Physical interactions among plant MADS-box transcription factors and their biological relevance

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Propositions

1. The ability to visualize and monitor physical interactions between proteins in a living cellular environment enables biologists to see the basis of life.
this Thesis

2. FRET-FLIM (Fluorescence Resonance Energy Transfer) - (Fluorescence Lifetime Imaging) has proven to be a very robust method to detect direct physical interaction between molecules of interest.
this Thesis (Gadella *et al* 1995, Gadella *et al.* 1999, Wallbarbe and Periasamy, 2005)

3. Proteins in living cells work as part of molecular networks that have specific functions, such as gene expression, energy transduction or membrane transport. One of the next objectives of cell biology is to quantify the flow of materials, information and energy through these molecular networks.
Sako and Yanagida (2003) Nature Reviews Molecular Cell Biology

4. Brightness encodes the stoichiometry of protein complexes.
Chen and Müller (2007) PNAS

5. Science should be autonomous, but is not value-free.
Drenth (2006) Science and Engineering Ethics

6. Most eminent women prefer to be remembered for their achievements rather than their X chromosomes.
Fara (2007) Nature

7. Deadlines are meant to qualify subordinates and not superiors.

8. To be successfully adapted to The Netherlands, one should have a precise watch, a busy agenda and being constantly complaining about the bad weather.

Propositions belonging to the thesis:

"Physical interactions among plant MADS-box transcription factors and their biological relevance"
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Contents

Chapter 1

Dynamics of the eukaryotic transcription process

Isabella A. Nougalli Tonaco, Gerco C. Angenent and Richard G.H. Immink

Transcription is universal

Making use of four nucleotides only, the genetic code, which is the greatest combinatorial matrix of life, defines the majority of the cellular processes existing in nature. Transcription is basically how organisms are able to read and interpret this genetic code, and therefore stands at the basis of life. The general mechanism of transcription is generic among different eukaryotes, and being nevertheless, far more complex in comparison to prokaryotes. One elementary difference between prokaryotes and eukaryotes relates to their cellular complexity and organization. Eukaryotes have their genomic DNA localized within a nucleus, instead of having the nuclear material spread in the cytoplasm, like prokaryotes. The organization of DNA within a nucleus makes transcription simple on one hand, because the target DNA and the proteins involved in the transcription process are kept together at a specified location; however, on the other hand the tight packaging of DNA within the nucleus requires a dynamic, complex, and efficient transcription machinery. Since transcription is universal and an essential process, cell and molecular biologists have studied it for decades by sophisticated molecular, cellular and micro-spectroscopy techniques, and made great advances in the understanding how this process works. Here, we will highlight the recent literature that reports about scientific achievements in the field of the eukaryotic transcription machinery, with a special focus on transcriptional dynamics in plants.

The nucleus and its components

Transcription takes place in the nucleus of eukaryotes and in a simplistic interpretation this organelle can be divided into three main parts: the nuclear membrane, the nucleolus and the genetic material (DNA). The nuclear membrane, or nuclear envelope, is formed by two membranes that contain pores, which permit the flow of transcription factors, chromatin remodeling factors and other molecules into the nucleus, as well as the transport of ribosomal particles, transcribed mRNA and other factors from the nucleus into the cytoplasm, where protein synthesis takes place. Further, it has been recently reviewed by Akhthar and Gasser (2007) that, at least in yeast, the more active part of the chromatin is physically organized close to the nuclear pores, whereas most of the heterochromatin is located on the inner membrane of the nuclear envelope.

The second easily recognizable nuclear structure is the nucleolus, which is involved in several nuclear functions, like the synthesis of ribosomal RNA (rRNA), assembly of the ribosomal subunits and finally in the generation of RNAs and RNA polymerases (reviewed in Raška *et al.*, 2006). Recently, Andersen and colleagues (2005) have investigated the proteome of the human nucleolus by mass spectrometry, which provides various perspectives for further investigation of this multifunctional organelle. Furthermore, in this study the flux of multiple endogenous nucleolar proteins was followed, making use of GFP-tagged proteins and triggering of cells by inhibitory compounds that affect nucleolar morphology. This analysis revealed that the nucleolus is a very dynamic organelle, with significant changes in protein content over time and in response to various stimuli and growth conditions. In plants, similar investigations have been done by Pendle and colleagues (2005) and a large number of nucleolar proteins could be identified. Interestingly, EJC-like (exon-junction complex) proteins were found, suggesting a possible role for the plant nucleolus in mRNA processing, since these EJC proteins are suggested to be involved in processing pre-mRNA in animals (Custodio *et al.* 2004). Encouraged by these findings, Brown and colleagues (2005) established the Arabidopsis nucleolar proteome database, which is a good starting point for studies within this field.

Last but not least, the cell nucleus contains the genetic material, or DNA. In eukaryotic cells, the DNA is organized in a very compact manner and is present as chromatin (reviewed in Pederson, 2004). Nucleosomes are the basic sub-units of chromatin and are composed of eight histone molecules, around which the DNA is wrapped. Nuclear regions where the chromatin shows higher density are called chromosome territories. These chromosome territories and interchromatin compartments (CT-IT, see Lanctot *et al.*, 2007) have been identified for the first time in the 70's in mammalian cells (for review see Cremer and Cremer, 2001).

From genes to the genome and vice-versa

Throughout the cell cycle and developmental stages, DNA is present within the nucleus at different forms of organization. This organization, which is mediated by packaging and condensation of chromatin, may be essential not only for genome replication, but also for the regulation of gene expression, i.e. making parts of the genome accessible to the transcription machinery when a specific set of genes needs to be transcribed. Generally, chromatin can be present in two forms; compact,

condensed and mainly inactive heterochromatin, and the more open and dynamic euchromatin. Changes in the chromatin state are achieved by chromatin modifications and chromatin remodeling. During chromatin modifications the changes are covalent either on DNA, histone tails, or in the histone core, whereas during remodeling the changes alter DNA-histone interactions, often as reaction on a modification (Seob Kwon and Wagner, 2007). The best known chromatin modifications are DNA methylation and histone post-translational modifications (histone PTM), like (de-)acetylation, (de-)ubiquitination, (de-)phosphorylation, and (de-) methylation. None of these modifications are unidirectional related to either activation or repression of gene expression; in contrast, they can cooperate in both ways, i.e. not only acting as repressor or activator, but also vice-versa (Berger, 2007). For example DNA methylation as well as histone PTM can contribute to the disruption of DNA-histone interactions, facilitating the assembly of the RNA polymerase and transcription machinery onto the DNA during gene regulation. Another remarkable point is that such modifications can occur not only to the core promoter site and the transcribed regions, but also to other regulatory sites which are localized distant from the gene locus. Recently, a very elegant experiment has been published by Zhang and colleagues (2006), to identify methylation sites in the genome of the model plant *Arabidopsis thaliana*. In this study, a DNA methylation map could be obtained for the whole Arabidopsis genome, which provides evidence that most methylation occurs within coding regions, whereas surprisingly, methylation of promoter regions appeared to be much less abundant. A nice example of the role of chromatin modification and its interference in gene regulation of developmental processes in plants has been described by Perales and Mas (2007), who identified that histone acetylation and deacetylation of the *TOC1* locus (one of the components of the plant oscillator) is rhythmically controlled by the circadian clock. The current hypothesis is that all these histone and DNA modifications lead to chromatin remodeling and that the association or dissociation of DNA-histone complexes contributes to the movement of nucleosomes and the packaging or release of genomic DNA, enabling repression or activation of genes, respectively. Recently, an elegant model for chromatin remodeling has been proposed by Seob Kwon and Wagner (2007), which includes a description of the SWI/SNF ATPase family of proteins. These important chromatin remodellers are found in several eukaryote

organisms and most likely hold similar functions related to chromatin organization (Figure 1).

Zooming in at the DNA part of chromatin, genes can be identified that might consist of exons and introns, long stretches of non-coding DNA that include basic promoter elements, like the TATA-box binding site, and enhancers (activators), repressor elements, and insulator sequences that set specific chromatin boundaries (Green, 2000; Wei *et al.*, 2005). The exact organization of all these regulatory elements within the genome of eukaryotes plays a significant role in transcription regulation and in this respect there is a clear difference with prokaryotes. Even though, a lot of research has been done on genome organization, a great part of the current information regarding genome size and organization is not yet complete, making further studies necessary. A nice example of this has been described for human chromosomes 21 and 22, from which the detectable number of transcribed exons in some cell lines appeared to be approximately ten times more than the number of exons that are annotated at the moment (Kapranov *et al.*, 2002). Like in animals, plants have similar nuclear organization and compartmentalization and definitely, research of the plant model species *Arabidopsis thaliana* has contributed to our further understanding in this field. Besides many similarities, also differences between plants and animals have been notified and an interesting feature of the plant genome structure and organization that differentiates plants from animals, regards how the non-coding regions are positioned in the chromosomes and how genes are organized. In animals, as well as in *C. elegans*, highly transcribed genes contain in general just a few introns. In contrast, plants, like Arabidopsis and rice for instance, have their highly transcribed genes in a less compact form (Ren *et al.*, 2006). This difference in intron size and abundance might be linked to their function in gene expression, although the relationship between gene regulation and size and number of introns is currently poorly understood. Further, the existence of introns might be linked to differences in mRNA stability, even though no strong evidence is found in this respect for yeast; however, Arabidopsis genes which code for the most unstable mRNA's appeared to have fewer intronic regions in comparison to other genes (Wang *et al.*, 2007).

Eukaryotic genome organization and transcription

The eukaryotic genome contains many sets of genes that need to be transcribed simultaneously and that are under very tight control. Considering that genome organization plays an important role during gene regulation, the next question that we can ask ourselves is: "How does the genome make available certain stretches of DNA, which allows transcription factors to specifically recognize their binding sites and promote gene expression?" From the discussion above, it is clear that chromatin modifications and remodeling determine the balance between euchromatin and heterochromatin, and in this way have an effect on the activation or repression of genes. A nice example of this has been described by Tessadori and colleagues (2007), who observed a clear change in chromatin decondensation during the transition to flowering, mediated by the blue-light receptor CRY2. Recent studies making use of sophisticated techniques like FISH and chromosome capture (3C) provided the first experimental basis, how active or inactive genes that are far from each other in the chromosomal range, are able to come in close contact (reviewed in Cremer and Cremer 2001; and Cremer *et al.* 2005). Another elegant example of this has been described by Simonis and colleagues (2007), who developed a (4C) chromosome conformation capture method to investigate the chromosome surrounding of the mouse β-globin locus. The method is based on PCR amplification of DNA fragments, which enables the identification of different loci within the nuclear environment. With this new technology, they could identify signals from 5 up to 10 Mbp apart from the chromosomic region where the β-globin locus is present, which clearly indicates that a set of genes quite distantly located, can be in close contact during the transcription process and hence, be co-regulated. Further, it has been shown that chromosome with a large number of very active genes are in general located in a more interior part of chromosomes territories (Tanabe *et al.*, 2002). However, how this is structurally related to the transcriptional activity is unclear at this moment. In the plant nucleus, the chromosomes are also organized in territories and these dense regions are surrounded by chromatin loops. In *Arabidopsis* for instance, the chromosomes contain the so-called chromocentres. These six to ten clearly defined regions are generally targeted by DNA methylation and constitute an organizing center from which chromatin loops emanate (Fransz *et al.*, 2002). A nice example of such loops has been found on the small arm of chromosome 4, where a very large chromatin loop is formed (Fransz *et al.*, 2002, van Driel and Fransz, 2004).

Obviously, all these structural organizations and modifications have an influence on gene regulation and by this directly affect developmental processes.
From all this work it is clear that genes are able to move outside the chromosome territories and that the regulation of gene expression goes beyond the chromatin and requires dynamic rearrangement of chromosomal domains (Shavtal *et al.*., 2006, Manderuzzo *et al.*, 2007). Like chromatin, also other parts of the transcription machinery seem to be in constant movement. Elements like the DNA polymerase and specific transcription factors appeared to be very mobile, meaning that transcription is spatially dynamic. In the next paragraph an overview will be given about the functioning of these trans-factors in the transcription process.

Trans - factors

During transcription, specific transcription factors bind to DNA (*cis* elements) and in this way activate or repress genes (Riechmann, 2002) (Fig 1). Transcription factors are very mobile and can be found associated to so-called transcriptional centers within the cell nucleus. However, these transcription factors are not immobile at these transcription centers "waiting for" transcription to occur, but very likely will be recruited to these centers together with the genomic regions, when transcription takes place (Jackson, 2003). Besides these transcription centers that are also known as "transcription factories" (Jackson, 2003), the genome consists of regions where several transcription factors are bound, and therefore have been termed "hot spots". The existence of transcription factor "hot spots" has been described by Moorman and colleagues (2006) based on experiments aiming at the development of a map of *in vivo* binding sites for seven different transcriptional regulators using chromatin immunoprecipitation (ChIP) on the genome of *Drosophila melanogaster*. So far, it is not clear why these "hotspots" exist and what specific function these structures have, nevertheless, three hypotheses were presented in their study. The first model suggests that the "hot spots" are functioning like "sinks" or "buffers", requesting many regulatory molecules, which would allow a fast response upon an inducing stimulus. The second model proposes that these regions may function similar to enhancers that promote transcription. Finally the third model predicts that the "hot spots" play a role as mediators of physical interactions between loci that are far away from each other within the genome. More detailed investigations in the near future will hopefully shed light on the exact function of these "hot-spots".

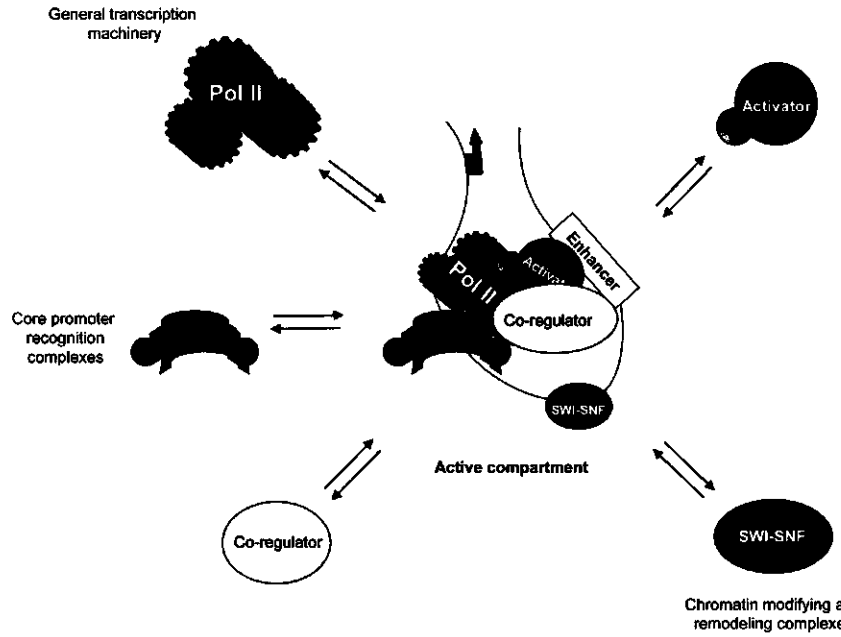

Figure 1. Schematic drawing of the transcription machinery and its components
The transcription machine is composed of: RNA polymerase II and its subunits, the core promoter elements, activators (transcription factors), co-regulators and chromatin-modifiers and remodelers (adapted from Isogai and Tjian 2003).

Another important *trans* factor present in the transcription machinery is RNA polymerase II, which can be seen as the molecular motor of the transcription process. Its mobility has been nicely shown by Kimura and colleagues (2007), who followed up the dynamics of this enzyme in living mammalian cells. For this purpose, the largest (catalytic) sub-unit of RNA polymerase II was tagged with GFP. Subsequent, microscopic analyses based on photo bleaching techniques, like FRAP (Fluorescence Recovery After Photo Bleaching) and FLIP (Fluorescence Loss In Photo Bleaching), revealed that approximately 75% of the tagged protein is mobile, meaning that the polymerase is in movement for the largest part of a transcription cycle. However, following the dynamics of each individual component of the transcription machinery and monitoring protein-DNA interactions may not be sufficient to unravel the dynamics of transcription. To get a better understanding of this aspect, we need to go beyond that and monitor how genome architecture acts in space and time in conjunction with the main components of the transcription

machinery (Jackson, 2003; Cook, 2003). In the next paragraph, we will present and discuss the present models that explain action at a distance during gene regulation.

Transcription evokes dynamic models

Considering that the genome organization within the chromosome territories is definitely not random and that the major elements of the transcription machinery such as RNA polymerase II and transcription factors are very mobile, our next question to be answered reflects one of the greatest paradigms in molecular genetics: "How do transcription factors search for their DNA binding sites and finally regulate gene expression in a dynamic manner?". To answer this question, many studies were performed and several models have been proposed in order to explain how regulatory proteins are seeking for their binding sites at distance and further, how finally DNA-protein and protein-protein interactions are able to regulate gene expression. Originally, the "looping", "twisting", "sliding", and "oozing" models (Fig. 2) have been suggested in order to exemplify how transcriptional regulators identify their binding sites (Ptashne, 1986); and more recently, another model: "hoping" (Fig. 2), has been integrated. In this respect, it is important to emphasize that these models are not only proposed for gene activation but might also explain the spreading of chromatin silencing (Talbert and Henikoff, 2006; Phair *et al.*, 2004). The different models for site-specific DNA-binding proteins can be summarized by the "facilitated diffusion model", in which non specific DNA-protein interactions mediate "walking" over the genome in search for specific target sequences (*cis* elements). In this overall model, either a one-dimensional or a three-dimensional ('sliding' or 'hoping') diffusion occurs during the search for specific binding-sites. Finally proteins are able to move between "protein–DNA" binding sites by the formation of loops in the DNA (Halford and Marko, 2004). To be more precisely, in the looping model, which is the most favorable for action at distance, distant chromosomic regions are brought together during gene transcription. Most likely these loops are formed upon protein-protein interaction and DNA-binding, which mediate the loop formation within the chromosomes territories. The twisting model requires a conformational change of DNA for the binding of the regulatory protein and this change may occur either by direct protein-DNA interactions, or alternatively, by enzymatic action of a protein that triggers subsequently other protein-DNA binding interactions. Sliding is another favorite; in this case one protein binds to DNA at one specific site and moves along

the DNA until it reaches a strong binding site or another protein elsewhere on the genome, which may be an interacting partner. In the 'oozing' model, the binding of one regulatory protein at a particular site of the DNA mediates the binding of other proteins in adjacent regions, until the complete protein complex is formed and transcription can be initiated. Finally, the hoping model, in which the protein moves from one site to another in a three dimensional space, associating and dissociating with nearby sites, which could even be another chromosome (reviewed by Halford and Marko, 2004). From the different models, the looping has been the one which explains interactions over chromosomal distances and this loop formation gave rise to the term "molecular ties", which are basically the so-called "transcription factories" where during transcription, DNA may form a loop around it (Cook, 2003; Manderuzzo *et al.*, 2007).

Most of the evidence for the above mentioned models comes from studies with fixed material and up till recently, hardly any real time *in vivo* experiments have been described that provide additional proof for any of the proposed models. Although demonstrated for prokaryotic cells, Elf and colleagues (2007) reported for the first time the specific DNA binding time, or time of residence, for the Lac1 transcription factor and its DNA binding sites in living cells. In this study, the Lac1 protein was labeled with the Yellow Fluorescent Protein (YFP) *Venus*, followed by single molecule detection FCS (Fluorescence Correlation Spectroscopy). Their experiments showed that approximately 90% of its life time, the Lac1 dimer was not specifically bound to its DNA binding sites, but instead, freely sliding along the DNA, which directly supports the "sliding" model. Nevertheless, the real situation is probably reflected the best by a combination of the various proposed models. The transcriptional mechanism appeared to be based on the cooperative dynamic association between *CIS* and *TRANS* elements. On one hand chromatin loops are formed within the chromosome territories, which enable distant loci to come in close contact and in this way facilitate gene regulation, whereas on the other hand transcription factors and other *TRANS* elements are able to move freely within the nucleus.

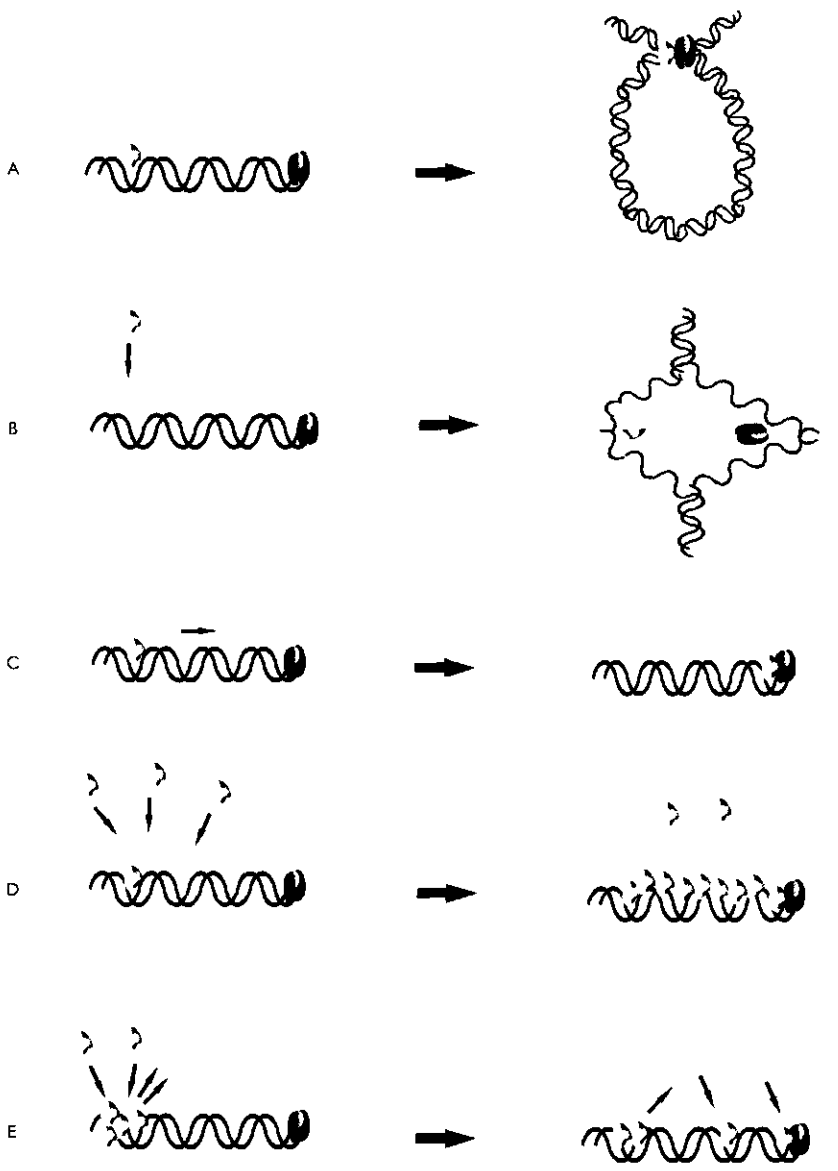

Figure 2. Different models for gene regulation at distance.
Looping model: distant loci are brought together by the formation of a DNA loop and interaction between regulatory proteins; (b) Twisting model requires a conformational change of the DNA during transcription; (c) Sliding: protein binds to one site of the DNA and slides through until it finds a strong binding site or its interaction partner at the right transcriptional site; (d) Oozing: the binding of one protein facilitates the other's binding; (e) Hopping: when the protein binds to one transcription site and 'hops' to another. Yellow spheres code for activating or silencing proteins and red spheres code for the RNA polymerase II or other proteins (adapted from Talbert and Henikoff, 2006).

Plant transcription factors: MADS-box family as model to study transcriptional regulation

In Arabidopsis, around 1800 genes, or approximately 6% of the total number of genes, encode for transcription factors, which can be subdivided into different families according to their DNA binding domain (Riechmann, 2002). Among these transcription factor families in plants, MADS-box proteins are of great importance for plant architecture and flower development (for review see Ferrario *et al.,* 2004). A model describing the molecular mode of action for MADS-box transcription factors was initially proposed based on experiments with *Anthirrinium,* and additional supportive evidence came from studies in Arabidopsis and other species. According to the model, known as "quaternary model", two independent dimers (homo- or heterodimers) are able to assemble into a higher order complex, which upon specific DNA binding, bend the DNA and promote the regulation of target genes (Egea-Cortines *et al.* 1999; Theißen and Saedler, 2001, Theißen 2001). This direct interaction and complex formation of transcription factor proteins appeared to be a general mechanism by which proteins with very similar DNA binding domains achieve regulatory specificity and regulate transcription (Riechmann and Meyerowitz, 1997). Based on this knowledge, we can hypothesize that the "looping" model is applicable for MADS-box proteins and that the understanding of how these proteins interact to each other might elucidate how specificity and proper transcriptional regulation is organized. MADS-box proteins have shown to dimerize in the cytoplasm, followed by transport to the nucleus, where probably specific higher order complexes are assembled (MacGonigle *et al*, 1996; Immink *et al.*, 2002; Nougalli-Tonaco *et al.*, 2006, chapter 2). Whether the assembly occurs on the DNA or independent from the binding site is not known, although yeast 3- and 4-hybrid studies demonstrated that higher order complexes can be formed in the absence of native DNA binding sites. We have demonstrated indirectly the formation of a higher-order complex between the *Petunia hybrida* MADS-box proteins FLORAL BINDING PROTEIN2 (FBP2), FBP11 and FBP24 in living plant cells, by the use of FRET-FLIM (Fluorescence Resonance Energy Transfer-Fluorescence Lifetime Imaging). In this study, we analyzed whether the FBP2 protein could function as a ternary factor that stabilizes the transient interaction between the proteins FBP11 and FBP24 (Nougalli-Tonaco *et al.*, 2006). FBP11 and FBP24 appeared to interact in sub-nuclear spots only, whereas interaction between these two proteins could be detected over the

whole nucleus after addition of the FBP2 protein, which strongly suggests that FBP2 binds to the transient heterodimer. As discussed before, in mammalian cells transcription most likely occurs in so-called "transcription factory", regions within the nucleus where the transcription machinery is present (RNA polymerase II, activators and other co-factors). From our FRET results we could speculate that the transient interaction between FBP11 and FBP24 probably occurs at places within the nucleus where these "transcription factories" are localized (Nougalli-Tonaco *et al.*, 2006). The ability of MADS-box proteins to assemble into higher order complexes has been monitored by several independent methods, and a recently performed gel-filtration experiment revealed that the FLC (FLOWERING LOCUS C) MADS-box protein is present in large multimeric complexes *in vivo* (Helliwell *et al.*, 2006). The exact stochiometry of these complexes is not known, but it becomes more and more clear that MADS-box proteins are able to interact not only with members of the family but also with different types of regulatory proteins. One example of this kind of interactions has been recently shown by Brambilla and colleagues (2007), who were able to detect interactions between the homeodomain transcription factor BELL1 and the ovule identity MADS-box proteins.

Conclusions

This overview summarizes the latest advances in our understanding of the transcription machinery in eukaryotes and the dynamic components of this 'machine'. It is clear that these components are much more mobile and dynamic than has been thought for many years. This dynamics allows the transcription factor to search for its specific binding site on the DNA, although it is not known how such a factor recognizes the right target site. The presence of the target sequence motif (e.g. CArG box for MADS-box proteins) is not sufficient to explain the specificity of binding, because these motifs are very abundant in the genome. Probably, small differences in TF-DNA binding affinities, reflected in the "time of residence", determine whether transcription occurs or not. A second mode of dynamics in the nucleus concerns the movement and bending of the DNA (e.g. DNA ties), which allows a close contact between distant genes in transcription factories, and brings together proteins that are essential for transcription initiation. Using novel live imaging technologies, researchers will further endeavor the various aspects of the transcription machinery that has been laid down in the physical models describing the dynamic interaction

between DNA and protein complexes. These studies will in combination with novel live imaging technologies, help biologists to solve this complex life's puzzle in the near future.

Outline of this thesis

As described in this chapter, our understanding of transcriptional regulation and its dynamics in eukaryotic cells is still fragmentary and we need advanced technologies to monitor the action of transcription factors in living cells. The goal of the work described in this thesis was to get a better understanding of the molecular action of transcription factors in living plant cells and for this we focused on the genetically well-characterized MADS-box transcription factor family. As a strategy of choice, we used various non-invasive sophisticated micro spectroscopy techniques, which are predominantly based on FRET (Fluorescence Resonance Energy Transfer) that allows the analysis of inter-molecular dynamics in living plant cells.

In chapter 2, we describe the analysis of the interactions between three different *Petunia hybrida* MADS-box proteins involved in ovule development, by means of FRET-FLIM (Fluorescence Resonance Energy Transfer-Fluorescence Lifetime Imaging). In this chapter, we provide evidence for the formation of stable MADS-box transcription factor heterodimers in vivo, and the possible stabilization of a weak dimer by a third protein through the formation of a higher order protein complex. Furthermore, we speculate about a potential function for the formed complex during ovule development.

In chapter 3, similar analyses were performed, but now with *Arabidopsis thaliana* MADS-box transcription factors involved in petal and stamen formation. Here, we could demonstrate clear differences in interaction strength between the various tested homo- and heterodimers and we hypothesized that this must be a crucial aspect of the partner selection mechanism, finally giving rise to the formation of only a selective set of specific stable complexes.

The results described in chapter 3 pointed to differences in interaction strength depending on the pairs of proteins available. A limitation of the pair-wise FRET assay is that the analysis of competition for dimerization and the formation of higher-order complexes involving at least three labeled proteins are not possible. Therefore, we developed a new FRET-based method designated "Competition- FRET", and this method and the results obtained are described in chapter 4.

Furthermore, we tried to implement the BiFC (Bimolecular Fluorescence Complementation) methodology in plants for the analysis of protein-protein interactions (chapter 5). In our case, the fluorescent molecule EYFP (Enhanced Yellow Fluorescent Protein) was divided into two non fluorescence parts and each part was fused to the MADS-box proteins under study. Upon protein-protein interactions, the two fluorescent molecules will be brought into close proximity, leading to the recovery of the fluorescent molecule and hence, a fluorescent signal at the location of interaction. For this purpose, several constructs were generated and tested, using different split positions of the YFP molecule, as well as different linker lengths between the fluorophore domains and the MADS-box proteins. Despite its theoretical simplicity and easiness, this methodology still needs to be further developed.

To get a better view on the stoichiometry of the MADS-box protein complexes, we decided to investigate the diffusion of complexes containing the MADS-box proteins AGAMOUS (AG) and SEPALLATA 3 (SEP3) by means of FCS (Fluorescence Correlation Spectroscopy) *in vitro* (Chapter 6). In these preliminary experiments, we observed that AGAMOUS diffuses two times slower than SEP 3. Comparison of the diffusion time with free YFP indicates that the AG protein is able to form multimeric complexes on its own. Furthermore, the co-translation of both proteins resulted in a decreased diffusion time, which is probably due to the formation of complexes of high molecular weight.

Finally, we performed the first experiments but not positive results on the analysis of direct physical interaction between protein partners in stably transformed plants by FRET-FLIM analyses (chapter 7). These plants expressed the MADS-box genes under the control of the endogenous promoters. The experiments revealed that the state-of- the art in the micro-spectroscopy field is not yet suitable for this type of experimental set-up. However, with the continuous advances in engineering new fluorescent molecules and new FRET-couples it is expected that monitoring protein interactions *in planta* will be possible in the near future.

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Chapter 2

In vivo imaging of MADS-box transcription factor interactions

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ABSTRACT

MADS-box transcription factors are major regulators of development in flowering plants. The factors act in a combinatorial manner either as homo- or heterodimers and they control floral organ formation and identity and many other developmental processes, through a complex network of protein-protein and protein-DNA interactions. Despite the fact that many studies have been done to elucidate MADS-box protein dimerisation by yeast systems, only little information is available on the behaviour of these molecules *in planta*. Here we provide evidence for specific interactions between the petunia MADS-box proteins FBP2, FBP11 and FBP24 *in vivo*. The yeast identified dimers for the ovule specific FBP24 protein have been confirmed in living plant cells by means of Fluorescence Resonance Energy Transfer (FRET)-Fluorescence Lifetime Imaging Microscopy (FLIM) and in addition, some, most likely, less stable homo- and heterodimers were identified. The followed *in vivo* approach revealed that particular dimers could only be formed in specific sub-nuclear domains. Moreover, we provided evidence for the *in planta* assembly of these ovule-specific MADS-box transcription factors into higher-order complexes.

INTRODUCTION

MADS-box genes represent a large multigene family in flowering plants and are involved in numerous developmental processes. In angiosperms, many of the genes belonging to this transcription factor family are involved in flower development, most notably in the determination of floral meristem and floral organ identity (Ferrario *et al.*, 2004a; Riechmann and Meyerowitz, 1997). The complete Arabidopsis genome sequence revealed the existence of over 100 MADS-box genes (Parenicová *et al.*, 2003; Martinez-Castilla and Alvarez-Buylla, 2003; Kofuji *et al.*, 2003; de Bodt *et al.*, 2003). During the last decade many members of the family have been subject to genetic studies in various plant species, which has led to the robust 'ABC' model as the paradigm for flower development in angiosperms. In addition, the functional characterization of a number of MADS-box genes has revealed regulatory roles for other MADS-box genes in flower induction, meristem formation and fruit development. In contrast to the enormous effort that has been put into this kind of analyses over the last decade, giving rise to detailed knowledge about MADS-box gene functions, virtually nothing is known about the molecular mode of action of the encoded proteins.

Analyses of MADS-box proteins have been mainly restricted to the MIKC type, which has a characteristic modular structure. From the N to the C terminus of the protein, four domains can be identified: the MADS-box (M), intervening (I), keratin-like (K), and C-terminal domains (Riechmann and Meyerowitz, 1997). The M-domain is the most conserved among all domains and consists of approximately 56-58 amino acids. It plays an important role in DNA binding and probably a minor role in dimerization. The I-domain is less conserved, varies in length, and is important for determining the dimerization specificity (Riechmann and Meyerowitz, 1997). The K-domain (~80 amino acids) contains several heptad repeats that most likely fold into amphipathic α-helices, which mediates dimerization (Yang *et al.*, 2003). The C-terminal domain is the least conserved and it has been shown that it is able to act as a transactivation motif for some of the plant MADS-box proteins and furthermore, it appears to be involved in higher-order complex formation (Egea-Cortines *et al.*, 1999; Honma and Goto, 2001, Yang and Jack, 2004).

