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

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Over the last decade, the yeast two-hybrid system has become the tool to use for the identification of protein–protein interactions and recently, even complete interactomes were elucidated by this method. Nevertheless, it is an artificial system that is sensitive to errors resulting in the identification of false-positive and false-negative interactions. In this study, plant MADS box transcription factor interactions identified by yeast two-hybrid systems where studied in living plant cells by a technique based on fluorescence resonance energy transfer (FRET). Petunia MADS box proteins were fused to either cyan fluorescent protein or yellow fluorescent protein and transiently expressed in protoplasts followed by FRET-spectral imaging microscopy and FRET-fluorescence lifetime imaging microscopy to detect FRET and hence protein–protein interactions. All petunia MADS box heterodimers identified in yeast were confirmed in protoplasts. However, in contrast to the yeast two-hybrid results, homodimerization was demonstrated in plant cells for three petunia MADS box proteins. Heterodimers were identified between the ovule-specific MADS box protein FLORAL BINDING PROTEIN 11 and members of the petunia FLORAL BINDING PROTEIN 2 subfamily, which are also expressed in ovules, suggesting that these dimers play a role in ovule development. Furthermore, the role of dimerization in translocation of MADS box protein dimers to the nucleus is demonstrated, and the nuclear localization signal of MADS box proteins has been mapped to the N-terminal region of the MADS domain by means of mutant analyses.

Many proteins are functional in a combinatorial manner and interact specifically with other proteins. Determination of these interaction patterns is an essential part of functional genomics research.

Yeast two-hybrid systems have shown to be powerful techniques to identify high-throughput protein–protein interactions (1). Both Uetz *et al.* (2) and Ito *et al.* (3) used the yeast two-hybrid GAL4 system successfully to analyze the complete yeast interactome. Remarkably, the data from these two studies do not largely overlap; among other causes for this situation are limitations of the genetic yeast two-hybrid GAL4 system. Therefore, interactions identified by yeast systems require confirmation by other methods in a more natural environment. Recently, a noninvading method based on fluorescence resonance energy transfer (FRET) (4, 5) became available for monitoring protein–protein interactions in living cells (6, 7). FRET is the phenomenon of energy transfer between a donor fluorescent molecule and a neighboring chromophore, the acceptor, when the two fluorescent molecules come in very close proximity. This will actually be the case when two proteins tagged with two different fluorescent dyes are physically interacting. Recently, Más *et al.* (8) used the FRET technique to show specific interaction between the *Arabidopsis* photoreceptors PHY-B and CRY2 and translocation of the formed complex to nuclear speckles in a light-dependent manner.

The occurrence of FRET can be determined by fluorescence lifetime imaging microscopy (FLIM, ref. 9). FRET decreases the fluorescence lifetime of the donor fluorescent molecule independently of local chromophore concentration and absorption of donor fluorescence by chlorophyll.

We aimed to study MADS box transcription factor interactions in living plant cells. In *Arabidopsis*, this large family of transcription factors is comprised of at least 80 members (10). MADS box proteins form specific homo- and/or heterodimers (refs. 11–13 and R.G.H.I., S.F., M.B., and G.C.A., unpublished data), which direct a variety of developmental processes (14). Recently it has been shown that even higher-order ternary complexes are formed between members of this transcription factor family (15, 16). Although the ability to form a particular dimer or complex has been demonstrated in yeast for several MADS box proteins, no information is available about these physical interactions in living plant cells. Furthermore, it is not clear where exactly these specific interactions occur and how MADS box proteins are translocated to the nucleus. In this work the petunia MADS box protein FLORAL BINDING PROTEIN (FBP)11, which is involved in proper ovule formation, has been studied. Cosuppression of the closely related MADS box genes *FBP7* and *FBP11* has resulted in the formation of carpeloid structures instead of ovules, which demonstrates that these genes are involved in ovule identity specification (17). Nevertheless, ectopic expression of *FBP11* under control of the constitutive cauliflower mosaic virus 35S promoter revealed ovule formation on the adaxial side of sepals and rarely on the petals only (18), indicating that besides FBP11 additional factors are needed for proper ovule formation. Because MADS box proteins are functional as dimers, the specific cofactors essential for ovule development are probably represented by heterodimerization partners of FBP11. Therefore, putative interaction partners of FBP11 were isolated by means of the yeast two-hybrid GAL4 system. Subsequently, the novel FRET-FLIM technology was applied to gain insight in MADS box transcription factor interactions in living plant cells, and the intracellular localization of the MADS box protein monomers and dimers was determined.

Materials and Methods

Yeast Two-Hybrid GAL4 System. The ovary-specific cDNA expression library was constructed from poly(A)$^+$ RNA from young petunia ovaries, according the manufacturer's instructions (Stratagene, no. 235601). In parallel, the FBP11-encoding ORF was generated by PCR and cloned in-frame in the bait vector (pBD-GAL4). Subsequently, yeast cells containing the bait construct were transformed with "ovary" phagemid library DNA, according the CLONTECH protocol. In total, $1.5 \cdot 10^6$ individual yeast transformants were generated. Screening for protein–protein interaction events was performed according the Stratagene manual.

