Visualization of protein interactions in living plant cells using bimolecular fluorescence complementation

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Summary

Dynamic networks of protein–protein interactions regulate numerous cellular processes and determine the ability to respond appropriately to environmental stimuli. However, the investigation of protein complex formation in living plant cells by methods such as fluorescence resonance energy transfer has remained experimentally difficult, time consuming and requires sophisticated technical equipment. Here, we report the implementation of a bimolecular fluorescence complementation (BiFC) technique for visualization of protein–protein interactions in plant cells. This approach relies on the formation of a fluorescent complex by two non-fluorescent fragments of the yellow fluorescent protein brought together by association of interacting proteins fused to these fragments (Hu et al., 2002). To enable BiFC analyses in plant cells, we generated different complementary sets of expression vectors, which enable protein interaction studies in transiently or stably transformed cells. These vectors were used to investigate and visualize homodimerization of the basic leucine zipper (bZIP) transcription factor bZIP63 and the zinc finger protein lesion simulating disease 1 (LSD1) from Arabidopsis as well as the dimer formation of the tobacco 14-3-3 protein T14-3c. The interaction analyses of these model proteins established the feasibility of BiFC analyses for efficient visualization of structurally distinct proteins in different cellular compartments. Our investigations revealed a remarkable signal fluorescence intensity of interacting protein complexes as well as a high reproducibility and technical simplicity of the method in different plant systems. Consequently, the BiFC approach should significantly facilitate the visualization of the subcellular sites of protein interactions under conditions that closely reflect the normal physiological environment.

Keywords: bimolecular fluorescence complementation, protein–protein interaction, intracellular localization, bZIP transcription factor, 14-3-3 proteins, LSD1.

Introduction

The regulation and execution of biological processes requires specific interactions of numerous proteins. Tightly regulated protein interaction networks mediate cellular responses to environmental cues and direct the implementation of developmental programs. The selectivity of protein–protein interactions and their appropriate temporal and spatial regulation determine the developmental potential of the cell and its response to endogenous and exogenous signals. On the molecular level differential protein–protein interactions are thought to determine the operation of complex regulatory circuits and signal transduction systems.

The complete sequencing of an increasing number of eukaryotic genomes has provided a wealth of information about the number and complexity of protein functions.
required to build up an organism. However, the regulation and interplays of these proteins remain to become explored in order to appreciate the molecular mechanisms of their action. Several methods have been developed to identify, examine and visualize protein interactions and protein complexes in living cells. Among them, the yeast two-hybrid system has significantly advanced the speed and extent of protein interaction studies. However, this system bears intrinsic limitations as for example systematic ‘false-positive’ and ‘false-negative’ interactions and, moreover, usually combines protein pairs in a heterologous environment (Field and Song, 1989; Stephens and Banting, 2000). The most widely used approach for the visualization of protein interactions in living cells is fluorescence resonance energy transfer (FRET) between spectral variants of the green fluorescence protein (GFP) fused to the associating proteins (Chen et al., 2003; Periasamy, 2000). However, to enable observation and quantification of small alterations in fluorescence emission, the GFP fluorophores have to join in close spatial proximity and the fusion proteins generally have to be expressed in high levels. Furthermore, verification, whether changes in fluorescence emission are caused by energy transfer, requires complicated irreversible photobleaching or fluorescence lifetime imaging techniques (Chen et al., 2003; Periasamy, 2000). However, the instrumental equipment necessary for these techniques is not widely available and FRET requires intensive methodical training. For these reasons reports about FRET-based protein-protein interaction investigations in living cells have remained rare especially in plant science (Immink et al., 2002; Más et al., 2000; Shah et al., 2002; Vermeer et al., 2004).

Alternatively, protein interactions can also be investigated in vivo if the protein complex formation can be visualized by the restoration of a detectable activity. In this regard, the principle of intragenic complementation of the lacZ locus from Escherichia coli was adapted to detect protein interactions (Rossi et al., 1997; Ullmann et al., 1967). In this experimental system the detection of protein–protein interactions by restoration of β-galactosidase activity was enabled by using β-galactosidase fragments, which could associate only when fused to interacting proteins. Similarly, fragments of the dihydrofolate reductase have been used in protein interaction studies based on complementation of protein function (Pelletier et al., 1998). However, these techniques require the application of extrinsic fluorophores to visualize the complex formation.