The first studies aiming at the elucidation of the molecular mechanisms underlying MADS-box protein functioning using in vitro DNA binding approaches revealed that these transcription factors form specific dimers (Schwarz-Sommer *et al.*, 1992; Krizek

and Meyerowitz, 1996; Riechmann *et al.*, 1996; West *et al.*, 1998; Egea-Cortines *et al.*, 1999). In addition, the yeast two-hybrid system has been adopted very frequently, to obtain information about MADS-box protein-protein interactions. Comprehensive matrix based screens for petunia and Arabidopsis MADS-box transcription factor interactions have shown that these factors form specific homo- and heterodimers and that these interactions are conserved between different plant species (Immink *et al.*, 2003; de Folter *et al.*, 2005). A further complexity was proposed based on results obtained with Antirrhinum and Arabidopsis MADS-box proteins in yeast experiments (Egea-Cortines *et al.*, 1999; Honma and Goto, 2001). These experiments revealed that additional MADS-box proteins may bind to a dimer at the C-terminus forming a ternary or quaternary complex. Like dimerisation, this complex formation seems to be conserved, because similar complexes could be identified for Petunia, Arabidopsis and Chrysanthemum MADS-box proteins using yeast three- and four-hybrid screenings (Ferrario *et al.*, 2003; Favaro *et al.*, 2003; Shchennikova *et al.*, 2004). This ability of MADS-box proteins to form multimeric complexes suggests that they are active in a combinatorial manner and based on these findings the "quartet model" for MADS-box transcription factor functioning was hypothesized. According to this model, two dimers within a higher-order tetrameric complex recognize two different binding sites in the DNA sequence, which are brought into close proximity by DNA bending. Pursue on this model the control of floral organ identity is supposed to be driven by four different tetrameric transcription factor complexes composed of the "ABC" - MADS-box proteins (Theißen, 2001; Theißen and Saedler, 2001).

Despite that the yeast screenings can be performed in a high-throughput manner and offer a first glimpse on dimerization patterns and complex formation, they have many drawbacks, specially when it concerns transcription factors that often contain intrinsic transcriptional activation domains. Because of this, yeast methods give rise to false-positive and false-negative results and therefore, should be verified by *in-planta* studies (Immink and Angenent, 2002). Moreover, the ability to visualize and follow molecules and events in living cells has become an important aspect in cell biology (Lippincott-Schwartz and Patterson, 2003). Recently, innovative micro spectroscopic approaches have been developed in order to combine the high spatial resolution of microscopy with spectroscopic techniques to obtain information about the dynamical behavior of molecules (Gadella *et al.*, 1999; Hink *et al.*, 2002). Fluorescence Resonance Energy Transfer (FRET) based methods have become a key for the

detection of protein-protein interactions in living cells. In its principle, excited-state energy is transferred non-radioactively through space from a donor to an acceptor molecule. This energy transfer takes place only, if emission and excitation spectra of the fluorophore pair are overlapping and if the distance between the molecules is very small (within ~1 to ~10nm of each other). Hence, protein-protein interactions can be studied by fusing the proteins of interest to two fluorescent molecules with the right characteristics (Gadella *et al.*, 1999; Hink *et al.*, 2002). The combination of cyan (CFP) and yellow (YFP) fluorescent proteins has proven to be the best marriage for in-*planta* FRET studies (Immink *et al.*, 2002; Russinova *et al.*, 2004). FRET can be quantified by observing changes in the fluorescence lifetime of the donor using Fluorescence Lifetime Imaging Microscopy (FLIM) (Gadella *et. al.*, 1993; Borst *et al.*, 2003). In case of a protein-protein interaction, FRET will occur and the fluorescence lifetime of the donor molecule will decrease. The advantages of FLIM for the detection of FRET are that it is not dependent on changes in probe concentration, and that it is less sensitive to photo bleaching and other factors that limit intensity based steady state analyses (Chen and Periasamy, 2004).

With respect to MADS-box transcription factors, protein interactions in living cells have been shown only for a few petunia MADS-box proteins by means of FRET-Spectral Photo Imaging Microscopy (SPIM) and FRET-FLIM analyses (Immink *et al.*, 2002). The ovule specific FLORAL BINDING PROTEIN11 (FBP11) appeared to interact specifically with three closely related proteins, FBP2, FBP5 and FBP9 that belong all to the SEPALLATA clade of MADS-box proteins (Ferrario *et al.*, 2003). Recently, another ovule specific MADS-box gene *ABS* (*Arabidopsis B-sister* gene, Becker *et al.*, 2002) has been described, formerly known as *AGL32* and *TT16* (*Transparent Testa16,* Nesi *et al.*, 2002). The *abs* mutant is affected in seed coat pigmentation and probably to some extent in the integrity of the entire inner integument. The petunia *FLORAL BINDING PROTEIN24* (*FBP24*) gene appeared to be very close in sequence to *ABS* and is expressed in ovules, specifically (de Folter and Immink, unpublished results). Currently, it is unknown how the ovule specific FBP24 protein is acting at the molecular level and to which protein complexes it contributes. Therefore, we performed yeast two- and three-hybrid analysis to study FBP24 protein-protein interactions. Subsequently, FBP24 and its putative interacting partners were tagged with fluorescent proteins and expressed in protoplasts, which allowed the analysis of cellular localization and in-*planta* interactions using FRET-

FLIM imaging techniques. The obtained results improve our knowledge about plant MADS-box transcription factor functioning at the molecular level and provide information about their dynamics in living plant cells.

MATERIAL AND METHODS

Plant Material

The *Petunia hybrida* line W115 and *Cowpea* Black Eye California variety were grown under normal greenhouse conditions (16/8 hr light/dark, 20°C for petunia and 28°C for cowpea).

Plasmids construction

All the clonings were done following the Gateway™ system from Invitrogen (Carlsbad, CA). The complete ORFs of the MADS-box genes were PCR amplified using specific primers yielding entry clones. Vectors containing Cyan Fluorescent Protein (ECFP) and Yellow Fluorescent Protein (EYFP) under control of the CaMV 35S promoter (Immink *et al.*, 2002) were made Gateway compatible according to the Invitrogen manual. In addition, the coding region of the monomeric Red Fluorescent Protein (mRFP) (Campbell *et al.*, 2002) was cloned in the same vector backbone. Finally, expression vectors encoding the various MADS-box transcription factors tagged with a C-terminal fused fluorescent protein were obtained by an LR reaction.

Yeast two- and three-hybrid experiments

Two-hybrid analyses using the CytoTrap and the GAL4 system were performed as described previously (Immink *et al.*, 2003). For this purpose the entire FBP24 coding region was cloned in-frame in the pMYR, pSOSnes, and pADGAL4 and pBDGAL4 vectors. FBP24 was screened against 14 petunia MADS-box proteins in the GAL4 system (FBP2, FBP4, FBP5, FBP9, FBP23, pMADS12, FBP6, FBP7, pMADS3, FBP11, FBP26, FBP29, PFG, FBP24). Selection for interaction was performed, using the Histidine marker in combination with two diferent concentrations of 3 Amino-Triazole (3AT, 1 mM and 5mM), and by the Adenine marker. The three-hybrid experiments were done with a modified yeast two-hybrid GAL4 system as described by Ferrario *et al.* (2003).

Transient expression in Cowpea and Petunia protoplasts

Cowpea protoplasts were prepared and transfected according to Shah *et al.* (2002). Petunia protoplasts were obtained from W115 petunia leaves and transfected as described by Immink *et al.* (2002). Protoplasts were incubated overnight in protoplast medium at 25°C in the light for Cowpea, and in the dark for Petunia and subsequently imaged for fluorescence.

Localization studies in living cells

The imaging of the fluorescent fusion proteins was done by a Confocal Laser Scanning Microscope 510 (Carl Zeiss, Jena, Germany). Protoplasts were excited by 458 and 514 nm Ar laser lines for CFP and YFP, respectively. In addition, a 543 nm He laser line was used to excite mRFP. The pinholes were set at one Airy unit which corresponds to a theoretical thickness (full width at half-maximum) of 1μm. Images and data analyses were performed with Zeiss LSM510 software (version 3.2).

Fluorescence Lifetime-Imaging Microscopy

For FRET-FLIM analyses, cowpea protoplasts were analysed as described by Russinova *et al.* (2004), using a Bio-Rad Radiance 2100 MP system (Hercules, CA) in combination with a Nikon TE 300 inverted microscope (Tokyo, Japan). Two–photon excitation pulses were generated by a Ti:-Sapphire laser (Coherent Mira) that was pumped by a 5-W Coherent Verdi laser. The excitation light was directly coupled to the microscope and focused to the sample by the use of a CFI Plan Apochromat 60x water immersion objective lens (N.A. 1.2).

The heterodimer between FBP2 and FBP11 and the combination FBP2 and PFG (Petunia Flowering Gene) were used as positive and negative controls, respectively (Immink *et al.*, 2002).

In this study, a two-photon set-up was used and the donor fluorescence lifetime values were measured pixel by pixel. In all cases measurements were done for the central part of the nucleus where the fluorescence lifetime is not influenced by the auto fluorescence from chloroplasts. For each analysis at least ten representative cells were measured, expressing either a single CFP labeled MADS-box protein, or a combination of a CFP and an YFP labeled protein.

Images with a frame size of 64 X 64 pixels were acquired using the Becker and Hickl1 SPC 830 module, and for the data analysis, the SPCimage 2.8 software was used.

RESULTS

Yeast two- and three-hybrid analyses

To get a first impression about putative FBP24 interaction partners and to select candidates for future *in-vivo* studies yeast two- and three-hybrid analyses were performed. Initially, FBP24 has been tested for dimerization with the 23 known petunia MADS-box proteins (Immink *et al.*, 2003) in the CytoTrap two hybrid system. Remarkably, none of the tested couples resulted in growth of the yeast at 37°C, suggesting that a putative FBP24 heterodimerization partner is not present in the collection. Alternatively, FBP24 is able to interact weakly with one of the known MADS-box factors but it just can not be detected by the yeast CytoTrap system, due to the relative high assay temperature in this system. Therefore, FBP24 dimerization was tested in the yeast two-hybrid GAL4 system at room temperature. This analysis revealed that FBP24 interacts specifically with FBP2 and FBP4 and is neither able to dimerize in yeast with the ovule specific and very closely related FBP7 and FBP11 D-type proteins (Angenent *et al.*, 1995), nor the putative C-type proteins FBP6 and pMADS3 (Kater *et al.*, 1998).

Taking into account that for some MADS-box proteins higher-order complexes have been identified, we were wondering whether FBP24 may interact with the ovule specific D-type proteins in a higher-order complex. To test this ability a yeast three-hybrid analysis was performed. In this screen the FBP2 protein lacking the C-terminal domain (FBP2ΔC) was used, because FBP2 contains an intrinsic transcriptional activation domain in this region (Ferrario *et al.*, 2003). Although, the detected interactions were very weak and could be detected at room temperature and low concentrations of 3 Amino-Triazole (3AT) only, the combinations FBP24-FBP11-FBP2 and FBP24-FBP7-FBP2 gave clearly growth of yeast in comparison to the controls (Table 1).

pBDGAL4	pADGAL4	pRED	- HIS + 1mM 3AT	- HIS + 5mM 3AT
FBP24	FBP11	FBP2ΔC	+	-
FBP24	FBP11		-	-
FBP24	FBP7	FBP2ΔC	+	-
FBP24	FBP7		-	-
FBP24	FBP24	FBP2ΔC	-	-

Table 1. FBP24 higher-order complex formation.
Double and triple combinations were obtained by transformation and spotted onto the different selection media. After spotting the plates were incubated at room temperature for 5 days and subsequently, scored for growth of the yeast clones (+ = growth, - = no growth).

Localization of MADS-box proteins in living plant cells

In order to analyze the various MADS-box proteins *in vivo*, the proteins were labeled with Cyan Fluorescent Protein (CFP), Yellow Fluorescent Protein (YFP) and the monomeric Red Fluorescent Protein (mRFP) at their C-termini. It is known that tagging of proteins with fluorescent groups may disturb their physical properties, which may affect the localization and accumulation of the proteins. However, analyses of the MADS-box protein APETALA1 (AP1) labeled with GFP at its C terminus revealed an active protein that was able to rescue the *ap1* mutant. On the other hand, the N-terminal GFP::AP1 fusion appeared to be non-functional and its sub cellular localization was abnormal being mostly cytoplasmic (Wu *et al.*, 2003). Also C-terminal fusions with Arabidopsis proteins FRUITFULL and AGAMOUS do not affect the biological activity of these MADS proteins (Angenent and Urbanus, unpublished). Considering these data and the results from Immink *et al.* (2002), C-terminal fusions were generated.

Subsequently, the obtained fusion products were transiently expressed in both petunia and cowpea leaf protoplasts. Because similar localizations were obtained in petunia (not shown) and cowpea protoplasts and cowpea protoplasts are more amenable for transfections than petunia protoplasts, all further experiments were done with cowpea cells. Initially, we performed localization experiments with the single proteins, FBP11, FBP2 and FBP24 (Figure 1A-C). The proteins FBP2 and FBP24 appear to be nuclear localized, whereas FBP11 remains in the cytoplasm. Most likely, this can be explained by the inability of FBP11 to homodimerize, which

seems to be a prerequisite for movement into the nucleus (Immink *et al.*, 2002). Surprisingly, both FBP24 and FBP2, for which no homodimerization could be detected by the yeast two-hybrid experiments, were nuclear localized. FRET-FLIM analyses performed in the past for FBP2, revealed that this protein is able to homodimerize in protoplasts and hence, transported into the nucleus (Immink *et al.*, 2002).

In the next step, cells co-transfected with two labeled proteins, for which either dimerizaton or no interaction could be detected in yeast two-hybrid experiments, were analyzed. Nuclear co-localization has already been described for the partners FBP2 and FBP11 (Immink *et al.*, 2002). The combination FBP2 and FBP24 appeared to result in nuclear co-localization as well (Fig1D-G). Surprisingly, both proteins FBP11 and FBP24 were present in the nucleus in the double transfected cells (Fig H-K), while the single FBP11 protein was localized in the cytoplasm (Fig 1A). Taken into account the hypothesis that dimerization is essential for transport into the nucleus (Immink *et al.*, 2002), their co-localization suggests heterodimerization. Finally, all three proteins were imaged simultaneously by transient expression of FBP11, FBP2 and FBP24, labeled with different fluorescent molecules. In this case, all three proteins were present in the nucleus (Fig 1L-P).

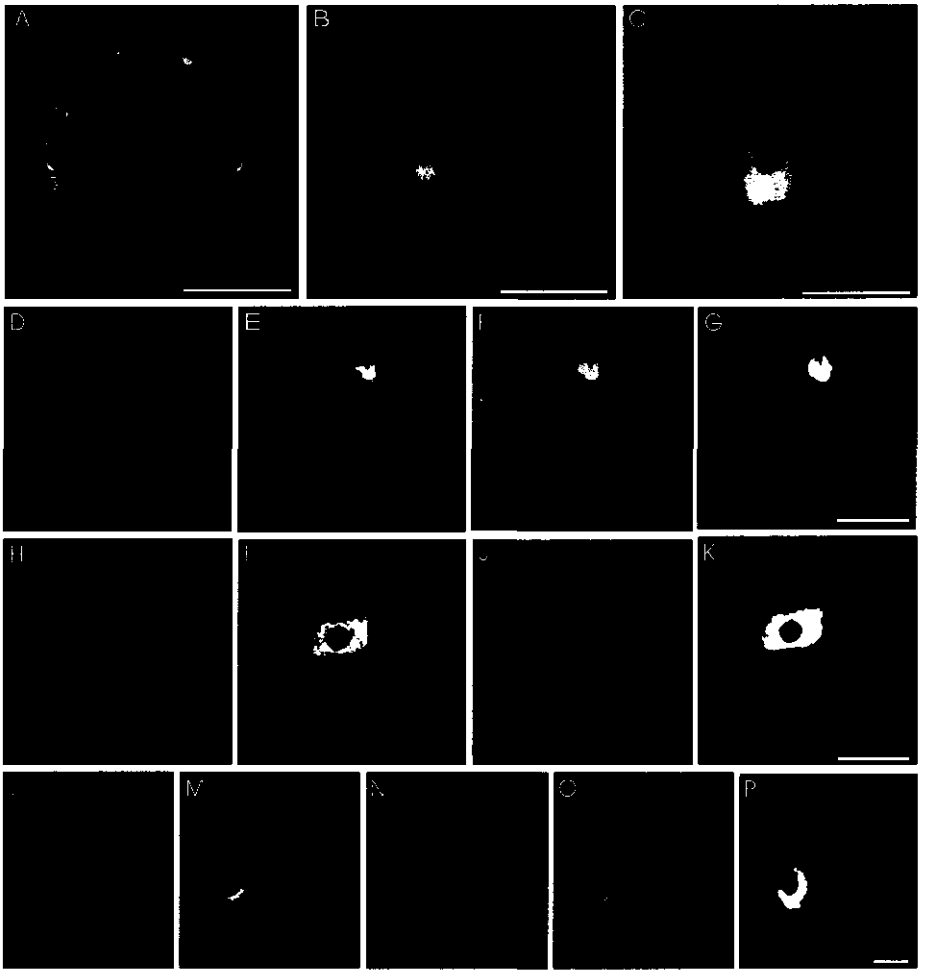
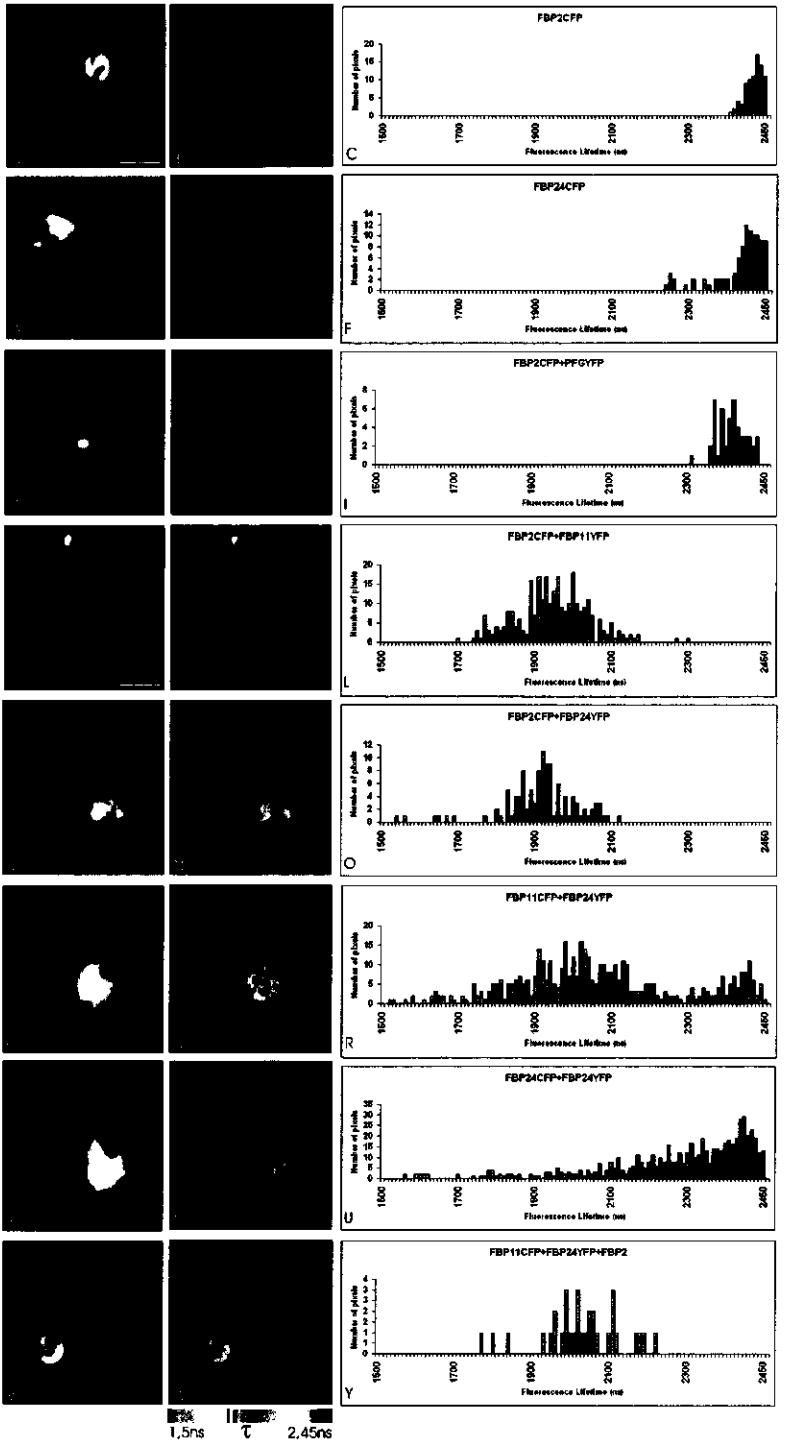


Figure1. Localization of MADS-box proteins in protoplasts.
Confocal images of cowpea leaf protoplasts transfected with single constructs: FBP11-CFP ***(A)****, FBP2-CFP* ***(B)****, and FBP24-YFP* ***(C)****, respectively.* ***(D)*** *to* ***(G)****, images of protoplast co-transfected with FBP2-CFP and FBP24-YFP. Each figure displays one respective channel: chlorophyll (red)* ***(D)****, YFP (yellow)* ***(E)****, CFP (cyan)* ***(F)****, and merged* ***(G)****.* ***(H)*** *to* ***(K)****, images of protoplast co-transfected with FBP11-CFP and FBP24-YFP: chlorophyll* ***(H)****, YFP* ***(I)****, CFP* ***(J)****, and merged* ***(K)****.* ***(L)*** *to* ***(P)****, Transient expression of the proteins, FBP2-CFP, FBP11-YFP, and FBP24-mRFP, in one cell: chlorophyll* ***(L)****, YFP* ***(M)****, CFP* ***(N)****, mRFP (orange)* ***(O)****, and merged* ***(P)****. Bars = 10μm.*

FRET-FLIM analyses reveal differences between dimers

Although sub-cellular co-localization may suggest dimerization and complex formation in living cells, evidence for physical interaction between proteins can only be obtained after application of an appropriate methodology. Therefore, we used FRET-FLIM analyses for the detection of homodimerization and heterodimerization of the ovule-specific MADS-box transcription factors described above. To calculate the fluorescence lifetime of CFP in the absence of the YFP acceptor, the single proteins FBP2 and FBP24 labeled with the donor molecule CFP were used for protoplast transfections. The obtained fluorescence lifetime values and distribution over the nucleus were used as reference for values obtained with the various double transfections. Cells transfected with either FBP2-CFP or FBP24-CFP show a limited variation in fluorescence lifetime values, with an average around 2,45 ns (Fig 2A-F). The fluorescence lifetime for the negative control, the combination of FBP2 and PFG, appeared to be in the same range, however, the variation in lifetime values for different cells is slightly larger (Fig 2G-I). For the positive control (FBP2-FBP11), the fluorescence lifetime drops to about 1,9-2,0 ns on average, which can be measured throughout the nucleus (Fig 2J-L).

Figure 2. Monitoring Fluorescence Resonance Energy Transfer (FRET) by Fluorescence Lifetime Imaging Microscopy (FLIM).
FRET-FLIM analyses of transfected cowpea leaf protoplasts, expressing single and various combinations of MADS-box proteins fused to CFP and YFP, respectively.
***(A)** to **(C)** FLIM analysis on protoplast transiently expressing FBP2-CFP. In **(A)** the fluorescence intensity image of the nucleus of a representative cell is shown, in **(B)** the fluorescence lifetime image of the same nucleus (by a false color code), and in **(C)** a histogram representing the distribution of fluorescence lifetime values over the nucleus. FLIM analysis for FBP24-CFP **(D)** to **(F)**; for FBP2-CFP+PFG-YFP **(G)** to **(I)**; for FBP2-CFP+FBP11YFP **(J)** to **(L)**; for FBP2-CFP+FBP24-YFP **(M)** to **(O)**; for FBP11-CFP+FBP24-YFP **(P)** to **(R)**; for FBP24-CFP+FBP24-YFP **(S)** to **(U)**; and for FBP11-CFP+FBP24-YFP+FBP2 **(V)** to **(Y)**. The fluorescence intensity is always shown in the left panel, the fluorescence lifetime in the middle panel and the distribution of fluorescence lifetime values in the right panel. Bars=10μm.*


FBP2CFP
C
FBP24CFP
F
FBP2CFP+PFGYFP
I
FBP2CFP+FBP11YFP
L
FBP2CFP+FBP24YFP
O
FBP11CFP+FBP24YFP
R
FBP24CFP+FBP24YFP
U
FBP11CFP+FBP24YFP+FBP2
Y
Number of pixels
Fluorescence Lifetime (ps)
1500
1700
1900
2100
2300
2450
1,5ns
τ
2,45ns

Subsequently, the combination FBP2-CFP and FBP24-YFP was analyzed. For this combination, heterodimerization was detected in yeast and co-localization of the proteins was observed in living plant cells (Fig 1D-G). The FLIM data depicted in Figures 2M-O show that this combination gives a strong reduction in fluorescence lifetime, demonstrating that these proteins interact in living plant cells. The reciprocal combination (FBP24-CFP and FBP2-YFP) has been tested as well and gave the same result (data not shown). Surprisingly, in the case of the combination FBP11-FBP24 a distribution of different fluorescence lifetime values over the nucleus was observed (Fig 2P-R), suggesting that there are sub-nuclear regions with and without interaction between the two proteins. Finally, we analyzed cells transfected with both FBP24-CFP and FBP24-YFP, in order to determine whether this protein is able to homodimerize. Interestingly, the same variation of fluorescence lifetime values distributed over the nucleus was found as described for FBP11-FBP24 (Fig 2S-U).

Stability of protein-protein interactions and higher-order complex formation

It has been proposed that MADS-box proteins are active as multimeric complexes, such as ternary or quaternary complexes (Egea-Cortines *et al.*, 1999). Information about the stability of the dimeric interactions and the influence of additional factors is limited to yeast experiments and is completely lacking for *in planta* interactions. Our FRET-FLIM analyses clearly revealed differences between dimers with respect to distribution over the nucleus and stability of interaction. Some combinations interact all over the nucleus while others interact, most likely in a more transient manner, in sub nuclear regions only. To get a possible explanation for this difference in distribution and stability of dimers, FLIM studies were done using a non-labelled third factor (FBP2) in combination with FBP11-CFP and FBP24-YFP. This experiment revealed a reduction in fluorescence lifetime with a more uniform distribution over the nucleus for the triple combination (Fig.2V-Y vs. Fig.2P-R).

DISCUSSION

During the last decade, many studies have been performed to identify the genes involved in regulation of important steps in plant development. Transcription factors belonging to the MADS-box family appeared to play pivotal roles in these processes and can be considered as the main regulators of plant development. Nevertheless, little is known about their behavior in plant cells at the molecular level and the

dynamic process of gene regulation in the nucleus. It has been hypothesized that the MADS-box proteins form specific dimers, which are further assembled into tetrameric complexes (reviewed in Theißen and Saedler, 2001). Intriguing questions remain whether these complexes are actually formed and how stable these complexes are. In this study, we have followed an *in vivo* approach to investigate the dynamics of MADS-box transcription factor interactions in a plant cell environment. For this purpose, the Petunia MADS-box proteins FBP11, FBP2 and FBP24 that are supposed to be involved in ovule development, were selected as object.

Surprisingly, no dimerization partner could be detected for FBP24 in the yeast CytoTrap two-hybrid system. In this system the selection for protein-protein interactions is based on the Ras signal transduction cascade and due to this the temperature sensitive yeast strain is able to growth at a relative high temperature of 37°C upon a protein-protein interaction (Aronheim *et al.*, 1997). A temperature dependent interaction has been reported for the class B proteins PISTILLATA and APETALA3 from Arabidopsis (Kohalmi *et al.*, 1996), which can be stabilized by the presence of additional MADS-box factors. *FBP24* has been designated as a "B-sister" gene (Becker *et al.*, 2002), based on its evolutionary relationship with the class B proteins. Our yeast two-hybrid results also point to weak and temperature sensitive interactions between FBP24 and other MADS-box proteins such as FBP2 and FBP11 and furthermore, a third protein facilitates the formation of a more stable dimer.

It has been hypothesized that dimerization is a prerequisite for nuclear localization of plant MADS-box transcription factors (Immink *et al.*, 2002; Ferrario *et al.*, 2004b). In line with this, FBP11 that is not able to homodimerize is localized in the cytoplasm, whereas FBP2 molecules form homodimers and are subsequently transported to the nucleus. Despite that FBP24 did not show homodimerization in yeast, it appeared to be nuclear localized in plant cells. FRET-FLIM analyses in living plant cells demonstrated however that in contrast to the yeast two-hybrid results, homodimerization could be detected for this protein. Like FBP24, homodimerization of FBP2, FBP5 and FBP9 (Immink *et al.*, 2002) could only be detected *in planta*, but not by a traditional yeast two-hybrid system, demonstrating the importance of in-*vivo* protein-protein interaction studies. Furthermore, it suggests that plant MADS-box transcription factor homodimers are in general less stable than heterodimers. The fact that only five homodimers have been identified in a large-scale yeast two-hybrid

screening with over 100 Arabidopsis MADS-box transcription factors (de Folter *et al.*, 2005) supports this observation.

For the putative FBP2-FBP24 heterodimer the yeast result were confirmed by the *in planta* analysis. A relative low fluorescence lifetime with little variation was observed for this combination, suggesting the formation of a "stable" heterodimer. On the other hand, the proteins FBP11 and FBP24 that are not interacting in yeast seem to interact in sub-nuclear regions in plant cells. A similar kind of fluorescence lifetime distribution over the nucleus, with regions with relatively low fluorescence lifetime values visualizing an interaction and regions with high fluorescence lifetime values representing no interaction, was observed for the FBP24 homodimer. Most likely, in these cases the proteins associate into dimers and dissociate all over the nucleus, but their interaction is stabilized in the specific sub-nuclear spots. At this moment it is not clear what these sub-nuclear regions represent. The stronger FRET signal in the sub nuclear spots is at least not due to a higher concentration of the fluorescence proteins at these places, because fluorescence intensity measurements showed a more or less equal distribution of signal over the nucleus. A number of studies indicate that homo- and/or heterodimerization facilitate the binding to specific DNA sequences (Pellegrini *et al.*, 1995: Shore and Sharrocks, 1995) and that higher-order complex formation of MADS-box transcription factors is stabilized by specific DNA binding (Egea-Cortines *et al.*, 1999). It might be possible that the sub-nuclear regions represent places where the chromatin is available for transcription and to which the transcription factors are recruited, resulting in stabilization of the less stable or "transient" interactions.

The triple transfection experiment that has been performed in this study, suggests that an instable dimer can be stabilized by a third factor, because addition of a non-labeled FBP2 to the combination FBP11-CFP + FBP24-YFP resulted in a drop in fluorescence lifetime values. Based on differences in FRET signal that were obtained for the individual dimers (FBP2-FBP2, FBP24-FBP24, FBP2-FBP11, FBP2-FBP24 and FBP11-FBP24), we assumed that competition for dimerization between the individual proteins will occur, when more than two factors are co-expressed. In the case that competition for dimerization is the only aspect that plays a role, addition of the non-labeled FBP2 to the combination FBP11-CFP + FBP24-YFP will result in less or no dimerization between FBP11 and FBP24 and hence, an increase in fluorescence lifetime. However, the fluorescence lifetime was decreased for this

specific triple combination demonstrating that FBP11 and FBP24 are still in one and the same complex. This observation and the results of the yeast three-hybrid experiments, suggest that higher-order complex formation plays a role. Considering these results, some hypotheses can be drawn about complex formation that probably occurs *in vivo* for the ovule specific MADS-box transcription factors (Fig.3). For example, a ternary complex might be formed between the monomers FBP24, FBP11 and FBP2. However, both FBP24 and FBP2 are able to homodimerize and hence a quaternary complex involving a heterodimer in combination with either a FBP2 or FBP24 homodimer could theoretically be formed. Nevertheless, the yeast and FRET-FLIM analyses suggest that these homodimers are less stable than the FBP2-FBP11 and FBP2-FBP24 heterodimers. Taken this into account, we hypothesize that it is more likely that *in vivo* a quaternary complex is formed by the two very stable dimers FBP24-FBP2 and FBP2-FBP11. A putative quaternary complex like this would fit perfectly in the proposed 'quartet model' of MADS-box transcription factor functioning (Egea-Cortines *et al.*, 1999; Honma and Goto, 2001, Theißen and Saedler, 2001).

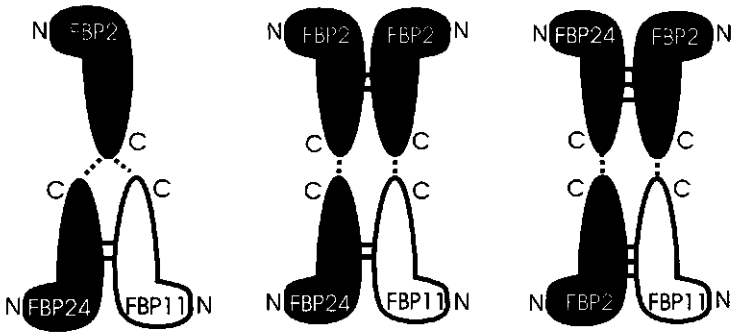


Figure 3 Putative higher-order complexes formed between the ovule specific MADS-box proteins.
Schematic representation of putative complexes. On the left a ternary complex formed by the monomer FBP2, and a heterodimer between FBP24 and FBP11. In the middle, a quaternary complex formed by the homodimer FBP2- FBP2 and the heterodimer FBP24-FBP11; and on the right a quaternary complex formed by the stable dimers FBP24-FBP2 and FBP2-FBP11. The dotted lines represent the interactions at the C-termini between monomers and dimers and the continuous lines indicate dimerization. For less stable dimers double continuous lines were used, and for stable dimers three lines were drawn.