Abbreviations: CSLM, confocal scanning laser microscopy; CFP, cyan fluorescent protein; FLIM, fluorescence lifetime imaging microscopy; FBP, floral binding protein; FRET, fluorescence resonance energy transfer; SPIM, *sp*ectral *i*maging *m*icroscopy; YFP, yellow fluorescent protein; NES, nuclear export signal; NLS, nuclear localization signal.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database [accession nos. AF335235 (FBP5) and AF335236 (FBP9)].

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Yeast Two-Hybrid CytoTrap System. The CytoTrap Vector kit from Stratagene was used. Instead of the enclosed pSOS vector, pSOS-NES (Stratagene) was used, containing a nuclear export signal (NES) fused in-frame between the SOS coding region and the multiple cloning site. FBP2, -5, and -9 were cloned in the pMYR and pSOS-NES vectors.

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

***In Situ* Hybridizations.** *In situ* hybridizations were carried out as described by Cañas *et al.* (19). All probes consist of specific cDNA fragments, lacking the conserved 5′ MADS domain-encoding sequence.

Construction of Cyan Fluorescent Protein/Yellow Fluorescent Protein (CFP/YFP) Plasmids. Complete ORFs of MADS box genes *FLORAL BINDING PROTEIN 2* (*FBP2*), *FBP5* (GenBank accession no. AF335235), *FBP9* (accession no. AF335236), *FBP11*, and *PETUNIA FLOWERING GENE* (*PFG*) were amplified with gene-specific primers to remove the stop codon and for C-terminal in-frame fusion with the coding region of *CFP* and *YFP* (from *pECFP* and *pEYFP*; CLONTECH catalog nos. 6075-1 and 6004-1, respectively). To obtain FBP2ΔN and FBP11ΔN, lacking 38 and 31 amino acids at the N terminus, respectively, a second set of gene-specific forward primers were designed for these two genes annealing 114 and 93 nucleotides downstream from the start codon, respectively, and introducing an in-frame new ATG start codon. Subsequently, "MADS box gene–CFP/YFP" products were cloned in the expression vector pGD120 (pUCAP containing the expression cassette: cauliflower mosaic virus 35SDE–AMV leader sequence–multiple cloning site–NOS terminator). The FBP2ΔNLS-YFP plasmid with the point mutation in the nuclear localization signal (NLS) was generated by site-directed mutagenesis on pGD120-FBP2YFP with primers PRI690 (5′-CAATAGACAAGTTACCTTTGCT**GC**G**GC**AAGAAATGGACTATTGAAAAAAGC-3′) and PRI691 (5′-GCTTTTTTCAATAGTCCATTTCTT**GC**C**GC**AGCAAAGGTAACTTGTCTATTG-3′) according the Stratagene QuickChange Site-Directed Mutagenesis kit manual.

Protoplast Transfection. The MADS box CFP/YFP fusion proteins were transiently expressed in protoplasts from full-grown leaves of W115 petunia plants grown in the greenhouse. Protoplasts were isolated and transfected as described by Denecke *et al.* (20).

Localization Studies. Localization of MADS box–CFP/YFP fusion proteins in petunia protoplasts was determined by confocal scanning laser microscopy (CSLM) analyses (CSLM 510, Zeiss). Excitation was provided by the 458- and 514-nm argon laser lines controlled by an acousto-optical tunable filter. Three dichroic beam splitters were used to separate excitation from emission and to divide the fluorescence emission into the CFP, YFP, and chlorophyll channels. Images were analyzed and adapted with Zeiss LSM510 software.

FRET-*S*pectral *I*maging *M*icroscopy (SPIM). For details of the set-up see Goedhart and Gadella (21). Single protoplasts expressing specific MADS box protein–CFP and MADS box protein–YFP fusion proteins were positioned by aligning them across the entrance slit of the spectrograph (set at 200 μm width corresponding with a line of 10 μm width in the object plane). Acquisition time was 1–2 s. Regions of the image spectrum corresponding to the nucleus or cytosol of labeled cells were distance-averaged (typically 5–10 rows of pixels), and the resulting fluorescence spectra were corrected for background fluorescence and camera bias by background subtraction with an extracellular region just next to the plasma membrane region from the same spectral image. For each specific MADS box protein combination at least five independent protoplasts were imaged.

FRET-FLIM. FLIM was performed on a wide-field frequency-domain instrument described in detail elsewhere (9). The cells were excited with the 457-nm argon-ion laser line modulated at 60.116 MHz, and the CFP fluorescence was selectively imaged by using an Omega Engineering (Stamford, CT) 470 DCLP dichroic mirror and an Omega 487RDF42 band-pass emission filter. Twenty phase images (1–3 s each) were taken (10 with increasing and 10 with decreasing the phase allowing correction for photobleaching, which was less than 10% in all cases). Used microscope set-up, calculations, and image processing for frequency-domain FLIM were described before (9).