An alternative experimental approach for the visualization of protein interactions is based on the formation of a fluorescent complex by fragments of the enhanced yellow fluorescent protein (YFP) when brought together by the interaction of two associating partners fused to these fragments. Recently, Kerppola and colleagues (2002) reported a proof-of-concept for such an approach for the investigation of protein interactions in living mammalian cells and designated this technique as bimolecular fluorescence complementation (BiFC). The unique characteristic of the BiFC approach is that the bright intrinsic fluorescence of the bimolecular complex allows direct visualization of the complex formation in living mammalian cells. Moreover, by analyzing the interactions between members of the basic leucine zipper (bZIP) and Rel transcription factor families, the BiFC approach provided direct evidence of the intracellular locations where the protein association occurs (Hu et al., 2002). The application of the BiFC approach has recently been extended to the investigation of the interaction pattern and intracellular localization of G-protein complexes in mammalian cells and Dictyostelium discoideum (Hynes et al., 2004) and to the visualization of 1-aminocyclopropane-1-carboxylase synthase heterodimer formation in E. coli (Tsusisaka and Theologis, 2004). Furthermore, by introducing a large number of different GFP variants the technique was extended to multicolor BiFC, which allows the direct visualization of multiple protein interactions within the same cell (Grinberg et al., 2004; Hu and Kerppola, 2003).

In this report we describe the generation of several sets of plant-compatible BiFC vectors. We used these vectors for investigating the interaction of plant nuclear and cytoplasmic proteins in different plant systems. Our study attests the general applicability of the BiFC technique and that this assay represents an efficient and convenient tool to investigate protein–protein interactions in living plant cells.

**Results**

**Generation of plant-compatible BiFC transformation vectors**

To develop the BiFC technology for the visualization of protein–protein interactions in living plant cells, we constructed four pairs of vectors (Figure 1; for further details see Experimental procedures and Supplementary material). These vectors have been designated pSPYNE and pSPYCE (for split YFP N-terminal/C-terminal fragment expression) respectively. Each vector pair enables the expression of proteins of interest fused either to the N-terminal 155 amino acids (YFPN) or to the C-terminal 86 amino acids of YFP (YFPC); Hu et al., 2002). Moreover the plasmids contain either a c-myc (pSPYNE) or HA (pSPYCE) affinity tag for detection of fusion protein expression in cell extracts (Figure 1). The binary pSPYNE-KAN and pSPYCE-BAR vectors enable the expression of YFP fragment-fused genomic DNA or of YFP fragment constructs driven by any promoter of interest (Figure 1). Strong and constitutive expression of fusion proteins in plant cells is ensured by the binary pSPYNE-35S and pSPYCE-35S plasmids which contain the 35S promoter of the cauliflower mosaic virus. For selection of transgenic plants pSPYNE-KAN and pSPYNE-35S carry a nos promoter-driven kanamycin resistance gene (nptII), whereas pSPYCE-BAR and pSPYCE-35S harbor the bar gene conferring
insensitivity to the herbicide glufosinate (Figure 1). In addition, we generated two additional sets of vectors based on pUC19 that are specially designed for transient plant cell transformation approaches. pUC-SPYNE and pUC-SPYCE contain the entire expression cassette of pSPYNE-35S or pSPYCE-35S, respectively, and harbor a more variable multi-cloning site (MCS) (Figure 1). Furthermore, in a second set the entire MCSs of pUC-SPYNE and pUC-SPYCE were replaced by the Gateway conversion cassette providing the attR1 and attR2 recombination sites for use with the Gateway cloning system (pUC-SPYNEx, pUC-SPYCEG, Figure 1).

BiFC analysis of Arabidopsis nuclear bZIP63

To address the feasibility of BiFC for visualization of protein–protein interaction in living plant cells we first choose a member of the Arabidopsis bZIP factor family (bZIP63, AGI: At5g28770) as a model protein. bZIP63 belongs to subfamily C of Arabidopsis bZIP factors (Jakoby et al., 2002). This transcription factor binds to promoter elements containing the CACGTG or GACGTC sequence in vitro and is localized to the nucleus of plant cells (Näke, 2001). Moreover, bZIP transcription factors are known to form homodimers and heterodimers via the C-terminal leucine zipper domain (Siberil et al., 2001).

