holes are indeed regulated at a post-transcriptional level and whether the F-box protein UFO is involved in these two processes. Although targeted SEP3 protein degradation seems to be the most likely cause of the SEP3:GFP black holes in developing petal and stamen primordia, we could not proof SEP3 protein degradation by the 26s proteasome. Furthermore, SEP3:GFP localisations in different genetic backgrounds did not give clear indications that UFO affects the subcellular localisation of SEP3:GFP protein or that UFO is involved in the formation of SEP3:GFP black holes.

## Results

### SEP3 presence during petal and stamen development

The spatio-temporal localisation pattern of a GFP-tagged genomic *SEP3* clone shows that SEP3:GFP protein in the inflorescence meristem and the first two flower bud stages is predominantly nuclear localized [9]. However, from flower bud stage 3 onwards SEP3:GFP protein suddenly becomes more cytoplasmically localized in the presumptive second and third whorl, while the SEP3:GFP protein in the centre of the floral meristem and in the epidermis of the first whorl sepal primordia remains predominantly nuclear localized. During the initiation of the petal and stamen primordia the cytoplasmically and nuclear localized SEP3:GFP starts to fade in the subepidermal and inner cell layers, until SEP3:GFP protein is mostly found in the epidermal cell layer in stage 5 flower buds (Figure 1A,C,E). We named this absence of SEP3:GFP fluorescence in the middle of the developing petal and stamen primordia 'black holes'. In comparison, the spatio-temporal localisation pattern of a GFP-tagged genomic *AGAMOUS* (*AG*) clone, expressed in the floral meristem at the position of the future third and fourth whorl, shows a more uniform AG:GFP presence in all three cell layers throughout development (Figure 1B,D,F) [9]. The confocal microscopic scans that



**Figure 1. Different behaviour of SEP3 and AG during petal and stamen development.** Simultaneous localisations of SEP3:GFP and AG:TagRFP in a stage 5 flower bud from a *gSEP3:GFP*, *gAG:TagRFP* plant. (A) Green channel 3-D projection showing SEP3:GFP presence in all three cell

layers in the three inner whorls and also in the epidermal cell layer of the first-whorl sepal primordia. The nuclei filled with SEP3:GFP protein are visible as big white dots, while the autofluorescence of the plastids are visible as smaller dots. **(B)** Red channel 3-D projection showing AG:TagRFP presence only in the developing third-whorl stamen primordia and the fourth whorl. The nuclei filled with AG:TagRFP protein are visible as big white dots, while the epidermal cell layer of the sepal primordia shows some autofluorescent background signal. **(C, D)** Optical sections showing that in stamen primordia SEP3:GFP protein is both cytoplasmically and nuclear localized and more restricted to the epidermal cell layer **(C)**, while AG:GFP protein is more nuclear localized and more uniformly present in all cell layers in the stamen primordia **(D)**. Note that the stamen primordium in the upper right corner is already developing a so-called SEP3:GFP black hole. **(E, F)** Optical sections showing a SEP3:GFP black hole in the stamen primordia and a developing black hole in the petal primordia **(E)**, while AG:GFP protein is more uniformly present in all cell layers in the stamen primordia **(F)**. (\*) black hole; (4) whorl 4; (p) petal primordium; (s) sepal primordium; (st) stamen primordium. Scale bars are 25  $\mu$ m.

show AG:GFP fluorescence in the same regions where SEP3:GFP black holes are observed, demonstrate that the SEP3:GFP black holes are specific for SEP3:GFP and not artefacts of confocal laser scanning microscopy through thick tissues. Unlike SEP3:GFP protein, AG:GFP protein is predominantly nuclear localized throughout development [9]. However, in initiating stamen primordia both AG:GFP protein and SEP3:GFP protein are more cytoplasmically localized in the same cells (Figure 1C-F). This could be the result of cell divisions in these initiating primordia, since nuclear localized MADS domain proteins show a more uniform distribution throughout the whole cell during the cell division process (Figure 2).

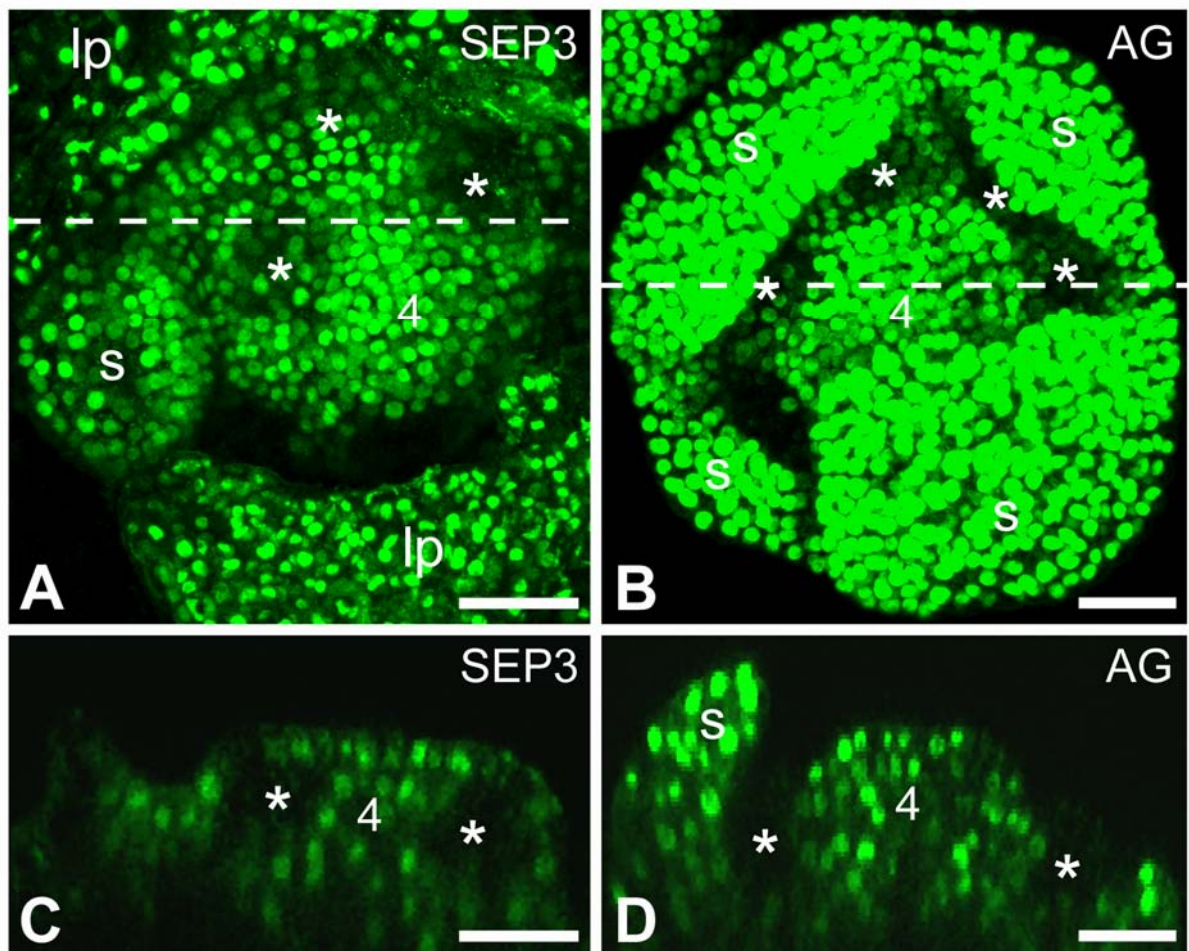
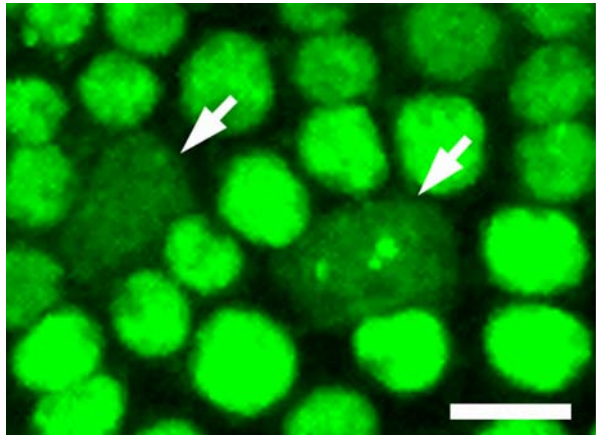
### Post-transcriptional regulation versus transcriptional regulation

The *SEP3* mRNA expression pattern during the early stages of petal and stamen development has been described as uniform [1], whereas the SEP3:GFP protein localisation pattern shows greatly reduced SEP3:GFP presence in the subepidermal and inner cell layers of initiating petal and stamen primordia in the so-called black holes (Figure 1A,C,E) [9]. This suggests that the observed SEP3:GFP black holes are the result of post-transcriptional processes, such as protein modifications that alter the behaviour of the protein or protein degradation.