Results

Yeast Two-Hybrid Analyses. A petunia ovary-specific cDNA expression library was screened with FBP11 (17) as bait to identify interacting partners for this ovule-specific MADS box protein. Yeast colonies positive for all three-reporter genes were selected as true interactors, and the prey vector was investigated further by sequencing analysis. Except for a small domain of a ferredoxin protein, which was isolated twice, all positive clones contained full-length coding sequences of either MADS box protein FBP2 (isolated 4×) (22) or one of the novel proteins FBP5 (4×) and FBP9 (3×). For all further analyses, only these full-length clones were investigated.

In a subsequent experiment, FBP11 and its isolated interacting MADS box proteins were tested for their ability to homodimerize. For FBP11, no homodimerization could be detected in the genetic yeast GAL4 system. The three related proteins, FBP2, FBP5, and FBP9, gave autoactivation of yeast reporter genes when expressed as bait proteins. This autoactivation by MADS box proteins can be abolished by deletion of the C-terminal domain containing the transcription activation domain (23). We followed this strategy for FBP2 and revealed specific heterodimers between the truncated protein and other MADS box proteins, including the heterodimer with FBP11 (R.G.H.I., S.F., M.B., and G.C.A., unpublished data). Nevertheless, homodimerization between this truncated protein and the full-length FBP2 could not be identified. Subsequently, the ability of FBP2, FBP5, and FBP9 to form homodimers was analyzed in the cytoplasmic yeast two-hybrid CytoTrap system, in which selection for interaction is not based on transcriptional activation (24). As in the yeast two-hybrid GAL4 system, no homodimerization could be identified.

Biological Characteristics of FBP11 Interactors. A first prerequisite for proteins to interact in a biological context is their presence in the same tissues and at the same developmental stage. To test whether this is the case for FBP11 and its putative interactors, expression analyses were performed. Because FBP11 has shown to be involved in petunia ovule development, we focused on this tissue for the expression analyses. *In situ* hybridizations demonstrate that both *FBP11* and *FBP2* have overlapping expression patterns in developing ovules (Fig. 1 *A–C*). In mature ovules, both genes are highly expressed in the endothelium surrounding the embryo sac. Northern blot analyses revealed that both *FBP5* and *FBP9*, which like *FBP2* belong to the *SEPALLATA* group of MADS box genes (Fig. 1*F*), are also strongly expressed in ovules (not shown). Transgenic petunia plants overexpressing *FBP11* under the control of the cauliflower mosaic virus 35S promoter resulted in the formation of ectopic ovules on sepals and rarely on the tube of the petals (18). Expression analyses on the sepals of these *FBP11* overexpression plants demonstrated that *FBP2* is expressed in the ectopically formed ovules and in the epidermal cell layer (Fig. 1*D*). In wild-type petunia flowers, *FBP2* is expressed in the epidermal cell layer on the

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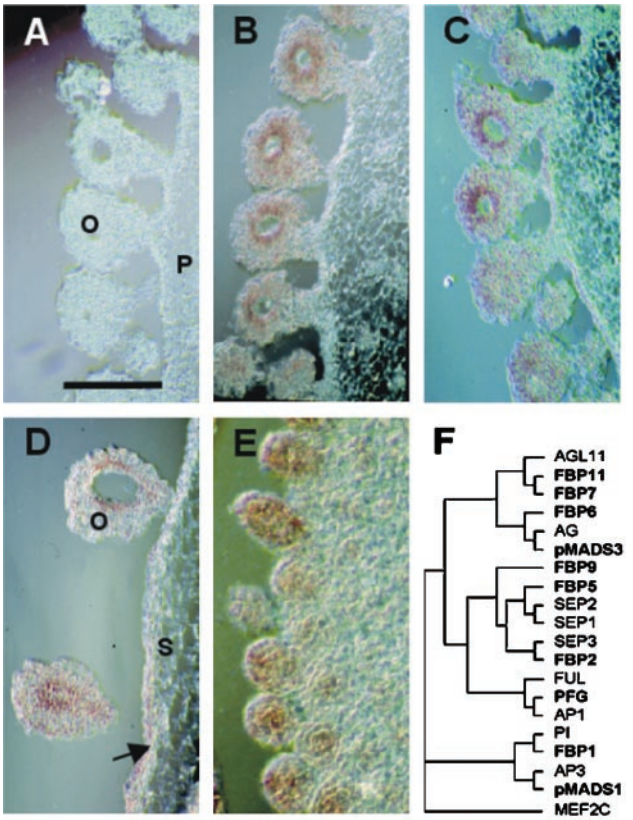