In conclusion, this report demonstrated that the *in vivo* analyses provide more detailed and reliable information about protein-protein interactions than the yeast systems. Even though, the proteins are transiently expressed at relatively high-levels in plant cells, the experiments performed here give a first glimpse about the native behavior of MADS-box transcription factors in plant cells. Certainly, more analyses are required to get a final proof for higher-order complex formation between plant MADS-box transcription factors and to understand the exact stoichiometry of these kind of complexes in the plant tissue where they are active. Remains the question, what the biological function is of the complex involving FBP2, FBP11 and FBP24. As mentioned before, *FBP24* has a high sequence similarity with *ABS* from *Arabidopsis,* which is supposed to play a role in seed coat pigmentation and probably is essential for the formation or maintenance of the endothelial cells (Nesi *et al.*, 2002). Probably, the petunia homolog FBP24 is required for late ovule development as well, in combination with FBP2 and FBP11. This suggests that FBP11 plays a dual role in ovule development, being involved in the initiation of ovules (Colombo *et al.*, 1995; 1997) and in late ovule development. The higher order complex identified between FBP11, FBP2 and FBP6 (unpublished results Immink and Angenent) and their Arabidopsis orthologs SEEDSTICK (STK), SEPALLATA3 (SEP3) and AGAMOUS (AG) (Favaro *et al.*, 2003), respectively, are supposed to be involved in the early ovule function, while the complex between FBP11, FBP2 and FBP24 identified in this study, might be responsible for the late ovule function.

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Chapter 3

Physical interactions and dimerization affinity *in vivo* for MADS-box proteins involved in petal and stamen formation

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ABSTRACT

MADS-box transcription factors are required for floral organ identity specification and based on genetic analyses the well known ABC-model for floral development has been established. For instance, stamen formation in Arabidopsis is driven by the MADS-box proteins PISTILLATA and APETALA3 (PI and AP3, B-function), SEPALLATA 3 (SEP3, E-function), and AGAMOUS (AG, C-function), whereas a similar combination is involved in petal formation, but with a role for APETALA1 (AP1, A-function) instead of AG. Yeast two-, three- and four-hybrid studies revealed that these MADS-box proteins are able to form specific hetero- and homodimers and furthermore, can assemble into higher-order complexes. Our goal was to characterize the physical interactions between these proteins in living plant cells. For this purpose, the various MADS-box proteins were labeled with the Green Fluorescent Protein (GFP) variants Cyan Fluorescent Protein (CFP) or Yellow Fluorescent Protein (YFP), followed by transient expression in leaf protoplasts. Subsequently, Confocal Laser Scanning Microscopy (CLSM) was used to determine the localization of the various proteins, while the physical interactions were studied by Fluorescence Lifetime Imaging Microscopy (FLIM) measurements in order to monitor Fluorescence Resonance Energy Transfer (FRET). This study revealed a clear difference in homodimerization capacity for the MADS-box proteins involved in stamen development. SEP3 and AG appear to homodimerize efficiently and a FRET signal was observed in the entire nucleus, whereas PI and AP3 display homodimerization only in specific sub-nuclear regions. Remarkably, homodimerization can not be detected for any of these proteins by the yeast two-hybrid system, which shows the sensitivity of the FRET technology. Furthermore, differences in FRET signal strength were detected between the various tested heterodimers, and this probably reflects differences in interaction affinity. As a consequence of this, most likely only a subset of all possible dimer combinations will be formed at a certain moment during petal and stamen development. The obtained results give new insights in the functioning of plant MADS-box transcription factors at the molecular level in their native environment.

INTRODUCTION

For flowering plants, *Arabidopsis thaliana* has become the model species for the analyses of developmental processes. For instance, the extensive use of genetics and the morphological and molecular studies on mutants have extended our understanding about flower formation and architecture enormously. The Arabidopsis flower consists of four concentric whorls of organs, from outside to inside: sepals, petals, stamens and carpels, and the initiation of their identity is driven by specific homeotic genes. More than a decade ago, genetic analyses of homeotic floral mutants have led to the postulation of the elegant and widely accepted ABC model, in which different classes of genes (A, B, C) determine the identity of the floral organs (Coen and Meyerowitz, 1991). With these studies, many of the questions about the "metamorphosis" raised by Goethe could be answered finally. He postulated that unknown factors are required for the transformation of the vegetative leaves into floral organs.

In Arabidopsis, the different functions of the ABC model are mainly defined by genes belonging to the MADS-box transcription factor family. 107 members of this family have been identified and many of them play a role as major regulators during floral organ formation (Parenicova *et al.,* 2003). The combination of different genes results in specific organ formation: the A-function is defined by *APETALA1* (*AP1)* and *APETALA2* (*AP2)*, B-function by *APETALA3* (*AP3)* and *PISTILLATA* (*PI),* and the C-function by *AGAMOUS* (*AG).* Later on, the model has been expanded, when D-function (Colombo *et al.,* 1995) and E-function (Pelaz *et al.,* 2000) were described, and added to the existing model. The E-function represented by the *SEPALLATA (SEP)* genes was considered as "the missing" factor for the complete homeotic change from leaves into floral organs, and vice versa (Goto *et al.,* 2001).

Within the ABC model, the B-type genes *AP3* and *PI* are involved in specifying petal and stamen identity in Arabidopsis, and their mutants cause alterations in the two middle whorls, with sepals instead of petals in the second whorl and carpels instead of stamens in the third whorl, respectively (Jack *et al.,* 1992; Goto and Meyerowitz, 1994). At the molecular level, AP3 and PI have shown to heterodimerize and auto-regulate their own expression (Goto and Meyerowitz, 1994). Furthermore, Honma and Goto (2001) nicely demonstrated that simultaneous ectopic expression of PI, AP3, SEP3, and AG is sufficient to convert cauline leaves into staminoid organs. In addition, their yeast results refined and extended the molecular model for functioning

of these proteins, in which SEP3 interacts with the AP3-PI heterodimer and can also act as an intermediate partner for the interaction with AG in the quaternary stamen identity complex, AG-SEP3-AP3-PI. For the specification of the petal identity, it was supposed that SEP3 interacts with AP1 in the petal complex: AP1-SEP3-AP3-PI.

In addition to protein-protein interactions, specific protein-DNA interactions have also proven to be essential for the proper functioning of these homeotic transcription factors. In the last decade several biochemical experiments contributed to the identification of specific DNA target sequences that are bound by the MADS-box proteins. For instance, Riechmann and colleagues (1996a,b) demonstrated in an elegant way that various dimers can be formed in vitro and specifically bind to so called CArG-boxes, the consensus target sequence for MADS-box transcription factors. In these experiments it was clearly demonstrated that "partner specificity" plays a role in selective DNA-binding, which shed light on the possible mechanisms behind target gene selection. However, important questions remain to be answered. How the formation of the various dynamic protein complexes occurs *in vivo*, and how this facilitates specific DNA-binding and subsequent regulation of target genes remain to be elucidated. Here we report a study, aiming the understanding of MADS-box proteins partner selection.

Our strategy of choice to address these questions was to analyze the homo- and heterodimerization capacity for the MADS-box proteins involved in determination of stamen and petal identity, in living cells. Fluorescence Lifetime Imaging (FLIM) in order to monitor FRET (Fluorescence Resonance Energy Transfer) was used in this study. FRET is a physical phenomenon that can occur when two fluorophores come into close proximity (less then 10Å) and energy is transferred from the excited donor fluorophore to a suitable acceptor fluorophore. When proteins of interest are fused to these fluorophores, the physical interaction between the proteins can be detected by FRET. The lifetime of a fluorophore is the average time it stays in the excited state before it falls back to the ground state and is decreased when FRET occurs (Lakowicz, 1999). For our FRET-FLIM analyses, we labeled the MADS-box proteins AP1, AP3, PI, AG, and SEP3 with Cyan Fluorescence Protein (CFP) and Yellow Fluorescence Protein (YFP), one of the best couples available for FRET studies. Subsequently, the single labeled proteins and several combinations were transiently expressed in Arabidopsis leaf protoplasts for localization and interaction analyses. The obtained results reveal that there is a clear difference in interaction affinity for the

analyzed proteins and that FRET-FLIM allows the detection of these differences in living cells. We hypothesize that these differences in interaction affinity are relevant for the functioning of the MADS-box proteins and that the dimer combination determines to a great extent their transcriptional activity in plants.

MATERIAL AND METHODS

Plant material

Protoplasts were obtained from *Arabidopsis thaliana* Col0 leaves, which were grown under normal greenhouse conditions (16/8h light /dark), 22°C, according to Aker *et al.*, 2006.

Plasmid constructions

The coding region of the MADS-box genes *APETALA1 (AP1), APETALA3 (AP3), PISTILLATA (PI), AGAMOUS (AG), SEPALLATA3 (SEP3)* and *SEPALLATA3* lacking the C-domain, were cloned as entry clones lacking stop codons in order to allow C-terminal fusions. For the *SEPALLATA3* lacking the C-domain, the reverse primer was designed just after the K-box eliminating the C-terminal domain (80 aa in total), including part of the putative last alpha helical structure. The entry clones were recombined into the Gateway compatible vectors pARC971 and pARC428, from which, expression is driven by the constitutive CaMV35S promoter and that contains the coding regions of the different fluorophores, Enhanced Cyan Fluorescent Protein (ECFP) and Enhanced Yellow Fluorescent Protein (EYFP), respectively (Nougalli-Tonaco *et al.*, 2006). Furthermore, N- terminal fusions were made for AP3 and PI. In this case, the destination vector was the pK7WGY2,0 from the VIB collection, containing the EYFP molecule. AP3 and PI entry clones including stop codons were taken from the REGIA collection (Parenicova *et al.*, 2003; de Folter *et al.*, 2005). LR reactions were done according to the protocols provided by Invitrogen. All plasmids were controlled by sequence analyses (DETT sequence kit, from Amersham).

Fluorescence microscopy in living cells

Arabidopsis leaf protoplasts were transfected as described by Aker and colleagues (2006). 15 -30 ug of plasmid DNA was used and the protoplasts were incubated overnight at 25°C before imaging. Images were made using a confocal laser

microscope 510 (Carl Zeiss, Jena, Germany). The Argon laser was used to excite at 458 and 514nm for CFP and YFP, respectively. Fluorescence was detected through a band pass filter of 470-500nm for CFP and 535-590nm for YFP.

FRET-FLIM measurements in living cells

FRET-FLIM analyses were done in Arabidopsis protoplasts according to Nougalli-Tonaco *et al.*, 2006 and Russinova *et al.*, 2004. As described previously, the donor fluorescence lifetime was measured on the central part of the nucleus of each single cell, pixel by pixel, and at least 10 cells were analyzed per combination. We fixed the donor lifetime at 2, 6 ns for further analyses. Images were acquired by using the "Becker and Hickl 1 SPC 830" module, and SPC image 2.8 software was used for the data analyses.

RESULTS

Localization of Arabidopsis MADS-box proteins involved in stamen development in living plant cells

To investigate the protein behavior in living cells, localization studies were performed. For this purpose, fusions were made between the proteins of interest and Enhanced Cyan Fluorescent Protein (ECFP) or Enhanced Yellow Fluorescent Protein (EYFP). Initially, the fluorophores were linked to the C-terminus of the MADS-box proteins, followed by transient expression of the fusion proteins in Arabidopsis leaf protoplasts. First, the Arabidopsis MADS-box proteins AP3, PI, AG, and SEP3 were expressed individually, and subsequently, double co-transfections have been analyzed. The single MADS-box protein transfections resulted in nuclear localization of the fluorescence in the majority of the analyzed cells (Fig 1A to D). Surprisingly, AP3 was mostly present in the nucleus, but also observed in the nucleus and cytoplasm in some cells, whereas PI was almost completely nuclear localized, when transfected individually. These results are not in accordance with previously results obtained with the same proteins in the work of MacGonigle and colleagues (1996). They observed that nuclear localization only occurs when both proteins are simultaneously expressed. However, in their case, the GUS reporter was used and N-terminally fused to the MADS-box protein, which may be the reason for the observed differences. We have shown previously that fusion of GFP-like fluorophores to the N-

terminus of MADS-box proteins can influence their nuclear import (Nougalli-Tonaco *et al.*, 2006). Therefore, we decided to analyze whether there is a difference between N- and C-terminal labeling with respect to localization. For this, we labeled AP3 and PI with YFP at the N-terminus and when the individual proteins YFP-AP3, or YFP-PI were transfected, most of the signal was localized in the cytoplasm (Fig. 1E), which is in accordance to the results of MacGonigle and colleagues (1996). Subsequently, we performed co-transfections using an N-terminal fusion for either AP3 or PI, and a C–terminal labeling for the other protein. Then, both proteins were mainly localized in the cell nucleus (Fig. 1F). Similar results were obtained by MacGonigle *et al.*, (1996) when they co-expressed one of the two B-type proteins labeled at the N-terminus and the other one without any fusion. Co-transfection with N-terminal fusions for both proteins resulted in an almost exclusive nuclear localized signal (Fig. 1G). In addition, we co-expressed various other combinations of the MADS-box proteins under study, and observed only nuclear localized signal (data not shown).

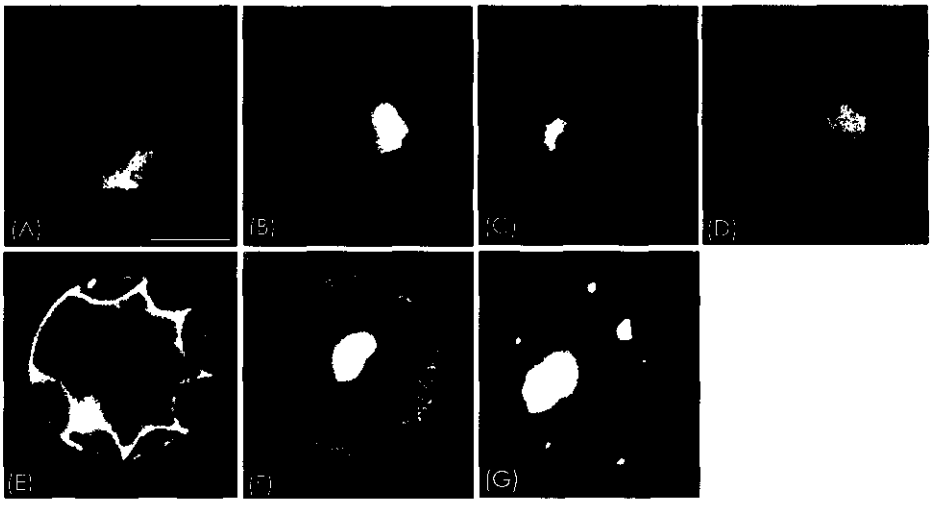

Figure 1. Localization of MADS-box proteins in Arabidopsis leaf protoplasts.

The figure displays the different MADS-box proteins fused to CFP or YFP and transiently expressed in protoplasts. ***(A)*** *SEP3-CFP,* ***(B)*** *AP3-YFP,* ***(C)*** *AG-YFP,* ***(D)*** *PI-CFP,* ***(E)*** *YFP-AP3,* ***(F)*** *YFP-AP3+PI-CFP,* ***(G)****YFP-AP3+YFP-PI. Bar =10 µM*

Homodimerization of MADS-box proteins in living cells

Previous studies provided evidence for the hypothesis that dimerization is a prerequisite for import of MADS-box proteins into the cell nucleus (MacGonigle *et al.*, 1996; Immink *et al.*, 2002). In line with this paradigm, all proteins that show nuclear localization when expressed on their own, should be able to homodimerize, or alternatively, interact efficiently with an endogenous factor. Based solely on localization experiments, we cannot elucidate the exact mechanism underlying the obtained nuclear localization of the analyzed proteins. Therefore, we used FLIM to determine FRET, to test for homodimerization of the MADS-box proteins under study. For the estimation of donor lifetime we used single transfections of the MADS-box proteins labeled with CFP only, like is depicted in Fig 2A. The combinations "SEP3-CFP + SEP3-YFP", "AG-CFP + AG-YFP", "AP3-CFP + AP3-YFP" and, "PI-CFP + PI-YFP" were analyzed for homodimerization and interestingly, a remarkable difference was detected among these proteins for their capacity to homodimerize. Clear homodimerization could be detected for two out of the four analyzed proteins. Both SEP3 and AG revealed a drop in fluorescent lifetime in the entire nucleus, suggesting efficient homodimerization (Fig 2A and 2B). In contrast, PI homodimerizes in some

specific regions within the nucleus (Fig 2E), while AP3 seems to form very weak or transient homodimers showing interactions in certain specific spots, only (Fig 2D).

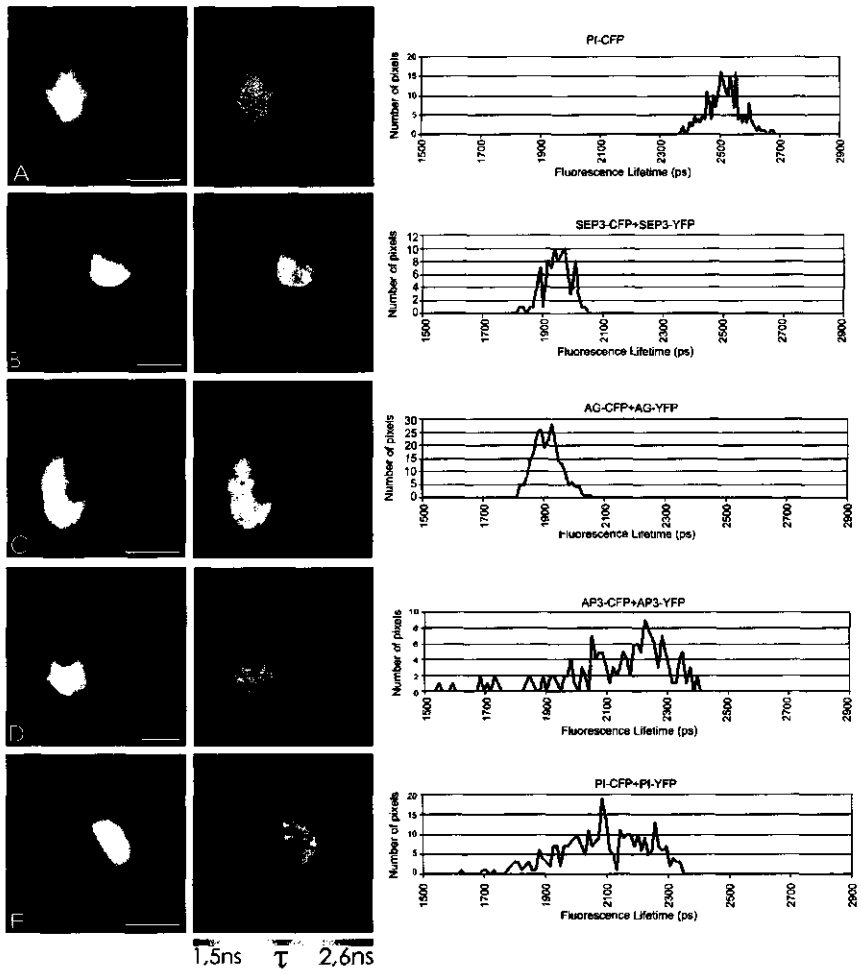

Figure 2. Detection of Fluorescence Energy Transfer (FRET) by Fluorescence Lifetime Imaging Microscopy (FLIM) for different MADS-box protein homodimers combinations

The analysis of transfected Arabidopsis leaf protoplasts, co-expressing MADS-box proteins fused to either CFP or YFP. One representative protoplast is shown for each combination. The left panels display the intensity channel, the middle panels show the fluorescence lifetime image of the same nucleus in a false color code, and the right panels depict histograms representing the distribution of fluorescence lifetime values within the nucleus.

***(A)** FLIM analysis on a protoplast transiently expressing PI-CFP (donor only), **(B)** SEP3-CFP+SEP3-YFP; **(C)** AG-CFP+AG-YFP; **(D)** AP3-CFP+AP3-YFP; **(E)** and PI-CFP+PI-YFP. Bars=10μm.*

Preference for different heterodimerization partners and protein-protein interaction dynamics.

In a following experiment, heterodimerization among the different proteins was tested by FRET-FLIM measurements. According to the information available from previous yeast two-hybrid analyses (de Folter *et al.*, 2005), some of the MADS-box proteins involved in stamen and petal development are supposed to dimerize, whereas others are expected to fail to interact (Table 1). Among all different heterodimers tested in protoplasts, some showed clear interaction all over the cell nucleus, whereas certain combinations displayed interaction in specific spots in the nucleus only, probably meaning that differences in affinity play a role in the selection of interacting partners (Table 1). For instance, the dimers AG-SEP3 and AP1-SEP3 (Fig 3A and 3B) showed fast decay in lifetime, indicating stable dimerization, whereas the decay in lifetime signals for the combinations AP3-SEP3 and PI-SEP3 were less contrasting than for the two previous mentioned combinations (Fig 3C and 3D). All these protein combinations revealed heterodimerization throughout the nucleus. In contrast, the combination AP3-AG seems to be very transient and its interaction can be observed only in specific spots (Fig 3E), whereas PI-AG seems to be stable (Fig 3F). Also the combination AP3-PI displayed an interesting pattern (Fig 3G). For this particular combination, most of protein was localized around the nucleolus and a stronger reduction in lifetime was recorded in this region. This observation was made for almost all transfected cells that were analyzed. As a negative control, we made use of the combination pECFP+PI-YFP, and no interaction was observed in this case (Fig 3J).

Figure 3. Detection of Fluorescence Energy Transfer (FRET) by Fluorescence Lifetime Imaging Microscopy (FLIM) for different MADS-box protein combinations

The analysis of transfected Arabidopsis leaf protoplasts, co-expressing MADS-box proteins fused to either CFP or YFP. One representative protoplast is shown for each combination. The left panels display the intensity channel, the middle panels show the fluorescence lifetime image of the same nucleus in a false color code, and the right panels depict histograms representing the distribution of fluorescence lifetime values within the nucleus.

***(A)** FLIM analysis on a protoplast transiently expressing AG-CFP+SEP3-YFP, **(B)** AP1-CFP+SEP3-YFP; **(C)** AP3-CFP+SEP3-YFP; **(D)** PI-CFP+SEP3-YFP; **(E)** AP3-CFP+AG-YFP; **(F)** PI-CFP+AG-YFP; **(G)** PI-CFP+AP3-YFP; and **(H)** pECFP + PI-YFP. Bars=10μm.*

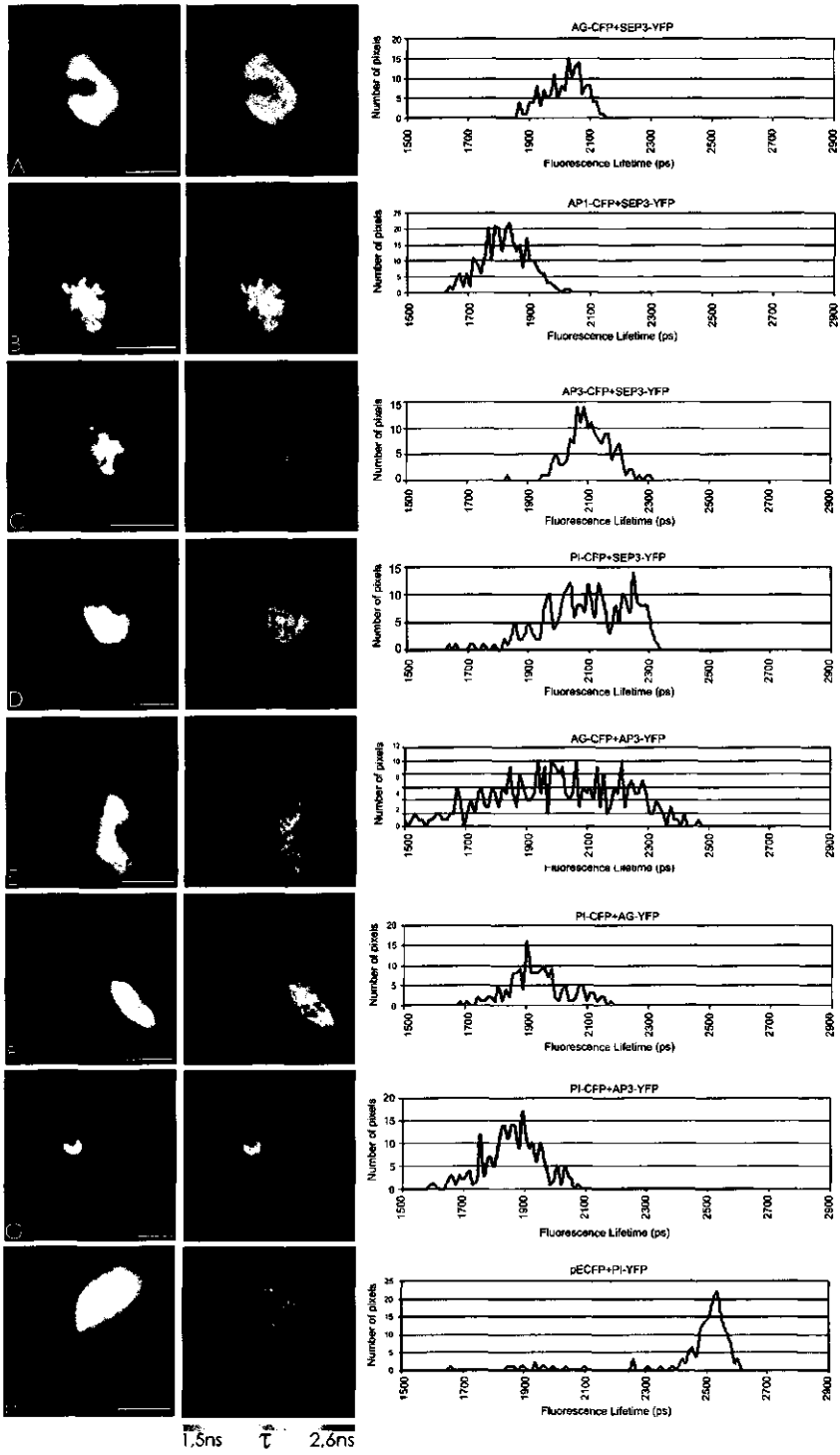
A
B
C
D
E
F
G
H
AG-CFP+SEP3-YFP
AP1-CFP+SEP3-YFP
AP3-CFP+SEP3-YFP
PI-CFP+SEP3-YFP
AG-CFP+AP3-YFP
PI-CFP+AG-YFP
PI-CFP+AP3-YFP
pECFP+PI-YFP
Number of pixels
Fluorescence Lifetime (ps)
1500
1700
1900
2100
2300
2500
2700
2900
1,5ns
τ
2,6ns

Table 1. MADS-box protein interactions analyzed by the yeast two-hybrid system and by FRET-FLIM analyses in Arabidopsis protoplasts.
(+) indicates interaction, (-) indicates no interaction, (+/-) indicates weak interactions (yeast), and transient or only in specific spots (FRET-FLIM). Note that the heterodimer between AP3 and PI in yeast could only be detected for clones lacking the MADS domain.

Combinations	Yeast	FRET-FLIM
SEP3-SEP3	+/-	+ +
AG-AG	-	+ +
AP3-AP3	-	+/-
PI-PI	-	+/-
SEP3-AG	+	+ +
PI-SEP3	-	+
PI-AG	-	+
AP3-PI	+	+ (+)
AP3-AG	-	+/-
AP3-SEP3	-	+
AP1-SEP3	+	+ +

Interactions of SEPALLATA 3 lacking the C-domain

To our surprise many more heterodimers were identified by FRET-FLIM *in-planta*, than by the yeast two-hybrid studies done in the past. Furthermore, many MADS-box proteins seem to homodimerize in living plant cells, while for only very few Arabidopsis MADS-box transcription factors homodimerization could be detected in yeast (de Folter *et al.*, 2005). Taking this into account and the fact that higher-order complexes of MADS-box proteins have been identified that consist of two different dimers (Egea-Cortines *et al.*, 1999; Homna and Goto, 2001), it can not be excluded that in living cells some of the identified interactions between two proteins do represent complex formation between two homodimers.

As an approach to address this hypothesis, we made use of a truncated version of SEPALLATA3 lacking the C-terminal domain (SEP3ΔC). This mutated version of SEPALLATA3 was previous tested in yeast and appeared to be still able to heterodimerize with for example AG, but higher-order complex formation was no

longer obtained, when SEEDSTICK (STK) was added to the AG-SEP3ΔC dimer (Immink, R.G.H. & Angenent, G.C., unpublished results). In contrast, the dimer between AG and the full-length SEP3 protein forms a strong higher-order complex with the STK protein (Favaro *et al.*, 2003). Starting from this point, the following combinations were tested: SEP3ΔC+AP1, SEP3ΔC+AP3, SEP3ΔC+PI, SEP3ΔC+AG and SEP3ΔC+SEP3 (homodimerization). In the case of SEP3ΔC+AG and SEP3ΔC+AP1, a strong decay of lifetime could be observed in the entire nucleus for most of the analyzed cells (Figs. 4B and 4D), indicating heterodimerization as was also shown with the full length SEP3 protein (Figs 4A and 4C). In contrast, when the combinations SEP3ΔC+AP3 and SEP3ΔC+PI were tested, deletion of the C-terminal domain of SEP3 seems to have a major effect (Fig 4F and 4H). The interaction was almost completely abolished, except for a few specific spots. Finally, we tested SEP3ΔC+SEP3, which displayed a similar result as SEP3ΔC+AP3 and SEP3ΔC+PI, i.e. no interaction anymore (Fig 4J). This suggests that the FRET signal observed for the combination SEP3-CFP and SEP3-YFP (Fig 4I) is in fact due to the formation of a higher order complex that is no longer formed when the C-terminus is deleted.

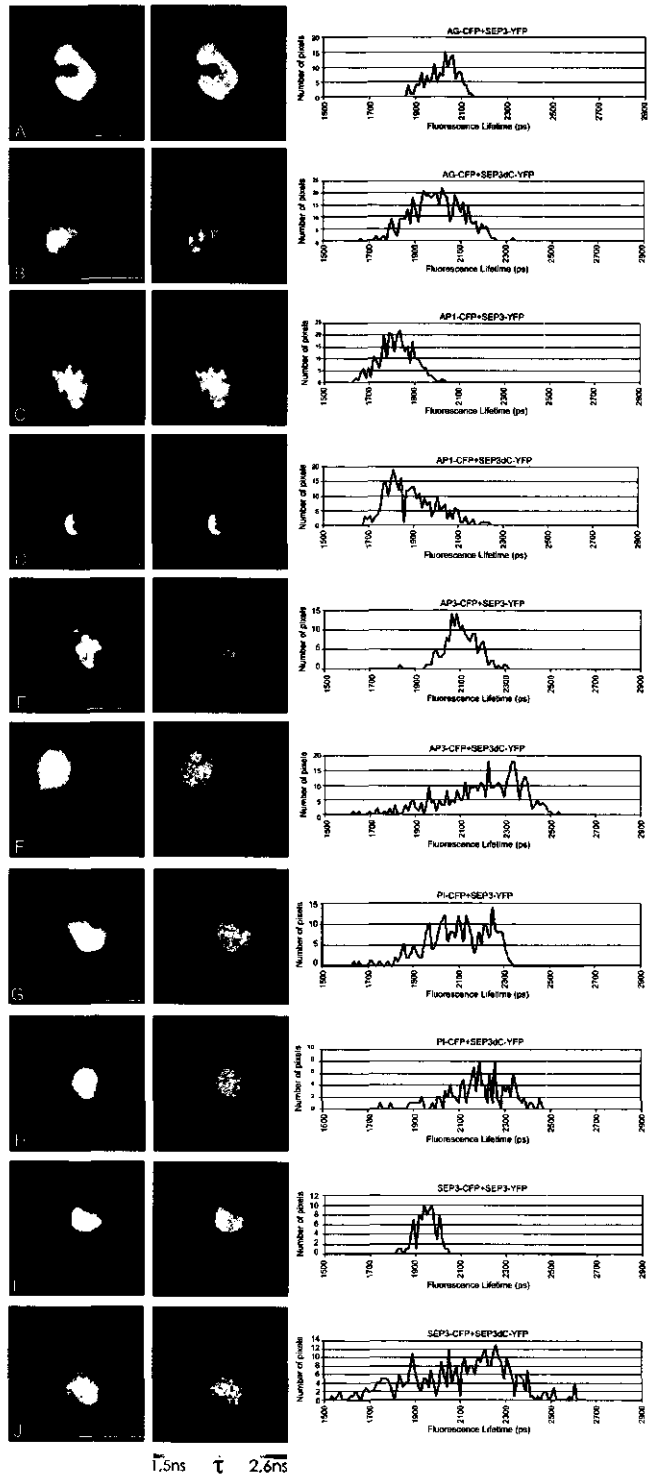
A
AG-CFP+SEP3-YFP
B
AG-CFP+SEP3dC-YFP
C
AP1-CFP+SEP3-YFP
D
AP1-CFP+SEP3dC-YFP
E
AP3-CFP+SEP3-YFP
F
AP3-CFP+SEP3dC-YFP
G
PI-CFP+SEP3-YFP
H
PI-CFP+SEP3dC-YFP
I
SEP3-CFP+SEP3-YFP
J
SEP3-CFP+SEP3dC-YFP
Number of pixels
Fluorescence Lifetime (ps)
1.5ns
τ
2.6ns

Figure 4. Interactions of SEPALLATA 3 lacking the C domain

The analysis of transfected Arabidopsis leaf protoplasts, co-expressing MADS-box proteins fused to either CFP or YFP. One representative protoplast is shown for each combination. The left panels display the intensity channel, the middle panels show the fluorescence lifetime image of the same nucleus in a false color code, and the right panels depict histograms representing the distribution of fluorescence lifetime values within the nucleus. ***(A)*** *FLIM analysis on a protoplast transiently expressing AG-CFP+SEP3-YFP,* ***(B)*** *AG-CFP+ SEP3ΔC-YFP;* ***(C)*** *AP1-CFP+SEP3-YFP;* ***(D)*** *AP1-CFP+SEP3ΔC-YFP;* ***(E)*** *AP3-CFP+SEP3-YFP;* ***(F)****AP3-CFP+SEP3ΔC-YFP;* ***(G)*** *PI-CFP+SEP3-YFP;* ***(H)*** *PI-CFP+SEP3ΔC –YFP;* ***(I)*** *SEP3-CFP+SEP3-YFP;* ***(J)*** *SEP3-CFP+ SEP3ΔC-YFP. Bars=10μm.*

APETALA3-PISTILLATA and SEPALLATA3 are able to form a higher-order complex in vivo

As described previously, the dimer between the B-type proteins AP3 and PI could not be identified in yeast with the full length proteins, but there is a clear interaction between the full-length proteins in living cells (Fig. 3G). In previous studies the AP3-PI couple showed to be interacting with SEP3 as a ternary factor (Honma and Goto, 2001), suggesting that this protein can mediate and stabilize the interaction between the two full-length B-type proteins.