To determine whether the observed SEP3:GFP behaviour during petal and stamen development is indeed regulated at post-transcriptional level and not simply due to a reduction in gene expression, we analyzed the localisation pattern of SEP3:GFP protein during second and third whorl development in plants constitutively overexpressing a *SEP3:GFP* cDNA clone. Since the spatio-temporal localisation pattern of genomic AG:GFP does not show the formation of AG:GFP black holes (Figure 1B,D,F) [9], we used plants constitutively overexpressing an *AG:GFP* cDNA clone as control plants. While the early-flowering *pCaMV35S::SEP3:GFP* plants commonly have only one incomplete terminal flower, the early-flowering *pCaMV35S::AG:GFP* plants usually produce a few more flowers that consist of carpelloid sepals, stamens or staminoid organs, and a pistil [27]. In *pCaMV35S::SEP3:GFP* flower buds we observed a clear reduction in GFP fluorescence in

**Figure 2. Altered subcellular SEP3:GFP localisation during cell division.**

A 3-D close-up of epidermal cells expressing SEP3:GFP proteins in an inflorescence meristem. Most cells have predominantly nuclear localized SEP3:GFP protein, but cells that are in the process of cell division (arrows) show a more uniform distribution of the SEP3:GFP signal over the whole cell. Scale bar is 5  $\mu$ m.



**Figure 3. Black holes in plants constitutively overexpressing SEP3:GFP or AG:GFP.**

Confocal microscopic analyses of the presence of so-called black holes in floral meristems with constitutive overexpression of either SEP3:GFP or AG:GFP. (A) 3-D projection of a terminal *pCaMV35S::SEP3:GFP* floral meristem of approximately stage 5, showing black holes in three initiating (presumably) third-whorl primordia. (B) 3-D projection of a *pCaMV35S::AG:GFP* stage 5 floral meristem, showing black holes in four initiating (presumably) third-whorl primordia. (C) Longitudinal section through the *pCaMV35S::SEP3:GFP* floral meristem as indicated with a dashed line in (A), showing the presence of two black holes on either side of whorl 4. (D) Longitudinal section through the *pCaMV35S::AG:GFP* floral meristem as indicated with a dashed line in (B), showing the presence of two black holes on either side of whorl 4. (\*) black hole; (4) whorl 4; (lp) dissected leaf primordium; (s) sepal primordium. All scale bars 25  $\mu$ m.

the second and third whorl primordia (Figure 3A,C), very similar to the genomic SEP3:GFP black holes that were found (Figure 1A,C,E) [9]. Surprisingly, the *pCaMV35S::AG:GFP* flower buds showed a similar reduction of GFP fluorescence in second and third whorl primordia (Figure 3B,D), which was unexpected based on the observed behaviour of genomic AG:GFP during flower development (Figure 1B,D,F) [9]. This suggests that both SEP3:GFP and AG:GFP proteins are post-transcriptionally regulated in these developing second and third whorl organs, for instance by targeted protein degradation. However, it was shown before that the *CaMV35S* promoter is not very active in petals until stage 8 flower buds and also drives lower levels of expression in stage 5 and 6 stamens [28]. Taking this information together with the unexpected absence of AG:GFP in the second and third whorl tissues, it seems more likely that the SEP3:GFP and AG:GFP black holes seen in these constitutive overexpressing plants are at least partly due to lack of strong activity of the *CaMV35S* promoter in these tissues at these developmental stages.

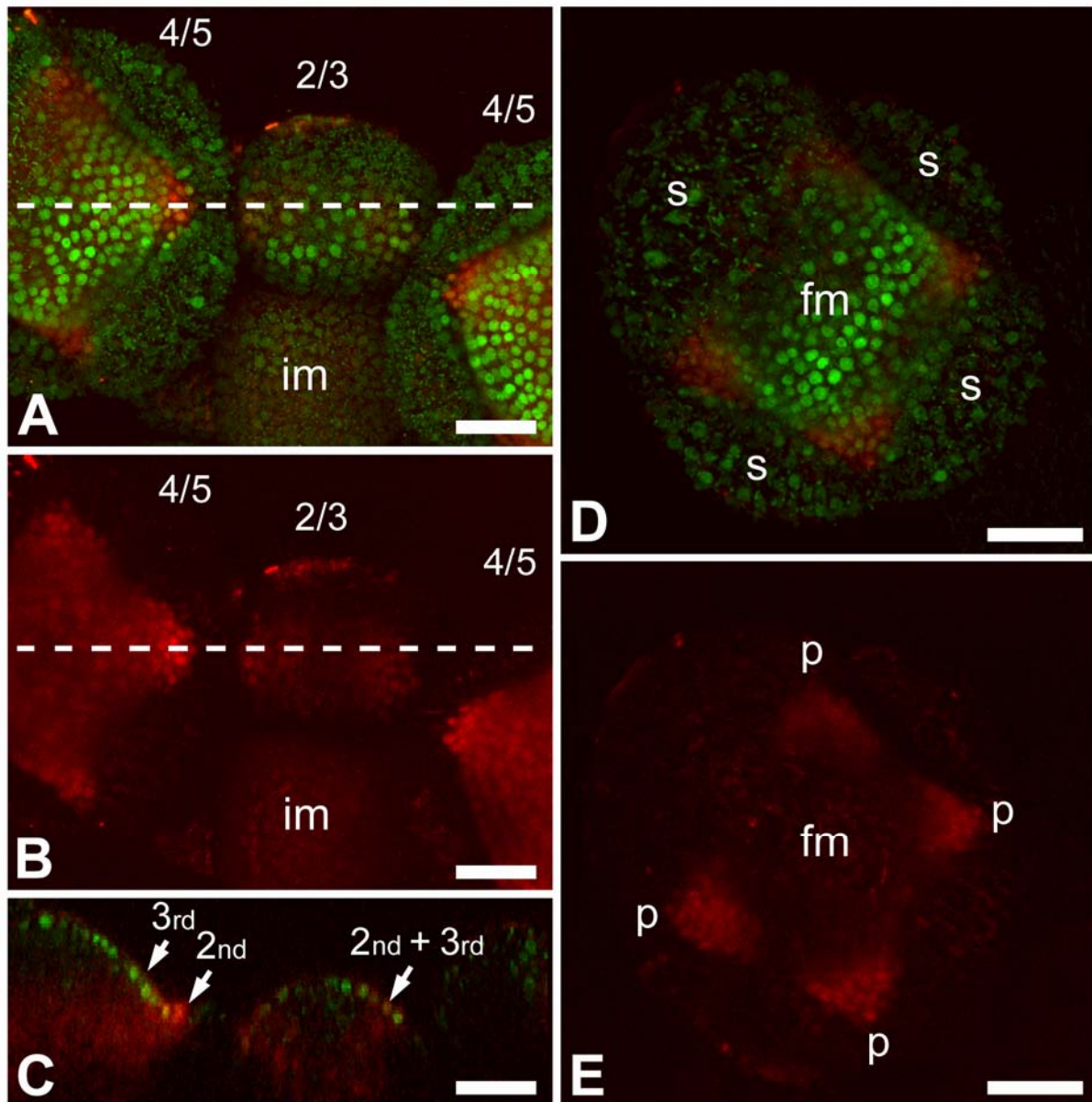
### **Proteasome inhibitors do not affect the black holes**

We tried to determine if the SEP3:GFP and AG:GFP black holes are created by 26s proteasome mediated degradation of the GFP-tagged proteins by applying proteasome inhibitors to the flower buds. Three different proteasome inhibitors were tested at several concentrations and incubation times, namely the untagged proteasome inhibitor MG132 and two fluorescently-tagged epoxomicin analogs MVB003 and MV151 [29]. In all treated *pCaMV35S::SEP3:GFP*, *pCaMV35S::AG:GFP* and also *gSEP3:GFP* flower buds we still observed black holes in initiating second or third whorl primordia (results not shown). However, this assay does not only depend on the effectiveness of the proteasome inhibitors, but also on the ability of the sample tissue to produce enough *de novo* synthesized GFP-tagged proteins to detectably fill up the black holes.