Fig. 1. Expression of *FBP11* and the genes encoding its interaction partners. Longitudinal sections were hybridized with digoxygenin-labeled probes (red signal). (*A*) Almost mature W115 wild-type ovules, hybridized with a sense *FBP11* probe. (*B*) Same stage as *A*, hybridized with an *FBP11* antisense probe. (*C*) Same stage as *A*, hybridized with an *FBP2* antisense probe. (*D*) Sepal of cauliflower mosaic virus-35S::*FBP11* overexpression plant with ectopic ovule formation on adaxial side, hybridized with an *FBP2* antisense probe. The signal in the epidermal cell layer is marked with an arrow. (*E*) Young developing ovules, hybridized with a *PFG* antisense probe. (*F*) Phylogenetic tree of MADS box genes described in this study. As a reference *Arabidopsis* and petunia MADS box genes with a known function are included. Petunia MADS box genes are in bold. For the comparison of the proteins the MADS box, I region and K box domains were used. AP1, *APETALA1*; AP3, *APETALA3*; AG, *AGAMOUS*; AGL, *AGAMOUS*-like; FBP, *FLORAL BINDING PROTEIN*; FUL, *FRUITFULL*; MEF2C, *Myocyte Enhancer Factor 2C*; O, ovule; PFG, *PETUNIA FLOWERING GENE*; PI, *PISTILLATA*; P, placenta; S, sepal (adaxial side); and SEP, *SEPALLATA*. [Bar in *A* = 1.0 mm.]

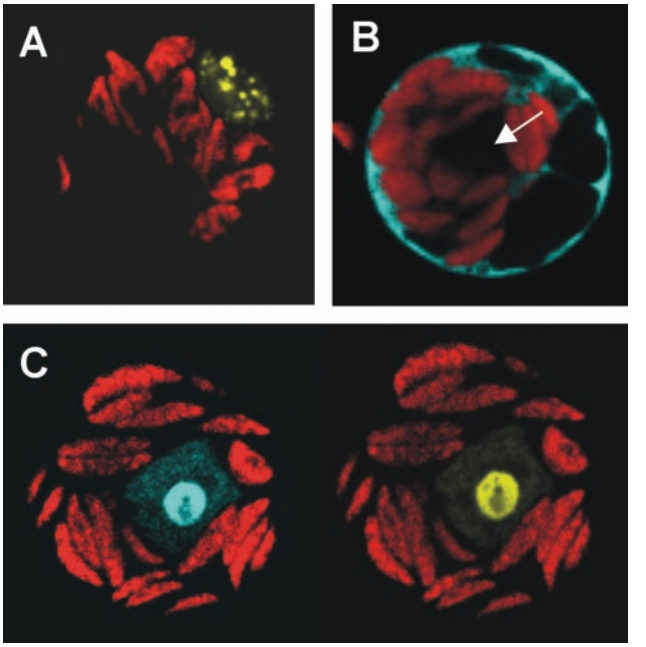

Fig. 2. Localization of MADS box proteins in petunia leaf protoplasts imaged by CSLM. Chlorophyll autofluorescence is shown in red. (*A*) Nuclear-localized FBP9-YFP. (*B*) Cytoplasmic-localized FBP11-CFP. The position of the nucleus is marked with an arrow. (*C*) Protoplast expressing FBP11-CFP in combination with FBP2-YFP. (*Left*) The nuclear-localized CFP signal. (*Right*) The YFP signal of the same protoplast.

adaxial side of sepals and in the petals (22). For comparison, the expression of the *PETUNIA FLOWERING GENE* (*PFG*) (25) in ovules of wild-type petunia plants is shown. Despite the coexpression of *PFG* and *FBP11* in ovules, no interaction was detected between PFG and FBP11 by the yeast two-hybrid GAL4 system (R.G.H.I., S.F., M.B., and G.C.A., unpublished data).

Localization of MADS Box Proteins in Protoplasts. To localize FBP11 and its putative interacting partners FBP2, FBP5, and FBP9 inside a living plant cell, fusions of the MADS box proteins with CFP and/or YFP were made. These fusion proteins were transiently expressed in petunia leaf protoplasts, and cells were imaged by CSLM. Fig. 2*A* and Movie 1 (which is published as supporting information on the PNAS web site, www.pnas.org) show the nuclear localization of the FBP9–YFP fusion protein. In addition to an overall fluorescence of the nucleus, sometimes YFP signals were concentrated in small nuclear spots. Except for FBP11–CFP and FBP11–YFP, all fusion proteins tested were nuclear-localized (results not shown). For the fusion proteins with FBP11, a fluorescent signal was observed only in the cytoplasm (Fig. 2*B*) and any nuclear import was absent. For all MADS box proteins analyzed, no differences were obtained between CFP and YFP fusions.