To first investigate the interaction potential of bZIP63 by an independent experimental approach, we performed an interaction analysis in the yeast two-hybrid system. As shown in Figure 2, bZIP63 formed homodimers in vivo as demonstrated by the growth of transformants on interaction selective medium and induction of β-galactosidase reporter activity above background level. To corroborate that the
Homodimer formation is mediated by the leucine zipper we introduced two point mutations in the bZIP63 sequence which changed Leu188 and Leu195 into Pro. Leu188 and Leu195 are the first two hydrophobic amino acids in the C-terminal zipper-forming amphipathic α-helix of the bZIP63 monomers. Therefore, conversion of these positions into prolines is likely to interfere with the dimerization potential of the protein (Siberil et al., 2001). The combination of wild type bZIP63 with the mutated version (bZIP63PP) reduced expression of the reporter genes (Figure 2). It is noteworthy that in yeast mutation of Leu188 and Leu195 to proline does not completely abolish homodimerization of bZIP63 (Figure 2). In summary, these data indicate that the leucine zipper domain is predominantly responsible for bZIP homodimer formation in yeast.

We next attempted the direct visualization of homodimerization in living plant cells. To this end, we transiently transfected Arabidopsis cell culture protoplasts with various pUC-SPYNE/pUC-SPYCE constructs of bZIP63 and, in addition to microscopic analysis, quantified the fluorescence intensity. Whereas cells transfected with single plasmids and any combination with empty vectors produced no or only background fluorescence, a strong signal was observed when bZIP63-YFPN was co-expressed with bZIP63-YFPN (Figure 3a,b). Significantly weaker fluorescence signals were observed when combinations of bZIP63PP with wild type bZIP63 were transfected, thereby reflecting reduction in homodimerization by these mutations (Figure 3a,b). Generally, the number of BiFC signal-emitting protoplasts was about the half compared with cells expressing full-length GFP fusion proteins. This difference corresponds to the reduced efficiency when more than one construct is used for transfection.

To test the functionality of the binary BiFC vectors in planta, the wild type bZIP63 and bZIP63PP cDNAs were cloned into pSPYNE-35S and pSPYCE-35S, respectively. The constructs were delivered into leaf cells of tobacco (Nicotiana benthamiana) by Agrobacterium infiltration (Voinnet et al., 2003; Witte et al., 2004). Similar to the situation in Arabidopsis protoplasts strong YFP fluorescence was observed when wild type combinations of bZIP63 were expressed (Figure 4a, panels I, II). Pairwise expression of bZIP63 and bZIP63PP, bZIP63PP alone or in combination with the YFP fragments induced no or only weak fluorescence signals (Figure 4a, panels I, II and data not shown). Using HA- and c-myc-tag-specific antibodies the expression of all fusion proteins in tobacco cells was demonstrated (Figure 4a, panel III). Notably, we observed that the transformation efficiency of Agrobacterium-infiltrated tobacco cells strongly depends on the constructs used. For instance, whereas 80% of epidermal cells which have been infiltrated with bZIP63-YFPN and bZIP63-YFPN-carrying Agrobacteria...
showed BiFC-induced fluorescence, LSD1 homodimer formation (see Figure 7) was observed in only 20% of the cells.

In transfected Arabidopsis protoplasts and infiltrated tobacco leaves the homodimerization-induced YFP fluorescence appeared exclusively inside the nucleus which is in agreement with the observation that bZIP63-GFP and bZIP63PP-GFP are nuclear proteins (Figures 3c and 4b).

BiFC analysis of proteins in the cytoplasm of plant cells

To further extend the applicability of BiFC beyond bZIP transcription factors we analyzed a 14-3-3 protein (isoform T14-3c from N. tabacum; GenBank: NTU91724) and the zinc finger protein LSD1 from Arabidopsis thaliana (Dietrich et al., 1997) by BiFC analyses in Arabidopsis protoplasts and Agrobacterium-infiltrated tobacco leaves respectively.