### **Does UFO regulate the presence of SEP3?**

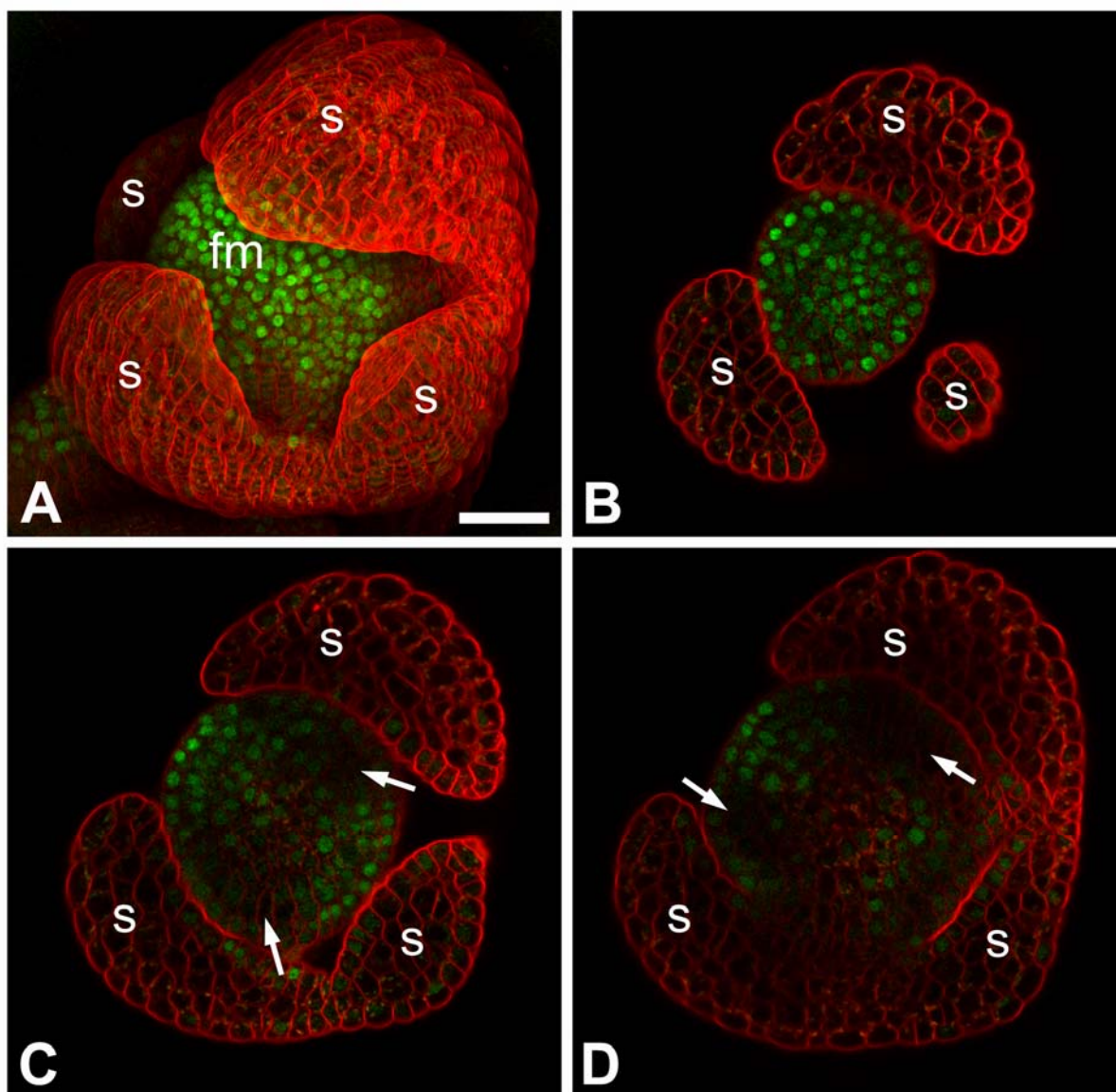
We investigated whether the second and third whorl expressed F-box protein UFO, which controls petal and stamen development together with the transcription factor LFY, is involved in the observed change in SEP3:GFP subcellular localisation and the formation of SEP3:GFP black holes during petal and stamen initiation and outgrowth. Given the fact that UFO seems to be a subunit of an SCF E3 ubiquitin ligase complex [20-22], it is possible that SEP3 is a target for post-translational modification by this SCF E3 ubiquitin ligase complex. Mono-ubiquitination could alter the behaviour of the SEP3 protein, while poly-ubiquitination could target SEP3 proteins for degradation by the 26s proteasome [23, 26].

We first determined the localisation of UFO protein in inflorescence tissues from transgenic plants that express a fluorescently-tagged genomic *UFO* construct under the control of its own promoter. These plants showed that UFO:TagRFP protein was both nuclear and cytoplasmically localized and present in the inflorescence meristem and in the presumptive second and third whorl of flower bud stage 2/3 onwards (Figure 4). At later developmental stages the UFO signal reduced in the stamen primordia and became more restricted to the petal primordia (Figure 4D,E). This UFO localisation pattern corresponds



**Figure 4. UFO:TagRFP localisation in inflorescence tissues.**

Spatio-temporal localisation pattern of UFO:TagRFP in *gUFO:TagRFP*, *gSEP3:GFP* inflorescences. (A) 3-D projection showing the presence of both UFO:TagRFP (red) and SEP3:GFP (green) in inflorescence tissues. In the green channel the nuclei filled with SEP3:GFP proteins are visible as big green dots, while the autofluorescence of the plastids is visible as smaller green dots. (B) Only the red channel 3-D projection showing the UFO:TagRFP fluorescence. (C) Longitudinal section as indicated with a dashed line in (A), showing that UFO:TagRFP is present in flower buds in the presumptive second and third whorl tissue, but not in the presumptive fourth whorl tissue. (D) 3-D projection showing the presence of UFO:TagRFP (red) and SEP3:GFP (green) in a stage 5 flower bud, and (E) only the red channel 3-D projection showing that UFO:TagRFP is more restricted to the petal primordia at this stage. (2) stage 2 flower bud; (2<sup>nd</sup>) second whorl; (3<sup>rd</sup>) third whorl; (4/5) stage 4 to 5 flower bud; (fm) floral meristem; (im) inflorescence meristem; (p) petal primordium; (s) sepal primordium. All scale bars are 25 µm.



**Figure 5. SEP3:GFP localisation in *ufo* mutant background.**

Localisation of SEP3:GFP in a *gSEP3:GFP, ufo-2* flower bud of approximately stage 5. SEP3:GFP protein in the nuclei is visible as big green dots, while the red dye FM4-64 stains the cell membranes. (A) 3-D projection of the flower bud showing strong SEP3:GFP signal in all three cell layers in the floral meristem (fm) and in the epidermis of the sepal primordia (s). (B-D) Progressive optical sections through the flower bud in (A) from top to bottom, indicating clusters of cells where SEP3:GFP is almost absent (arrows). Scale bar is 25  $\mu$ m.

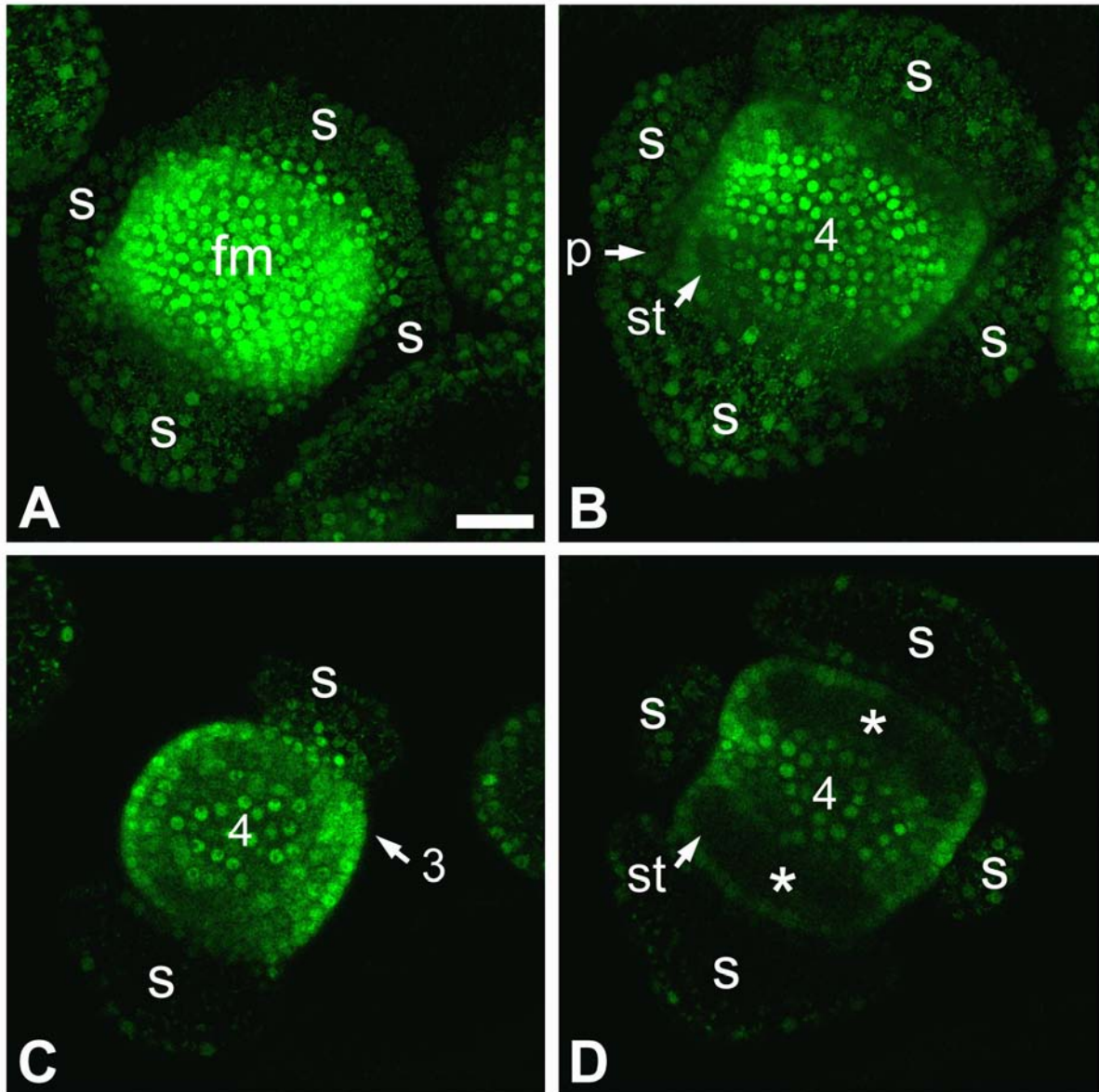
with the previously reported mRNA pattern, although the withdrawal of UFO:TagRFP protein to the petal primordia seemed to occur a little bit later in development than the withdrawal of the *UFO* mRNA to the petal primordia [18]. These localisations demonstrate that UFO protein is indeed present in the second and third whorl tissues where more cytoplasmic SEP3:GFP is detected and during the formation of SEP3:GFP black holes in the developing petals and stamens.