Our results (Figs. 3C and 3D) revealed that the interactions between SEP3-AP3 and SEP3-PI are weaker when compared to SEP3-AG and SEP3-AP1 (Figs. 3A and 3B), whereas the dimer PI-AP3 (Figs. 3G and 5A) showed a strong decay in lifetime in some specific areas of the nucleus only. Furthermore, upon co-transfection of PI and AP3 a reproducible localization of these proteins around the nucleolus was observed (Figs 3G and 5A). So far, it is not clear whether this localization has any biological relevance.

To verify if the protein complex involving AP3, PI and SEP3 can be stably formed in living cells we performed FRET-FLIM analysis between PI -CFP and SEP3 -YFP in the presence of a non labeled AP3 protein. As expected based on the yeast results, the weak or transient interaction between SEP3 and PI turns into a more stable interaction. Remarkably, also in this case a preference for localization around the nucleolus was observed (Fig 5C).

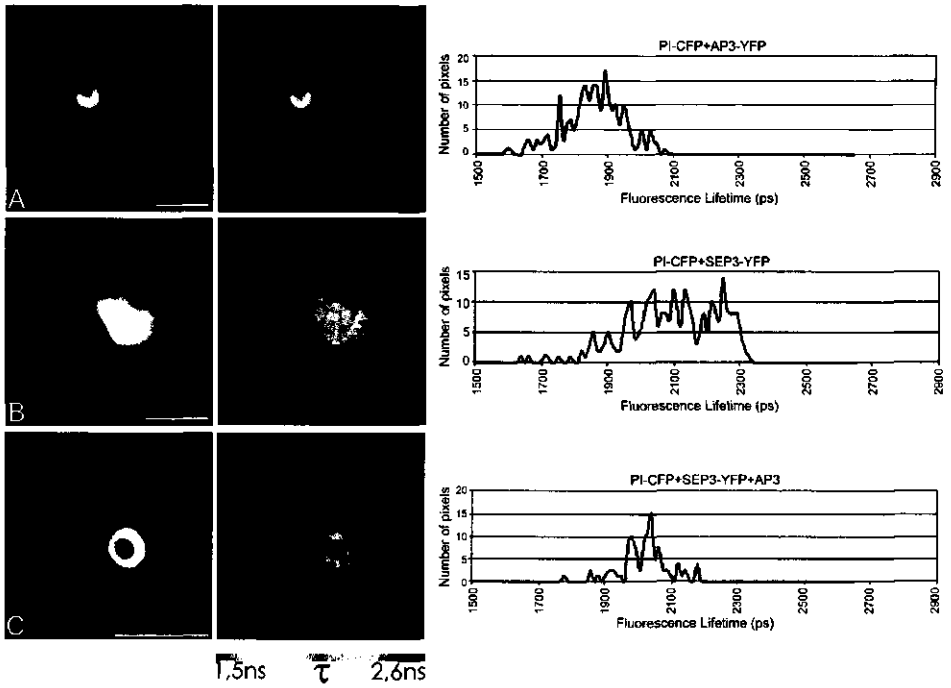

Figure 5. APETALA3-PISTILLATA and SEPALLATA 3 form a higher-order complex in vivo

The analysis of transfected Arabidopsis leaf protoplasts, co-expressing MADS-box proteins fused to either CFP or YFP. One representative protoplast is shown for each combination. The left panels display the intensity channel, the middle panels show the fluorescence lifetime image of the same nucleus in a false color code, and the right panels depict histograms representing the distribution of fluorescence lifetime values within the nucleus.

***(A)** FLIM analysis on protoplast transiently expressing PI-CFP and AP3-YFP; **(B)** PI-CFP+SEP3-YFP; **(C)** PI-CFP+SEP3-CFP+AP3. Bars=10μm.*

DISCUSSION

MADS-box transcription factors play essential roles during development in flowering plants. Their functioning and specificity are mainly determined by direct physical protein-protein and protein-DNA interactions. Although, many genetic studies have yielded a wealth of information about how members of this family act in flower architecture, there is a lack of knowledge concerning their molecular functioning and mechanisms of regulation. In order to shed light on the mode of action, we have made use of the sophisticated micro spectroscopy technique FRET-FLIM (Fluorescence Resonance Energy Transfer-Fluorescence Lifetime Imaging Microscopy), to analyze protein-protein interactions in living cells. This technique enables measurements of molecular distances between two fluorophores that are in close proximity, and in this way gives a good indication for dynamic protein complex formation.

Initially, localization studies were done for the MADS-box proteins AP3, PI, AG and SEP3, and for all of them nuclear localization was observed. The result for AP3 and PI was in disagreement with the results previously obtained by MacGonigle *et al.*, 1996, but we showed that the reason for the obtained differences is in the labeling of the proteins at either the C- or N-terminus. Our results indicate that N-terminal labeling of the B-type MADS-box proteins can indeed affect their localization. The same was observed for the MADS-box protein AP1 upon N-terminal labeling with GFP, and additional functional analysis revealed that the N-terminal fusion was unable to rescue the *ap1-15* mutant (Wu *et al.,* 2003). When C-terminal fusions were used, all proteins were localized in the nucleus and homodimerization was detected for all tested proteins, which is in line with the hypothesis that dimerization is needed for nuclear localization (MacGonigle *et al.*, 1996; Immink *et al.,* 2002. Nevertheless, AP3 and PI showed homodimerization only in specific spots, suggesting that the interactions are very weak or very transient. This may also explain why we failed to detect many homodimers of MADS-box proteins in yeast 2-hybrid screenings (de Folter *et al.*, 2005), where probably some other important cellular components, for instance protein modifiers, that might contribute to the direct interactions are not present when compared to experiments done in living cells, for example. Currently, it is still unclear whether these very transient interactions are biologically relevant. However, the ability of B-type proteins to homodimerize is supposed to be the ancestral status, which afterwards evolved in obligatory heterodimerization in the

core eudicots (Winter *et al.*, 2002). In line with this, it could well be that these very transient interactions identified for the individual Arabidopsis B-type proteins are remnants of their former ability to homodimerize that has been partly lost during evolution. Apparently, this remnant potential to homodimerize can only be detected by a very sensitive method like FRET-FLIM.

Besides homodimers, various heterodimers were identified. As expected, the combinations that were positive in yeast two-hybrid screens and by co-immunoprecipitations (de Folter *et al.*, 2005; Honma and Goto, 2001), such as for example SEP3-AG and SEP3-AP1, showed a strong decay in lifetime, indicating an interaction between the MADS-box proteins. Furthermore, the well-studied heterodimer AP3-PI (Honma and Goto, 2001) was clearly detected by FRET-FLIM. Remarkably, this heterodimerization could not be detected in yeast when the full length proteins were used (Yang *et al.*, 2003; de Folter *et al.*, 2005). In the FRET-FLIM experiments, the AP3-PI heterodimer was specifically observed around the nucleolus. Although we have observed this reproducible localization in all transfected cells, it is not clear whether this interaction pattern has any biological relevance; even though the protein localization is clear it might be possible that the observed interactions could be stabilized by co-factors that are present around the nucleolus only. Furthermore, we observed that when both AP3 and PI were present in the same cell, the fluorescent signals increased enormously in comparison with single transfections of the same proteins. This strongly suggests that heterodimerization stabilizes the B-type proteins. Besides the expected dimers discussed above, several combinations that were not interacting in yeast, were tested for heterodimerization in plant cells, like: SEP3-AP3, SEP3-PI, AG-AP3, and AG-PI. To our surprise, SEP3 and AG interacted with all tested floral organ identity proteins *in vivo,* but interestingly, there was a clear preference for some specific partners. SEP3 seems to interact weakly with both AP3 and PI for example, while AG prefers PI to AP3. This preference has also been demonstrated by co-immunoprecipitations in earlier experiments by Riechmann *et al.*, (1996). Besides that, AG could partially substitute for AP3 in the nuclear localization of PI-GUS, suggesting that AG is able to interact with PI (MacGonigle *et al.,* 1996). Strikingly, *PI* is expressed at very early developmental stages in the centre of the floral meristem during carpel development, where also AG is present (Goto and Meyerowitz, 1994). Taking this all into account,

PI may have a function together with AG in the fourth floral whorl and hence, the detected interaction could be of biological relevance.

For some combinations, e.g. AG-AP3, the FRET analysis revealed a spotty pattern in the nucleus, probably representing a weak and/or dynamic interaction. One could speculate that these spots mark areas in the nucleus where these interactions are stabilized, e.g. by DNA binding. Alternatively, these sub-nuclear spots are so called 'hot-spots', where transcription factors and co-factors are stored while still associated to the DNA (Moorman and colleagues, 2006). At this point, it is essential to perform experiments that would shed light on the specific interaction between MADS-box proteins and genomic DNA *in vivo*. An approach would be to perform *in-situ* FLIM measurements according to Cremazy *et al.*, 2005; however in this case, the cells need to be fixed, which will exclude the detection of dynamic, or more transient interactions.

Another aspect of the MADS-box protein interactions concerns the exact composition of the complexes for the various identified positive combinations. Could some of the unexpected interactions be explained by higher-order complex formation between two homodimers for example? To test this possibility, a mutated version of SEP3 lacking the C-domain (SEP3ΔC) was generated. It is known that the C-region of the MADS-box proteins is the domain which presents a large degree of variation and this variation may drive the specificity for partner affinity in higher-order complex formation (Vandebussche *et al.*, 2003; Lamb and Irish, 2003). Therefore, the use of a mutated version of the SEP3 protein that lacks ternary interaction capacity in yeast would provide us with evidence for this hypothesis. When using SEP3ΔC, we expected that only real heterodimers would give a clear FRET signal. In line with our expectations, deletion of the SEP3 C-terminus had hardly any effect on the interactions with AG and AP1. These results give strong evidence for heterodimerization between SEP3 and AG, and SEP3 and AP1, without the interference of a third factor. The SEP3-AG interaction is supposed to be involved in determination of carpel and stamen identity and according to the "quaternary model" (Theißen & Saedler, 2001) two of these dimers form a higher-order complex for carpel development. Based on our results we can not rule out that AG and SEP3 associate into higher-order complexes, but our results suggest that at least stable heterodimers can be formed *in planta*. The same holds for SEP3 and AP1. In contrast, and very interestingly, were the results obtained for the combinations

SEP3ΔC-AP3 and SEP3ΔC-PI. These interactions were strongly affected when compared to the combinations with the full length SEP3 protein. Based on these observations, we hypothesize that in the case of SEP3-AP3 and SEP3-PI, a FRET signal is obtained because of higher-order complex formation between two homodimers. Whether these kinds of complexes between two homodimers are functional in plants is not known at the moment. However, in eudicots, only homodimerization of the B-type proteins seems not to be sufficient for their homeotic functions in petal and stamen development (Winter *et al.*, 2002). Although they are not sufficient for complete homeotic changes (Jack *et al.*, 1994; Goto and Meyerowitz, 1994) it can not rule out that the complexes between homodimers fulfill some more subtle functions in floral organ development.

In conclusion, our results revealed that the physical interactions between MADS-box proteins show partner selectivity and affinity. It is likely that only very stable dimers will be assembled into multimeric complexes in an *in vivo* environment; however, some of the transient and very dynamic interactions might be biologically relevant, but on the other hand difficult to be detected by conventional techniques. Thus, the next challenging step to broaden our understanding on the molecular mechanisms of transcription activity will be to unravel the interaction affinities and competition effects that play a role during transcription in plant cells.

ACKNOWLEDGEMENTS

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Chapter 4

Competition-FRET: a new method to unravel protein dynamics in living cells

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ABSTRACT

In the cell, various dynamic processes are determined by physical interactions among proteins, interactions between proteins and other molecules and post-translational modifications of proteins, like glycosylation and phosphorylation. Together, these interactions and modifications are key components of the regulatory mechanisms that allow a rapid response to adjacent cells or environmental signals. Currently, our knowledge about various cellular paradigms increases substantially due to the use of sophisticated microscopic techniques and the great choice of different fluorophores to label and follow molecules in space and time. FRET (Fluorescence Resonance Energy Transfer) based methods, for example, allow the visualization of temporal and spatial cellular processes on a nanometer and nanosecond scale. Nevertheless, the use of FRET based techniques has been restricted for monitoring direct protein-protein interactions and was never applied to analyze competition for interaction or protein dynamics in an intact cellular environment. In this study, we developed a new method based on the FRET principle, "Competition – FRET", which can be used to monitor competition and/or higher-order complex formation in living cells. To allow unidirectional FRET measurements, we monitored FLIM (Fluorescence Lifetime Imaging) between EYFP and mRFP (donor-acceptor pair) in the presence of a third molecule labeled with ECFP (competitor or ternary factor). We applied the novel method to detect competition for the formation of homo-, heterodimers or higher-order complexes for the MADS-box transcription factor proteins SEPALLATA3 and AGAMOUS; which are key players during floral organ development in the model plant species *Arabidopsis thaliana*.

INTRODUCTION

Physical interactions between proteins play a key regulatory role in the coordination of cellular processes. Fluorescent tagging in combination with a number of sophisticated micro spectroscopic techniques allows the non-invasive visualization and monitoring of cellular processes at spatial and temporal scales (Lippincott-Schwartz and Patterson, 2003). Among several techniques, FRET-FLIM (Fluorescence Resonance Energy Transfer) - (Fluorescence Lifetime Imaging) has proven to be a very robust method to detect direct physical interaction between molecules of interest (Gadella *et al* 1995; Gadella *et al.* 1999; Wallbarbe and Periasamy, 2005).

FRET is based on the physical phenomenon that energy can be transferred from a fluorescent molecule to another chromophore that is in close proximity through a dipole-dipole coupling (Stryer, 1978). In such case, upon excitation of the donor, energy will be transferred to the acceptor in the nanometer range. Prerequisites for the occurrence of FRET are an overlap between the emission spectrum of the donor and the absorbance spectrum of the acceptor, satisfactory quantum yield for the donor, and the right spatial orientation and proximity of the two fluorescence molecules (Gadella *et al.*, 1999). FRET has become a general tool to identify protein interactions in mammalian cells, but this technique is still not commonly used in plant studies (Immink *et al.*, 2002; Shah *et al.*, 2000; Russinova *et al.*, 2004, Nougalli-Tonaco *et al.*, 2006). Possibly high implementation costs and high level of expertise to operate such a sophisticated micro spectroscopic set-up hampered general introduction into the plant sciences. The usage of FRET has been restricted to analyze the interaction between two labeled proteins only, whereas it would be extremely interesting to study the interaction and competition among more molecules at a time. The possibility of using a 3-chromophore FRET has been demonstrated by Galperin and colleagues (2004). In their case, FRET was monitored by the donor-acceptor couples ECFP-EYFP and EYFP-mRFP. However, when all three molecules are present, FRET efficiency between ECFP-EYFP increases (From E=0.42 to E=0.46). This increase in FRET efficiency is due to the fact that also direct FRET occurs between ECFP and mRFP, although at lower efficiency. This interferes with the measurement of the interaction between the ECFP and EYFP tagged proteins and hence the analysis of competition with a third protein. Only a few studies are known, where they have used FRET as a method to detect possible higher order

protein complexes. One example of its use for this purpose has been described recently by our group (Nougalli-Tonaco *et al.*, 2006). However, in this case a third non-labeled protein has been used for the detection of possible ternary or quaternary complex formation between plant MADS-box proteins. Hence it is not possible to study the expression level of this third protein, preceding quantitative analyses. As an attempt to address these kinds of questions, we developed a new method based on FRET, "Competition-FRET"; in which dynamics of partner's selection, competition among protein partners, and eventually the formation of higher-order complexes can be monitored in living cells. In our concept competition will cause a reduction in FRET signal, i.e. an increase in donor fluorescence lifetime upon the presence of the donor-acceptor pair and a competing molecule.

In the plant model species *Arabidopsis thaliana,* the MADS-box transcription factor family consists of 107 members and functional analyses revealed important roles for these proteins in the regulation of flower development and plant architecture (Parenicova *et al.,* 2003). Their functioning is dependent on direct physical protein-protein and protein-DNA interactions, to tightly regulate target gene expression in a temporal and spatial manner. Recently, a network of protein interactions has been established for this family of transcription factors by a matrix-based yeast two-hybrid approach and surprisingly, out of the 274 dimers obtained, only 5 are homodimers (de Folter *et al.*, 2005). Recent *in vivo* analyses demonstrated that in a plant cell environment homodimerization occurs much more frequently than could be monitored by the yeast two-hybrid system (Nougalli-Tonaco *et al.*, 2006). Out of the 107 members of the MADS-box family, two transcription factors, AGAMOUS (AG) and SEPALLATA3 (SEP3) were selected, which are important players involved in the development of reproductive floral organs, and the genes encoding these two proteins have been studied extensively (Yanofsky *et al.*, 1990; Pelaz *et al.*, 2000). Furthermore, these two proteins have been proposed to act in a higher-order complex for the determination of carpel identity (Theißen and Saedler, 2001). In yeast, these proteins heterodimerize, but no homodimerization could be detected for either of them (de Folter *et al.*, 2005). Interestingly, *in vivo* studies have shown that these proteins are able to form homodimers besides heterodimers, but most likely with different interaction affinities (Chapter 3; Nougalli-Tonaco I.A. and Immink R.G.H. unpublished results). Based on this, we hypothesized that partner selection and specificity plays an important role in defining which complexes will be formed

and hence, what the final developmental outcome will be. Nevertheless, virtually nothing is known about the mechanism for the selection of different homo- or heterodimerization partners *in vivo*. In line with all these findings we selected these two proteins as a model for the development of the "Competition-FRET method".
To set-up the system and verify the broad usefulness of this technique, we made fusion proteins consisting of the two MADS-box proteins of interest (AGAMOUS and SEPALLATA3) and fluorescent molecules (ECFP, EYFP and mRFP). Subsequently, we transiently expressed the generated fusion constructs in *Arabidopsis* leaf protoplasts and analysed for competition between homo- and heterodimerization, or alternatively higher-order complex formation, *in-vivo*. Based on the experiments we concluded that there is a strong preference for homodimerization of AG, whereas SEPALLATA3 seems to prefer to heterodimerize with AGAMOUS. Furthermore, the results obtained, suggest differences in specific homo- and heterodimerization protein-protein interaction domains for AGAMOUS and SEPALLATA3.

MATERIAL AND METHODS

Plant material

Leaf protoplasts were obtained from *Arabidopsis thaliana* Col0 leaves, which were grown under normal greenhouse conditions (16/8h light /dark), 22°C, according to Aker *et al.*, 2006.

Plasmid constructions

The coding regions of the MADS-box genes *AGAMOUS (AG)* and *SEPALLATA3 (SEP3)* were cloned as Gateway entry clones lacking stop codons in order to allow C-terminal fusions. The entry clones have been recombined into Gateway compatible pGD120 vectors, from which expression is driven by the constitutive CaMV35S promoter, and that contain either the coding region of Enhanced Cyan Fluorescent Protein (ECFP), Enhanced Yellow Fluorescent Protein (EYFP), or Monomeric RFP (mRFP), respectively (Nougalli-Tonaco *et al.*, 2006). All plasmids were controlled by sequencing analyses (DETT sequence kit, from Amersham).

Fluorescence microscopy in living cells

Protoplasts obtained from *Arabidopsis thaliana* leaves were transfected with the plasmids (38µg final concentration) and incubated overnight at 25°C in light before imaging as described by Aker and colleagues (2006).

FLIM acquisition

Fluorescence lifetime imaging was performed using the wide-field frequency domain approach on a home-build instrument (Van Munster and Gadella, 2004a) using a RF-modulated image intensifier (Lambert Instruments II18MD) coupled to a CCD camera (Photometrics HQ) as detector. A 40x objective (Plan Apochromat NA 1.3 oil) was used for all measurements. The modulation frequency was set to 75.1 MHz. Twelve phase images with an exposure time of 50-100 ms seconds were acquired in a random recording order to minimize artifacts due to photo bleaching (van Munster and Gadella, 2004b). An argon-ion laser was used for excitation at 514 nm, passed onto the sample by a 525DCXR dichroic (Chroma Inc., Rockingham, VT, USA) and emission light was filtered by a Chroma HQ545/30 nm emission filter. All components are controlled by self written macros and c-code in the Matlab (the Mathworks, Natick, MA, USA), running on a PC under Windows 98 (Microsoft, Redmond, WA, USA). Each FLIM measurement is calibrated by a reference measurement of the reflected laser light using a modified filter cube (Van Munster and Gadella, 2004a) for correcting the phase and modulation drift of the excitation light. The reference is calibrated by averaging three to five FLIM measurements of a freshly prepared 1 mg/ml solution of erythrosine B (cat # 198269, Sigma-Aldrich, Zwijndrecht, The Netherlands) in H_2O, which has a known short fluorescence lifetime of 0.086 ns (Bastiaens *et al.*, 1992; van Munster and Gadella, 2004b). This extra calibration corrects for path-length differences and possible optics-related reflections that are different between the FLIM and reference measurements.

From the phase sequence an intensity (DC) image and phase and modulation lifetime image are calculated using Matlab macros. For the latter two images a 3x3 pixel averaging operation was performed on the original phase sequence images. The three resulting images were processed for display using the Image J macro *'lifetimes6'*. This macro generates false color lifetime maps, 1 and 2 D histograms and intensity-weighted lifetime maps.

Next to taking lifetime images, 3 additional wide-field images were taken of the same specimen. Here, the mercury lamp was used as excitation source and the same modulated detector was used. The following filter settings (excitation, dichroic, emission) were used for the 3 images: 'CFP'-image (D436/20 nm, 455DCLP, D480/40), 'YFP'-image (HQ500/20, 525 DCXR, HQ545/30) and RFP (D546/10 nm, 600DCXR, 600FS20-25). All filters were from Chroma Inc. (Rockingham, VT, USA) except for the last filter, which was from Andover corp. (Salem, NH, USA). The 3 images were collected automatically (with equal exposure times (approx. 20-100 ms duration each)) and background fluorescence (excitation off) was subtracted, all controlled by a Matlab macro *'take_CYR'*.

Image processing

For quantitative analysis, the 3 lifetime images (DC, Tau(phi) and Tau(mod)) and the 3 'CFP', 'YFP', and 'RFP' images were concatenated into a single image stack of 6 images and were further processed in Image J. The background fluorescence was subtracted from the DC image and from the CFP, YFP, RFP images using a rolling ball algorithm (15 pixels diameter). Then a threshold was applied to the DC image, and this image was subsequently thresholded to 15% of the maximum intensity of the image. Then a mask was created for all pixels with higher intensities for identifying objects in the image (labeled nuclei). This mask was applied to all 6 images in the stack. Typically 5-20 nuclei could be identified for each image stack. For each object with a size larger than (50 pixels), the average intensity in the DC image, the average phase lifetime, modulation lifetime and the average 'CFP', 'YFP' and 'RFP' intensity were determined. These average values were exported to Microsoft Excel for further calculation. In Excel the 'CFP', 'YFP' and 'RFP' intensities were corrected for relative brightness of the fluorophores and differences in detection efficiencies based on a calibration experiment with purified proteins. In the calibration experiment, the three fluorescent proteins (ECFP, EYFP, and mRFP1) were loaded at identical concentrations in 3 microcuvettes. They were subsequently imaged with the three filter settings and their relative intensities were quantified. At identical concentration the detected brightness was 11.76 for ECFP, 4.05 for EYFP and 0.553 for mRFP1. The differences in the values are most determined by the microscope optics and presence/absence of mercury lamp emission lines. The corrected 'CFP', 'YFP' and 'RFP' intensity values (using division by the above mentioned numbers) were used

for calculating the fraction of acceptor to donor (RFP/(YFP+RFP)) and the fraction of competitor to acceptor (CFP/(CFP+RFP)). These fractions were plotted against the average lifetime being the square root of the phase lifetime times the modulation lifetime.

ECFP and EYFP were purified as described (Kremers *et al.*, 2006). Recombinant mRFP1 (cDNA kindly provided by dr. R.Y. Tsien) was isolated and purified from E coli transformed cultures essentially using the same protocol and kindly provided by dr. Ir. J. Goedhart (UvA, Amsterdam).

RESULTS

The principle of Competition-FRET

Like other FRET (Fluorescence Resonance Energy Transfer) based methods, Competition-FRET is based on the energy transfer principle of Förster. Our strategy for monitoring FRET was to make use of FLIM (Fluorescence Lifetime Imaging). In this case, the decay of the donor fluorescence lifetime is monitored in the presence of the acceptor molecule with or without competitor molecules. In our set-up we made use of EYFP (Enhanced Yellow Fluorescent Protein) as donor and mRFP (monomeric Red Fluorescent Protein) (Campbell *et al.*, 2002) as acceptor, and the competitor molecule was labeled with ECFP (Enhanced Cyan Fluorescent Protein) (Figure 1). The basic difference between FRET and Competition-FRET is that a third molecule, which is also labeled with a fluorophore, cannot function as an acceptor molecule for the donor; however it is either able to compete out one of the protein partners of the dimer, or alternatively, it is able to assemble into a higher-order complex together with the donor and acceptor molecules. Normally, in case of dimerization between donor and acceptor molecules, a reduction in fluorescence lifetime of the donor molecule can be detected. Once a third molecule is added that can compete out one of the protein partners, the reduction of the fluorescence lifetime will diminish (less FRET) or completely disappear. In contrast, upon higher-order complex formation, a dimer is probably stabilized and hence, a stronger reduction in fluorescence lifetime will be detected instead (increased FRET). Figure 1 shows a schematical representation of the Competition-FRET principle.

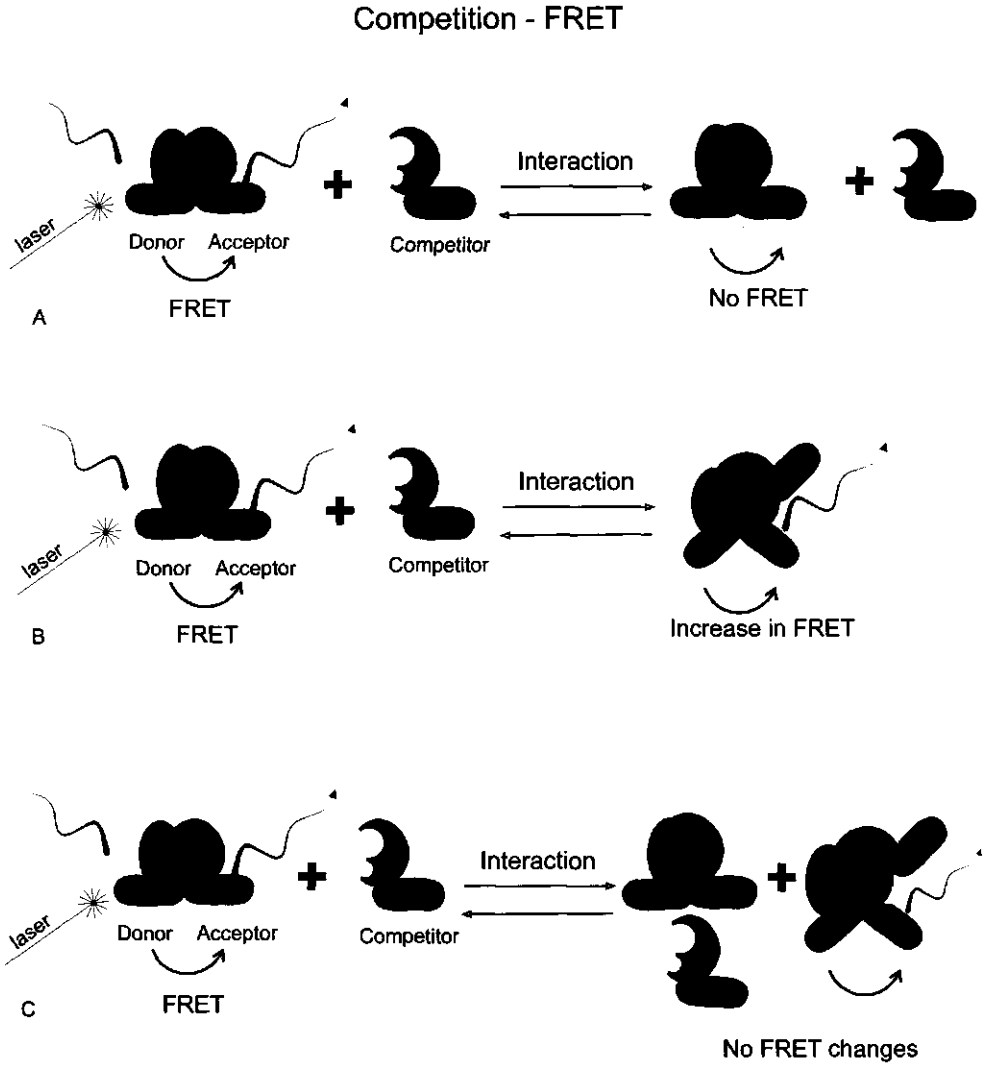


Figure 1- Competition-FRET can be used for the detection of differences in dimerization affinity and competition effects between molecules. *Upon an interaction between the two proteins of interest the YFP donor molecule and mRFP acceptor come into close proximity, which will result in FRET. Upon presence of a third molecule (competitor labelled with CFP), differences in FRET can be monitored. A) Fluorescence lifetime of the donor will increase when competition between proteins "W" and "Y" takes place. B) As a result of adding the third protein stronger FRET will occur in case of stabilization of the dimer by higher-order complex formation, and hence the fluorescent lifetime of the donor will decrease. Alternatively, a mixture of different complexes may occur simultaneously, in this case the donor lifetime will be unaltered (C).*

FRET does not occur in the EYFP- ECFP direction

The novel technique explained above can work only, when no energy can be transferred between the donor (EYFP) and the competitor (ECFP); this can be easily achieved and verified by a lack of overlap between the donor emission spectrum and the competitor absorbance spectrum. For EYFP and ECFP, respectively, this is the case. To further demonstrate the lack of FRET for EYFP to ECFP in our system, we started our analysis by testing the possibility of energy transfer from EYFP to ECFP, when EYFP is excited as donor molecule. For that, we analysed a few combinations of labelled MADS-box proteins, for which previously interaction has been detected in living plant cells by FRET-FLIM measurements (Chapter 3). The combinations AGAMOUS-EYFP + AGAMOUS-ECFP and AGAMOUS-EYFP + SEPALLATA3-ECFP were analysed and in both cases, the fluorescence lifetime (Tau) of the donor, in this case AGAMOUS-EYFP, was not reduced in the presence of the proteins fused to ECFP. Average values of 2.5ns for Tau phi (phase) and 2.7ns for Tau mod (modulation) were found for the pool of cells that co-expressed the EYFP and ECFP fusion proteins. These values are identical to the values found when the single EYFP labelled proteins were expressed and subsequently, analysed (Fig 2).