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

Identification of MADS Box Protein Interactions in Living Cells. Colocalization of proteins, as identified for specific petunia MADS box protein combinations, is a first indication for physical interaction between the concerned proteins. However, all MADS box proteins contain the highly conserved DNA binding MADS box domain and are therefore expected to be functional as transcription factors and hence nuclear-localized. To determine whether there is a physical interaction between specific MADS box proteins, both FRET-FLIM and FRET-SPIM measurements were performed on petunia protoplasts expressing a specific MADS box protein–CFP/YFP combination. Based on the results of the yeast two-hybrid analyses, the putative heterodimers FBP11-FBP2, FBP11-FBP5, and FBP11-FBP9 were selected. In addition, the combination FBP2–PFG (25), for which no dimerization was scored in yeast (R.G.H.I., S.F., M.B., and G.C.A., unpublished data), was evaluated by the fluorescence microspectrometry methods. Both FBP2 and PFG are nuclear-localized in petunia protoplasts (not shown) and their corresponding genes are expressed in ovules (Fig. 1 *C* and *E*).

In Fig. 3, the results from the FRET-FLIM analyses for the combinations FBP2–CFP/FBP11–YFP (Fig. 3 *A* and *C*) and FBP2–CFP/PFG–YFP (Fig. 3 *B* and *C*) are shown. Interaction, and

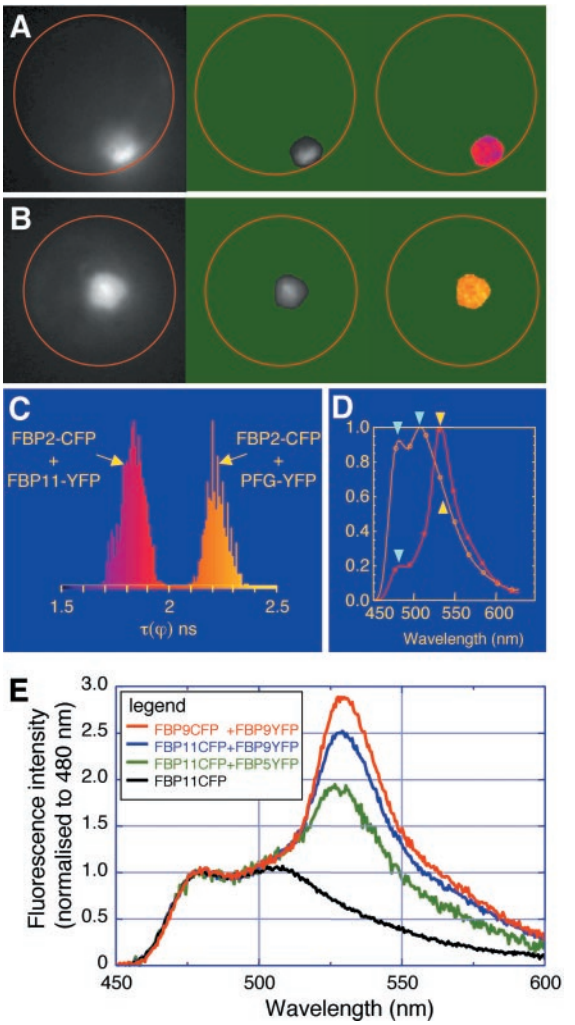

Fig. 3. FRET-FLIM and FRET-SPIM analyses of protoplasts expressing petunia MADS box proteins fused to CFP and YFP. (*A*) Protoplast expressing FBP2-CFP + FBP11-YFP. The border of the protoplast is artificially marked with a red circle. (*Left*) Fluorescence image of a protoplast. (*Center*) Fluorescence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

hence FRET, results in a reduction of fluorescence lifetime of the donor fluorescent molecule (CFP). FRET-FLIM measurements on petunia protoplasts expressing only a single MADS box protein–CFP fusion protein revealed a fluorescent lifetime of about 2.3 ns for the CFP chromophore (not shown). In case of the combination FBP2–CFP/FBP11–YFP, a large reduction in fluorescence lifetime from 2.3 to 1.8 ns was obtained (Fig. 3*C*), demonstrating FRET between CFP and YFP. In contrast, imaging of protoplasts expressing the combination FBP2-CFP/PFG-YFP yielded an average lifetime of about 2.3 ns (Fig. 3*C*), similar to what was observed when a single CFP construct was expressed. In a parallel experiment, FRET-SPIM analyses were performed for the same two combinations. After excitation of CFP, interaction (FRET) leads to quenching of CFP emission (donor), whereas YFP (acceptor) will be sensitized, resulting in a strongly increased fluorescence at 525 nm as compared with 475 nm. This altered fluorescence-emission ratio clearly occurs for the combination FBP2–CFP/FBP11–YFP (Fig. 3*D*), whereas the emission spectra of the cells expressing the combination FBP2–CFP/PFG–YFP is reminiscent of a single MADS box–CFP fusion protein. The spectral change obtained for the combination FBP2–CFP/FBP11–YFP and the strong reduction in fluorescence lifetime demonstrate the molecular interaction between these two MADS box proteins in a living plant cell. In contrast, no interaction was detectable for the MADS box proteins PFG and FBP2, despite their nuclear colocalization, which confirms the yeast two-hybrid results. Because similar results were obtained with FRET-FLIM and FRET-SPIM analyses, only FRET-SPIM measurements were done for the two other putative FBP11 complexes. For both FBP11–FBP5 and FBP11–FBP9, a very clear spectral shift was obtained (Fig. 3*E*), demonstrating physical interactions between these proteins in plant protoplasts.