14-3-3 proteins form a conserved family of eukaryotic polypeptides that were the first signaling molecules identified as discrete phosphoserine/phosphothreonine-binding modules. They associate to homodimers or heterodimers with a saddle-shaped structure, with each monomer forming an extended groove that allows binding of the phosphorylated sequence motif (Rittinger et al., 1999; Wütele et al., 2003). Dimerization of 14-3-3 proteins occurs via their N-terminal region. Accordingly, yeast two-hybrid analyses revealed that an N-terminally truncated version of T14-3c is

Figure 3. BiFC visualization of bZIP63 dimerization in transiently transfected Arabidopsis thaliana cell culture protoplasts.
(a) Epifluorescence (I) and bright field images (II) images of Arabidopsis cell culture protoplasts co-transfected with constructs encoding the indicated fusion proteins.
(b) Quantification of fluorescence intensities in transiently transfected Arabidopsis cell culture protoplasts. Fluorescence intensity (arbitrary units) was determined using the Metamorph software. The mean and standard deviation of three independent measurements are shown.
(c) bZIP63-GFP and bZIP63PP-GFP are localized to the nucleus. Epifluorescence (I) and bright field (II) images of protoplasts transfected with constructs expressing the indicated fusion proteins. Scale bars, 20 μm.

no longer able to homodimerize (Jaspert and Oecking, 2002). For BiFC studies the cDNAs encoding wild type T14-3c (T14) and the mutant version (T14ΔN) were cloned into pUC-SPYNE and pUC-SPYCE or pSPYNE-35S and pSPYCE-35S, respectively, and transformed into either Arabidopsis protoplasts or tobacco leaf cells. Upon co-expression of T14-

YFPN and T14-YFPc, strong YFP fluorescence was detected throughout the cytoplasm and the nucleus in both systems indicating that homodimerization occurs in both compartments (Figures 5a and 6a). In transformed epidermal cells of tobacco the cytoplasmic fluorescence typically appears as a thin area between the cell wall and the turgent vacuole. Plasmolysis of the cells by treatment with 500 mM mannitol demonstrated the localization of the BiFC signal in the cytoplasm (data not shown). Importantly, the localization of homodimer formation corresponds to the subcellular distribution of T14-3c, when expressed as GFP fusion in protoplasts or tobacco leaf cells under the control of the 35S promoter (Figures 5b and 6b). In contrast, expression of neither the N-terminal truncated form T14ΔN fused to the YFP fragments nor any combination of wild type with the truncated version resulted in a YFP signal (Figures 5a and 6a). Western blot analysis confirmed that the expression of
the non-interacting T14-3c fusion proteins was comparable with the expression of the interacting form of T14-3c (Figures 5c and 6c).

LSD1 is a small zinc finger protein that forms homodimers in vivo and functions as a negative regulator of programmed cell death in plants (Dietrich et al., 1997; Epple et al., 2003). As observed for the T14-3c homodimer, co-expression of LSD1-YFPN and LSD1-YFPC induced strong fluorescence in the cytoplasm and the nucleus of infiltrated tobacco cells, whereas control pairs gave no or only a very weak YFP signal (Figure 7a and data not shown). Again, the location of LSD1 homodimer formation is identical to the localization of LSD1-YFP when expressed under the control of the 35S promoter in transgenic Arabidopsis plants (Figure 7b). To test the specificity of LSD1 protein association, we analyzed the interaction of bZIP63 and LSD1 in co-transformed tobacco leaf cells. This protein pair was chosen because in the yeast two-hybrid system bZIP63 does not interact with LSD1 (Näke, 2001). As shown in Figure 7, co-expression of bZIP63 and LSD1 in any combination revealed no fluorescence, although the fusion proteins were expressed.