To determine if the absence of UFO has an effect on the subcellular localisation of SEP3:GFP or the formation of the SEP3:GFP black holes, we analyzed the presence and subcellular localisation of SEP3:GFP in a *ufo-2* mutant background. *Ufo-2* flowers have impaired petal and stamen development and commonly have a variety of second and third whorl organs with mixed identities or homeotic conversions, such as sepals, petaloid organs, petals, filamentous organs, staminoid organs, stamens and carpelloid organs [17]. Additionally, the fourth whorl gynoecium often consists of more than two carpels. The localisation pattern of SEP3:GFP in the *ufo-2* inflorescence meristem and the early flower bud stages was similar to the localisation pattern of SEP3:GFP in a wild type background (Figure 1A,C,E) [9]. However, in *ufo-2* flower buds of approximately stage 4/5 there was less cytoplasmic SEP3:GFP visible in the second and third whorl cell layers (Figure 5). In *ufo-2* flower buds of approximately stage 5, when petal and stamen primordia are visible in wild type flowers, there were often no distinct bulges of second and third whorl organ primordia visible in the floral meristem. However, at these positions in the floral meristem we did observe clusters of cells with less SEP3:GFP signal (Figure 5B-D), resembling the black hole phenotype described for SEP3:GFP during wild type flower development. This suggests that UFO is not involved in the formation of SEP3:GFP black holes during second and third whorl development. However, UFO could be involved in the change in subcellular localisation of SEP3:GFP, since SEP3:GFP protein in the *ufo-2* mutant plants seemed to be less cytoplasmically localized in the second and third whorl than in wild type plants. To study this in more detail we analyzed the spatio-temporal localisation pattern of SEP3:GFP in a *pCaMV35S::UFO* background. *pCaMV35S::UFO* flowers develop supernumerary petals and stamens at the expense of sepals and carpels [18]. No obvious difference was found between the spatio-temporal localisation pattern of SEP3:GFP in a *pCaMV35S::UFO* background (Figure 6) and in a wild type background (Figure 1A,C,E) [9]. This indicates that UFO does not influence the subcellular localisation of SEP3:GFP protein during petal and stamen development.

### Discussion

In *Arabidopsis thaliana* the E-type SEPALLATA MADS domain transcription factors play an important role as 'glue' proteins in the different multimeric MADS domain protein complexes that specify the identity of the four different floral organs, namely sepals, petals, stamens and carpels [4-8]. During the initiation of petal and stamen primordia, the spatio-temporal localisation pattern of a GFP-tagged genomic *SEP3* clone shows an unexpected SEP3:GFP pattern when compared to the previously described *SEP3* mRNA pattern [1, 9]. In contrast to the uniform *SEP3* mRNA expression in early stages of petal and stamen development, SEP3:GFP protein becomes restricted to the epidermal cell layer of initiating petal and stamen primordia and disappears in the subepidermal and inner cell layers. We named this phenomenon SEP3:GFP 'black holes'. Prior to the development of these SEP3:GFP black holes, the subcellular localisation of SEP3:GFP in cells of the presumptive second and third whorl changes suddenly from predominantly nuclear to more cytoplasmically localized and





**Figure 6. SEP3:GFP localisation in constitutive UFO overexpressing plants.**

Spatio-temporal localisation pattern of SEP3:GFP in *gSEP3:GFP*, *pCaMV35S::UFO* plants. The nuclei filled with SEP3:GFP proteins are visible as big green dots, while the autofluorescence of the plastids is visible as smaller green dots. (A) 3-D projections of a stage 4 flower bud and (B) a stage 5 flower bud with initiating petal and stamen primordia. (C) Optical section through the stage 4 flower bud from (A), demonstrating that in third whorl cell layers the SEP3:GFP protein is more cytoplasmically localized than in fourth whorl cell layers. (D) Optical section through the stage 5 flower bud from (B), showing the presence of SEP3:GFP black holes in the developing stamen primordia. (\*) black hole; (3) whorl 3; (4) whorl 4; (fm) floral meristem; (p) petal primordium; (s) sepal primordium; (st) stamen primordium. All scale bars are 25  $\mu$ m.

remains that way throughout later developmental stages. Assuming that the spatio-temporal localisation pattern of SEP3:GFP is a good reflection of the localisation pattern of endogenous SEP3, the black holes would suggest that after primordium initiation SEP3 is required mostly in the epidermis of the developing organs and less in the subepidermal and inner cell layers. The predominant SEP3:GFP localisation in the epidermis of older petals, stamens, but also the gynoecium and sepals, is in agreement with the hypothesis that SEP3 is mainly active in the epidermal cell layer during floral organ outgrowth and differentiation [9] (unpublished data).

The fact that *SEP3* mRNA expression is uniform during early petal and stamen development [1] while *gSEP3:GFP* and also *pCaMV35S::SEP3:GFP* plants show SEP3:GFP black holes, suggests that the SEP3:GFP black holes are caused by post-transcriptional processes. However, in the case of the *pCaMV35S::SEP3:GFP* plants it cannot be excluded that the black holes appear due to the lack of strong expression from the *CaMV35S* promoter in particular stages of petal and stamen development [28]. Since we have no indication that SEP3 presence is controlled through post-transcriptional inhibition by miRNAs [30, 31], we focused on post-translational processes that could cause the SEP3:GFP black holes. One explanation for the formation of the SEP3:GFP black holes could be the targeted degradation of SEP3 proteins in the subepidermal and inner cell layers in the second and third whorl tissues by the 26s proteasome from flower bud stage 4 onwards. Unfortunately, we were not able to show involvement of the 26s proteasome in the formation of the black holes in the experiments where we treated inflorescences with proteasome inhibitors. An alternative explanation for the formation of SEP3:GFP black holes could be that the sudden change to more cytoplasmically localized SEP3:GFP proteins from flower bud stage 3 onwards makes the SEP3:GFP proteins available for intercellular transport towards the epidermal cell layer [32], where they accumulate.

One known regulator of petal and stamen development that could potentially alter the behaviour of a protein through ubiquitin modification and mark proteins for degradation by the 26s proteasome, is the F-box protein UFO that is thought to be a subunit of an SCF E3 ubiquitin ligase complex [20-22]. We therefore investigated whether the sudden change in SEP3:GFP subcellular localisation and the formation of SEP3:GFP black holes during petal and stamen development is due to UFO-mediated modifications of SEP3 proteins. The spatio-temporal localisation pattern of a TagRFP-tagged genomic *UFO* clone demonstrated that UFO protein is indeed present in flower buds from stage 2/3 onwards in the presumptive second and third whorl tissues from which the petals and stamen will develop. However, UFO does not seem to be required for the formation of SEP3:GFP black holes, since SEP3:GFP localisations in a *ufo-2* mutant background still showed clusters of cells with less SEP3:GFP in the positions where second and third whorl organs would eventually arise. On the other hand, there was less cytoplasmically localized SEP3:GFP in these *ufo-2* mutant plants, which could indicate that UFO does play a role in the subcellular localisation of SEP3. However, the localisations of SEP3:GFP in a *pCaMV35S::UFO* background did not reveal overall increased cytoplasmic SEP3:GFP localisation, which would be expected if UFO indeed regulates the cytoplasmic localisation of SEP3 proteins. Therefore, our experiments

did not provide evidence for a direct role of UFO in the sudden change in SEP3:GFP subcellular localisation and the formation of SEP3:GFP black holes during petal and stamen development.

## Material and methods

### Plant materials and growth conditions

All *Arabidopsis thaliana* plant lines were grown at 22 °C in growth chambers under a long-day light regime (16 h light/8 h dark). The previously described *gSEP3:GFP* (pARC423) plant line [27] was crossed into the *ufo-2* mutant line N6294 [17, 18]. Homozygous *ufo-2* mutant plants carrying the *gSEP3:GFP* construct were selected from the F2 progeny based on the presence of *ufo-2* mutant phenotypes and the presence of GFP fluorescence. The *gUFO:TagRFP* construct (CZN695) and the *gAG:TagRFP* construct (CZN690) were transformed into Columbia-0 (Col-0) wild type plants and *gSEP3:GFP* expressing plants. The *pCaMV35S::UFO* (CZN684) construct was transformed into the Col-0 wild type plant line and the *gSEP3:GFP* plant line. Additionally, we used two plant lines expressing *pCaMV35S::AG:GFP* (pARC276) and *pCaMV35S::SEP3:GFP* (pARC277) in *sgs2-1* mutant background that were previously described in chapter 5.