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

Role of NLS. To predict the sequence motifs involved in nuclear import of MADS box proteins, analyses with the program PROSITE (http://www.expasy.ch/prosite/) were performed. These analyses revealed that both FBP2 and FBP11 contain a putative conserved bipartite NLS consensus sequence in the MADS domain (Fig. 4*A*). N-terminal-truncated FBP11-CFP and FBP2-YFP constructs were generated to determine the involvement of these NLS motifs in nuclear import. The first 31 and 38 amino acids of the MADS box DNA-binding domains were removed from FBP11 and FBP2, respectively, yielding FBP11ΔN-CFP and FBP2ΔN-YFP (Fig. 4*A*). These truncated proteins were transiently expressed in petunia leaf protoplasts, resulting in a cytoplasmic fluorescent signal in both cases (Fig. 4 *B* and *C* right). In parallel, double transfections were performed of the truncated fusion proteins in combination with either the full-length or N-terminal-truncated interacting partner (FBP11ΔN-CFP + FBP2ΔN-YFP, FBP11ΔN-CFP + FBP2-YFP, and FBP11-CFP + FBP2ΔN-YFP). All three combinations, each comprising at least one interacting partner with a truncated MADS box, appeared to result in a colocalization of CFP and YFP signals in the cytoplasm only (Fig. 4*E*, not all combinations shown). None of the cells showed a fluorescent signal in the nucleus, as was seen for the full-length proteins (Figs. 2*C* and 4*D*). In contrast to protoplasts expressing the full-length fusion proteins, many cells accumulating the truncated fusion proteins produced fluorescent aggregates in the cytoplasm. Because of this clustering of fusion proteins, a dramatic reduction of fluorescence lifetime was observed and hence no reliable FRET-FLIM and FRET-SPIM measurements were possible for the determination of protein–protein interactions. Cells without aggregates expressed the chimeric proteins at very low levels and therefore could not be used for microscopical measurements.

These results indicate that the NLS of MADS box proteins is positioned in the N-terminal-located MADS domain and, furthermore, that this domain should be present in both interacting