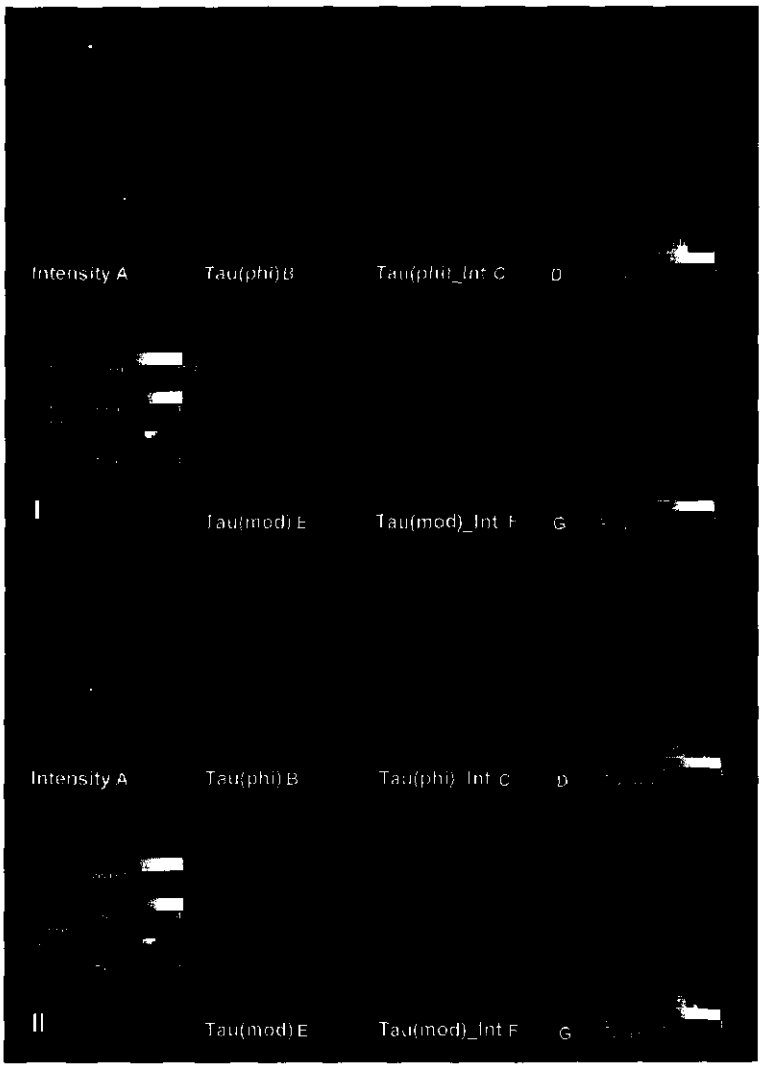


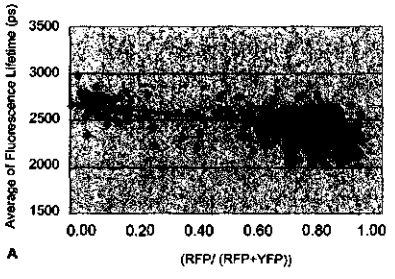
Figure 2 – FRET analysis of EYFP to ECFP.
Panel I) *AGAMOUS was labelled with EYFP (donor) and was transiently expressed in protoplasts. A) Displayed is the EYFP intensity. The nuclei of various protoplast can be seen as bright spots; B the EYFP lifetime based on the phase [Tau (phase)] for the different nuclei, indicated by false colour, and C the intensity weighted EYFP lifetimes based on the phase [Tau (phase)] for the different nuclei; D) Histogram of the EYFP phase-lifetime. E and F) In these panels the EYFP lifetime is calculated based on the modulation [Tau (mod)] and represented by false colour codes; G) the histogram from the lifetime based on modulation. The legend in the left bottom panel shows the color bars for the intensity and lifetime values.* ***Panel II)*** *AGAMOUS labelled with EYFP and SEPALLATA3 labelled with ECFP were transiently expressed. The EYFP lifetime remained unchanged as compared to panel I (note unaltered horizontal position of the histograms D) and G) as for Panel II.*

Homo and heterodimerization for AGAMOUS and SEPALLATA3 in living cells

Since no FRET could be detected from EYFP to ECFP when EYFP was excited as donor molecule, the next step was the identification of homo- and heterodimers between AGAMOUS (AG) and SEPALLATA3 (SEP3) using EYFP-mRFP as donor-acceptor FRET pair. By means of these experiments, we should detect homodimerization for both AG and SEP3, as well as heterodimerization between these two MADS-box proteins, in a similar way as has been reported before (Chapter 3), where we made use of ECFP-EYFP as donor-acceptor FRET couple. For all three combinations the donor fluorescence lifetime dropped on average from 2,5ns to 2,0ns for Tau phi and from 2,7ns to 2,4ns for Tau mod, confirming the expected homo- and heterodimerization.

AGAMOUS homodimer can not be competed out by SEPALLATA 3 protein

Once that homodimerization could be detected for both AGAMOUS and SEPALLATA3, as well as heterodimerization in the case that both proteins are present, the next question to be addressed is if there is a possible preference for either homo- or heterodimer formation inside living cells for the AGAMOUS and SEPALLATA3 proteins? For this, the competition-FRET method was applied and we screened for FRET changes when the competitor (i.e., heterodimer partner) is simultaneously transfected with the homodimer acceptor-donor combination. For accuracy in the data analysis, we combined the intensity values for the three fluorophores, and the two different lifetimes, Tau phase and Tau modulation, into relative fractions, fraction of acceptor to donor: [acceptor/(acceptor + donor)] and fraction of the competitor to the acceptor: [competitor/(competitor + acceptor)] against an average of the lifetimes. For this quantitative analysis, the transfected combinations: "donor" only, "donor – acceptor", and "donor- acceptor – competitor", were combined.



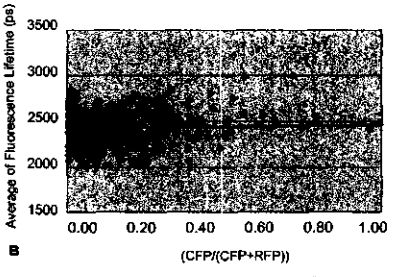


Figure 3. AGAMOUS homodimer can not be competed out by SEPALLATA 3 protein. *The graphs display all data points obtained from 200 up to 400 different cells that have been analysed with the combinations AG-EYFP + AG-mRFP, AG-EYFP+AG-mRFP + SEP3-ECFP, and AG-EYFP alone. A) The square root of Tau phase and Tau modulation is plotted against the acceptor-donor fraction. B) An average of Tau phase and Tau modulation lifetimes is plotted against the competitor-acceptor fraction.*

For the first competition experiment, we tested whether the homodimer formed by AGAMOUS protein could be competed out by its heterodimeric partner SEPALLATA3. Surprisingly, the homodimerization of AGAMOUS (Fig 3A) was not affected by its heterodimerization partner SEPALLATA3 (Fig 3B). Neither competition (increase in lifetime), nor clear higher-order complex formation (decrease in lifetime) could be detected in this case.

AGAMOUS can compete out the SEPALLATA3 homodimer and the heterodimer AGAMOUS-SEPALLATA3

The following competition experiment was designed to investigate whether the homodimer formed by SEPALLATA3 could be competed by the AGAMOUS protein. For this purpose, AGAMOUS-ECFP was co-transfected in combination with SEPALLATA3-EYFP and SEPALLATA3-mRFP. In contrast to what has been seen for the AGAMOUS homodimer, in this experiment a major competition effect could be observed between the heterodimer partner AGAMOUS and the SEPALLATA3 homodimer (Fig 4A and 4B), reflected by an increase in SEPALLATA3-EYFP fluorescence lifetime when the competitor (AGAMOUS-ECFP) was added. Additionally, we tested if AGAMOUS is able to compete the heterodimer AGAMOUS-SEPALLATA3. In this case, a clear competition was shown by a strong increase in fluorescence lifetime upon co-transfection with the competitor (Fig 4C-D), indicating that an AGAMOUS homodimer is preferred over an AGAMOUS-SEPALLATA3 heterodimer.

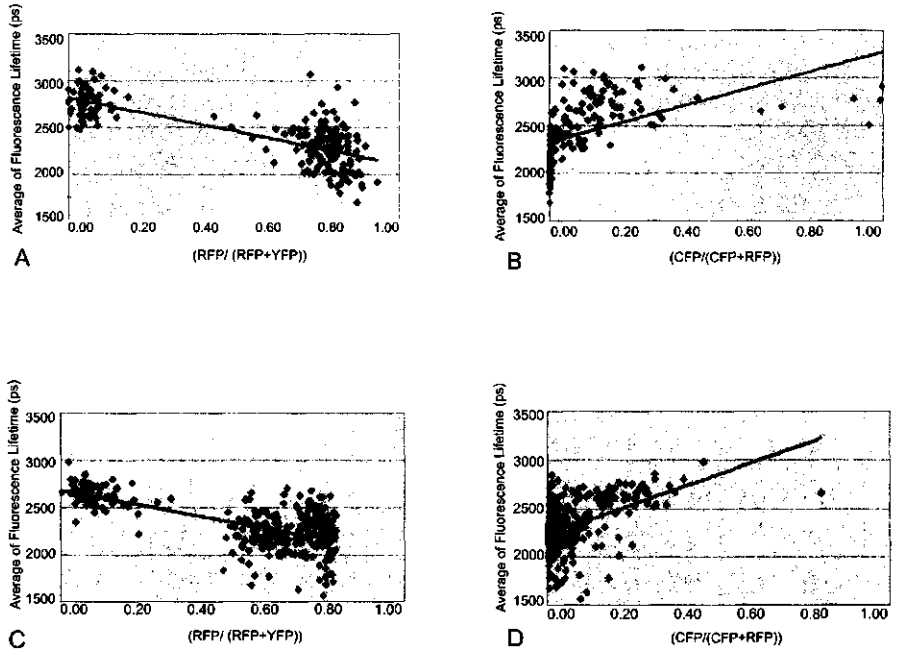


Figure 4. AGAMOUS protein can compete out the homodimer formed by SEPALLATA3 and the heterodimer AGAMOUS-SEPALLATA3.
The graphs display the following combinations A, and B: SEP3-EYFP, SEP3-mRFP and competitor AG-ECFP; and C and D: AG-EYFP, SEP3-mRFP and competitor AG-ECFP. A and C show the square root of Tau phase and Tau modulation plotted against the acceptor-donor fraction. B and D graphs display an average of Tau phase and Tau modulation lifetimes plotted against the competitor-acceptor fraction.

The role of the SEPALLATA 3 C-terminus in complex formation

The C domain of MADS-box proteins has been reported to be a mediator of higher-order complex formation between proteins of this transcription factor family (Egea Cortines et al., 1999; Honma and Goto, 2001), whereas the last part of the MADS domain, the I-region and the K-box are supposed to be involved in determining dimerization specificity (Yang et al., 2003 and Yang and Jack, 2004). Taking this into account, we tried to monitor differences in dimerization preference for a SEPALLATA 3 protein with a truncation of the C-terminal tail (SEP3ΔC). Analysis of this truncated protein by the yeast two-hybrid system revealed that it is still able to form a heterodimer with AGAMOUS, but that in contrast to the full-length SEPALLATA3, it lacks the capacity to form a higher-order complex with AGAMOUS (AG) and SEEDSTICK (STK) (Immink and Angenent, unpublished results). In this experiment

we monitored FRET changes between the homodimer formed by SEPALLATA3 upon presence of a third full-length SEPALLATA3 protein, or the SEPALLATA3ΔC protein labeled with ECFP. As depicted in Fig 5A and 5C, we were able to detect clear reduction of fluorescence lifetime, i.e. homodimerization of SEPALLATA3 (SEP3-Y+SEP3-R); however, upon addition of the third SEPALLATA3 molecule (competitor), competition among SEPALLATA3 molecules takes place (Fig 5B), which results in an increase of the lifetime. This suggests that a dimer rather than a ternary complex is preferred. Similar results were obtained when SEPALLATA3ΔC as competitor was used instead, indicating that the C-terminal domain is not involved in homodimerization of this protein (Fig 5D).

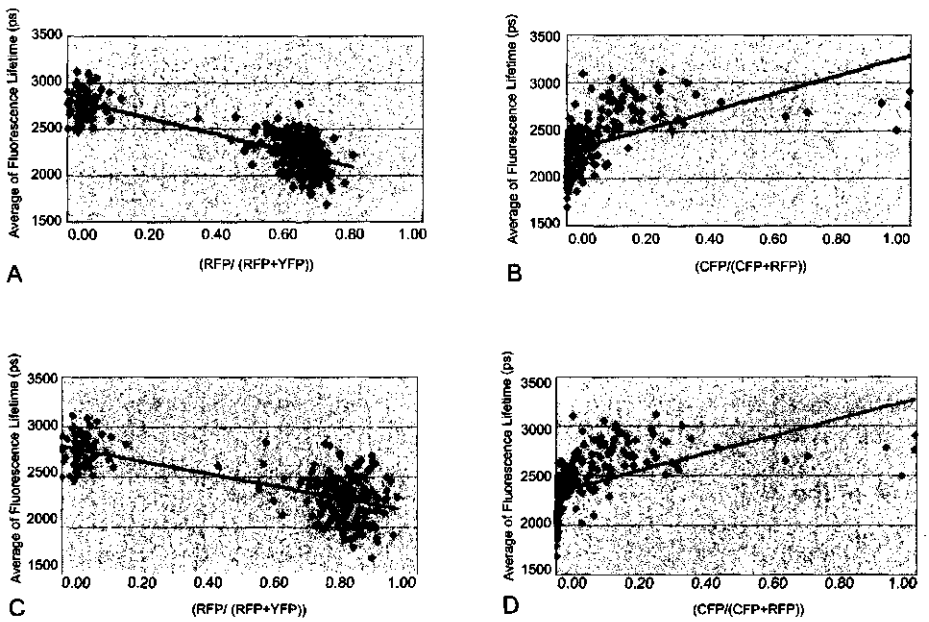


Figure 5. The role of the C-terminus of SEPALLATA3 in homodimerization. A and B: *SEP3-EYFP, SEP3-EYFP + SEP3-mRFP, SEP3-EYFP + SEP3-mRFP + SEP3-ECFP; and* ***C*** *and* ***D****: SEP3-EYFP, SEP3-EYFP + SEP3-mRFP, SEP3-EYFP + SEP3-mRFP +SEP3ΔC-ECFP.* ***SEP3ΔC*** *encodes for SEPALLATA3 lacking the C domain. Graphs A and C show the square root of Tau phase and Tau modulation plotted against the acceptor-donor fraction. B and D graphs display an average of Tau phase and Tau modulation lifetimes plotted against the competitor-acceptor fraction*

DISCUSSION

The ability to visualize and monitor physical interactions between proteins in a living cellular environment enables biologists to visualize major molecular mechanisms that are at the basis of biological processes. FRET (Fluorescence Resonance Energy Transfer) based methods report on direct physical interactions between proteins (Gadella *et al.*, 1999; Walrabe and Periasamy, 2003). While FRET based methods have proven to be useful in the analysis of two-component physical interactions, it remains a challenge to expand the technique towards more complex interactions. For three components, three labeled proteins and knowledge about the concentrations of the labeled proteins are required to monitor FRET. Recently, Galperin and colleagues (2004) have shown the use of three-chromophore FRET with ECFP, EYFP and mRFP in the detection of protein complexes in living cells. Although the method shows a number of applications, its robustness is affected by the fact that direct FRET, besides ECFP to EYFP and EYFP to mRFP, can also occur between ECFP and mRFP and thereby complicate the data analysis.

In this study, we developed a novel method, "Competition-FRET"; which allows quantitative detection of a third molecular component on the interaction between protein dimer partners. The method is based on the detection of FRET changes between the donor-acceptor pair and the donor-acceptor + competitor combination. In the Competition-FRET method, the donor molecule used is EYFP, the acceptor molecule is mRFP and the competitor molecule is tagged with ECFP, where the latter does not contribute to the FRET. Making use of EYFP as a donor has several advantages in this case. Due to its physical properties, unidirectional FRET can be detected between EYFP and mRFP, while no energy can be transferred from EYFP to ECFP. Furthermore, the use of EYFP as a donor in plant cells has an extra advantage when compared to ECFP because it causes fewer problems with auto-fluorescence originating from the chloroplasts.

To test the method, we selected two important players in floral organ formation in *Arabidopsis thaliana* AGAMOUS (AG) and SEPALLATA3 (SEP3), and tried to determine in living cells whether homodimerization is preferred instead of heterodimerization. Homodimerization among MADS-box proteins seems to be rare, but that is mainly based on yeast two-hybrid data (de Folter *et al.* 2005). In contrast, homodimerization could be easily detected in living plant cells by FRET, although in the absence of potential heterodimerization partner (Immink *et al.*, 2002; Nougalli-

Tonaco *et al.*, 2006). Our results reported here demonstrate the formation of both homo and heterodimers for the AGAMOUS and SEPALLATA3 proteins, but interestingly, we could detect preference for homodimerization for the AGAMOUS protein above heterodimerization with SEPALLATA3. This result is surprising, since in yeast no homodimer could be detected for the AGAMOUS MADS-box protein and it is generally believed that homodimers interact weakly (de Folter *et al.*, 2005). In contrast, SEP3 prefers to form a heterodimer with AGAMOUS, because AGAMOUS was able to compete out the SEPALLATA3 homodimer. In a similar experiment, AGAMOUS could compete out the heterodimer AG-SEP3, although in this case we could not distinguish whether the AGAMOUS competitor is titrating out the AGAMOUS donor or the SEPALLATA3 acceptor, since AGAMOUS is able to homo- and heterodimerize with both proteins. However, taking into account the strong homodimer formation of AGAMOUS molecules, it is most likely that AGAMOUS-AGAMOUS homodimers are formed.

We also studied the formation of a ternary complex between a dimer and a third molecule. According to the quartet model for MADS-box proteins (Theißen and Saedler), MADS-box proteins are able to form higher order complexes composed of three molecules or two dimers. In previous FRET-FLIM experiments (Nougalli-Tonaco *et al.*, 2006), we were able to detect a stabilization of a dimer when a third protein was added. This strongly indicated (in an indirect way) that a higher order complex was formed in living plant cells, although we could not monitor the third protein because it was not tagged by a fluorescence protein. Here we labelled SEPALLATA3 with three chromophores and we did not observe an indication for higher order complex formation by this protein on its own. Addition of SEPALLATA3 competitor disturbed the donor-acceptor homodimer, by titrating out the donor or acceptor and hence diminishing FRET. When avoiding higher order complex formation by deleting the C-terminal domain, a similar reduction in FRET was observed when compared to full-length SEPALLATA3. All together, it is tempting to conclude that there is a preference to form a dimer rather than a ternary complex. However, we can not exclude that certain ternary complexes and/or dimers can be formed that can not be detected as a FRET couple, because of unfavourable distance/orientation of the chromophores. Furthermore, it is possible that tagging of all three proteins at the C-terminus with a fluorescent protein prevents the formation of a ternary complex.

Based on genetic and yeast n-hybrid studies it was shown (Pelaz *et al.*, 2000; Honma and Goto, 2001) that both SEPALLATA3 and AGAMOUS are in one and the same complex that is responsible for the identity specification of the fourth whorl. It was assumed (for review, see Ferrario *et al.*, 2006) that such a complex is composed of two heterodimers AG-SEP3; however based on the results presented here, it is more likely that an AGAMOUS homodimer is an essential component of such a complex. What the role of SEPALLATA3 is in this complex and whether it forms a homodimer as well, or binds as a single molecule in a ternary complex structure, remain to be elucidated. .

In conclusion, the novel method developed here, reveals to be a robust method for the detection of protein interaction dynamics and competition between proteins in living cells.

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Chapter 5

The use of BiFC to detect protein-protein interactions between MADS-box transcription factors

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ABSTRACT

The investigation of cellular behavior as well as dynamic processes such as protein-protein interactions with spatial and temporal resolution has become an important issue in biology. To follow these processes and mechanisms, preferably in a living cell environment, an enormous variety of imaging techniques has been developed in the last few years. Among several methods, the most robust technologies to detect protein-protein interactions in living cells are based on the FRET (Fluorescence Resonance Energy Transfer) principle, although they are very laborious and require sophisticated and expensive equipment. Here, we implemented the BiFC (Bimolecular Fluorescence Complementation) assay as a method to detect protein-protein interactions in living plant cells, which is a more simple way. We made use of the well studied heterodimer FBP2 - FBP11 as a strategy of choice to set-up the method. These two petunia MADS-box proteins have been characterized as physical interacting proteins in living plant cells by FRET-FLIM (Fluorescence Lifetime Imaging Microscopy) and FRET-SPIM (Spectral Imaging Microscopy). A dozen of different constructs were made, based on the fragmentation of the Yellow Fluorescence Protein (YFP) into two non-fluorescent parts, and tested by transient expression in cowpea and Arabidopsis protoplasts. Many different variations were tested, such as changing the split position of the YFP molecule, making use of various peptide sequences as linkers, and finally, different incubation temperatures for the transfected protoplasts. Furthermore, we explored the possibilities to use the method in combination with FRET to detect higher-order complex formation for MADS-box proteins. Our results demonstrate that although the BiFC approach is simple and can be broadly used, the method has limitations and therefore, some important aspects of experimental design should be considered. Finally, we concluded that for MADS-box proteins, this method seem to be sensitive to different experimental conditions and hence, FRET based methods provide more robust data.

INTRODUCTION

Knowledge about the dynamics of the cell and its processes has become a crucial point for many biologists in recent years. Methods to observe the main processes inside a living cell, and preferable in intact tissues and under native conditions, will provide the key to understand regulatory processes and pathways. Substantial improvements have been accomplished by the use of sophisticated imaging techniques, which allow an appropriate spatial and temporal observation of the cellular components and their behavior. In addition, the use of fluorescent proteins such as GFP (Green Fluorescent Protein) and its variants had a tremendous impact on cell biology. GFP allows studying biological processes in vivo, such as trafficking of molecules, protein-protein interactions, and expression of genes (Chudakov *et al.*, 2005). The microscopic techniques based on FRET (Fluorescence Resonance Energy Transfer) provide elegant ways to determine physical interactions between fluorescently labeled molecules (Gadella *et al.*, 1999). Despite the fact that these novel technologies such as acceptor photo bleaching, FLIM (Fluorescence Lifetime Imaging), and SPIM (Spectral Imaging Microscopy) are very powerful and reliable, an expensive microscopy set-up, as well as laborious data analyses are required (Gadella *et al.*, 1993; Immink *et al.*, 2002; Nougalli-Tonaco *et al.*, 2006).

Recently, a novel technology has been established to identify protein-protein interactions in living cells in a more simple way. This approach, called BiFC (Bimolecular Fluorescence Complementation) uses the complementation of two "split" parts of any variant of the Green Fluorescence Protein (GFP) for the detection of protein-protein interactions (Ghosh *et al.*, 2000; Hu *et al.*, 2002). In case of Yellow Fluorescent Protein (YFP) for instance, the molecule is split into two non-fluorescent and complementary fragments: YFP/N and YFP/C. Fusion proteins are generated by cloning the genes encoding for the proteins of interest in-frame with either the YFP/N or the YFP/C encoding parts. The two fragments of YFP are not fluorescent on their own, but upon protein–protein interaction between the proteins of interest, the two parts of YFP are brought together, followed by reassembly of the protein and recovery of fluorescence (Hu *et al.*, 2002). This method has been successfully used for the determination of protein-protein interactions in various living cells. This has been demonstrated repeatedly for interactions between transcription factors in mammalian cells, e.g.the interaction of bZIP and Rel family transcription factors (Hu *et al.*, 2002; Hu and Kerpolla, 2003). Subsequently, the method was

further developed into multicolor BiFC to demonstrate dimerization and competition for interaction between Myc/Max/Mad family members (Grinberg *et al.*, 2004). More recently, a few examples were reported on the use of this technology in living plant cells (Bracha-Drori *et al.*, 2004; Walter *et al.*, 2004; Bhat *et al.*, 2006), indicating that this technology can be broadly used for many organisms.

MADS-box proteins are very important players in the regulation of various developmental processes in higher land plants. At the molecular level, they interact physically and form dimers and higher-order complexes that are supposed to regulate specifically different pathways. Over 100 members of the MADS-box family have been identified in the Arabidopsis genome (Parenicová *et al.*, 2003), and recently, a comprehensive network of Arabidopsis MADS-box protein-protein interactions has been established by yeast 2-hybrid studies (de Folter *et al.*, 2005). Furthermore, studies using FRET-SPIM and FRET-FLIM have been performed for some petunia MADS-box proteins and revealed physical interactions in living plant cells (Immink *et al.*, 2002; Nougalli-Tonaco *et al.*, 2006).

In this study, we aimed to establish the BiFC technology to study dimerization and higher-order complex formation for plant MADS-box transcription factors *in vivo*. A tool to better study the behavior of these transcription factors *in vivo* will provide new insights in the functioning of these proteins in their natural context. Advantages and drawbacks of the use of this technique for MADS-box proteins will be discussed.

MATERIAL AND METHODS

Plant material

Leaf protoplasts were obtained from the cowpea variety Black Eye California and from young Arabidopsis thaliana wild type plants (Col0, rosette stage). Both were grown under normal greenhouse conditions (16/8h light /dark), at 28°C (cowpea) and 22°C (Arabidopsis) according to Nougalli-Tonaco *et al.*, 2006 and Aker *et al.*, 2006, respectively.

Plasmid construction for BiFC analysis in living plant cells

For the BiFC analysis in living plant cells, different sets of constructs have been tested and a schematic representation of them is provided in Figure 1. Initially, the coding sequence of the Enhanced Yellow Fluorescence Protein (EYFP) was fragmented into two parts, encoding for an N-terminal 154 amino acids fragment (YFP/N154) and for a C-terminal 84 amino acids fragment (YFP/C84), respectively (Figure 1 and 2). Four different fragments have been cloned separately into the pGD120 high copy plasmid under control of the CaMV35S promoter (Nougalli-Tonaco *et al.*, 2006), two for N-terminal fusions without stop codons and two for C-terminal fusions with stop codons, for both YFP/N and YFP/C. Subsequently, the vectors were made Gateway compatible by introducing the RFB Gateway cassette from Invitrogen (Carlsbad) into the *Bam*HI (for N-terminal fusions) and *Xba1-Bam*HI (for C-terminal fusions) digested and blunted vectors. In addition, the Open Reading Frames (ORFs) of the MADS-box genes *FBP2* and *FBP11* were cloned as Gateway entry clones in pDONOR207 (Invitrogen) with and without stop codon, in order to allow N- and C-terminal fusions with the YFP fragments, respectively. Finally, expression vectors have been obtained by LR reactions following the Gateway protocol.

Besides this, another set of clones has been made by restriction enzyme based cloning. However, in this case the fragmentation of the fluorescent molecule was made one amino acid downstream, yielding a 155 amino acids YFP/N encoding fragment (YFP/N155) and an 83 amino acids YFP/C encoding fragment, designated YFP/C83 (Figure 2). For N-terminal fusions, the coding sequences of the YFP fragments (YFP/N155 and YFP/C83) were cloned into the *BamHI* digested pGD120 vector as *Bgl* II–*Bam*HI fragments. For C-terminal fusions of YFP/N155 and YFP/C83 to the genes of interest, the YFP fragments were cloned into the same vector but using *Bam*HI and *Xho*I. For these constructs, we used two peptide sequences as linkers, RSIAT and KQKVMNH, according to the work of Hu and collegues (2002). The RSIAT linker sequence was used between the YFP/N155 fragments and the protein of interest, while KQKVMNH was used in combination with YFP/C83. Both linker sequences were introduced during the PCR step by using primers with the specific extensions. In addition, the complete ORF sequences of the MADS-box genes *FBP2* and *FBP11* without stop codon, and with a 5' *Xba*I site and a 3' *Bam*HI site, were obtained by PCR with PFU proofreading polymerase, and subsequently

cloned into the pGEM-T easy vector (Promega). In a next step these ORFs were cloned as *Xba*I-*Bam*HI fragments into the generated BiFC vector set for C-terminal fusions. The ORFs of both MADS-box genes including the stop codon were cloned before (Immink *et al*, 2003) and these were isolated as *Bam*HI-*Sal*I fragments and introduced into the generated BiFC vector set for N-terminal fusions.

Finally, we also tested the plant vectors kindly provided by Walter and co-workers (2004). These vectors were named pSPYNE and pSPYCE (SPlit YFP N-terminal/C-terminal fragment expression). The pSPYNE vector contains a Multiple Cloning Site (MCS) in front of a fragment encoding for the first 155 amino acids of EYFP, whereas the pSPYCE vector contains a MCS in front of a fragment encoding for the last 83 amino acids of EYFP. The MADS-box genes *FBP2* and *FBP11* as well as *AGAMOUS* and *SEPALLATA3* were cloned into these vectors making use of restriction enzymes, as mentioned previously for the C-terminal fusion constructs.

Furthermore the ORFs of *SEEDSTICK (STK)* and *APETALA1 (AP1)* were cloned without stop codon as Gateway entry clones and recombined with ECFP destination vector according to Nougalli-Tonaco *et al.*, 2006 to allow our SPLIT-FRET studies.

All generated constructs have been checked by sequencing using the DETT sequencing kit (Amersham).

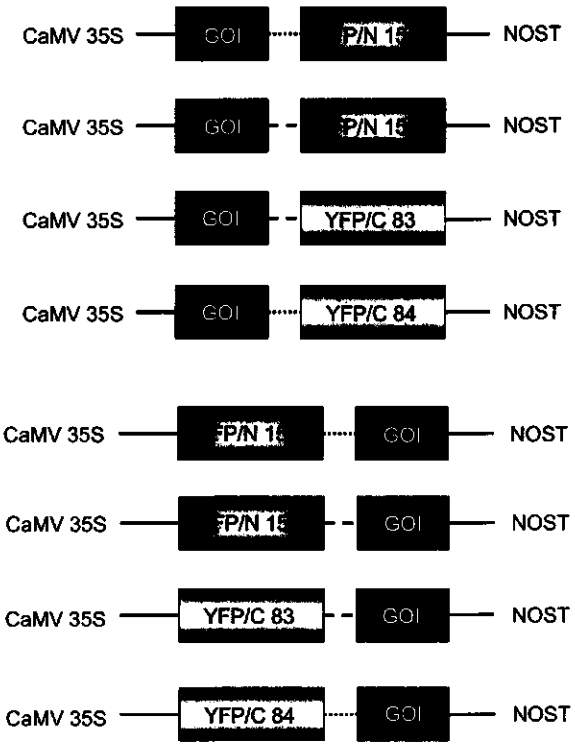

Figure 1. Schematic representation of the constructs made for BiFC in plants. *CaMV 35S is the constitutive 35S Cauliflower Mosaic Virus promoter; GOI is the gene of interest; YFP/N 154 and YFP/N 155 encode for the N-terminal parts of the fragmented YFP molecule and the numbers refer to the number of aa residues of each respective fragment; YFP/C 83 and YFP/C 84 encode for the C-terminal fragments of the YFP molecule and the meaning of the numbers is as mentioned for YFP/N; the different lines between GOI and the fragments of YFP represent the different linker sequences used. The tiny dashed line represents the Gateway sequence as a linker and the broader dashed line represent the linkers RSIAT for fusions to YFP/N and KQKVMNH for fusions to YFP/C. NOST is the Nopaline Synthase terminator sequence.*

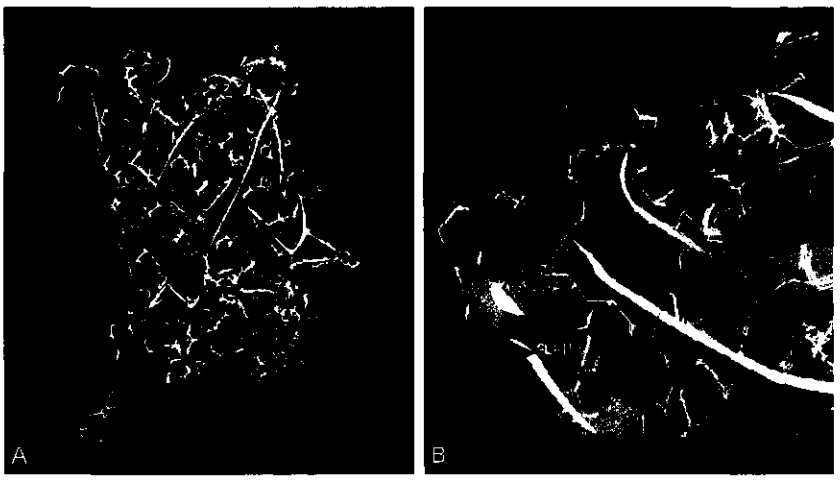

Figure 2. Schematic 3D-representation of the YFP molecule.
A) Structure of Yellow Fluorescence Protein (YFP). B) Close-up of the representation of the EYFP protein structure in (A) around the fragmentation positions. The "split" positions are marked by a light ellipse and the position of ALA 155 *by a red arrowhead.*

Fluorescence microscopy in living cells

Cells obtained from cowpea and Arabidopsis leaf protoplasts were transfected with plasmid DNA (15 -30ug) and incubated overnight before imaging as described before (Nougalli-Tonaco *et al.,* 2006). Images were made using a confocal laser microscope (Carl Zeiss, 510) and by using the Ar laser (514nm) to excite YFP. Furthermore, FRET-FLIM analyses were performed for the combination SPLIT-FRET according to Nougalli- Tonaco *et al.*, 2006 and Aker *et al.*, 2006.

RESULTS

BiFC analysis for FBP2 and FBP11 using the split YFP/N154- YFP/C84 vectors

We took advantage of the known interactions for the two petunia MADS-box proteins FBP2 and FBP11 to test the BiFC technology in living plant cells. For this, we used a transient assay in cowpea and later on, in Arabidopsis leaf protoplasts. Based on previous work we expected to be able to detect homodimerization for FBP2 and heterodimerization for FBP2 and FBP11, whereas FBP11 should not give homodimerization (Immink *et al.*, 2002; Nougalli-Tonaco *et al.*, 2006). Initially, the Gateway compatible YFP/N154 and YFP/C84 vectors were tested. In this case, the linker sequence between the YFP fragments and the MADS-box coding regions was

originated from the Gateway cloning procedure. For this experiment, both N- and C-terminal fusions of the YFP fragments to FBP2 and FBP11 were used (N-N; C-C; and, N-C combinations). The putative FBP2 homodimer and FBP2-FBP11 heterodimer were tested, and the expected lack of FBP11 homodimerization was used as negative control. For each transfection experiment, the construct pGD120-FBP2-YFP (Nougalli-Tonaco *et al.*, 2006) was used as a control to determine the transfection efficiency. All combinations were analyzed in both cowpea and Arabidopsis leaf protoplasts. After transfection the cells were incubated overnight at 28°C, followed by analysis using the confocal microscope. Surprisingly, no fluorescence was detected for any of the various tested combinations, although, the control transfection was giving strong nuclear localized fluorescent signal in a high percentage of analyzed cells, as expected.

One of the reasons for the lack of a positive result could be that we fragmented the EYFP molecule after amino acid (aa) residue 154, whereas Hu *et al.,* 2002, Walter *et al.,* (2004), and, Bracha-Drori and colleagues (2004) split the EYFP molecule at aa position 155. Because of this, we decided to follow a different strategy in line with the published work.

BiFC analysis for FBP2 and FBP11 using the split YFP/N155-YFP/C83 vectors.

The following approach was to test the BiFC technology, using the split of the EYFP molecule after aa position 155. Furthermore, for these vectors different peptide sequences were used as linkers between the fragments of YFP and the proteins of interest, to determine whether this influences the reconstitution of the fluorescent molecule. Vector sets were made to allow both N- and C- terminal fusions, and with the RSIAT sequence for YFP/N155 and the KQKVMNH linker for the fragment YFP/C83, according to Hu and colleagues (2002). As before, the petunia FBP2 and FBP11 proteins were chosen as proteins of interest to test the vectors. All possible homo- and heterodimer combinations with both C- and N-terminal fusions to the YFP fragments were tested in cowpea and Arabidopsis protoplasts. After transfection, the protoplasts were incubated overnight at 28°C. Despite the usage of constructs that are very similar to the ones that were successfully used by Hu *et al* (2002), once more no fluorescence was detected except for the control transfection.