### Construction of binary vectors and plant transformation

To generate a *TagRFP*-tagged genomic clone of *UFO*, a 6152 bp genomic fragment was made from Col-0 gDNA by proofreading PCR with the primer PDS1274 (5'-TCAAGGATGTTACCGGAGAG-3') 4826 bp upstream of the START codon and the primer PDS1269 (5'-ACAGACTCCAGGAAATGGAAG-3') immediately upstream of the STOP codon. After adding a T/A overhang, this genomic fragment was introduced into the pCR8/GW/TOPO TA vector by using the pCR8/GW/TOPO TA Cloning kit (Invitrogen), after which the right orientation of the genomic *UFO* insert was ensured. An LR reaction between this *gUFO* entry clone and the destination vector CZN652 containing the expression cassette *GW\_TagRFP\_NOS terminator*, created the expression clone *gUFO:TagRFP* (CZN695). A similar *TagRFP*-tagged genomic clone of *AG* (CZN690) was created by LR reaction between a *gAG* entry clone (pARC353) and the same destination vector CZN652. These two expression clones were introduced into *Agrobacterium tumefaciens* strain GV3101 and transformed to Col-0 wild type plants and *gSEP3:GFP* plants by floral dip method [33].

An untagged overexpressor *UFO* construct was created by LR reaction between the *UFO* cDNA clone CZN300 (pENTR221-AT1G30950 from ABRC DNA Stock Center) and the destination vector pARC959 (pB7WG2,0) [34]. The resulting expression clone *pCaMV35S::UFO* (CZN684) was introduced into *Agrobacterium tumefaciens* strain GV3101 and transformed to Col-0 wild type plants and *gSEP3:GFP* plants by floral dip method.

### Confocal laser scanning microscopy

To observe the localisation of the GFP-tagged protein in rosette leaves, inflorescence meristems and floral meristems, plant tissues were dissected and embedded as previously

described [9, 27]. Confocal Laser Scanning Microscopy (CLSM) of the living plant tissue was performed with a Leica SPE DM5500 upright microscope with a 63x ACS APO (NA 1.15 CORR) lens, using the LAS AF 1.8.2 software. The fluorescent tag GFP was excited with the 488 nm solid state laser and the emission scanned at a bandwidth of 505-530 nm. The red dye FM4-64 (Molecular Probes) was excited with the 488 nm solid state laser and the emission scanned at a bandwidth of 600-700 nm. The fluorescent tag TagRFP was excited with the 532 nm solid state laser and the emission scanned at a bandwidth of 560-650 nm. The obtained confocal z-stacks were median-filtered and converted to 3-D maximum projections or cross section images. All images were adjusted with Adobe Photoshop version 5.0.

### **Proteasome inhibitor experiments**

Inflorescences from *gSEP3:GFP* plants, *pCaMV35S::SEP3:GFP* and *pCaMV35S::AG:GFP* plants were treated with three proteasome inhibitors, namely MG132 and the two BODIPY-labelled epoxomicin analogs MVB003 and MV151 [29]. After initial experimentation with different incubation methods, the preferred method was to submerge dissected inflorescences in different proteasome inhibitor solutions in multiwell plates. The inflorescences were prepared by removing the older flower buds until the relevant flower buds and inflorescence meristem were visible, while a long enough stem was kept for easy handling of the samples. After incubation in the proteasome inhibitor solutions the inflorescence samples were further prepared for imaging as previously described [27]. The three proteasome inhibitor solutions were made in tap water or 0.5 x MS, and ranged in concentration from 0.5  $\mu$ M to 500  $\mu$ M. To reduce the surface tension of the solutions and aid the infiltration of the proteasome inhibitors into the inflorescence samples the surfactants Triton X-100, Tween-20 and Tween-80 were also sometimes added to the proteasome inhibitor solutions at an end concentration of 0.03%. Inflorescences in the same solution without proteasome inhibitor were used as control samples. The incubation times ranged from 30 minutes to six hours.

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# Chapter 7

Concluding remarks and perspectives

### **MADS domain transcription factors**

The MADS domain transcription factor family plays an important regulatory role in the development of angiosperm flowers. Although previous studies report the mRNA expression patterns of most of the MADS box genes involved in *Arabidopsis thaliana* floral development, often the more important information on the proteins encoded by these MADS box genes is missing. Protein localisation patterns can differ substantially from mRNA expression patterns, due to post-transcriptional and post-translational processes such as translational inhibition by miRNAs, protein degradation, and intercellular protein transport [1-3]. Intercellular transport of proteins and other macromolecules is thought to be mediated by dynamic channels that connect neighbouring cells, called plasmodesmata [4, 5]. Only recently it has been realized that transport of non-cell-autonomous proteins through plasmodesmata is a highly dynamic process that can play an important regulatory role in developmental processes. The only two known intercellularly transported MADS domain proteins are the B-type proteins DEFICIENS (DEF) and GLOBOSA (GLO) in *Antirrhinum majus* that are able to move towards the epidermis in the floral meristem [6]. Intriguingly, their respective *Arabidopsis thaliana* orthologues APETALA3 (AP3) and PISTILLATA (PI) do not seem to have the same ability [7]. Moreover, AP3, PI and also the A-type protein APETALA1 (AP1) seem to lack the ability for inward movement in floral meristems [7-9]. Nevertheless, it has been suggested that certain MADS domain transcription factors have non-cell-autonomous functions, such as the C-type protein AGAMOUS (AG) in floral meristem integrity [10-12] and FRUITFULL (FUL) in pistil development [13].

### **Tagging of MADS domain proteins**

Since the discovery of the GREEN FLUORESCENT PROTEIN (GFP) and the subsequent development of similar fluorescent tags, it has become possible to observe the subcellular localisation and behaviour of fluorescently-tagged proteins in living tissues with confocal laser scanning microscopy [14-16]. In this thesis we show that fluorescent tagging of MADS domain proteins for *in planta* imaging should be used with some caution. Adding a fluorescent tag or a small peptide tag to MADS domain proteins easily leads to transgene silencing, which often results in specific loss-of-function mutant phenotypes. This is not only the case with constructs overexpressing tagged MADS box gene versions, but also with tagged MADS box gene constructs that are expressed under the control of the endogenous regulatory elements by using full genomic clones [17-20]. In the case of GFP-tagged MADS box gene constructs this silencing problem could be circumvented by using plants with an *sgs2-1* mutant background that are defective in gene silencing [21, 22]. However, with the photoconvertible *mEosFP*-tagged MADS box gene constructs even the *sgs2-1* mutant background did not help in the recovery of stable primary transformants.

Of course it should be taken into account that the presence of a tag can interfere with the functioning of the protein. We demonstrated that C-terminal GFP-tagged MADS domain proteins in stable transgenic plants are functional and able to complement their respective



mutant backgrounds, but we cannot exclude that the GFP tag interferes with certain functions or in specific complexes. Especially if several MADS domain proteins in a multimeric complex are tagged, it is possible that this will hinder the functioning or even the formation of the complex. For instance, we provide some evidence that the presence of both GFP-tagged AP3 and GFP-tagged PI in one complex might make this complex non-functional. Nevertheless, when the tag is thoughtfully placed and stable transgenic plants are obtained, *in planta* imaging of fluorescently-tagged proteins by confocal laser scanning microscopy is a valuable tool to investigate protein behaviour during development.

### **Intercellular transport of MADS domain proteins**

The spatio-temporal localisation patterns of GFP-tagged AG, AP1, FUL, and the E-type protein SEPALLATA3 (SEP3) during floral development demonstrated that there are several tissues, often the epidermal cell layer, where MADS domain proteins could be detected while the available literature describes an absence of mRNA in those tissues. In plants that specifically overexpress GFP-tagged MADS domain proteins in the epidermal cell layer, we were able to demonstrate with a photobleaching technique that AG, SEP3, and the obligate heterodimer AP3/PI are all able to move at least short distances within the epidermal cell layer. This lateral epidermal movement of MADS domain proteins, which seems to be passive diffusion through primary plasmodesmata that connect the epidermal cells, provides an explanation for most of the unexpected MADS domain protein localisations that we found in the spatio-temporal localisation analyses. The amount of passive diffusion of MADS domain proteins that occurs, might be limited by nuclear targeting and sequestration of dimeric MADS domain protein complexes [23-25]. For instance, in roots it was shown that the movement of the GRAS transcription factor SHORT ROOT (SHR) from the stele is limited by nuclear sequestration through the interaction with the GRAS transcription factor SCARECROW (SCR) in the adjacent cell layer [26-28]. According to the extended 'ABC' model, the homeotic MADS domain transcription factors are active in clearly defined regions in the floral meristem [29-35]. If MADS domain proteins are allowed to flow freely through the whole epidermis, this could indicate that they lack the capacity to change organ identity from this layer. Supporting this hypothesis is the fact that epidermal-expressed GFP-tagged AP3 is not sufficient for full complementation of the *ap3* mutant.

Additionally, we demonstrated that epidermis-expressed GFP-tagged AG is able to move from the epidermis to the subepidermis in the centre of the floral meristem. This observation and the demonstrated lateral movement of AG in the epidermis of the floral meristem provide proof for the previous suggestions that AG acts non-cell-autonomously [10-12]. The transport of AG from the epidermis to the subepidermis through the secondary plasmodesmata that connect these two cell layers seems to be an active process. Perhaps the AG protein possesses a domain that enables it to interact with (secondary) plasmodesmata, while SEP3, AP3 and PI proteins lack this domain. For KNOX transcription factors, which can actively enlarge the aperture of plasmodesmata to allow the passage of large molecules, it was shown that the homeodomain is essential for the interaction with the plasmodesmata [36-39].