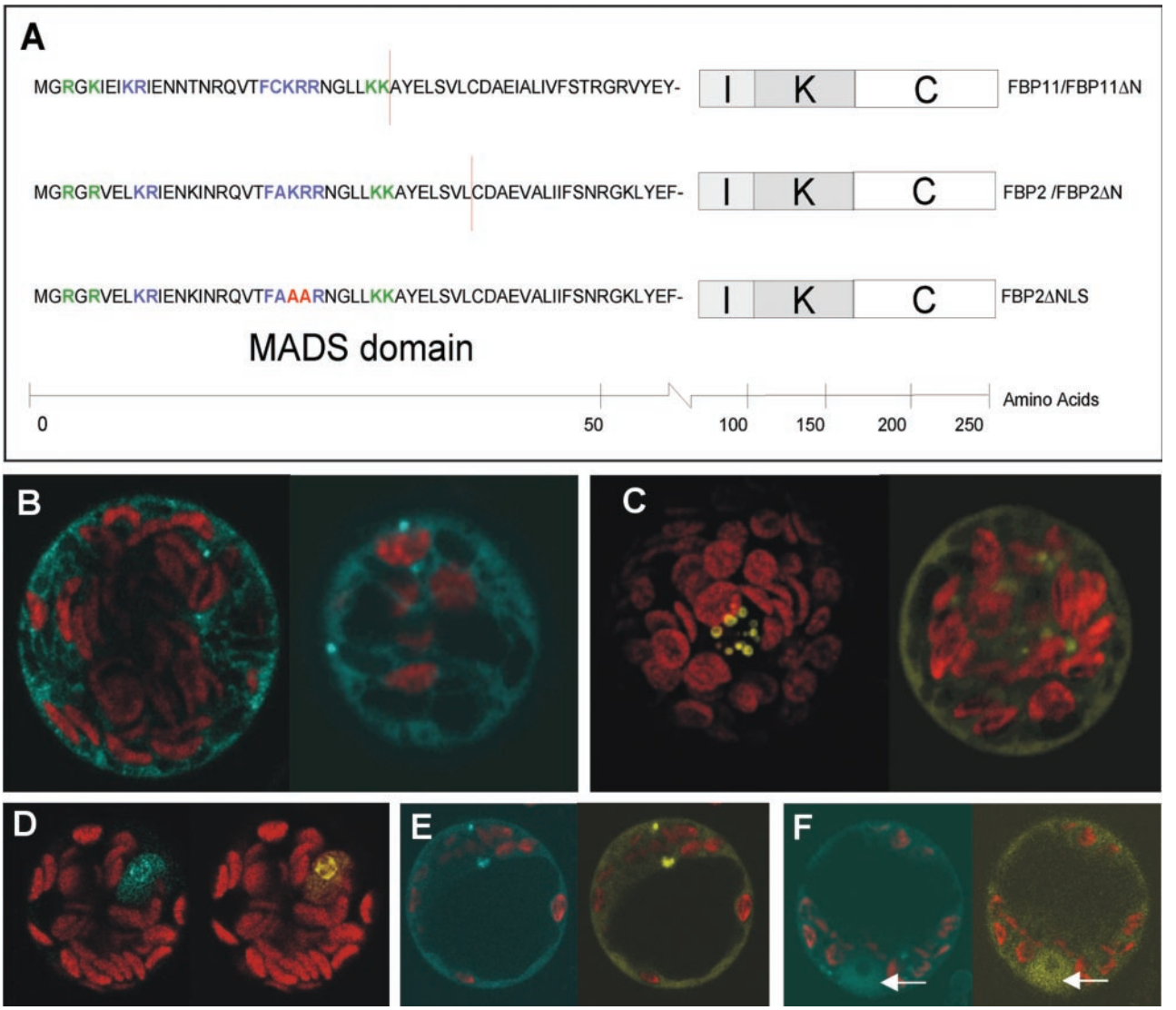

Fig. 4. Role of NLS in MADS box protein translocation. (*A*) Representation of MADS box protein structure and the predicted position of NLSs. The bipartite NLS localized in the N-terminal MADS domain is marked blue, and conserved basic amino acids in the MADS domain are marked in green. I, intervening region; K, K-box; C, C-terminal region. All images (*B–F*) were obtained by CSLM. Chlorophyll autofluorescence is shown in red. (*B*) Localization of FBP11-CFP (*Left*) and FBP11ΔN-CFP (*Right*). (*C*) Localization of FBP2-YFP (*Left*) and FBP2ΔN-YFP (*Right*). (*D*) Nuclear colocalization of FBP11-CFP and FBP2-YFP. (*Left*) The CFP signal. (*Right*) The YFP signal of the same protoplast. (*E*) Cytoplasmic colocalization of FBP11ΔN-CFP and FBP2-YFP. (*Left*) The CFP signal. (*Right*) The YFP signal of the same protoplast. (F) Colocalization of FBP11-CFP and FBP2ΔNLS-YFP. (*Left*) The CFP signal. (*Right*) The YFP signal of the same protoplast. The nucleus is marked with an arrow.

partners for transport of the dimer to the nucleus. The consensus sequence of the bipartite NLS in this domain is "$PP(N)_{10}(P_3N_2)$," in which "P" is a positively charged amino acid and "N" represents any amino acid (26). To test whether the predicted bipartite NLS sequence in the MADS domain is an essential signal for nuclear localization, point mutations were generated in this motif. For this purpose, two of three positively charged amino acid residues in the NLS of FBP2 were changed in the uncharged amino acid alanine, resulting in the construct FBP2ΔNLS-YFP (Fig. 4*A*). Expression of this construct in leaf protoplasts revealed a cytoplasmic signal exclusively (not shown), whereas unmodified FBP2 is translocated to the nucleus (Fig. 4*C Left*). However, expression of FBP2ΔNLS-YFP in combination with FBP11-CFP resulted in a fluorescent signal in both cytoplasm and nucleus (Fig. 4*F*). Therefore, nuclear translocation of the FBP11-FBP2ΔNLS heterodimer seems to be impeded in these cells but not completely blocked.

Discussion

Yeast Two-Hybrid vs. *in Planta* Methods. In this study, MADS box protein–protein interactions were analyzed in living plant cells, making use of advanced fluorescent microscopy techniques. All heterodimers identified by the yeast two-hybrid GAL4 system, using FBP11 protein as bait, were confirmed in petunia protoplasts, indicating that the yeast system gives a reliable result. However, in addition to the FBP11 heterodimers, homodimers of FBP2, FBP5, and FBP9 were formed in living plant cells, which could not be detected by the yeast two-hybrid systems. In the yeast two-hybrid GAL4 system, protein-protein interaction events are monitored at 30°C and in the CytoTrap system at 37°C. These relatively high nonambient temperatures may influence the folding and hence interaction capability of some proteins. Temperature-dependent interaction has been reported for the *Arabidopsis* class B MADS box proteins APETALA3 (AP3) and PISTILLATA (PI) (27), which may also be the case for FBP2, FBP5, and FBP9 homodimers. The lack of homodimerization in the CytoTrap system seems at least not to be the result of general problems related to this system, because it was used successfully to determine the interactions between 23 petunia MADS box proteins, including two homodimers (R.G.H.I., S.F., and G.C.A., unpublished data). Alter-

natively, the discrepancy between the results obtained with the yeast systems and the *in planta* system is because of plant-specific factors or modifications that are required for homodimerization of these three MADS box transcription factors.