Discussion

In this report we establish BiFC as a very efficient technology for the analysis of protein–protein interactions in living plants cells. The vectors described are readily suitable for BiFC analyses in Arabidopsis protoplasts and Agrobacterium-infiltrated tobacco leaves. They also allow determination of protein expression levels by Western blot analysis. The binary BiFC vectors will also enable interaction and protein complex formation studies in transgenic plants. However, at high expression levels the free YFP fragments sometimes tend to associate non-specifically, thereby

Figure 6. BiFC visualization of T14-3c dimerization in tobacco leaves. (a) Confocal (I) and bright field (II) images of epidermal leaf cells from Nicotiana benthamiana infiltrated with a mixture of Agrobacterium suspensions harboring constructs encoding the indicated fusion proteins. For technical details of infiltration see Experimental procedures. (b) T14-3c-GFP is localized to the cytoplasm and nucleus. Confocal (I) and bright field (II) images of stably transformed N. tabacum epidermal cells are depicted. (c) Demonstration of protein expression by immunodetection with anti-HA (α-HA) antibodies for YFPN fusions and anti-c-myc (α-c-myc) for YFPC fusions. Extracts from tissue co-transformed with constructs co-expressing either T14-YFPN and T14-YFPC (lane 1) or T14ΔN-YFPN and T14ΔN-YFPC (lane 2) are shown.

Figure 7. LSD1 forms homodimers in planta but does not interact with bZIP63. (a) Epifluorescence (I) and bright field (II) images of epidermal leaf cells infiltrated with a mixture of Agrobacterium suspensions harboring constructs encoding the indicated fusion proteins. For technical details of infiltration see Experimental procedures. The expression of the proteins (III) is demonstrated by immunodetection with anti-HA (α-HA) antibodies for YFPN fusions and anti-c-myc (α-c-myc) for YFPC fusions. (b) LSD1-YFP is localized to the nucleus and cytoplasm of Arabidopsis cells. Epifluorescence (I) and bright field (II) images of hypocotyl cells from transgenic Arabidopsis plants which express LSD1-YFP are depicted. Scale bars, 50 μm.
generating background fluorescence (Figure 3a,b). This problem can be circumvented, when a non-interacting fusion protein is used in the control experiments as exemplarily shown for the T14AN and bZIP63/LSD1 pairs, or if the expression level is reduced by the use of a less active promoter.

Our BiFC analyses of interactions among bZIP63, T14-3c and LSD1 in living plant cells illustrate several significant advantages of this technique (Hu et al., 2002). (i) Protein interactions using BiFC are visualized in the normal environment of the plant cell. Several restrictions inherent to interaction approaches in heterologous systems (e.g., yeast), as for instance missing plant-specific post-translational protein modifications or incorrect subcellular localization, are overcome by BiFC. From a technical point of view the detection of BiFC-generated interactions does not require accessibility of the protein complex to extrinsic fluorophores and does not require the instrumental equipment necessary for FRET including subsequent complex data processing. (ii) Compared with published FRET data (Immink et al., 2002; Mäss et al., 2000; Shah et al., 2002; Vermeer et al., 2004) the BiFC signals observed in our study using the strong viral 3SS promoter for the expression of the fusion proteins appear to be very intense. Comparison of the microscopic exposure times suggest that for a given protein interaction the BiFC fluorescence intensity can reach about 30% of the signal intensity of the corresponding full-length GFP. From these data we conclude that BiFC is very sensitive and may allow the detection of interactions when the proteins are expressed at lower levels under the control of native promoters. As demonstrated by our study and recent reports (Grinberg et al., 2004; Hynes et al., 2004) BiFC-generated fluorescence signals can also be quantified. Thus, the ability of a protein to form different complexes with different interaction partners can be quantitatively determined at the cellular or even the subcellular level (Grinberg et al., 2004). (iii) The most appealing advantage of BiFC is that protein interaction occurs in the genuine compartment of the proteins examined. *Arabidopsis* bZIP63 is a nuclear transcriptional regulator and homodimer formation is exclusively detected by BiFC inside the nucleus. When expressed under the control of the viral 3SS promoter, tobacco T14-3c and *Arabidopsis* LSD1 accumulate in the cytoplasm and in the nucleus. Again, BiFC dimer formation strictly coincides with observed localization in these compartments. This should enable the identification of the subcellular distribution of interaction between regulatory proteins like transcription factors and signaling components and may immediately provide information about the functional role of the association. For instance, in mammalian HEK-293 cells BiFC analyses revealed that the different β subunits of G proteins direct the corresponding γ signaling complexes to alternative subcellular locations (Hynes et al., 2004). Furthermore in, mammalian cell lines using BiFC Kerppola and colleagues demonstrated that the cytoplasmic transcription factor Mad4 was recruited to the nucleus through dimerization with Max (Grinberg et al., 2004) and were able to identify the intramolecular region responsible for the differential intracellular distribution of ATF2/Jun heterodimers (Hu et al., 2002). (iv) Formation of the BiFC protein complex occurs through a multistep pathway in vitro and very likely in vivo (Hu et al., 2002). The initial steps are mediated by contacts between the proteins fused to the YFP fragments and are reversible. At this initial state complex formation can be competed by alternative interaction partners. Afterwards the initial complex is stabilized by the association of YFP fragments and further exchange of protein components is inhibited (Hu et al., 2002). Therefore, the kinetics of BiFC formation allows the detection of weak and transient protein complexes in vivo but has the disadvantage that shifts in the equilibrium between putative alternative complexes may hardly be detectable after initial formation. However, we expect that the dynamics of BiFC protein complex formation is still maintained or can be still modified by the cellular protein folding and degradation machinery. (v) The structural background of protein complex formation can likewise be investigated by BiFC. For instance, truncation of the N-terminal region of the tobacco T14-3c protein or point mutations within the C-terminal leucine zipper of *Arabidopsis* bZIP63 identified these domains to be responsible for homodimerization in plant cells. (vi) In perspective, the BiFC approach enables the identification of signals that induce the formation of protein complexes or modulate their intracellular distribution in planta. Accordingly, it has been shown that a BiFC complex consisting of the ATF2-Jun heterodimer is translocated into the nucleus of mammalian COS cells after expression of the stress-activated p38 SAPK protein kinase (van Dam et al., 1995; Hu et al., 2002).