Because all expression cassettes have been sequenced and no mistakes were found, the lack of fluorescent signal could not be explained by a possible mistake

during plasmid constructions. Nevertheless, we decided to extend our analyses, using a set of vectors that were successfully used in plants. In this way, it could be verified whether the problems are caused either by the specific MADS-box proteins analyzed, or due to an unexpected problem with the set of vectors generated by us.

BiFC analysis for FBP2 and FBP11 using pSPYNE and pSPYCE vectors.

As an alternative to verify whether the system could be applied for MADS-box proteins, we decided to test the plant vectors pSPYNE and pSPYCE (Walter *et al.*, 2004) that were kindly provided by Karin Shumacher and Klaus Harter. These vectors were successfully used for the detection of homodimerization of the Arabidopsis basic leucine zipper (bZIP) transcription factor bZIP63 and the zinc finger protein Lesion Simulating Disease 1 (LSD1). For the construction of these vectors the split position was made after aa 155 and the linker sequences used between the fluorescent fragment and the protein of interest consisted of a 24 aa long HA tag for YFP/C and a 26 aa long c-myc tag for YFP/N. *FBP2* and *FBP11* were cloned into both vectors and a couple of combinations were tested in Arabidopsis protoplasts that were after transfection incubated overnight at 28°C. Surprisingly, we were able to observe emission of YFP for the combination pSPYNE-FBP2/pSPYCE-FBP11 and for pSPYNE-FBP2/pSPYCE-FBP2 (Figure 3A and B). Nevertheless, we also detected some fluorescent signal, most likely due to protein aggregates for the combination pSPYNE-FBP11/pSPYCE-FBP11 (Figure 3C). This signal was observed in a few cells only.

Figure 3. Detection of protein-protein interactions for the MADS-box proteins FBP2 and FBP11 in living cowpea protoplasts by BiFC, making use of the pSPYNE and pSPYCE vector set.
From left to right : A) Overview of protoplasts co-transfected with pSPYNE-FBP2 and pSPYCE-FBP11; B) Close-up displaying the nuclear localized signal; C) Overview of protoplasts co-transfected with pSPYNE-FBP11 and pSPYCE-FBP11, showing a few cells with fluorescent structures of irregular form, which are probably fluorescent aggregates.

The effect of temperature on BiFC analysis for MADS-box proteins in plant cells

Although, we were able to obtain fluorescence upon heterodimerization between FBP2 and FBP11 and homodimerization of FBP2 using the pSPYNE and pSPYCE vectors, we also observed fluorescent signal for the negative control combination (FBP11-FBP11). Therefore, we decided to further optimize the method. As described above, so far all variations to get the system working were made at the level of vector construction, and not for any other aspect of the method, like the incubation conditions of the protoplasts. Recently, it has been demonstrated by Shyu and colleagues (2006) that chromophore maturation of enhanced YFP is sensitive to high temperatures; thus, for BiFC analyses in mammalian cells a pre-incubation at lower temperatures is recommended. Based on this report, we decided to test a lower temperature for incubation of the protoplasts after transfection: 23°C instead of 28°C, as has been used before. For this experiment, we only tested combinations of constructs with C-terminal fusions. We made this choice because MADS-box proteins appeared to be sensitive to different labeling positions and N-terminal fusions of fluorescent proteins can lead to mis-location of MADS-box transcription factors (see for example Wu *et al.*, 2003; Nougalli-Tonaco *et al.*, 2006). To our great surprise, we recorded fluorescent signal for the combination FBP2-YFP/N + FBP11-YFP/C, as well as for FBP2-YFP/N + FBP2-YFP/C in our own Gateway compatible vectors with the split after aa 154. Furthermore, positive results were obtained for the

combination FBP2-FBP11 in the pSPYNE/pSPYCE vectors, as before. No fluorescence was observed when we tested for FBP11 homodimerization (negative control) with these vectors at this lower incubation temperature, which is in line with previous experiences from our lab (Immink *et al.*, 2002; Immink *et al.*, 2003). Similar positive results were obtained when the Arabidopsis MADS-box proteins AGAMOUS (AG) and SEPALATA3 (SEP3) were tested in the pSPYNE/pSPYCE vectors, two proteins for which heterodimerization has been shown by the yeast two-hybrid system (de Folter *et al.*, 2005).

Combination of BiFC and FRET approaches to detect higher-order complex formation for MADS-box proteins.

Egea-Cortines and colleagues reported in 1999 for the first time that Antirrhinum MADS-box proteins can form higher-order complexes. Shortly after, this phenomenon was also discovered for MADS-box proteins from Arabidopsis and petunia (Honma and Goto, 2000; Favaro *et al.*, 2003, Ferrario *et al.*, 2003) and hence, it seems to be a common and conserved feature of these proteins. However, almost all the evidence for higher-order complex formation originated from yeast three- and four-hybrid assays. Recently, we provided indirect evidence for higher-order complex formation in living plant cells and the stabilization of a dimer by a third MADS-box protein (Nougalli-Tonaco *et al.*, 2006). Nevertheless, the state-of-art of the FRET method at that moment did not allow direct detection of interaction between three proteins. The positive results obtained for BiFC with MADS-box proteins prompted us to further extend its use into a combinatorial assay with the FRET-FLIM methodology for the detection of higher-order complexes in plant cells. In this case, two out of the three proteins have their interaction monitored by the BiFC method, resulting in the recovery of the YFP molecule, while the third protein partner is labeled with the fluorophore CFP, which is a suitable FRET donor for the recovered YFP molecule. To test the proposed BiFC-FRET method, a third CFP-labeled MADS-box protein has been co-transfected with the before mentioned SEPALLATA3 and AGAMOUS BiFC constructs. The SEP3-YFP/N and AG-YFP/C constructs were combined with pCaMV35S::*SEEDSTICK (STK)-CFP* and pCaMV35S::*APETALA1 (AP1)-CFP (not shown)*. The combination AG-SEP3-STK should be positive according to Favaro *et al.* (2003) and the ternary complex AG-

SEP3-AP1 has recently been identified by a yeast three-hybrid screen in our own lab (Immink *et al.*, in preparation).

For each combination all three plasmids were co-transfected in Arabidopsis protoplasts and incubate at 23°C overnight. After incubation, in many cells a nuclear co-localized CFP and YFP signal was obtained. However, for none of the combinations FRET could be monitored.

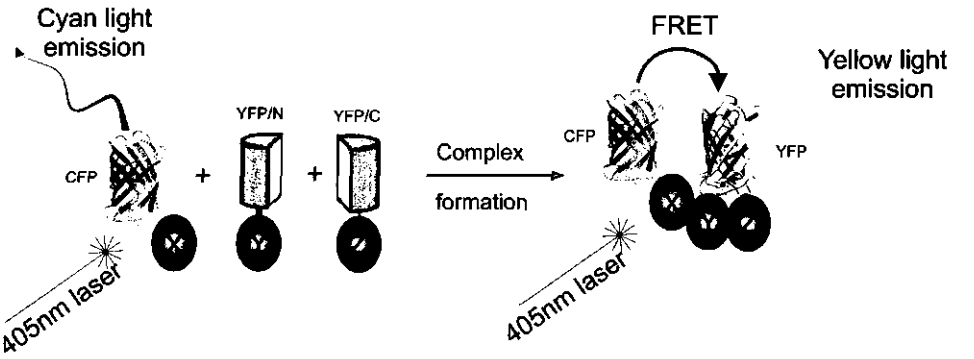

Figure 4. Scheme to represent the BiFC-FRET approach.
In this scheme, we present the combination of two approaches in order to monitor higher-order complexes in living cells. Dimerization is detected by the use of the BiFC and the third protein can therefore be detected by direct FRET between the single protein labeled with CFP and the dimer formed which is able to recover the YFP molecule.

DISCUSSION

In this study, we adapted the BiFC technology to enable the detection of MADS-box protein-protein interactions in living plant cells. The BiFC method was our strategy of choice, because it is a simple, inexpensive and relative fast approach; and therefore, might be very useful for studying protein-protein interactions *in vivo*. Furthermore, it may allow the detection of protein interactions in tissues, which was not successful yet with FRET-based methods (Nougalli-Tonaco, unpublished results).

The two petunia MADS-box proteins FBP2 and FBP11, which have been characterized previously by different methods as physical interaction partners (Immink *et al.*, 2002; Immink *et al.*, 2003; Nougalli-Tonaco *et al.*, 2006), were selected as protein pair to set-up the assay. Various different constructs and

experimental conditions were tested and pointed to important criteria that should be considered for the experimental design.

We have used two different positions for the fragmentation of EYFP: after aa MET 154 and after aa ALA155. Initially, it seemed that all our own constructs were not successful for MADS-box proteins, irrespective of the split position. However, when the pSPYNE and pSPYCE vectors were used, which were fragmented at position ALA155, we could detect interactions for the same proteins and at lower protoplasts incubation temperatures the Gateway compatible vectors with the fragmentation after aa 154 were also working. Furthermore, these Gateway compatible vectors (YFP/154-84) have been used successfully to detect protein interactions for several CDK's proteins (E. Russinova, VIB Ghent, personal communication). In addition, other groups have used different positions of fragmentation for various variants of GFP based fluorescent molecules (Ghosh *et al.*, 1999; Hu and Kerppola, 2003; Grinberg *et al.*, 2004). Thus it appeared that the used fragmentation positions for the EYFP molecule does not have a major effect on refolding of the fluorescence molecule upon a protein-protein interaction event.

A second aspect that is different between the vector sets used, is the peptide linker sequence. In the Gateway compatible YFP/154-84 vectors the linkers were originated from the Gateway recombination sites, while the vectors YFP/155-83 have the peptide linker sequence as suggested by Hu and colleagues in 2002. But no interaction could be detected at all in this case. In contrast, however, a positive result was obtained at this relatively high temperature with the pSPYNE and pSPYCE vectors. For these vectors, the peptide linker sequences consist of a recognition tag and part of the multiple cloning sites, which results in very long peptide sequences of 24-26 aa residues. Besides this difference in size, all linkers encode for a complete different aa sequence. Based on these observations, we suggest that the peptide linker sequence may have an affect on refolding of the fluorescent protein, or alternatively on the stability of the generated fusion proteins.

Another important aspect concerns the sensitivity of the method to high temperatures. Our results demonstrated that the temperature could have a strong effect on the proper refolding of the fluorescence molecule, as well as on the specificity of the signal. The positive results obtained for our own Gateway compatible vectors with incubation of the protoplasts at 23°C were striking. Furthermore the specificity of the fluorescent signal increased, when the pSPYNE

and pSPYCE vectors were used at this lower temperature. The supposed aggregates formed in the case of the test for homodimerization of FBP11 at 28°C, which according to yeast two-hybrid and FRET-FLIM analyses should not occur (Immink *et al.*, 2002), disappeared at 23°C. The question remains what is the reason for all these obtained effects? For EYFP it has been described that the maturation of the chromophore is very sensitive to higher temperatures (Tsien, 1998). Furthermore, in 2006 Shyu and colleagues clearly demonstrated that pre-incubation of cells at 30°C, 4 hours before imaging, after 24 hours at 37°C (normal temperature conditions for mammalian cells), increases significantly the BiFC signal for EYFP fragments. This all, clearly points towards temperature dependence on the properties of the EYFP molecule. To circumvent problems due to this temperature sensitivity, Shyu *et al* (2006) proposed the use of alternative chromophores, like the *Venus* and the *Citrine,* which are less sensitive to higher temperatures and also show higher specificity and faster refolding.

Another important aspect that needs to be taken into account when this method is selected for the analysis of protein-protein interactions *in vivo* is the fact that transient interactions are most likely stabilized by the recovery of the fluorescent molecule. This means that after recovery of YFP, the process is irreversible, and due to this the method is less suitable to investigate transient or dynamic interactions in cells. In these cases, the use of FRET based methods would most likely result in more reliable data sets and therefore, should be the method of choice.

As a novelty, we also tried to implement the BiFC-FRET method, in order to monitored higher order complexes in living cells. Despite the recovery of YFP by the dimer and co- localization with the third protein labeled with CFP, no FRET signal could be obtained. For MADS-box proteins, the C domain has shown to be involved in higher-order complex formation (Egea-Cortines *et al.*, 1999). Probably, when two partners are fixed at their C-terminal tails by the recovered YFP molecule, it will lead to steric hindrance towards the third protein, which will prevent higher-order complex formation. Alternatively, complex formation occurs, but the CFP and YFP fluorescent groups will remain too far from each others to allow energy transfer. Although we were not successful, it would still be very interesting to test BiFC-FRET with other proteins that have a different structure, or that are labeled at different positions.

Even though the BiFC assay still shows some technical limitations that should be overcome, it is a powerful and simple approach to analyze protein-protein

interactions *in vivo*. In combination with the usage of plant specific promoters the technology may enable to study interactions in intact tissues in the near future.

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Chapter 6

Diffusion of MADS-box proteins and complexes

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Sacco C. de Vries, Gerco C. Angenent and Richard G.H Immink

ABSTRACT

MADS-box transcription factors are key regulators of various plant developmental processes. Their molecular mode of action is dependent on the formation of dimers (homo and/or heterodimers) followed by the assembly into higher-order complexes that regulate target gene expression. We have previously analyzed a dozen of protein-protein interactions involving MADS-box transcription factors in living plant cells by means of Fluorescence Resonance Energy Transfer-Fluorescence Lifetime Imaging Microscopy (FRET-FLIM). Differences in affinity and interaction specificity play an important role for the selection of interaction partners and formation of multimeric complexes *in vivo*. These complexes determine to a large extent how these MADS-box proteins select their target genes for transcriptional control. To further investigate the dynamics of the MADS-box transcription factor protein complexes, we made use of Fluorescence Correlation Spectroscopy (FCS). FCS measurements enable the analysis of diffusion time at single molecule level. The well known and thoroughly studied MADS-box proteins AGAMOUS (AG) and SEPALLATA3 (SEP3) were selected to investigate the diffusion time of these proteins in plant cells and *in vitro* by the FCS technology. For this purpose, the two MADS-box proteins were labeled with the fluorescent molecule Enhanced Yellow Fluorescence Protein (EYFP). The *in vitro* experiments showed that the AG protein diffuses two times slower than SEP3 and in addition, the co-translation of both transcription factors suggests the formation of high molecular weight complexes.

INTRODUCTION

Plant MADS-box transcription factors are important regulators of flower development and plant architecture in higher eudicots. In the model plant species *Arabidopsis thaliana*, at least 107 members of this family could be identified (Parenicova *et al.*, 2003) and many of them have been functionally characterized. Insight in the molecular mode of action of plant MADS-box proteins has been gained from several yeast two- and three-hybrid studies, and more recently by the use of FRET (Fluorescence Resonance Energy Transfer) based methods to determine their protein-protein interaction patterns in plant cells (Egea-Cortines *et al.*, 1999; Honma and Goto, 2001; Immink *et al.*, 2002; Nougalli-Tonaco *et al.*, 2006). Based on these experiments, it has been hypothesized that their molecular mode of action relies strongly on their ability to homo- or heterodimerize and to assemble into higher-order protein complexes (Theißen and Saedler, 2001). The formation of these kinds of multimeric complexes seems to be very dynamic and differences in affinity and partner selection are most likely important determinants of the final interaction network. Although, FRET based methods have provided a realistic overview of MADS-box protein complex formation and protein interaction preferences *in vivo*, the number of molecules involved in such complexes cannot be determined by these methods. In order to address some of these questions, Fluorescence Fluctuation Spectroscopy (FFS) techniques can be applied (Hink *et al.*, 2002; Aker *et al.*, 2007). With FFS, the detection of physical parameters of fluorescent molecules is feasible at high spatial and temporal resolution, allowing the visualization of protein dynamics and the estimation of stoichiometry of molecule complexes. By means of Fluorescence Correlation Spectroscopy (FCS) for example, the diffusion time of excited single molecules can be monitored within a determined observation volume (Hess *et al.*, 2002; Hink *et al.*, 2002). We decided to use FCS as our strategy of choice, in order to determine the approximate diffusion time for individual and combinations of MADS-box proteins. AGAMOUS (AG) and SEPALLATA3 (SEP3) were selected for these studies. These two proteins are able to homo and heterodimerize *in vivo* (Chapter 3) and clearly show differences in preferences for homo- or heterodimerization (Chapter 4). For the *in vitro* FCS analyses, the two MADS-box proteins were labeled with the Enhanced Yellow Fluorescent Protein (EYFP) and synthesized by *in vitro* transcription/translation. Further, we performed gel filtration chromatography analysis for the SEP3 protein, in order to verify the

molecular size of the complex formed by this protein. Our results revealed significant differences in protein diffusion for AG-EYFP compared to SEP3-EYFP. In combination with the observations from the gel-filtration experiment, the obtained FCS results give a first indication about the stoichiometry of AG and SEP3 complexes *in vitro*.

MATERIAL AND METHODS

Plasmids construction

For the *in vitro* FCS experiments, the full length ORFs of *SEP3* and *AG*, fused to the ORF of the *Enhanced Yellow Protein (EYFP),* were cloned behind the T7 promoter in the pSPUTK vector, by introducing *Nco*I and *Bam*HI sites according to Kaufmann *et al.*, 2005. For the controls, we cloned the ORF of *EYFP* into this vector, as described previously.

FCS measurements set-up

FCS measurements were performed using the CLSM (Confocal Laser Scanning Microscope) LSM510, from Carl Zeiss (Germany), equipped with a C-Apochromat water immersive lens 63X with a numerical aperture of 1.2. To calibrate the pinhole settings, Rhodamine green (R6G) 10nM (Invitrogen) was used. Rhodamine green and EYFP, as well as the fusion proteins SEPALLATA3-EYFP and AGAMOUS-EYFP, were excited by the 514 nm Argon laser, followed by detection of emission between 530 and 560nm. Laser power excitation intensity was ~ 2µW. Measurements were acquired during 120 seconds. At least ten measurements were performed for each combination, in five independent experiments, including the controls Rhodamine and EYFP in solution. For the data analysis we used the FCS-data processor software version 1.5 from Scientific Software Technologies Software Centre Belarus, according to Skakun *et al* (2005). The autocorrelation curves were adjusted using an autocorrelation function (eq 1) assuming a three-dimensional

diffusion of the proteins with triplet state kinetics (Aker *et al.*, 2007).

$$G(\tau) = 1 + \frac{1}{N}\left(\left(1+\frac{\tau}{\tau_{dif}}\right)\sqrt{1+\left(\frac{\omega_{xy}}{\omega_z}\right)^2\frac{\tau}{\tau_{dif}}}\right)^{-1}\left(1+\frac{F_{trip}e^{\frac{-t}{T_{trip}}}}{1-F_{trip}}\right) \quad (1)$$

In this model $G(\tau)$ is the autocorrelation function, N is the average number of fluorescent molecules in the observation volume, τ_{dif} is the average diffusion time of the particles, ω_{xy} is the equatorial radius and ω_z the axial radius of the detection volume. F_{trip} and T_{trip} represent the fraction and the average time in which molecules are present in the triplet-state.

The diffusion time τ_{dif} describes the time in which particles stay in the observation volume and that is related to the diffusion coefficient (D) according to equation number 2.

$$\tau_{dif} = \frac{\varpi_{xy}^2}{4D} \quad (2)$$

For the calculations of the detection volume ω_{xy}, we made use of equation 2 and a diffusion coefficient (D) of the Rhodamine green of 280 µm2/s. For the estimation of the approximate molecular weight based on the diffusion coefficient, in this case considering globular particles, equation number 3 was used.

$$D^{-1} \approx \frac{6\pi\eta}{kT}\sqrt[3]{M} \quad (3)$$

Where D is the diffusion coefficient, M is the molecular weight, *k* is the Boltzmann constant, *T* is the absolute temperature and η is the viscosity of the solution.

The curves from the different experiments have been fitted using a fixed structure parameter (ω_z/ω_{xy}) obtained from the respective Rhodamine green control measurements performed with 95% confidence interval. The robustness of the fits was qualified by the Chi^2 values and the shapes of the fits and residuals.

In vitro protein synthesis

For the protein production, we subjected the above described plasmids to *in vitro* transcription and translation using the TNT Quick Coupled Transcription/Translation System, according to the manufacturer's instructions (Promega, Madison, WI). One microgram of template DNA was used in a 50µl reaction volume and the reaction mixture was incubated at 30°C for 75 min. Finally, 50 µl of the obtained mixture has been used for the FCS measurements.

Gel filtration chromatography analysis

The *in vitro* produced SEP3 protein has been loaded onto a Sephadex 200 gel filtration column (50 µl reaction product). The column running buffer was sodium phosphate buffer pH 7.5, 150 mM NaCl. The fractions obtained from the column were analyzed by Western blot analysis using a SEP3 specific peptide antibody (Eurogentec).

RESULTS

Diffusion of AGAMOUS and SEPALLATA3 proteins in vitro

Our goal was to determine protein diffusion of MADS-box proteins AGAMOUS (AG), SEPALLATA3 (SEP3), and the dimer combination of AG and SEP3, by FCS. The determination of diffusion time for these proteins could provide indications about their complex sizes, i.e whether the single proteins are able to associate into homodimers or higher-order complexes. Differences in diffusion time would imply the presence of different numbers of molecules in the complexes formed by these two proteins and hence, differences in oligomerization capacity for SEP3 and AG. Initially, we tried to perform the experiments in Arabidopsis leaf protoplasts upon transient expression of the labeled MADS-box genes. Although, nuclear fluorescent signals were obtained as expected (see Chapter 3), we failed to obtain reproducible data from these in vivo FCS measurements (data not shown), probably because of too high expression levels that could not be circumvented by pre-bleaching prior to the measurements. Further, we obtained very low diffusion times for both expressed proteins (around 3 ms in both cases), which could be related to the association of these transcription factors to other elements in the nucleus, or simply due to the formation of aggregates. Therefore, it was decided to perform in vitro experiments and for this purpose, the single AG-EYFP and SEP3-EYFP fusion proteins and a mixture of co-

translated proteins were generated. From the obtained results, we calculated the average diffusion coefficients of the possible heterogenic complexes formed between these two proteins. As controls, FCS measurements for the free EYFP protein were performed, which showed a similar behaviour as reported in literature (diffusion coefficient ~ 80 µm2/s. Widengren et al., 1999; Jung et al., 2000). Interestingly, the diffusion coefficients obtained for the two individual MADS-box proteins showed a clear difference. AG-EYFP appeared to diffuse twice as slow as SEP3-EYFP, while when both proteins were co-translated the diffusion time obtained was four times slower than for SEP3-EYFP (Fig 1). Considering that the diffusion coefficient is approximately inversely proportional to the cubic root of the mass (eq 3), and supposing that only one type of complex will be formed in each case, the possible complex formed by AG-EYFP is eight times bigger than the SEP3-YFP complex, whilst the complex formed by the combination of the two proteins is even much larger (~64 times).

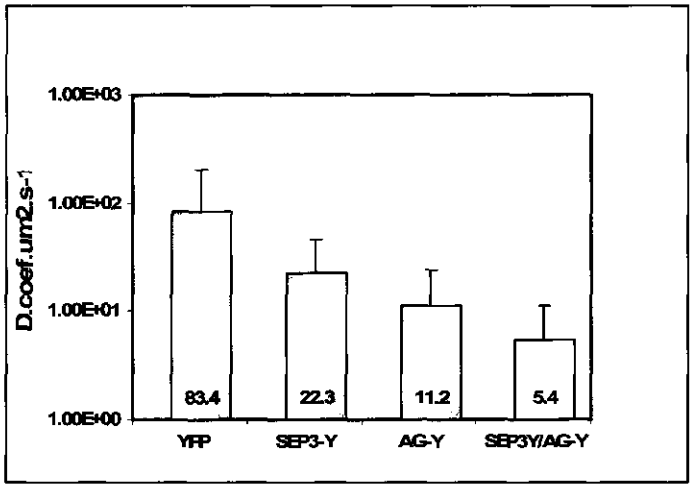

Figure 1. Diffusion coefficients for AGAMOUS and SEPALLATA3 determined by FCS. *The graph shows the diffusion coefficient for EYFP, SEP3-EYFP, AG-EYFP, and the co-translated SEP3-EYFP+AG-EYFP proteins in solution.*

Gel filtration chromatography of the SEPALLATA3 protein

In order to estimate the molecular sizes of the complexes formed by the MADS-box proteins based on the obtained FCS data, it is essential to have a good reference point. In theory, the FCS data obtained for the free EYFP can be used as reference, assuming that this molecule is present as a monomer in solution. However, it would

be much more accurate to estimate complex sizes based on experimental data from a particular MADS-box transcription factor complex obtained by an alternative method. Therefore, we decided to perform gel filtration chromatography analysis for the in vitro produced SEP3 protein. The obtained fractions were subjected to Western blot analysis using a SEP3 specific antibody (figure 2). The expected mass of a single SEP3 molecule is 30 kD. Interestingly, we observed that SEP3 is present both as a dimer (fraction 21, ~ 60 kD) and in a higher molecular weight form of around 500 kD (fraction 10). The complex present with a molecular weight of more then 600 kD (fraction 6) is most likely an agregate due to e.g. the association of the SEP3 protein with the ribosome units in the in vitro transcription/translation mixture. Based on solely these data we can not exclude that the same holds for the before mentioned complex of approximately 500 kD.

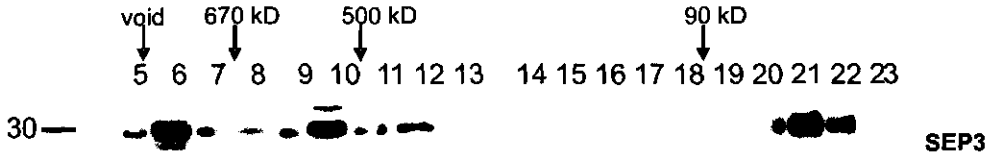

Figure 2. Gel filtration analyses reveal that SEPALLATA3 is present in solution as dimer and in a large complex of approximately 500 kD

DISCUSSION

MADS-box proteins form dimers and are most likely assembled into higher-order complexes. According to the quartet model (Theißen and Saedler, 2001), these transcription factors act as tetrameric complexes. However, this model is based on yeast studies, which do not give any information about the stoichiometry of the complexes. Therefore, we used Fluorescence Correlation Spectroscopy (FCS) analyses for the Arabidopsis MADS-box proteins AGAMOUS (AG) and SEPALLATA3 (SEP3) to get insight about the sizes of the complexes formed by these proteins. Our preliminary data showed that AG-YFP diffuses two times slower than SEP3-YFP in vitro and that the two proteins together diffuse four fold slower than SEP3-YFP. Based on a comparison of the diffusion time for the EYFP labelled MADS-box proteins a comparative estimation of the various complex sizes can be made in theory. To do so, a reference size is needed and therefore, we tried to estimate the molecular size of the SEP3 protein complex using a gel filtration chromatography assay. According to the gel filtration analysis, it is likely to assume

that SEP3 is present in its dimeric form and as a high molecular weight complex. We can not rule out that the observed large complex is not biological relevant and an artifact due to the usage of in vitro transcription/translation. Based on the results for SEP3, we may also speculate that the AG-EYFP protein, which diffuses twice as slow as SEP3-EYFP, assembles preferably into higher order complexes. When upon co-translation of both proteins only separate AG and SEP3 complexes would be formed, an intermediate diffusion coefficient was expected, but in contrast a strong additive effect was obtained. This suggests that large complexes are formed, which contain both SEP3 and AG molecules. Unfortunately, calculation of the exact size of a complex composed of SEP3 and AG proteins is difficult by FCS. That is because more complexes of various sizes are formed simultaneously, e.g. AG complexes and higher order complexes containing both SEP3 and AG. Furthermore, the in vitro produced proteins might not be folded properly due to the lack of co-factors. When the proteins are unfolded in a formed complex, it will result in very slow diffusion coefficients, which not necessarily correlate with a higher molecular weight. The situation in a plant cell is even far more complex, simply because more competing MADS-box proteins can be present in different concentrations (see also chapter 4). It is clear that for a complete overview, information from different types of experiments should be combined. Recently, the power of FCS measurements in combination with FRET-FLIM and biochemical approaches has been nicely illustrated by Aker and colleagues (2007). They were able to show that the CDC48A protein is present not only in its hexameric form, but rather in a larger protein complex in living plant cells. Reverting to MADS-box transcription factors, alternative and preferably in vivo methods are needed for the appropriate measurements of the exact number of molecules in a complex. An option could be PCH (Photo Counting Histogram), in which the molecular brightness of individual molecules and protein complexes can be determined. However the PCH technology is not completely established yet and only a few reports have shown its application so far (Chen et al., 1999; Chen et al., 2004). Furthermore, structural data from the proteins under study would also facilitate a better understanding of the assembly of MADS-box proteins into complexes.

Regardless the limitations of FCS for the exact size of protein complexes, the obtained data provide the first indication about the stoichiometry of particular MADS protein complexes. According to the quartet model MADS-box transcription factors

are active as tetramers (Theißen and Saedler, 2001), however, our data suggest that larger complexes are formed by these proteins in vitro.

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Chapter 7

Concluding remarks and perspectives of lifetime imaging for MADS-box transcription factor complexes

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MADS-box transcription factors

In this thesis, we attempted to unravel the molecular mechanisms behind plant MADS-box transcription factor functioning in the model species petunia and Arabidopsis making use of fluorescent microscopy techniques. Over 100 members of this family have been identified in Arabidopsis thaliana and many of them have been functionally characterized (Kofugi et al., 2003; de Bodt et al., 2003; Parenicova et al., 2003). As part of complex genetic networks that underlie plant development, they play essential roles in a plethora of developmental processes, such as: embryogenesis (e.g. AGL15; Perry et al., 1996); repression of flowering (e.g. FLC; Burn et al., 1993); transition to flowering (e.g. SOC1; Simon et al., 1996), floral meristem determination (e.g.AP1; Mandel et al., 1992) and last but not least, floral organ identity specification (reviewed in Ferrario et al., 2004). In the early 90's genetic studies that made use of various floral homeotic mutants elegantly provided evidence for the concept model in flower development, called the "ABC-model" that, a few years later, was extended with the D- and E-functions (Coen and Meyerowitz, 1992); Colombo et al., 1995 and Pelaz et al., 2000). Subsequently, biochemical and yeast 2-hybrid studies have shown that MADS-box proteins are physically interacting, forming dimers and most likely, are functional in large protein complexes (Egea-Cortines et al., 1999; Honma and Goto, 2001). The 'quartet model' for MADS-box transcription factor functioning proposes that at least two dimers assemble and direct the oriented and specific bending and binding to the DNA target sequences that regulates transcriptional activity (Theißen & Saedler, 2001). In line with these findings and the proposed molecular model, the objective of this thesis was to get a better understanding of the molecular mechanism behind MADS-box transcription factor functioning by analyzing their behavior and protein-protein interactions in a living cell environment.

Methods to analyze protein-protein interactions in living cells

At the start of this thesis research, many putative MADS-box transcription factor dimers were known based on *in vitro* and yeast two-hybrid experiments, however for only a very few combinations the interaction has been confirmed in living plant cells (Immink *et al.*, 2002). Therefore, we invested in the further development and implementation of sophisticated micro-spectroscopy techniques, which enable to visualize physical interactions and their dynamics *in planta*. In this work, several

methodologies were described such as: Fluorescence Resonance Energy Transfer – Fluorescence Lifetime Imaging (FRET-FLIM), Fluorescence Correlation Spectroscopy (FCS), and Bimolecular Fluorescence Complementation (BiFC), each with their specific advantages and drawbacks. FRET based methods are very reliable techniques, even though they may require extensive imaging analysis and a sophisticated set up. From different FRET based methods, FRET-FLIM is the most robust since it is independent of concentration; nevertheless its application in intact plant tissues that express an endogenous amount of the protein under study is not yet possible. FCS is another powerful technique which enables the monitoring of protein dynamics and by that may provide a first indication about protein complexes stoichiometry. It is a very sensitive and sophisticate approach, but just few examples of its use are available so far, being still under development. Another method that has been recently adopted to detect protein-protein interactions in living plant cells is called "Split-YFP" or bimolecular fluorescence complementation (BiFC). In this case, the Yellow Fluorescent Protein (YFP) is split into two domains that themselves are not fluorescent. These two inactive molecules are fused to two proteins of interest and upon interaction; the two halves of YFP come together and refold into a functional molecule. Only in case of an interaction a yellow signal will be visible and this can be monitored easily with a simple fluorescence microscope. Although, this method is relatively simple when compared with the other methods described above, it seems very much dependent on the structure and stability of the proteins under study.

Besides these methods mentioned above, several other techniques have been described in literature. For instance, the BRET (bioluminescence resonance energy transfer) method is very similar to the FRET based techniques but uses LUCIFERASE as donor molecule. BRET is predominantly used for in vitro experiments, but has been established in plants recently (Xu *et al.*, 2007). A big advantage of BRET is the simplicity of the method that does not require laborious imaging analysis like in the case of FRET. Furthermore, this method can be applied using a simple luminometer and does not require a very expensive and sophisticated set up.

In our work, we have demonstrated protein interactions and dynamics in leaf protoplasts, making use of confocal laser scanning microscopy, FRET-FLIM, split-YFP and FCS. Our results revealed that FLIM (Fluorescence Lifetime Imaging

Microscopy) is the most robust method to analyze protein interactions in a biological sample so far, even though the use of the split-YFP can be easily applicable. Furthermore, FCS demonstrates to be a very promising technique to answer dynamic processes; nevertheless its use in vivo it is not so trivial yet.