Nuclear Translocation of MADS Box Proteins. FBP11 fused to either CFP or YFP is localized in the cytoplasm of petunia leaf protoplasts despite the presence of the conserved bipartite NLS. After expression of one of its interaction partners, FBP11 is transported to the nucleus where a physical interaction between the two expressed proteins was demonstrated by FRET-FLIM. In contrast to FBP11, the MADS box proteins FBP2, FBP5, and FBP9 are nuclear-localized when individually expressed. This nuclear transport can be driven by a specific interaction with an endogenous MADS box protein present in the leaf protoplasts or, alternatively, the nuclear localization of FBP2, FBP5, and FBP9 in the living plant cell is driven by their homodimerization. The presence of FBP11 in the cytoplasm, a MADS box protein which is not able to homodimerize, is in line with the latter option. Based on the results we hypothesized that dimerization of MADS box proteins occurs in the cytoplasm and is essential for nuclear localization. More evidence for this hypothesis came from experiments with truncated proteins. Expression of FBP11-CFP, lacking 31 amino acids of the MADS domain, in combination with FBP2-YFP, resulted in a colocalized CFP/YFP signal in the cytoplasm. That FBP2 is retained in the cytoplasm implies that there is interaction in the cytoplasm between FBP2 and the truncated FBP11, lacking the NLS. Because of the lack of a proper nuclear localization signal, this heterodimer remains in the cytoplasm. Because no FBP2-YFP signal was observed in the nucleus, homodimerization of FBP2 proteins seems to be less efficient than the formation of heterodimers between FBP2 and the truncated FBP11. Dimerization as a prerequisite for nuclear localization of MADS box proteins has been suggested before by McGonigle *et al.* (28), who did similar localization studies with the *Arabidopsis* B-type MADS box proteins AP3 and PI. However, they were not able to show a direct interaction between these two proteins in living plant cells as is demonstrated here for the petunia MADS box proteins. The necessity for dimerization to obtain nuclear localization has been reported for other transcription factors as well (29–31) and seems to be a common fine-tuning mechanism for transcriptional regulation.

The domains or signals that are exactly involved in nuclear translocation of MADS box proteins are unknown. McGonigle *et al.* (28) mapped the NLS to the first 69 amino acids of AP3 and to the first 105 amino acids of PI. We were able to fine-map the NLS to the first 40 amino acids of FBP2 and FBP11, and nuclear localization was even abolished when this N-terminal domain was missing from either one of the two interacting partners, demonstrating once again that the presence of one complete bipartite NLS is not sufficient. To map the nuclear localization signal more exactly, a point mutation was generated in the conserved bipartite NLS of FBP2. This mutation seemed to be sufficient to abolish nuclear localization of the FBP2 homodimer and to drastically impair the translocation of the heterodimer with the full-length FBP11. This result suggests that the complete bipartite NLS in both protein partners is required for efficient nuclear localization.

Biological Function of Identified MADS Box Protein Dimers. In this study, we have demonstrated that FBP11 interacts specifically with the three very closely related SEPALLATA-like petunia MADS box proteins FBP2, FBP5, and FBP9. Expression analyses showed that all corresponding genes are highly expressed in ovules. FBP11 is responsible for the determination of ovule identity as has been demonstrated by suppression and overexpression mutants (22, 23). *In situ* hybridization on sepals of the *FBP11* overexpression plants revealed the presence of *FBP2* mRNA in the ectopically formed ovules. These results together suggest a role for the identified FBP11 heterodimers in the development of ovules, and that the very closely related proteins FBP2, FBP5, and FBP9 most likely play a redundant role in this process. *FBP2* and *FBP11* are also coexpressed at early developmental stages during wild-type ovule initiation and in the epidermal cells on the adaxial side of sepals of the *FBP11* overexpressor, which suggests a role for the FBP2-FBP11 heterodimer in ovule initiation. Nevertheless, *FBP2* is also expressed in other floral organs (22), whereas no ectopic ovules were identified on these organs in the *FBP11* overexpression plant (18). Therefore, additional factors are needed or, alternatively, another complex is involved in the initiation of ovule formation. The FBP2, FBP5, or FBP9 homodimers do not seem to be these ovule-initiation factors, because ovule formation was completely blocked in the *fbp11* cosuppression mutant (17) despite the expression of *FBP2*, *FBP5*, and *FBP9*. It has been demonstrated that FBP2 functions as an identity mediating MADS box protein involved in the development of the inner three floral whorls (32), like the *Arabidopsis* SEPALLATA proteins (33). The results obtained in this study suggest an additional function for FBP2-like proteins in ovule development, and that these two different functions probably are effected by heterodimerization of FBP2 with different MADS box protein partners.

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