From the studies in mammalian cells using a set of different Jun-YFP<sup>N</sup> and Fos-YFP<sup>C</sup> fusion polypeptides, it has been estimated that fluorescence complementation may occur when the YFP fragments are separated by an average distance of greater than 100 Å (Hu et al., 2002). A prerequisite for complementation over long distance is sufficient flexibility of the protein backbone, to which the fragments are fused. This then allows association of the YFP fragments to stabilize the initial BiFC complex. Therefore, the dynamics of BiFC complex formation in combination with sufficient complementation over long distance may enable the identification of novel protein interactions in plant cells by genetic screens based, for example, on high-efficiency protoplast transformation accompanied by fluorescence-activated cell sorting (Birnbaum et al., 2003; Galbraith, 2004). Furthermore, by generating large populations of transgenic plants expressing distinct YFP<sup>N</sup> or YFP<sup>C</sup> fusion proteins, interaction screens can also be performed on whole plant level by crossing individual lines.
Experimental procedures

Generation of plant BiFC vectors and plant expression constructs

Molecular techniques were performed using standard protocols according to Sambrook and Russell (2001) and Kudla et al. (1999). To generate the vector system for BiFC analysis we amplified fragments of eYFP coding for the N-terminal 155 and C-terminal 84 aa, thereby introducing Xmal and Sac restriction sites for cloning and sequences for c-myc and HA affinity tags respectively. The PCR products were cloned into the Xmal and Sac sites of pGPTVII.Kan and pGPTVII.Bar resulting in pSPYNE-Kan and pSPYCE-Bar. The same PCR products were cloned via Xmal/Sac into pGPTVII.GFP, Kan and pGPTVII.GFP, Bar resulting in pSPYNE-35S and pSPYCE-35S (for pGPTVII vector series see Figure S1 and S2). The pUC19 derivatives were generated by replacing the entire expression casette of psmGFP4 with the expression cassettes of pSPYNE-35S and pSPYCE-35S via HindII and EcoRI, resulting in pUC-SPYNE and pUC-SPYCE. These vectors were further modified to obtain the Gateway-compatible vectors pUC-SPYNEG and pUC-SPYCEG. To this end, the entire multiple cloning site of pUC-SPYNE and pUC-SPYCE was excised with XbaI and Smal, and afterblunting replaced with the Gateway vector conversion cassette B (Invitrogen, Carlsbad, CA, USA). The cDNA regions encoding the proteins and polypeptides investigated in this study were amplified from plasmid templates containing the corresponding cDNA by PCR using gene-specific primers and cloned into pGEM-T (Promega, Madison, WI, USA) or pBluescript (Stratagene, La Jolla, CA, USA) respectively. Site-directed mutagenesis of the bZIP63 cDNA was carried out using the Quick-Change Site-directed Mutagenesis Kit according to the manufacturer’s protocol (Stratagene). Primer sequences can be obtained upon request. PCR products were verified by sequencing and subsequently cloned into each of the BiFC vectors, pUC-SPYNE/pSPYNE-35S and pUC-SPYCE/pSPYCE-35S by using the BamHI/Smal (T14-3c, N-terminal truncated 14-3c), BamHI/Xhol (bZIP63, bZIP63T°) or Smal/BamHI (LSD1) restriction sites. For the generation of GFP fusion constructs, the coding region of T14-3c was cloned via KpnI/BamHI into pCF203, those of bZIP63 and bZIP63T° via BamHI/Xhol into pGPTVII-GFP, and that of LSD1 via BamHI/Smal into pPCVB-YFP.