Interestingly, the KNOX transcription factor *SHOOTMERISTEMLESS* (*STM*), which can actively transport inwards in floral meristems and is involved in *AG* upregulation in the inner two whorls of the floral meristem, is specifically expressed in the tissues where we observed *AG* transport [37, 40-42]. It is therefore tempting to speculate that inward *AG* transport in the floral meristem is mediated by interactions with *STM*.

Intercellular transport might also play a role in the observed *SEP3* behaviour during petal and stamen development. The spatio-temporal localisation pattern of GFP-tagged *SEP3* showed that during early stages of petal and stamen development the GFP-tagged *SEP3* presence reduced greatly in the subepidermal and inner cell layers, while the mRNA expression in these stages is described as uniform [43]. This seems to imply that *SEP3*-containing MADS domain protein complexes are needed in all three cell layers only during the initiation of petal and stamen primordia, while epidermal *SEP3* presence might suffice for further development and outgrowth. The absence of GFP-tagged *SEP3* protein in the subepidermal and inner cell layers in developing primordia, which we named 'black holes', could be caused at a post-translational level by targeted *SEP3* protein degradation. However, the GFP-tagged *SEP3* proteins change their subcellular localisation from predominantly nuclear to more cytoplasmic just prior to the initiation of the petal and stamen primordia. This change to a more cytoplasmic localisation might make the GFP-tagged *SEP3* proteins more available for intercellular transport towards the epidermis, where they accumulate [26, 44]. If intercellular transport of *SEP3* proteins towards the epidermis is indeed the cause of the black holes, it would indicate that the initiating petal and stamen primordia do not receive *SEP3* input from surrounding *SEP3*-containing tissues. Symplastically isolated primordia can be created by changing the gating properties of the plasmodesmata on the borders of the primordium [4, 45-47]. These symplastic borders could also play a role in limiting the observed lateral transport in the epidermis.

Although intercellular transport of MADS domain proteins in meristems might be only short distanced and often occurs in tissues where the genes themselves are expressed, this does not necessarily imply that this intercellular transport is not biologically relevant. On the contrary, the non-cell-autonomous action of some MADS domain proteins combined with the autoregulatory loops that many MADS proteins have [48-51] could be essential in establishing the observed gene expression patterns during floral development. Additionally, intercellular transport might be needed for the establishment of MADS domain transcription factor gradients that in a concentration-dependent way either initiate different developmental programs as a master regulator, or induce the differentiation of specific tissues as a morphogen [52]. Whether MADS domain transcription factors acquire a different or additional function by going through the process of plasmodesmal transportation is unclear at this moment [53].

### **Perspectives**

To complement the performed research on MADS domain protein transport in the floral meristem, it would be good to test whether MADS domain proteins are also able to move

laterally within the subepidermal cell layer or the inner cell layer, and whether they can move from these cell layers towards the epidermal cell layer as was shown for DEF and GLO in *Antirrhinum majus* [6]. The promoter sequence of *WUSCHEL* (*WUS*) or the promoter sequence of *CLAVATA1* (*CLV1*) [54, 55] could for instance be used to specifically express GFP-tagged MADS domain proteins in the centre of the floral meristem in the subepidermal and inner cell layers. This approach might also demonstrate whether there is a limit to the lateral transport in these cell layers, because it is conceivable that developing floral organ primordia represent symplastic domains that are (partially) isolated from MADS domain protein input from surrounding tissues. To investigate the possible role of intercellular SEP3 transport in developing petal and stamen primordia it might be useful to express fluorescently-tagged SEP3 proteins specifically in these developing petal and stamen primordia, for instance under the control of the *UNUSUAL FLORAL ORGANS* (*UFO*) promoter [56, 57]. To demonstrate SEP3 transport towards the epidermis, a GFP tag could be used in combination with a photobleaching technique, or photoconvertible fluorescent tags could be used. However, to make this approach successful there should be sufficient precision in the confocal microscopic set-up to target only the desired cell layers for photobleaching or photoconversion, respectively. Additionally, for AG it might be worthwhile to investigate whether active AG transport between cell layers in the floral meristem is indeed mediated by interactions with the KNOX transcription factor STM, by determining the degree of AG transport between cell layers under varying amounts of STM and associated proteins.

In conclusion, the work performed in this project shows the importance of studying transcription factor localisation and dynamics at the protein level in living tissues. Furthermore, the fluorescent tag GFP has highlighted various aspects of MADS domain protein functioning that we currently do not fully understand. The challenge for the near future will be to determine the biological relevance of the observed MADS domain protein behaviour and movement.

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In this thesis we investigated the behaviour of fluorescently-tagged MADS domain proteins during floral development in the model plant *Arabidopsis thaliana*, and explored the importance of intercellular transport via plasmodesmata for MADS domain transcription factor functioning. The MADS domain transcription factor family plays an important regulatory role in the development of flowers, among others by establishing the identities of the different floral organs. Although genetic screens and *in vitro* and *in vivo* studies on protein-protein and protein-DNA interactions provide important information on how MADS domain transcription factor complexes are able to regulate downstream target genes, understanding of the behaviour of MADS domain transcription factors *in planta* is still limited. Also, the extent to which intercellular movement of MADS domain transcription factors via plasmodesmata plays a role in developmental processes is poorly understood. Since the discovery of the GREEN FLUORESCENT PROTEIN (GFP) and the subsequent development of similar fluorescent tags, it has become possible to observe the subcellular localisation and behaviour of fluorescently-tagged proteins in living tissues with confocal laser scanning microscopy.

In Chapter 2 of this thesis, different methods of tagging the MADS domain transcription factors AGAMOUS (AG), SEPALLATA3 (SEP3), and FRUITFULL (FUL) for chromatin immunoprecipitation, chromatin affinity purification and *in planta* imaging are described. This research shows that the addition of a small peptide tag or a fluorescent tag to MADS domain proteins easily leads to transgene silencing and specific loss-of-function mutant phenotypes, especially when the tagged MADS box genes are expressed under the control of the constitutive *CaMV35S* promoter. Plants that express tagged MADS box genes from genomic fragments that include all or most of the regulatory elements, and therefore mimic the natural expression pattern as much as possible, show lower levels of loss-of-function phenotypes. In addition, these plants are also more useful for investigating biological relevant behaviour of the MADS domain proteins.

In Chapter 3, the spatio-temporal localisation patterns of GFP-tagged MADS domain transcription factors AG, SEP3, FUL and APETALA1 (AP1) during floral development are reported. These analyses demonstrate that there are several tissues, often epidermal cell layers, where MADS domain proteins could be detected, while the available literature describes an absence of mRNA in those tissues. This could indicate that there is intercellular transport of MADS domain proteins in meristematic tissues during floral development. The implications of the observed behaviour of the different MADS domain proteins for MADS domain protein functioning are discussed in this chapter.

In Chapters 4 and 5 we describe the different methods that were used to investigate whether MADS domain proteins are indeed able to transport between cells during floral development. The difficulties that we encountered in our attempts to investigate intercellular MADS domain protein transport with microinjection techniques and by using the photoconvertible fluorescent mEosFP-tag are discussed. In plants that specifically overexpress GFP-tagged MADS domain transcription factors AG, SEP3, APETALA3 (AP3), or PISTILLATA (PI) in the epidermis, we demonstrated with a photobleaching technique that all tested proteins were able to move within the epidermal cell layer. This mechanism of



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lateral epidermal movement provides an explanation for most of the unexpected MADS domain protein localisations that we found in the spatio-temporal localisation analyses in Chapter 3. Additionally, we demonstrate that epidermis-expressed GFP-tagged AG is able to move from the epidermis to the subepidermis in the centre of the floral meristem, which provides proof for the suggestions that AG acts non-cell-autonomously in the floral meristem. In these plants we also analyzed the effects of epidermal MADS domain protein expression on the plant phenotype. This showed, among others, that epidermis-expressed AG is able to fully complement its own mutant background, while epidermis-expressed AP3 is not.

In Chapter 6, we explore the mechanisms underlying the behaviour of GFP-tagged SEP3 during petal and stamen development that was observed in the spatio-temporal localisation studies described in Chapter 3. Just prior to the initiation of petal and stamen primordia GFP-tagged SEP3 proteins change their subcellular localisation from predominantly nuclear to more cytoplasmic, and at later stages GFP-tagged SEP3 protein seems to disappear in the middle of the primordia without the loss of *SEP3* mRNA expression. These two processes could be regulated at a post-transcriptional level by two mechanisms that are discussed, namely 26s proteasome mediated SEP3 protein degradation and epidermal-oriented intercellular transport of SEP3 proteins. Additionally, we demonstrate that there are no clear indications that the observed GFP-tagged SEP3 behaviour is due to the presence of F-box protein UNUSUAL FLORAL ORGANS (UFO), which regulates petal and stamen development.

In Chapter 7, this thesis finishes with some concluding remarks on *in planta* imaging of MADS domain transcription factors and the possible mechanisms of MADS domain protein movement in the floral meristem. Furthermore, we speculate on the importance of MADS domain protein movement in establishing MADS box gene expression patterns and MADS domain protein gradients, and on the need for symplastically isolated domains for proper floral development.