Perspectives for real time imaging of MADS-box protein interactions

In this thesis, we have identified several complexes in living cells that are involved in floral organ formation and we were able to formulate some hypotheses about the molecular mechanisms of MADS-box protein functioning. For flowering plants, *Arabidopsis thaliana* is the best model species for studying organ development and a wealth of genetic data is available. In addition, in vitro biochemical studies and yeast two-, three- and four-hybrid analyses provided information about the molecular action and interactions between the MADS-box proteins. The ABC (DE) model for floral organ formation was initially a genetic model (for review see: Ferrario *et al.*, 2004), but has been translated into the quartet model that describes the formation of tetrameric MADS-box complexes (Theißen 2000). Our *in vivo* results suggested that several of these complexes, for instance: A+E (AP1 and SEP3), B+C+E (AP3+PI+AG+SEP3) and C+E (AG and SEP3), are very stable and could be the basis for floral organ formation. Nevertheless, it must be said that most of these results were obtained using a transient assay system (protoplasts) and the constitutive 35 CaMV promoter to achieve high expression levels. A next improving step would be to analyze protein-protein interactions in tissues with native expression levels of the proteins. There are currently several ways to identify *in vivo* protein-protein interactions. A promising technique is the 'split-YFP' (BiFC) method (Hu *et al.*, 2002), although it has several drawbacks: (i) the proteins of interest fused to the YFP parts can not be detected by fluorescence, (ii) the interaction between the proteins of interest can be stabilized by the reassembly of the YFP molecules, which makes it not the best approach for studying dynamic processes, (iii) the reassembly of the YFP molecule depends on the conformation of the attached proteins. Nevertheless, positive results were obtained in mammalian and plant cells where different types of protein have been tested in different conditions (Hu *et al.*, 2003; Grienberg *et al.*, 2004 and Walter *et al.*, 2004). The studies mentioned above showed that progress has been made in the isolation and characterization of protein complexes under native circumstances. However, methods that can be used to monitor physical

interactions in intact living tissues of plants by direct observation under the microscope remain to be optimized. In mammalian systems, progress has been made in the detection of protein interactions by FRET assays (Chen *et al.*, 2003) but, up till now, the only proof that FRET measurements can be successfully performed in intact plant tissues was recently described by Deuschle *et al.*, 2006. In their studies, several FRET nanosensors were developed in order to monitor glucose metabolism in Arabidopsis plants. However, these nanosensors contain both CFP and YFP within a single molecule and are expressed from the strong constitutive Cauliflower Mosaic Virus 35S promoter.

As an approach to evaluate the feasibility of live cell imaging for protein-protein interactions, we performed donor lifetime measurements for the MADS-box protein AGAMOUS (AG) fused to GFP in intact tissues. As a control measurement, we transiently expressed the AGAMOUS protein fused to GFP under the control of the 35S promoter in Arabidopsis leaf protoplasts, following the protocols described in chapter 3 of this thesis. For the intact tissues, we made use of homozygous transgenic lines (T2 generation) in which the construct *pAG::AG-GFP* was introduced in *Arabidopsis thaliana* ecotype Col0 (Columbia) by *Agrobacterium* mediated transformation (Urbanus *et al.*, in preparation). These lines have a clear nuclear localization of AG-GFP in the young floral organ primordia of the inner two whorls, which is in accordance with *in situ* data of *AG* expression (Bowman *et al.*, 1991). Besides that, we used the transgenic lines MSG 1 (meristem – specific GFP), kindly provided by Patricia Zambryski (see Kim *et al.*, 2005), as an additional control. These plants have very bright GFP expression in young meristematic tissues. Flower buds from the transgenic plants were dissected and their meristematic tissues, as well as ovules, were analyzed by using a Bio-Rad Radiance 2100 MP system (Hercules, CA) in combination with a Nikon TE 300 inverted microscope (Tokyo, Japan). The FLIM measurements to detect the donor lifetime of GFP in these samples were done as described in chapters 2 and 3 of this thesis.

The analyses of the protoplasts, constitutively expressing AG fused to GFP, revealed donor fluorescence lifetime values which were similar to those obtained by Peter *et al.* (2005) with the same technical settings (Fig 1A). Subsequently, we performed analyses of young floral meristems and intact ovules from the transgenic plants, containing *pAG::AG-GFP*, as well as for meristematic tissues from the control line with soluble GFP (MSG 1). An important issue was the proper fixation of the flower

buds, in such a way that it would be possible to visualize at least the first two cell layers of the floral meristem, where AG is expressed, without damaging the tissue and keeping the material in focus. For that, we tried to immobilize the tissue by using low concentrations (0.08-0.2%) of agar. A concentration of 0.08% agar appeared to be the best and gave a sharper view from the tissue. To get a better resolution of the images, we also tried different objectives (20X, 40X and 60X) with different numerical apertures For the final analysis a 20X dry lens and 60X water lens were chosen. After the optimalization of the imaging procedure we performed the donor lifetime measurements. For that, we analyzed the control plants MSG1 and a good decay curve and a satisfactory number of photo counts were obtained (Fig 1C). In contrast, we were not able to measure donor lifetime in the *pAG::AG-GFP* transgenic plants, most likely due to the lower number of photo counts and the signal-to-noise ratio (Fig 1B).

Taken these preliminary data together, we may conclude that the expression level of AG:GFP under the control of the endogenous promoter was too low for FRET-FLIM measurements. These results were disappointing, since good confocal images could be made indicating that the expression levels were high enough for imaging (Urbanus *et al.*, in preparation). Usage of the constitutive 35S promoter is no option to solve the problem, because it would not reflect the native behavior of this MADS-box protein.

Another important aspect is the choice of the appropriate fluorescent protein that should be in accordance with the technique and type of instrument that will be used. For our experiments described in chapter 4, we made use of a triple fluorescent set-up in order to detect FRET-FLIM. The combination YFP → mRFP as FRET couple eliminates substantial signal-noise problems due to the autofluorescence from the chloroplasts, and that is because of the use of the YFP as donor molecule instead of CFP, which has a lower quantum yield. Based on this, the combination YFP-mRFP may have more potential for in planta interaction analyses of proteins expressed from the endogenous promoters. Furthermore, several new FP's have been engineered recently (Giepmans *et al.*, 2006) and many putative new FRET couples are currently tested.

Finally, it is worthwhile to mention that autofluorescence depend on the tissues analyzed. In some tissues, e.g. ovules in our case, the signal-to-noise ratio is very low, which requires substantial technical improvements to enable reliable

measurements. Using other tissues or imaging of interactions in epidermal or sub epidermal cell layers combined with new FRET couples will be the next step in developing *in planta* protein interaction methods. Once established, these methods will open avenues to study protein behavior and interaction dynamics in intact plants under native conditions.

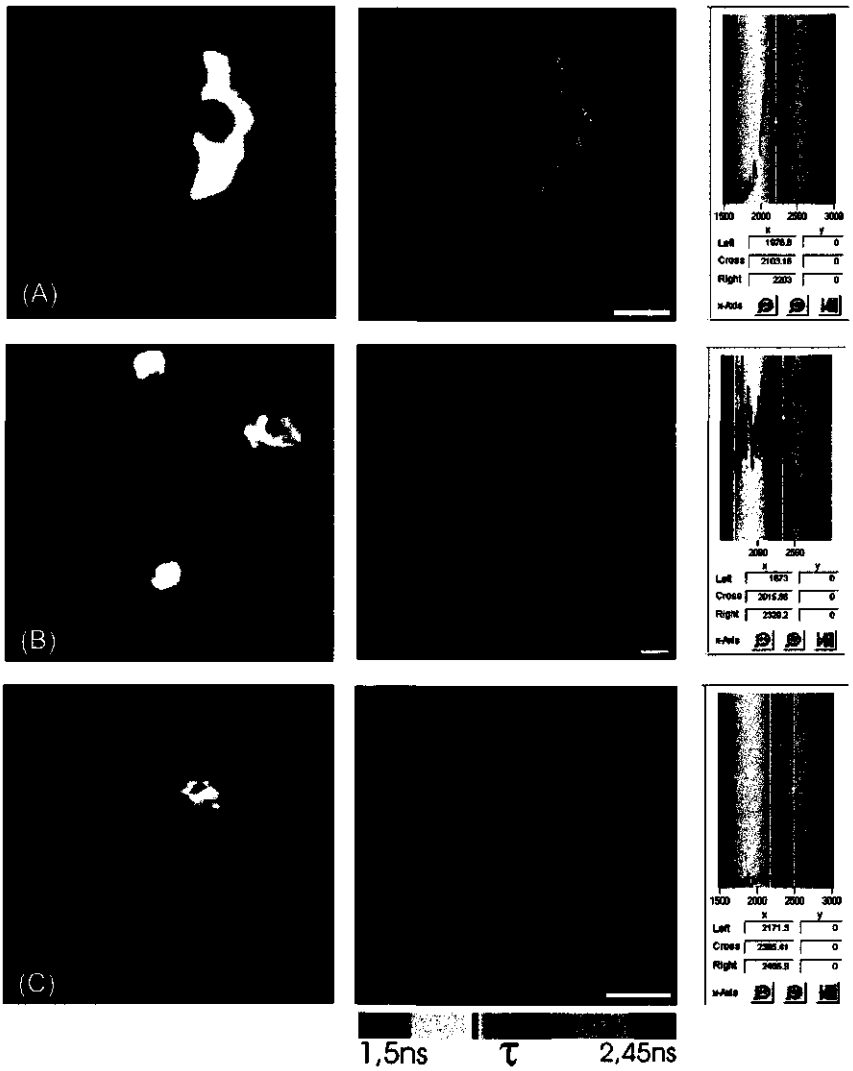

Figure1. FLIM measurements in Arabidopsis protoplasts, ovules and intact flower buds. *Left panels: Fluorescence Intensity Image, middle panels: Fluorescence Lifetime Image (by a false color code) and right panels: histograms presenting the distribution of fluorescence lifetime values..*
A) FLIM measurements for donor lifetime of 35S::AG:GFP in Arabidopsis protoplasts; B) FLIM measurements for donor lifetime of ovules obtained from the plants transformed with the construct pAG::AG-GFP; C) FLIM measurement for flower buds expressing 1X free GFP (MSG 1). The picture shows the intensity and lifetime of the central region of a stage one of the flower meristem. Bars=10μm

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Samenvatting

De biologische interpretatie van het genoom begint met het aflezen van het DNA, het zogenaamde transcriptie proces. Dit belangrijke biologische proces is vaak een onderdeel van signaal transductie routes en kan verschillende externe of interne signalen integreren tot de juiste respons. In het proces van transcriptie spelen transcriptiefactoren een essentiële en belangrijke rol en in hogere eukaryoten zijn zeker tweeduizend verschillende transcriptiefactoren aanwezig, die op basis van o.a. hun geconserveerde DNA bindingsdomein geclassificeerd kunnen worden in verschillende families. De MADS box familie is een van de belangrijke families van transcriptiefactoren voor planten en genetische analyses hebben uitgewezen dat deze eiwitten betrokken zijn bij verschillende ontwikkelingsprocessen, zoals de inductie van bloei, de vorming van bloemorganen en de vruchtzetting. In tegenstelling tot de gedegen kennis betreffende de functie van de verschillende MADS box transcriptiefactoren, is er weinig bekend over het werkingsmechanisme van deze klasse van eiwitten op moleculair niveau. Biochemische experimenten en analyses in gist hebben aangetoond dat MADS box eiwitten onderling aan elkaar kunnen binden en gebaseerd op deze bevindingen is het zogenaamde "quarternary model" ontwikkeld dat beschrijft hoe MADS box eiwitten mogelijk werken. Volgens dit model vormen MADS box eiwitten dimeren, die vervolgens aan elkaar binden zodat een complex van vier eiwitmoleculen ontstaat. Dit quarternaire complex kan dan aan DNA binden op specifieke plaatsen in het genoom en bepaalde "target genen" aan- of uitschakelen. Ondanks dat dit een aannemelijk model is voor het moleculaire werkingsmechanisme van MADS box transcriptiefactoren, is er geen enkel experimenteel bewijs voor aanwezig.

In dit proefschrift wordt beschreven hoe fysische interacties tussen verschillende MADS box transcriptiefactoren van Petunia hybrida en de model plant Arabidopsis thaliana (zandraket) zijn bestudeerd in levende plantencellen. Om dit te bewerkstelligen zijn een aantal nieuwe geavanceerde microspectroscopische technieken ontwikkeld en uitgetest. In het eerste hoofdstuk wordt in detail ingegaan op het transcriptieproces in eukaryote organismen en de rol van transcriptiefactoren hierin. De centrale vraag die gesteld wordt, is hoe transcriptiefactoren in staat zijn om hun specifieke bindingsplaats, de zogenaamde cis-elementen, te vinden in de enorme hoeveelheid en wirwar van DNA in de celkern. De verschillende

voorgestelde mechanismen hiervoor, zoals "looping" en "sliding" worden besproken, evenals het belang van interacties tussen transcriptiefactoren voor hun functioneren. Vervolgens wordt in hoofdstuk twee ingegaan op een eerste set experimenten, die zijn uitgevoerd om interacties tussen verschillende combinaties van petunia MADS box eiwitten aan te tonen in levende plantencellen. Om dit te bewerkstelligen zijn de betreffende MADS box eiwitten gelabeld met verschillende kleur varianten van het groen fluorescerend eiwit GFP. Deze gelabelde eiwitten zijn tot expressie gebracht in blad protoplasten, die vervolgens zijn geanalyseerd met behulp van FRET-FLIM (Fluorescence Resonance Energy Transfer-Fluorescence Lifetime Imaging) microspectroscopie om een fysische interactie aan te tonen tussen de gelabelde eiwitten. Het bleek goed mogelijk om met deze methode dimerisatie aan te tonen voor de MADS box transcriptiefactoren en daarnaast is het eerste bewijs verkregen voor de vorming van complexen van MADS box eiwitten bestaande uit meer dan twee moleculen. Uit dit laatst genoemde experiment is gebleken dat de MADS box eiwitten Floral Binding Protein2 (FBP2), FBP11, en FBP24, die gezamenlijk tot expressie komen in zaadknoppen, een complex kunnen vormen in levende plantencellen. Vergelijkbare analyses in blad protoplasten, voor Arabidopsis MADS box eiwitten die betrokken zijn bij de vorming van kroonbladeren en meeldraden, lieten zien dat er een duidelijk verschil is in bindingssterkte tussen verschillende MADS box eiwitten (Hoofdstuk 3). Verder bleek uit deze experimenten dat veel Arabidopsis MADS box eiwitten in staat zijn om met zichzelf te interacteren en zogenaamde homodimeren kunnen vormen. Deze homodimeren konden echter niet worden aangetoond met behulp van de analyses in gist. Uit deze analyses blijkt dus dat de FRET technologie een gevoelige methode is en ook zeer zwakke binding kan aantonen. Verder kan verondersteld worden dat in een plantencel, waar vaak meerdere verschillende MADS box eiwitten aanwezig zijn, mogelijk alleen de sterkste interacties plaats vinden en er dus competitie is voor binding tussen de verschillende aanwezige eiwitten. Om dit soort effecten beter te kunnen bestuderen is er een nieuwe technologie ontwikkeld, die beschreven staat in Hoofdstuk 4. Deze ontwikkelde "Competition-FRET" methode maakt het mogelijk om de hiervoor genoemde competitie effecten zichtbaar te maken. Daarnaast is het ook mogelijk om met deze methode aan te tonen of de vorming van multimere complexen optreedt in plaats van competitie voor dimerisatie. De ontwikkelde methode is uiteindelijk gebruikt om te bepalen of de Arabidopsis MADS box eiwitten SEPALLATA3 (SEP3)

en AGAMOUS (AG) de voorkeur geven aan interactie met zichzelf (homodimerisatie), interactie met elkaar (heterodimerisatie) of de vorming van een groot complex van meerdere moleculen.

Alle hiervoor beschreven experimenten voor het aantonen van eiwit-eiwit interacties zijn gebaseerd op FRET. Voor deze technologie zijn geavanceerde en dure microscopische opstellingen nodig. Om deze reden is ervoor gekozen om een alternatieve, goedkopere en theoretisch eenvoudiger methode te ontwikkelen en te testen, die in het verleden goede resultaten heeft opgeleverd voor het aantonen van eiwitinteracties in dierlijke cellen. De methode wordt BiFC (Bimolecular Fluorescence Complementation) genoemd of "Split-YFP" en is gebaseerd op het opsplitsen van een fluorescerend molecuul in twee inactieve delen. Deze delen worden vervolgens gefuseerd aan de twee eiwitten van interesse. Indien de twee eiwitten aan elkaar binden komen de twee helften van het fluorescerende molecuul samen en kunnen hervouwen tot een actief eiwit wat een lichtsignaal zal geven. In onze studie hebben we gebruik gemaakt van het geel fluorescerende eiwit YFP en zijn we er in geslaagd om de al bekende interactie tussen AG en SEP3 aan te tonen in de kern van Arabidopsis blad protoplasten (Hoofdstuk 5). Net als de FRET methode geeft deze techniek dus inzicht in de specifieke interacties tussen eiwitten in levende planten cellen. Geen van de beide technieken kan echter duidelijk aangeven hoe een gevormd complex is opgebouwd en hoeveel moleculen er daadwerkelijk aanwezig zijn in een complex. Een mogelijke manier om meer inzicht te krijgen in de grootte van een gevormd complex is om bijvoorbeeld naar de diffusie van complexen te kijken. In theorie zal een complex bestaande uit meerdere eiwitten groter zijn en zich langzamer bewegen in een oplossing of cel. Deze diffusie van eiwit complexen kan gemeten worden met FCS (Fluorescence Correlation Spectroscopy) en dit is in deze studie gedaan voor de door middel van in vitro transcriptie/translatie geproduceerde AG-YFP en SEP3-YFP fusie-eiwitten (Hoofdstuk 6). De verkregen resultaten suggereren dat SEP3 mogelijk homodimeren vormt, terwijl AG in staat is om grotere complexen te vormen. Indien beide eiwitten tegelijk in oplossing werden gebracht, bleek er een complex gevormd te worden met een zeer lage diffusie snelheid, wat suggereert dat een groot multimeer complex gevormd wordt. Of in een plantencel vergelijkbare complexen gevormd worden blijft de vraag, want alle gegevens zijn verkregen met in vitro geproduceerde eiwitten.

In het laatste afsluitende hoofdstuk worden de voor- en nadelen van alle hiervoor beschreven technieken voor het aantonen van eiwit-eiwit interacties in levende plantencellen op een rijtje gezet. Hieruit blijkt duidelijk dat iedere techniek zijn eigen plus- en minpunten heeft. Daarnaast blijkt duidelijk dat een volgende stap in het onderzoek is om de bindingscapaciteit van MADS box eiwitten te testen in gehele weefsels of planten, waarbij de eiwitten in normale concentraties aanwezig zijn. Om de mogelijkheden hiervoor te testen is in een pilot experiment gedaan om de FRET-FLIM technologie toe te passen op groeipunten van Arabidopsis planten. Hieruit blijkt duidelijk dat de techniek op dit moment nog niet gevoelig genoeg is. Echter op basis van de resultaten behaald in deze studie en de snelle vooruitgang in de wereld van de fluorescerende eiwitten en microspectroscopie is te voorspellen dat dit zeer waarschijnlijk tot de mogelijkheden gaat behoren in de nabije toekomst.

Summary

The biological interpretation of the genome starts from transcription, and many different signaling pathways are integrated at this level. Transcription factors play a central role in the transcription process, because they select the down-stream genes and determine their spatial and temporal expression. In higher eudicot species around 2000 specific transcription factors are present, which can be classified into families based on conserved common domains. The MADS-box transcription factor family is an important family of transcription regulators in plants and genetic studies revealed that members of this family are involved in various developmental processes, like floral induction, floral organ formation and fruit development. In contrast to this wealth of information concerning MADS-box gene functions, the molecular mode of action of the encoded proteins is far from completely understood. Biochemical and yeast n-hybrid experiments performed in the past showed that MADS-box proteins are able to interact mutually, and based on these findings a hypothetical quaternary model has been proposed as molecular working mechanism. According to this model two MADS-box protein dimers assemble into a higher order complex, which binds DNA and regulates target gene expression. Although, this molecular mechanism sounds plausible, it still lacks evidence from *in vivo* studies.

In this study we investigated physical interactions among members of the *Petunia hybrida* and *Arabidopsis thaliana* MADS-box transcription factor families in living plant cells. For this purpose, sophisticated micro-spectroscopy techniques have been implemented and in addition, some novel fluorescent-protein-based tools were developed. The first chapter gives an introduction about the dynamic transcriptional process and describes our current knowledge about transcriptional regulation in eukaryotes. The central question of this chapter is how transcription factors are able to find their specific binding sites (*cis*-elements) within the huge genome. The various mechanisms, such as "looping" and "sliding", that have been proposed are discussed, as well as the relevance of direct interactions between transcription factors for the control of gene expression.

In a first attempt to detect protein interactions in living cells, we transiently expressed combinations of petunia MADS-box transcription factors labeled with different color variants of the Green Fluorescent Protein (GFP) in leaf protoplasts (Chapter 2). Subsequently, the transfected protoplasts were analyzed by means of FRET-FLIM

(Fluorescence Resonance Energy Transfer – Fluorescence Lifetime Imaging) to identify specific dimerization. In addition, we have obtained indirect evidence for higher-order complex formation of the petunia MADS-box proteins FLORAL BINDING PROTEIN2 (FBP2), FBP11, and FBP24 in living cells. Similar kind of analyses for Arabidopsis MADS-box proteins involved in petal and stamen development revealed clear differences in interaction affinities *in vivo* and furthermore, many homodimers were identified that could not be detected by yeast-based systems in the past (Chapter 3). This result demonstrated the robustness of the FRET-FLIM approach. Based on our observations, we hypothesize that 'partner selectivity' plays an important role in complex formation at particular developmental stages. To study differences in interaction affinity and selectivity and the consequences for complex formation in more detail, a novel method was developed (Chapter 4). The technique, designated "Competition-FRET", allows the verification of competition effects between proteins, and furthermore, it may provide information about the formation of higher-order complexes between different proteins under study. The developed method was implemented to investigate in depth the preference for homo- or heterodimer interactions of the Arabidopsis MADS-box proteins AGAMOUS (AG) and SEPALLATA3 (SEP3).

The detection of interactions in living cells by FRET as it has been done in the studies described above demands a sophisticated microscopy set-up, and therefore, we decided to test and implement an alternative and theoretically simple technique (Chapter 5). This method for the *in vivo* detection of protein-protein interaction is called BiFC (Bimolecular Fluorescence Complementation), or "Split-YFP". In this system, a fluorescent molecule is split into two inactive domains and these two non-fluorescent parts are fused to the proteins under study. Only upon interaction of the two protein partners the two non-fluorescent parts of the fluorescent molecule are brought into close proximity, which enables the recovery of fluorescence. We used the EYFP (Enhanced Yellow Fluorescence Protein) molecule as fluorescent molecule and were able to detect the interaction between AG and SEP3 in nuclei of Arabidopsis leaf protoplasts. Techniques like this and FRET-FLIM allow the analyses of interactions between proteins in living cells, but give no information about the size of the formed complexes. To get a first indication about the stoichiometry of protein complexes, we monitored the diffusion time of *in vitro* synthesized AG-EYFP and SEP3-EYFP fusion proteins by means of FCS (Fluorescence Correlation

Spectroscopy). From these experiments described in Chapter 6, we could speculate that SEP3 is present as a dimer and also as a higher order complex, whilst AG on its own is able to assemble into larger complexes. The diffusion time of the product formed upon co-translation of both AG and SEP3, suggests that a multimeric protein complex with a high molecular weight is formed upon interaction between AG and SEP3. Even though FCS is a powerful technique, these interpretations should be taken cautiously, mainly because these experiments were done *in vitro* instead of in living cells. Finally, in the last chapter we discuss the various methods that have been implemented and developed to monitor protein-protein interactions and complex formation of MADS-box transcription factors in living plant cells. Furthermore, we made a first step to monitor interactions in intact tissues under endogenous expression levels, and the preliminary results obtained from these *in planta* FRET-FLIM measurements are discussed.

Curriculum Vitae

Isabella Antonia Nougalli Tonaco was born on the 25th of April 1977 in Sao Paulo, Brazil. From 1995-2000, she studied Agronomy at Federal University of Lavras (UFLA), located at Minas Gerais state, Brazil. After her degree, she gave continuation on her studies following a Masters degree on Genetics and Plant Breeding at the same University. During her Master thesis, she studied the genetic mechanism controlling a natural existent mutant affecting flower development in *Eucalyptus* trees, where she got her first interest in developmental genetics. With this interest, she contacted Prof. dr. Gerco Angenent at Plant Research International (PRI) for the possibility to perform her PhD study in The Netherlands. At the end of the year 2002, she obtained a Brazilian grant (CAPES) to follow up her PhD study abroad. In December of the same year she moved to Wageningen, to do her PhD within the group of Prof. dr. Gerco Angenent (PRI), in collaboration with Prof. dr. Sacco de Vries from the Biochemistry department at Wageningen University. The present thesis is the result of her research within this collaboration.

List of publications and manuscripts in preparation

Nougalli-Tonaco IA, Immink RGH, de Vries SC, Angenent GC and Gadella TW Jr. Competition-FRET: a new method to monitor protein-protein interactions in living cells. *In preparation.*

Nougalli-Tonaco IA, Borst JW, de Vries SC, Angenent GC and Immink RGH. Physical interactions and dimerization affinity *in vivo* for MADS-box proteins involved in petal and stamen formation. *In preparation.*

Nougalli-Tonaco IA, Kaufmann K, Hink M, Engel R, de Vries SC, Angenent GC and Immink RGH. Diffusion of MADS-box proteins and complexes. *In preparation.*

Ciannamea S, Kaufmann K, Frau M, **Nougalli-Tonaco IA**, Petersen K, Nielsen KK, Angenent GC and Immink RGH. (2006). Protein features of MADS box transcription factors involved in flowering in *Lolium perenne.* J.Exp.Bot. 57, 3419–3431.

Borst JW, **Nougalli-Tonaco IA**, Hink MA, Hoek AV Immink RGH and Visser AJWG. (2006). Protein-protein interactions *in vivo*: Use of Biosensors based on FRET In: Reviews in Fluorescence 2006 / Geddes, C.D., Lakowicz, J.R. - New York: Kluwer Academic/Plenum Publishers, - p. 341 - 355.

Nougalli-Tonaco IA, Borst JW, de Vries SC, Angenent GC and Immink RGH. (2006). *In vivo* imaging of MADS-box transcription factors interactions. J. Exp. Bot. 57, 33-42.

Acknowledgements

Five years ago I came to the Netherlands aiming to do my PhD. During this time I have collected a number of good memories and unforgettable moments. It was my first time abroad and things were certainly very challenging for me. I had a good combination of feelings, as every foreigner elsewhere in the world. Lovely memories combined with tough periods, which gave me personal strength and a better understanding of myself.

Since doing a PhD thesis comprises not only an intellectual training, but also a personal achievement I would like to thank those, who I shared a great time during my stay in the Netherlands.

Brazilians are by default very communicative, and these communication skills helped me to get in touch oversea with Antonio Chalfun. Antonio, I would like to thank you very much for the great support that you and your family gave me before and after I did arrive in Wageningen.

Antonio was a PhD student in the group of Prof. dr. Gerco Angenent at Plant Research International (PRI) and helped me to get in touch with Gerco, from whom I got promptly help and support for my application as PhD fellow in his lab. I would like to thank you, Gerco, for this opportunity, the challenging project you suggested to me and the nice discussions during my PhD time. I have learned a lot from you and your group and this experience will definitely be very important for my professional development, thanks a lot!

In Gerco's group, I was supervised by Dr. Richard Immink, with whom I also shared my office and the lab bench. I have very good memories from the time we spent working together and I would like to thank you very much for your support, guidance, discussions and good atmosphere. I guess the office is less loud and more organized since I left, as counterpart I am finally able to use my agenda very efficiently and I quite often catch myself eating while working in front of the PC.

I also would like to thank all my colleagues from PRI: John, Marco and Jacqueline, Stefan and Nayelli, Jeroen, Ruud, Silvia, Patricia, Guadong, Martijin, Kim, Wilco, Chun Ming, Ronny, Michiel, Jan, Jannie and many visiting scientists that I had the opportunity to meet and exchange experiences with. Thank you all for the nice environment and good moments.

I would like to especially thank Susan and Kerstin for the nice time we spent together: working, eating and talking ... Thanks to both of you for being very nice and supportive friends. I really enjoy our friendship and I hope we keep in touch!

Just to make things a little bit more challenging, going abroad was in a certain way not enough for me; therefore I had to have a new subject as well, something quite different than I was used to, with this idea in mind I immediately accepted my PhD project which had several novel techniques such as FLIM and FCS as approaches to better understand the molecular mechanisms of plant MADS-box transcription factors. I really liked my project, and even though things did not always work so easily at the beginning I could keep my enthusiasm throughout the years. This exciting project put me in direct collaboration with several other people, with whom I shared a great time at the scientific and personal level as well.

To start with, I would like to thank Prof. dr. Dorus Gadella Jr., with whom I had the opportunity to work closely together while developing the Competition-FRET method. I would like to thank you Dorus for being a very inspiring and motivating scientist to me. It was a great pleasure for me to have worked directly with you, thanks a lot!

I would like to sincerely thank my promotor Prof. dr. Sacco de Vries, of the Biochemistry department at Wageningen University. I would like to thank you Sacco for giving me the nice opportunity to join your group as your student as well. I felt very supported by you and your group during these years. Further, I would like to thank you for your input and the nice discussions we had, and of course for the good scientific opportunities that you encouraged me to take and to follow in order to train and develop myself. Thank you so much!

Biochemistry has been my other nice working environment and there I also met a couple of nice people that I would like to thank.

I spent a very long time at the basement of Biochemistry doing my FLIM and FCS measurements. During this time I have been very supported and helped by Jan Willem, whom I would like to especially thank! I have learned a lot from you and I really enjoyed the time we worked together. Thanks for being always very helpful and a very good friend during these years.

I also would like to thank Mark Hink and Ruchira for the FCS measurements. I really learned a lot from both of you and I appreciate very much our discussions and your help.

I also spent quite some time at Biochemistry either preparing my samples, running up and down the stairs or during the embryo work discussions, for this I would like to thank many people that I had the opportunity to meet: Jacques, Adrie and Simon (thanks a lot for the gel filtration analysis!), Sanne, Jenny, Cathy, Dolf, Mark and Sofia (obrigada pelo apoio e muitas risadas em varios dias cinzentos!)

I would like to especially thank Rumyana and Jose for the nice friendship and good times that we had together, I wish for both of you happy days and lots of success and I hope we keep in touch in the future.

I also had a great chance to meet many foreigners outside work...From this community I would like to especially thank: Rafaela, Asaph, Teun, Tetti, Salva, Mazel, Rumyana and Nicolai, Alessandra, Cinzia, Rada, Radi, Giulia, Shital, Vassilis, Arjen (thanks a lot for helping me moving my stuff around every now and then!), Anna Shchennikova (my sister! Oh! ahahaa) and finally to Stefano (thanks a lot for your friendship, I wish you all the best).

But Wageningen has been quite tropical during these years, with many dinners, parties and get-togethers of the brazilian community. I would like to thank many brazilian friends that I met in Wageningen as well: Joana e Pim (obrigada pela sua amizade e carinho durante estes anos, fico muito feliz por voces!), Gilma, Paulo e Vera, JM e Cia, Wagner, Simone e Cristiano, Mario e Ana, Nilvanira, Marcia, Luciana, inesquecivel Tania, amigao querido Flavio, Anabele e turma, Michael, Ulisses (vulgo Juju, ahahaha), Saulo, Vanja, and many other friends that I met during this time. Thanks for the nice time together. And of course the unforgettable family from the Bornesesteeg 16+18 floor: Odair, Glaciela e Valentin (obrigada pelo carinho e atencao durante estes anos, adoro vcs!), Celso (ermao) e Ritinha (cunhada), Rubia (querida), Anderson (fala ai irmao...ahaha).

Tambem gostaria de agradecer meus familiares e amigos do Brazil, pelo apoio e suporte durante estes anos. Em especial, Fabio e familia, prof. Lisete (Lavras) e prof. Magno (Lavras), pelo grande apoio e incentivo durante minha vinda e meus estudos. Minha tese nao teria sido a mesma sem o constante apoio das queridas amigas Eunice e Rose! Muito obrigada pelo carinho, atencao e amizade, durante nossas interminaveis conversas telefonicas nestes cinco anos. Sem voces este doutorado teria sido muito mais dificil, muito obrigada por tudo!

My deepest thanks to Eike. Your love, care and truly friendship made me strong and confident again. From you I have learned that true love makes everything possible.

Finally I would like to thank my parents for giving me the necessary strength that I need to go on in my lifes dreams, even though physically separated I will always keep you both with me.