**“MADS eiwitten in beweging: een studie naar het functioneren van MADS domein eiwitten tijdens bloemontwikkeling in *Arabidopsis thaliana*.”**

Voor dit proefschrift is het gedrag van fluorescent gelabelde MADS domein eiwitten onderzocht tijdens bloemontwikkeling in de model plant *Arabidopsis thaliana*. Daarbij hebben we ook het belang van intercellulair transport via plasmodesmata voor het functioneren van deze MADS domein transcriptiefactoren verkend. De familie van MADS domein transcriptiefactoren heeft een belangrijke regulerende rol tijdens de ontwikkeling van bloemen, onder andere door de identiteit van de verschillende bloemorganen te bepalen. Genetische onderzoeken en *in vitro* en *in vivo* studies op het gebied van eiwit-eiwit en eiwit-DNA interacties hebben veel informatie opgeleverd over hoe MADS domein transcriptiefactor complexen in staat zijn om downstream target genen te reguleren. Echter, over het gedrag van MADS domein transcriptiefactoren *in planta* en de mate waarin intercellulair transport van transcriptiefactoren via plasmodesmata een rol speelt tijdens ontwikkelingsprocessen is nog maar weinig bekend. Sinds de ontdekking van het fluorescerende eiwit GREEN FLUORESCENT PROTEIN (GFP) en de daaropvolgende ontwikkelingen op het gebied van fluorescente labels, is het mogelijk geworden om het gedrag en de subcellulaire lokalisatie van fluorescent gelabelde eiwitten in levende weefsels te bestuderen met confocale laser scanning microscopie.

In Hoofdstuk 2 van dit proefschrift worden verschillende manieren besproken om de MADS domein transcriptiefactoren AGAMOUS (AG), SEPALLATA3 (SEP3) en FRUITFULL (FUL) te labelen voor chromatine immunoprecipitatie, chromatine affiniteitszuivering en *in planta* microscopie. Dit onderzoek toont aan dat de toevoeging van een kort peptide label of een fluorescent label aan MADS domein eiwitten makkelijk leidt tot transgen silencing en specifieke loss-of-function mutante fenotypes. Dit is vooral het geval als de gelabelde MADS box genen tot expressie komen onder de controle van de constitutieve *CaMV35S* promotor. In planten waarbij de gelabelde MADS box genen tot expressie worden gebracht vanaf genomische fragmenten, waardoor de natuurlijke expressiepatronen zoveel mogelijk nagebootst worden, komen deze loss-of-function fenotypes minder vaak voor. Door de nabootsing van de natuurlijke expressiepatronen zijn deze planten ook nuttiger voor het bestuderen van biologisch relevant gedrag van de eiwitten.

In Hoofdstuk 3 worden de lokalisatie patronen in plaats en tijd van de GFP-gelabelde MADS domein transcriptiefactoren AG, SEP3, FUL en APETALA1 (AP1) tijdens de bloemontwikkeling gerapporteerd. Deze analyses laten zien dat in een aantal weefsels, meestal de epidermale cellagen, MADS domein eiwitten aanwezig zijn, terwijl de beschikbare literatuur daar geen mRNA aanwezigheid rapporteert. Dit zou erop kunnen wijzen dat tijdens de bloemontwikkeling in meristematische weefsels intercellulair transport van MADS domein eiwitten plaatsvindt. De implicaties van het waargenomen gedrag van de verschillende MADS domein eiwitten voor hun functioneren worden besproken in dit hoofdstuk.

In Hoofdstuk 4 en 5 beschrijven we de verschillende methoden die gebruikt zijn om te onderzoeken of MADS domein eiwitten inderdaad in staat zijn om tussen cellen te bewegen

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tijdens de bloemontwikkeling. We bespreken de moeilijkheden die we tegen zijn gekomen bij onze pogingen om intercellulair transport van MADS domein eiwitten te onderzoeken met micro-injectie technieken en door gebruik te maken van het fotoconverteerbare fluorescente mEosFP-label. In planten die de GFP-gelabelde MADS domein transcriptiefactoren AG, SEP3, APETALA3 (AP3) of PISTILLATA (PI) specifiek in de epidermis overproduceren, hebben we met een fotoblekingstechniek gedemonstreerd dat al de geteste eiwitten in staat zijn om in de epidermale cellaag van cel naar cel te bewegen. Dit laterale bewegingsmechanisme geeft een verklaring voor het merendeel van de onverwachte MADS domein eiwit lokaliseringspatronen die we gevonden hebben in de gedetailleerde lokaliseringsanalyses in Hoofdstuk 3. Verder laten we zien dat epidermaal-geproduceerd GFP-gelabeld AG in staat is om van de epidermis naar de subepidermis te bewegen in het midden van het bloemmeristeem. Hiermee is de suggestie dat AG non-cel-autonoom functioneert in het bloemmeristeem voor het eerst van bewijs voorzien. In deze planten hebben we ook de effecten van epidermaal-geproduceerde MADS domain eiwitten op het fenotype van de plant geanalyseerd. Hieruit is onder andere gebleken dat epidermaal-geproduceerd AG in staat is om zijn eigen mutante achtergrond volledig te complementeren, terwijl epidermaal-geproduceerd AP3 dit niet kan.

In Hoofdstuk 6 worden de mechanismen achter het gedrag van GFP-gelabeld SEP3 tijdens bloemblad en meeldraad ontwikkeling onderzocht. Uit het werk beschreven in Hoofdstuk 3 blijkt dat de subcellulaire aanwezigheid van GFP-gelabelde SEP3 eiwitten net voor de initiatie van de bloemblad en meeldraad primordia verandert van hoofdzakelijk kern gelokaliseerd naar meer cytoplasmatisch gelokaliseerd. In latere stadia lijken de GFP-gelabelde SEP3 eiwitten in het midden van de primordia te verdwijnen zonder dat het *SEP3* mRNA daar verdwijnt. Deze twee processen zouden op een posttranscriptioneel niveau door twee mechanismen veroorzaakt kunnen worden, namelijk SEP3 eiwitafbraak door het 26s proteasoom en epidermaal-georiënteerd intercellulair transport van SEP3 eiwitten. Deze twee mechanismen worden nader besproken in dit hoofdstuk. Verder laten we zien dat er geen duidelijke aanwijzingen zijn dat het waargenomen gedrag van GFP-gelabeld SEP3 veroorzaakt wordt door de aanwezigheid van het F-box eiwit UNUSUAL FLORAL ORGANS (UFO) dat bloemblad en meeldraad ontwikkeling reguleert.

In Hoofdstuk 7 eindigt dit proefschrift met een aantal concluderende opmerkingen over *in planta* microscopie en de mogelijke mechanismen van MADS domein eiwit transport in het bloemmeristeem. Verder speculeren we over het belang van MADS domein eiwit transport voor het creëren van MADS box gen expressiepatronen en MADS domein eiwit gradiënten, als ook over de noodzaak van symplastisch geïsoleerde gebieden voor correcte bloemontwikkeling.

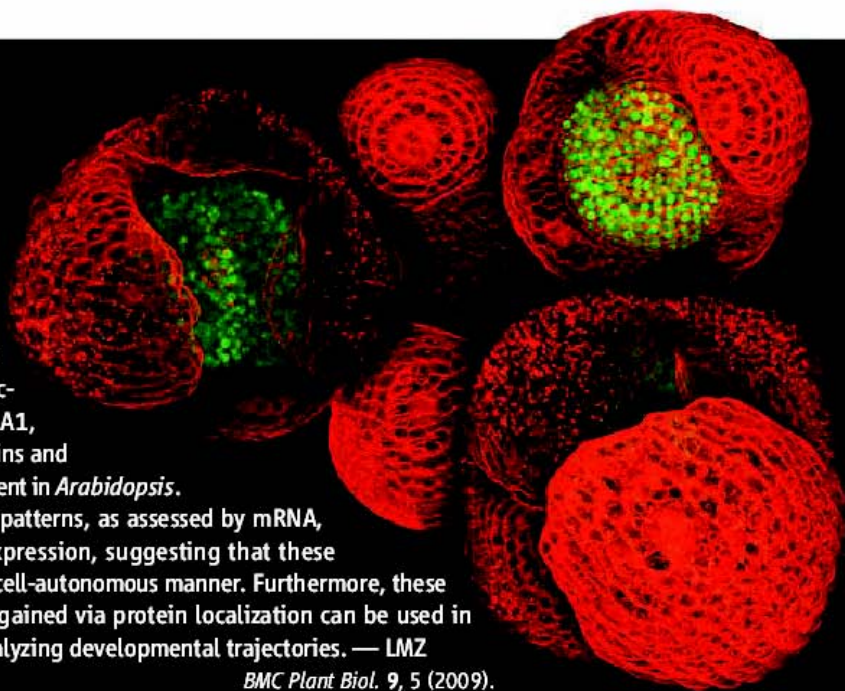


## GENETICS

### Focusing on Flowers

Transcription factors play many important roles in flowering plants, including the spatial and temporal regulation of highly conserved events in floral development. Urbanus *et al.* have mapped the localization of four such factors—AGAMOUS, SEPALLATA3, APETALA1, and FRUITFUL—that contain MADS domains and are associated with floral organ development in *Arabidopsis*. Their results show that gene expression patterns, as assessed by mRNA, are not fully correlated with protein expression, suggesting that these transcription factors are acting in a non-cell-autonomous manner. Furthermore, these data support the view that information gained via protein localization can be used in concert with mRNA measurements in analyzing developmental trajectories. — LMZ

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## Acknowledgements

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In my life I have had many variations of my name. I started out with the official name Susanna, which was rapidly changed into Susanneke when I was a baby and then shortened to Sanne. At a certain point in my life I foolishly decided to change my name to Susan, because there was this other girl in my class who was also called Sanne. Of course, not long afterwards it turned out there were actually a lot more girls called Susan (or a variation on this spelling). I decided to keep the name, although at home and with old friends I was still called Sanne or San. Suddenly people started to call me Suus, which I did not like very much. Several years later, during my PhD time, I had to get used to a nickname that was marginally better than Suus, given to me by a guy with an insatiable appetite for giving everybody a nickname. I tried to fight it, but a lot of people in the lab picked up on this nickname. In the end resistance was futile, so I gave up. Now I am known in the lab as Suzie. Thanks a lot!

I would like to thank Gerco Angenent for giving me the opportunity to do my PhD in his Plant Developmental Systems group at Plant Research International in Wageningen. In the beginning of my PhD I was also supervised in the lab by Stefan de Folter and later on Richard Immink became my main supervisor. During this time I learned a lot about being an independent scientist. I want to thank all three of you for the supervision and guidance during these years and I think we got some nice papers out of the research.

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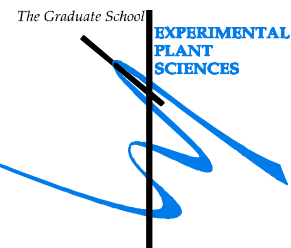
And of course I would like to thank Chiel for going through this with me and for not only being my partner in my personal life, but also my partner in science.





Susanna Leonora Urbanus was born on the 25<sup>th</sup> of December 1978 in Nijmegen, the Netherlands. At VU University in Amsterdam she studied Biology with specialisations in molecular plant biology, plant ecology, and developmental biology. She graduated *cum laude* in 2004. In this same year she started her part-time PhD in the Plant Developmental Systems group of Prof. Gerco Angenent at Plant Research International in Wageningen, affiliated with the Laboratory of Molecular Biology of Wageningen University. Here she studied MADS domain transcription factor function and movement during floral development in the model plant *Arabidopsis thaliana*. The results from that study are summarized in this thesis.





Issued to: **Susan L. Urbanus**  
 Date: **19 May 2010**  
 Group: **Plant Developmental Systems (Bioscience), PRI & Laboratory of Molecular Biology, Wageningen University and Research Centre**

<b>1) Start-up phase</b>	<u>date</u>
<ul style="list-style-type: none"> <li>▶ <b>First presentation of your project</b> Signal transduction by transcription factors: from synthesis to transcription regulation</li> <li>▶ <b>Writing or rewriting a project proposal</b></li> <li>▶ <b>Writing a review or book chapter</b></li> <li>▶ <b>MSc courses</b></li> <li>▶ <b>Laboratory use of isotopes</b></li> </ul>	<p>Sep 29, 2004</p>

*Subtotal Start-up Phase* 1.5 credits\*

<b>2) Scientific Exposure</b>	<u>date</u>
<ul style="list-style-type: none"> <li>▶ <b>EPS PhD Student Days</b>                      PhD student day 2005, Radboud University Nijmegen                      PhD student day 2006, Wageningen University                      PhD student day 2007, Wageningen University                      1st joint retreat of the PhD Students in Experimental Plant Sciences in Wageningen</li> <li>▶ <b>EPS theme symposia</b>                      Theme 1 Developmental Biology of Plants, Wageningen University                      Theme 1 Developmental Biology of Plants, Leiden University                      Theme 1 Developmental Biology of Plants, Wageningen University                      Theme 1 Developmental Biology of Plants, Leiden University</li> <li>▶ <b>NWO Lunteren days and other National Platforms</b>                      2nd Dutch Chromatin Meeting Wageningen                      Grand Opening Netherlands Proteomics Centre Utrecht                      1st Leica Confocal User Meeting                      ALW lunteren days 2005                      Netherlands Proteomic Centre Fall meeting: Protein networks                      ALW lunteren days 2006                      ALW lunteren days 2008                      Progress meeting of Netherlands Proteomics Centre</li> <li>▶ <b>Seminars (series), workshops and symposia</b>                      Systems Biology Symposium                      Flying seminar Prof Joseph Ecker                      Flying seminar Prof Philip Benfey                      Flying seminar Prof Jim Carrington                      Flying seminar Prof Hiroo Fukuda                      Flying seminar Prof Richard Vierstra                      seminar Prof Jan Lohmann                      seminar Prof Roger Tsien at the MPI for biochemistry in Munich                      EPS Symposium Intracellular Signalling                      Bioscience special seminars                      personal confocal workshop at Leica company in Mannheim                      personal confocal workshop at Nikon company in Badhoevedorp                      NVBMB spring symposium organized by Dolf Weijers                      KNBV Genetics, development and functioning of flowers</li> <li>▶ <b>Seminar plus</b>                      Seminar plus Prof. Hiroo Fukuda</li> <li>▶ <b>International symposia and congresses</b>                      International MADS meeting in Gargnano, Italy                      8th International Botanical Microscopy Meeting in Salzburg, Austria                      19th International Conference on Arabidopsis Research in Montreal, Canada</li> <li>▶ <b>Presentations</b>                      Gargano, Italy: In planta localization of endogenously expressed MADS box transcription factors                      Molecular Biology, WU: Inter/intracellular trafficking of MADS box transcription factors                      PRI Bioscience thematic meeting: transcription factor trafficking and complex formation                      Salzburg, Austria: In planta localization and dynamics of MADS box transcription factors                      PRI Bioscience thematic meeting: transcription factor localization and movement                      PRI Bioscience thematic meeting: transcription factor localization and movement                      WU Biochemistry: Transport and in planta localization of MADS box transcription factors                      Montreal, Canada: Poster "In planta localization and dynamics of fluorescently tagged MADS box transcription                      Wageningen, the Netherlands: MADS transcription factors                      Leiden, the Netherlands: Dynamic behaviour of MADS domain proteins in flowers                      PRI Bioscience thematic meeting: Transcription factor localization and movement</li> <li>▶ <b>IAB interview</b></li> <li>▶ <b>Excursions</b></li> </ul>	<p>Jun 02, 2005                      Sep 19, 2006                      Sep 13, 2007                      Oct 01-02, 2008                      Apr 26, 2005                      May 12, 2006                      Oct 11, 2007                      Jan 30, 2009                      Jan 21, 2005                      Feb 03, 2005                      Apr 28, 2005                      Apr 04-05, 2005                      Nov 04, 2005                      Apr 03-04, 2006                      Apr 07-08, 2008                      Feb 19, 2008                      Nov 04, 2004                      Sep 26, 2005                      Oct 24, 2005                      Apr 13, 2007                      Nov 26, 2007                      Apr 14, 2008                      Jun 26, 2008                      Sep 11, 2009                      Feb 02, 2006                      2004-2009                      Dec 21, 2006                      Feb 28, 2007                      May 18, 2007                      Sep 28, 2007                      Nov 26, 2007                      Oct 23-26, 2005                      Mar 31-Apr 05, 2007                      Jul 23-27, 2008                      Oct 23-26, 2005                      Feb 10, 2006                      May 02, 2006                      Apr 01, 2007                      May 01, 2007                      Apr 22, 2008                      May 28, 2008                      Jul 23-27, 2008                      Oct 02, 2008                      Jan 30, 2009                      Apr 14, 2009                      Sep 14, 2007</p>

*Subtotal Scientific Exposure* 22.4 credits\*

<b>3) In-Depth Studies</b>	<u>date</u>
▶ <b>EPS courses or other PhD courses</b> Systems Biology: principle of -omics data analysis Signalling in plant development and plant defence	Nov 07-10, 2005 Jun 19-21, 2006
▶ <b>Journal club</b> Plant Developmental Systems Literature Discussions	2004-2009
▶ <b>Individual research training</b>	

*Subtotal In-Depth Studies*      5.1 credits\*

<b>4) Personal development</b>	<u>date</u>
▶ <b>Skill training courses</b> Digital scientific artwork Advanced photoshop Scientific publishing workshop Time and project management PhD scientific writing Career orientation Grant writing workshop, Mennen Training & Consultancy NWO Talent Class Creative Thinking	Dec 14-15, 2004 Dec 16-17, 2004 Oct 13, 2005 Oct - Nov, 2006 Sep - Nov, 2007 May-Jun, 2008 Oct 08, 2009 Dec 08, 2009
▶ <b>Organisation of PhD students day, course or conference</b>	
▶ <b>Membership of Board, Committee or PhD council</b>	

*Subtotal Personal Development*      6.9 credits\*

<b>TOTAL NUMBER OF CREDIT POINTS*</b>	<b>35,9</b>
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Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS which comprises of a minimum total of 30 credits

\* A credit represents a normative study load of 28 hours of study

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