Education Statement of the Graduate School

Experimental Plant Sciences

The Graduate School EXPERIMENTAL PLANT SCIENCES

Issued to: Isabella Antonia Nougalli Tonaco
Date: 14 January 2008
Group: Biochemistry , Wageningen University

1) Start-up phase	date
▶ **First presentation of your project**	
Protein-protein interactions of MADS-box proteins in living cells	May, 2003
▶ **Writing or rewriting a project proposal**	
▶ **Writing a review or book chapter**	
▶ **MSc courses**	
Regulation of Plant Development	Jan-Mar, 2003
Cell biology and Advanced Imaging Technology	Nov-Dec, 2003
▶ **Laboratory use of isotopes**	
Course 'Safe Handling of Radioactive Materials and Sources, level 5B'	Oct, 2003
Subtotal Start-up Phase	*9.00 credits**

2) Scientific Exposure	date
▶ **EPS PhD student days**	
PhD student day 2004 (Vrije Universiteit Amsterdam)	Jun 03, 2004
PhD student day 2005 (Radboud Universiteit Nijmegen)	Jun 02, 2005
PhD student day 2006 (Wageningen Universiteit)	Sep 19, 2006
▶ **EPS theme symposia**	
EPS theme 2 symposia " Interactions between plants and biotic agents"	Dez 12,2003
EPS theme 1 symposia " Developmental biology of plants"	Feb 17, 2004
EPS theme 1 symposia " Developmental biology of plants"	Apr 26, 2005
EPS theme 1 symposia " Developmental biology of plants"	May 12, 2006
▶ **NWO Lunteren days and other National Platforms**	
ALW meeting Lunteren 2003, Plant Sciences	April 07-08, 2003
ALW meeting Lunteren 2004, Plant Sciences	April 06-07,2004
ALW meeting Lunteren 2005, Plant Sciences	April 05-06, 2005
ALW meeting Lunteren 2006, Plant Sciences	April 04-05, 2006
▶ **Seminars (series), workshops and symposia**	
GeNeYous symposium - Decisions in Genomics	Jan 20, 2004
Advanced Microscopy in Cell Biology	Mar 30, 2004
Flying seminar Dr. Steven Clark: Rec.Sign.Pathw. Regulating organ embryo and meristem devel. In A.t.	Jun 28, 2004
Symposia on Systems Biology	Nov 04, 2004
2nd Meeting of Dutch Chromatin Community	Jan 21, 2005
Workshop: Scientific publishing: An introductory worshop for PhD students and young authors	Oct 13, 2005
Flying seminar Dr. Jiri Friml: Cell biological determinats of polar auxin transport	Jun 14, 2006
▶ **Seminar plus**	
▶ **International symposia and congresses**	
16th International Conference in Arabidopsis Research - Madison-USA	Jun 15-19, 2005
International Workshop on MADS-box trasncription factors	Oct 23-26, 2005
XV Congress of the Federation of European Societies of Plant Biology FESPB	July 17-21, 2006
▶ **Presentations**	
EPS theme 1 symposia " Developmental biology of plants" (oral)	Apr 26, 2005
16th International Conference in Arabidopsis Research - Madison-USA (poster)	Jun 15-19, 2005
International Workshop on MADS-box trasncription factors (oral)	Oct 23-26, 2005
XV Congress of the Federation of European Societies of Plant Biology FESPB (poster)	July17-21, 2006
EPS theme 1 symposia " Developmental biology of plants" (oral)	May 12, 2006
▶ **IAB interview**	Jun 03, 2005
▶ **Excursions**	
Subtotal Scientific Exposure	*14.6 credits**

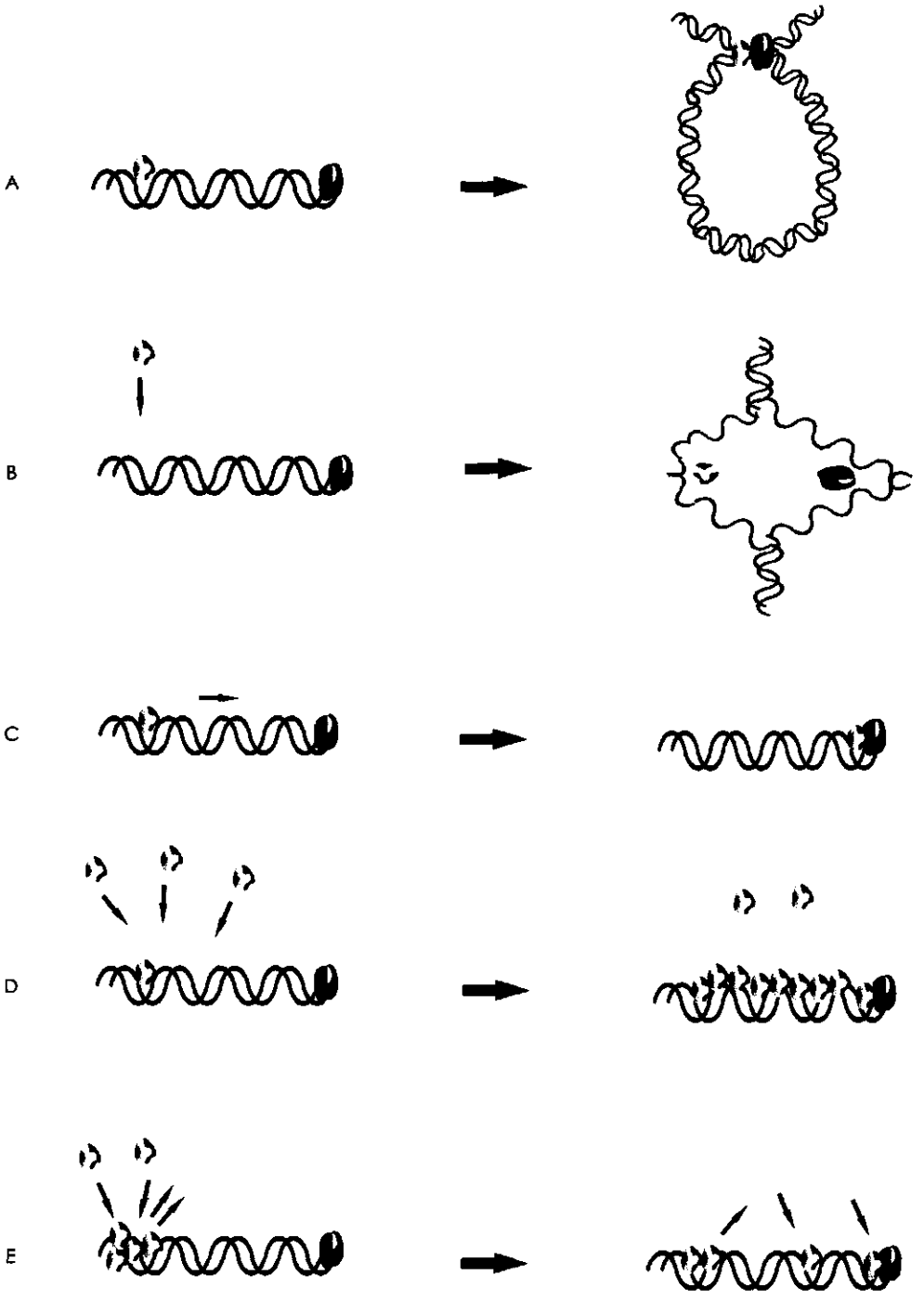
3) In-Depth Studies	date
▶ **EPS courses or other PhD courses**	
EPS summerschool: Functional genomics:theory and hands-on data analysis (Utrecht)	Aug 25-28, 2003
Homology Modelling Course (Nijmegen)	Oct 18-29, 2004
Bioinformation Technology 1 (Vlag, Wageningen)	Nov 08-16, 2004
▶ **Journal club**	
Biweekly Biochemistry progress report	2002-2006
Annual progress report Plant Research International (Bioscience cluster)	2002-2006
Weekly work discussions Plant Research International	2002-2006
Montly literature discussion Plant Research International	2002-2006
▶ **Individual research training**	
Subtotal In-Depth Studies	*10.5 credits**

4) Personal development	date
▶ **Skill training courses**	
English Academic Writing (CENTA, Wageningen)	Jan-Apr, 2005
French 1 (CENTA, Wageningen)	Sep-Dec, 2005
French 2 (CENTA, Wageningen)	Jan-Apr, 2006
English Scientific Writing (CENTA, Wageningen)	May-Jully, 2006
▶ **Organisation of PhD students day, course or conference**	
▶ **Membership of Board, Committee or PhD council**	
Subtotal Personal Development	*7.6 credits**

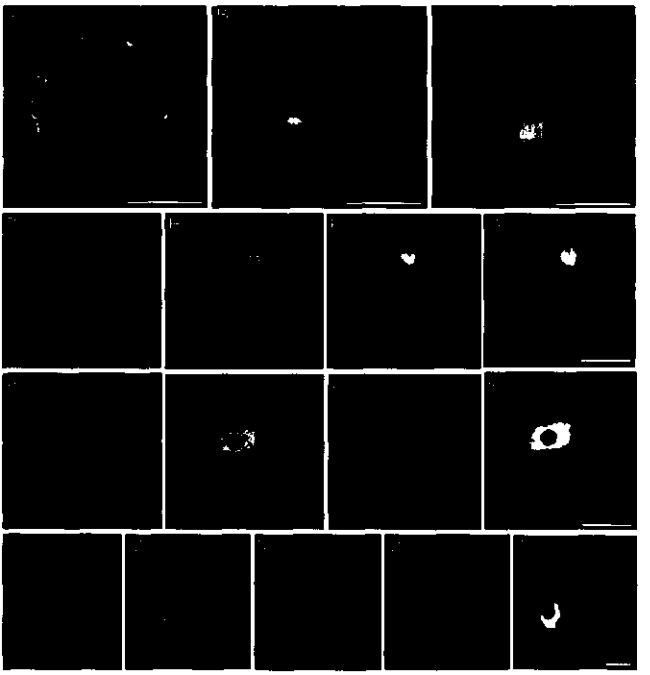
TOTAL NUMBER OF CREDIT POINTS*	**41.7**

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

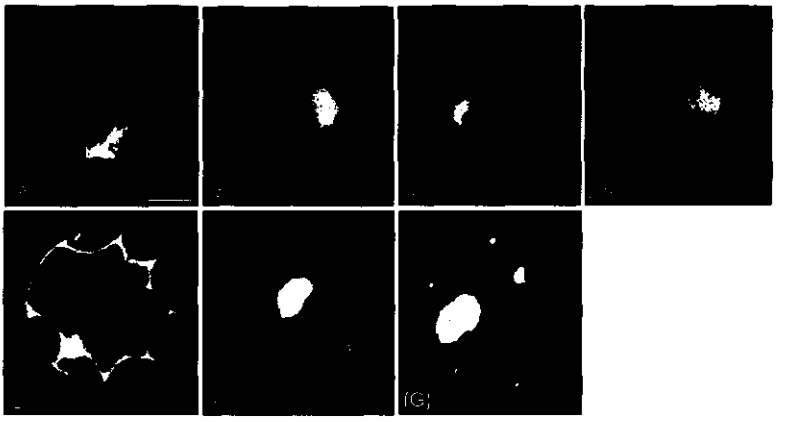
* A credit represents a normative study load of 28 hours of study



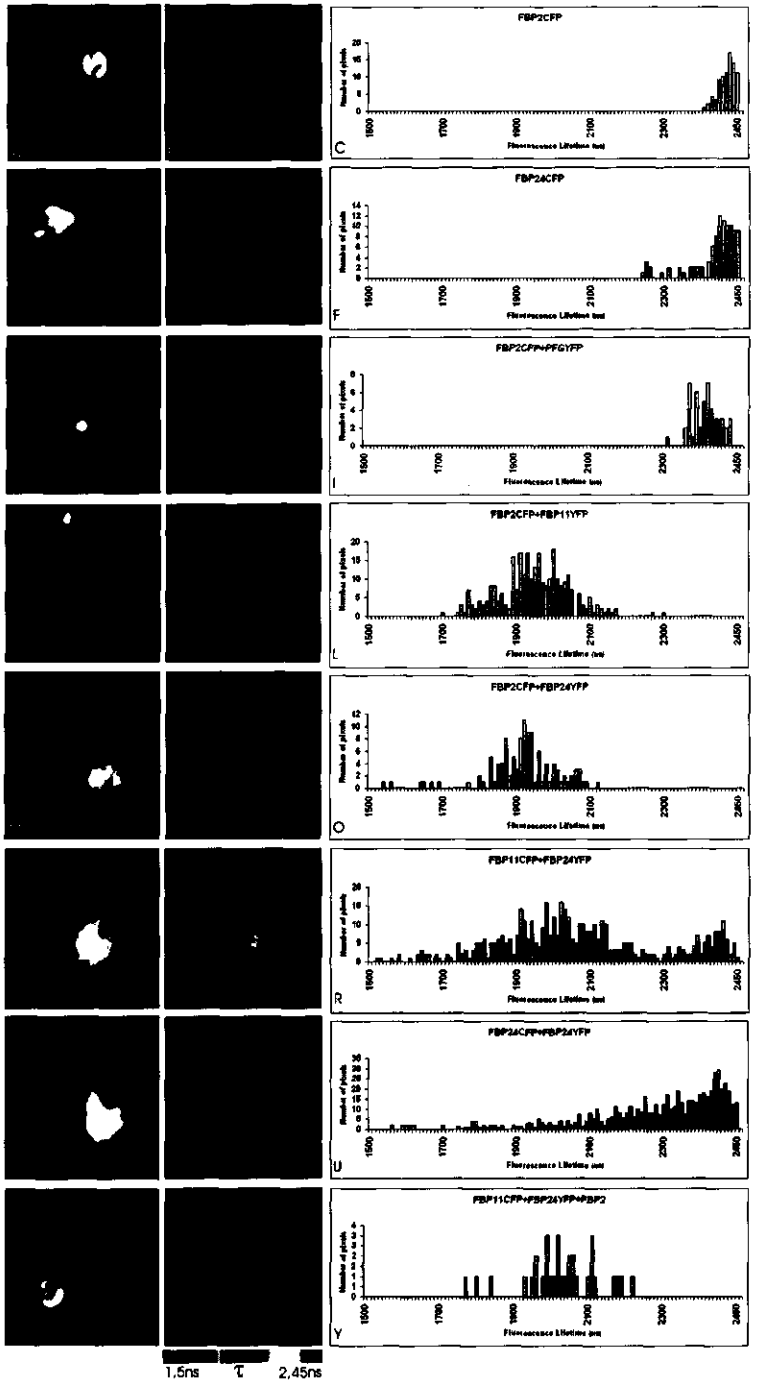
Chapter 1 - Figure 2. Different models for gene regulation at distance.



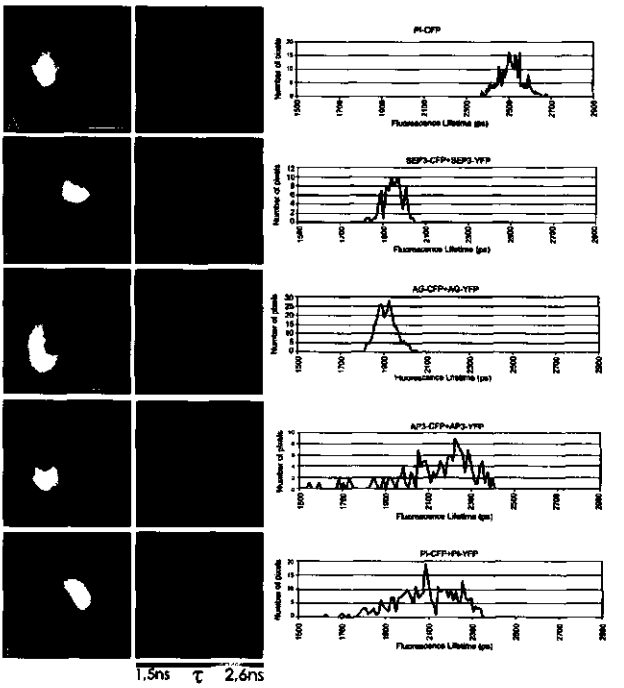
Chapter 2 - Figure 1. Localization of MADS-box proteins in protoplasts.



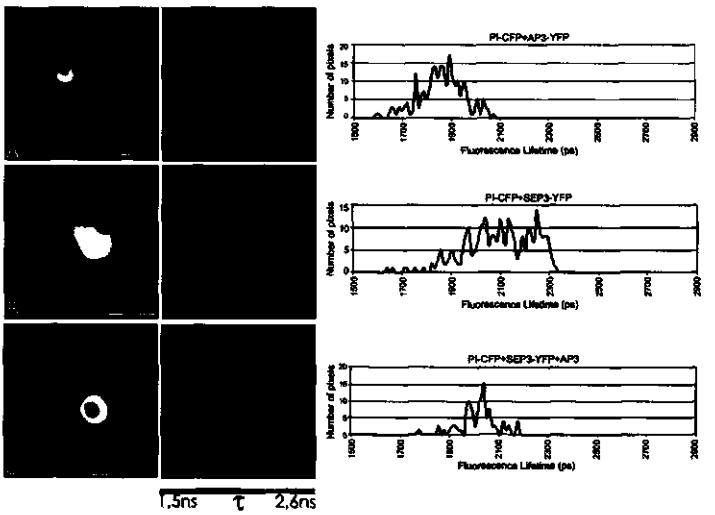
Chapter 3 – Figure 1. Localization of MADS-box proteins in Arabidopsis leaf protoplasts.



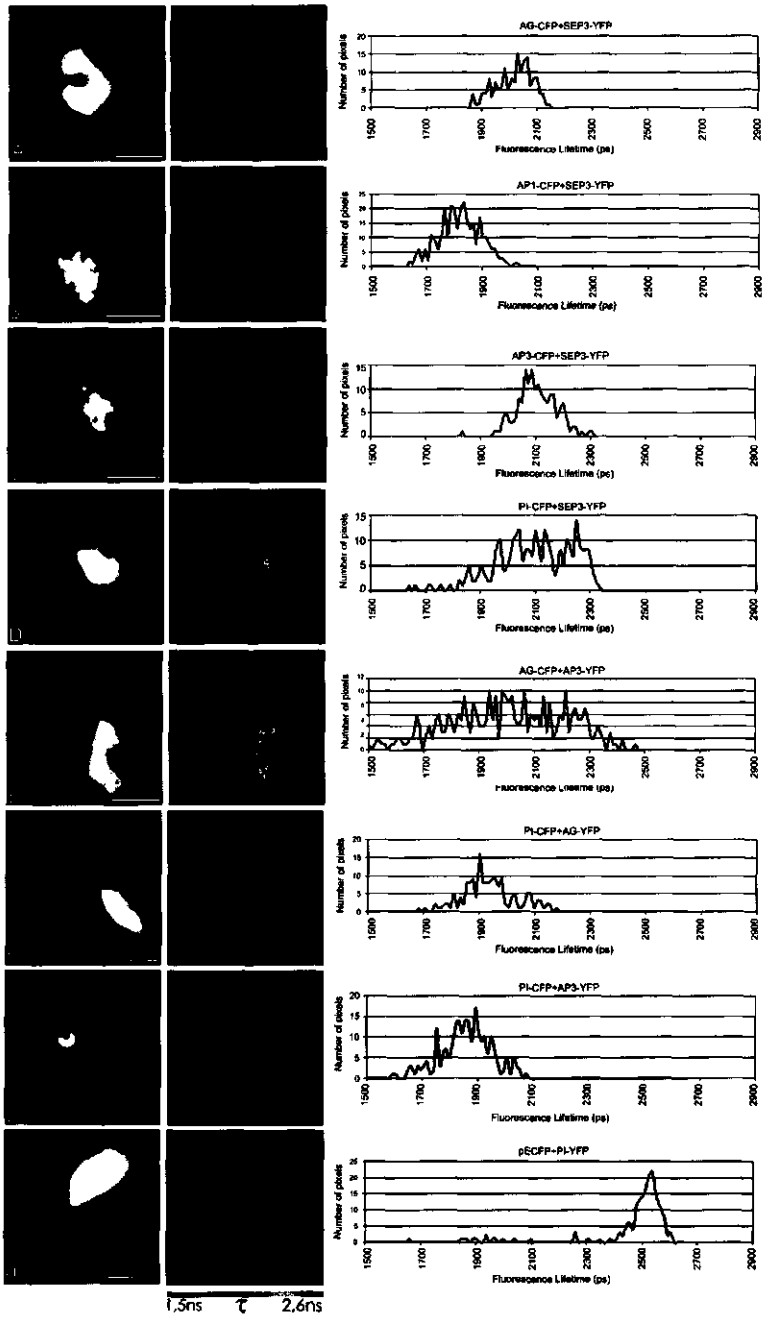
Chapter 2 - Figure 2. Monitoring Fluorescence Resonance Energy Transfer (FRET) by Fluorescence Lifetime Imaging Microscopy (FLIM).



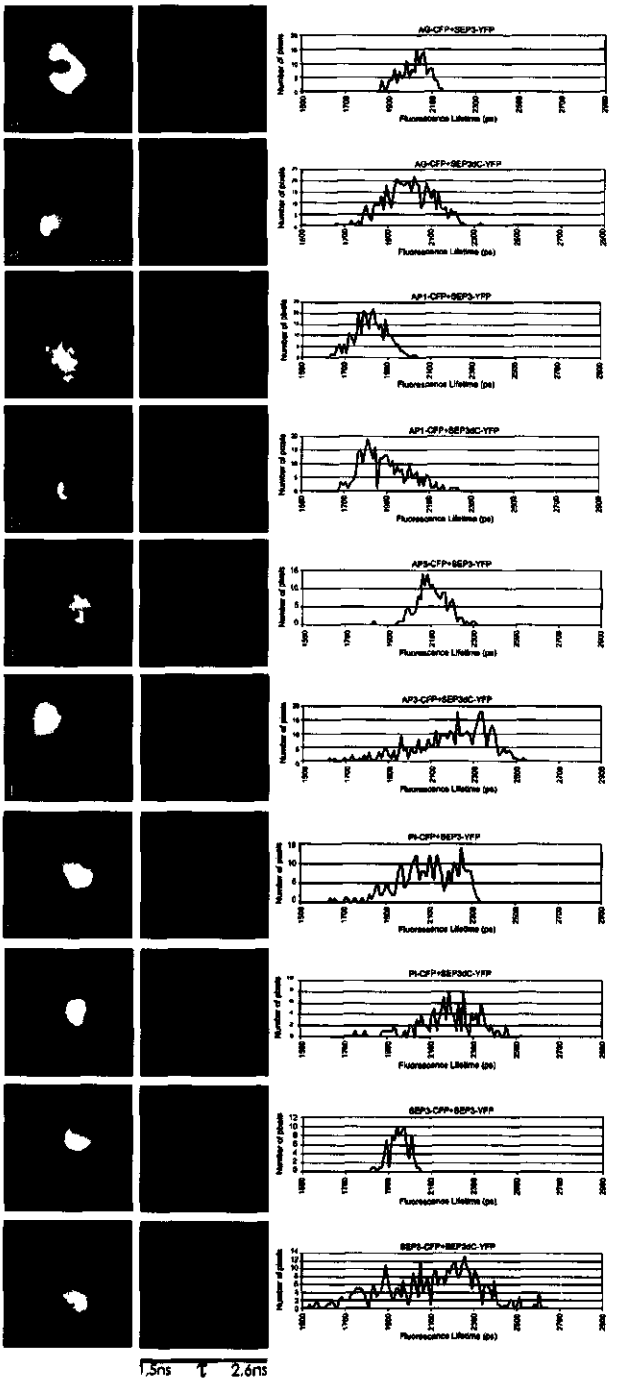
Chapter 3 - Figure 2. Detection of Fluorescence Energy Transfer (FRET) by Fluorescence Lifetime Imaging Microscopy (FLIM) for different MADS-box protein homodimers combinations



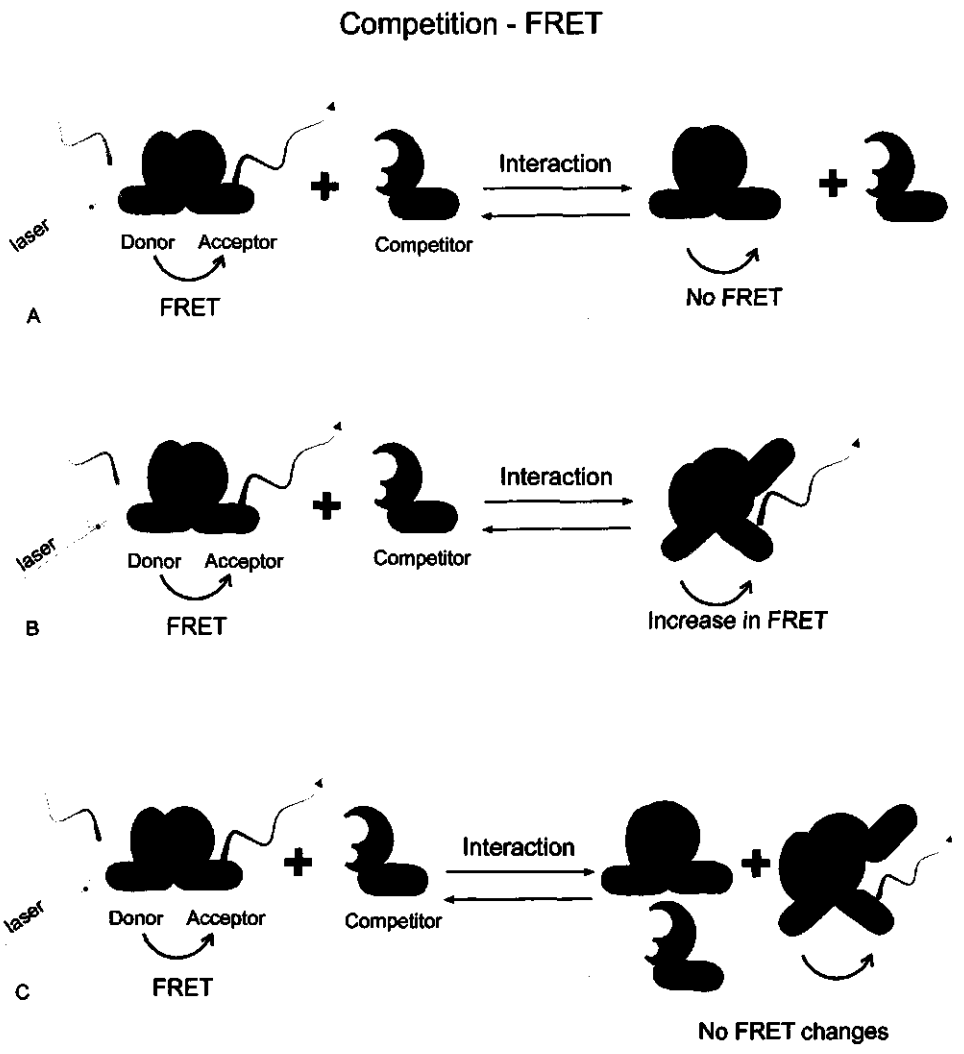
Chapter 3 - Figure 5. APETALA3-PISTILLATA and SEPALLATA 3 form a higher-order complex in vivo



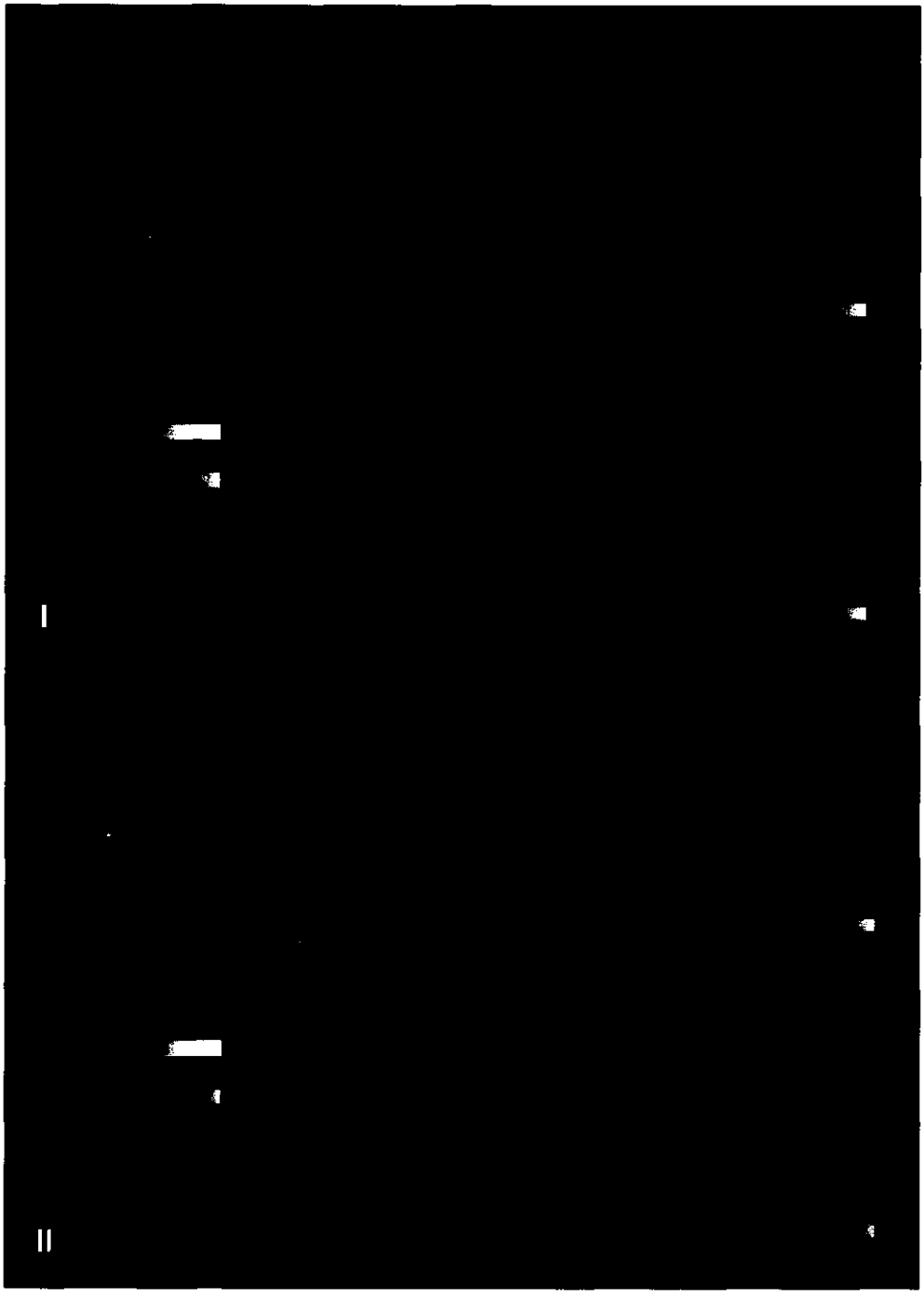
Chapter 3 - Figure 3. Detection of Fluorescence Energy Transfer (FRET) by Fluorescence Lifetime Imaging Microscopy (FLIM) for different MADS-box protein combinations.



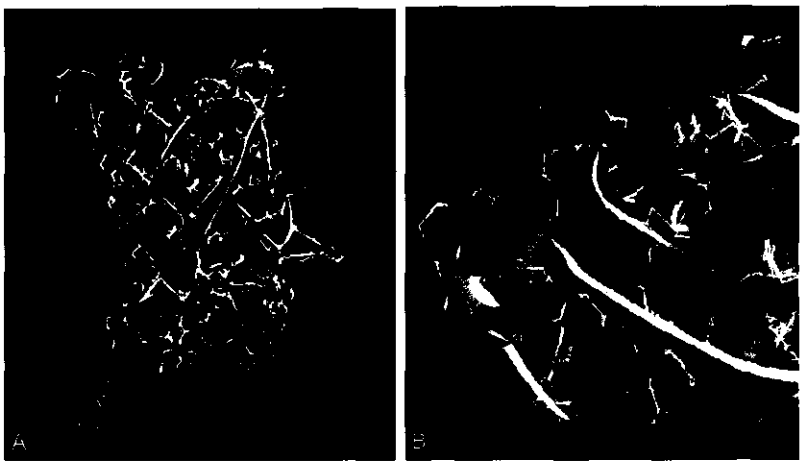
Chapter 3 - Figure 4. Interactions of SEPALLATA 3 lacking the C domain



Chapter 4 - Figure 1. Competition-FRET can be used for the detection of differences in dimerization affinity and competition effects between molecules

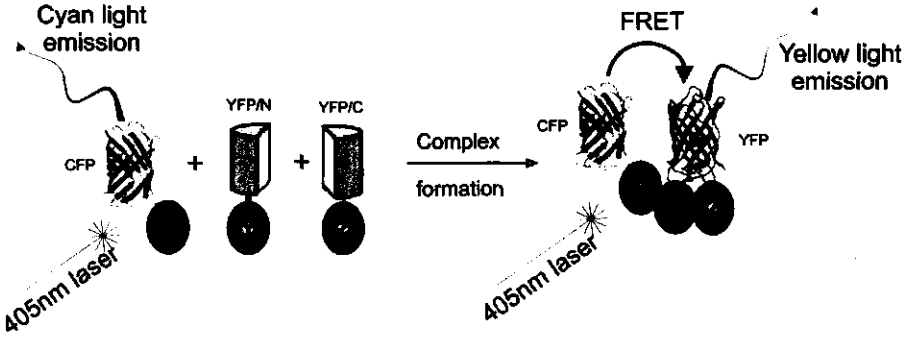


Chapter 4 - Figure 2. FRET analysis of EYFP to ECFP.

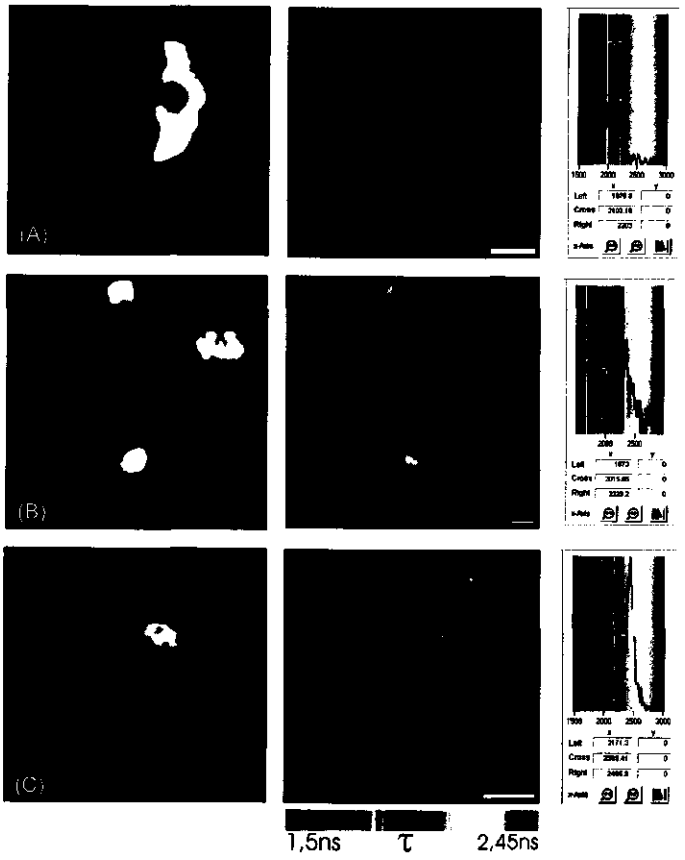


Chapter 5 - Figure 2. Schematic 3D-representation of the YFP molecule

Chapter 5 Figure 3. Detection of protein-protein interactions for the MADS-box proteins FBP2 and FBP11 in living cowpea protoplasts by BiFC, making use of the pSPYNE and pSPYCE vector set.



Chapter 5 - Figure 4. Scheme to represent the BiFC-FRET approach.



Chapter 7 – Figure 1. FLIM measurements in Arabidopsis protoplasts, ovules and intact flower buds.

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