Protoplast transfection, plant transformation, microscopic techniques, and quantification of fluorescence intensity

Protoplasts were generated from 1-week-old Arabidopsis Col-0 suspension cell culture (Liu et al., 2003). The cells were collected by centrifugation at 400 g for 5 min and the pellet was washed with 25 ml of cell wall digestion buffer lacking the digestion enzymes. Protoplasts were prepared and transformed according to the protocols of Merkle et al. (1996) and Negrutiu et al. (1987). Protoplasts were assayed for fluorescence 12–18 h after transformation. For infiltration of N. benthamiana, the Agrobacterium tumefaciens strain C58C1 carrying the pCH32 helper plasmid was infiltrated into the abaxial air space of 2–4-week-old plants as described (Voinnet et al., 2003; Witte et al., 2004). The p19 protein of tomato bushy stunt virus was used to suppress gene silencing. Co-infiltration of Agrobacterium strains containing the BiFC constructs and the p19 silencing plasmid was carried out at OD600 of 0.7:0.7:1.0. Epidermal cell layers of tobacco leaves were assayed for fluorescence 1–2 days after infiltration. Stable transformation of N. tabacum SNN and Arabidopsis thaliana (Col-0) with Agrobacterium was carried out as described previously (Bechthold et al., 1993; Martin et al., 1993). Microscopic techniques were performed according to Kircher et al. (1999) and images were processed using the Adobe Photoshop software package. Quantification of fluorescence signal intensity was carried out using the Metamorph software package (Universal Imaging Corporation Downington, PA, USA).

Yeast two-hybrid interaction assays, protein extraction and assay, SDS-PAGE, Western blot and immunodetection

Yeast two-hybrid interaction tests (in strain PJ69-4A) including growth and quantitative β-galactosidase reporter gene assays were performed as described previously (Albrecht et al., 2001; Lohmann et al., 2001). Transfected protoplasts and Agrobacterium-infiltrated tobacco leaf discs were extracted under denaturing conditions using boiling SDS-sample buffer supplemented with 4 M urea (Harter et al., 1993). Protein assay, SDS-PAGE, Western blot transfer and immunodetection using HA- (Roche, Basel, Switzerland) and c-myc-specific (Sigma, St. Louis, MO, USA) antibodies has been described elsewhere (Har ter et al., 1993).

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Note added in proof

The usefulness of BiFC for in planta-protein interaction studies is also presented in the paper by Bracha-Drori et al. (2004), in this issue.

Supplementary material

The following material is available from http://www.blackwellpublishing.com/products/journals/suppmat/TPJ/TPJ2219/TPJ2219sm.htm.

**Figure S1.** (a) Schematic depiction of the series of pGPTVII vectors (kan, bar, hyg). Unique restriction sites are given in bold letter, others in italics.

(b) Multiple cloning site of the pGPTVII vector series. Unique restriction sites (above the nucleotide sequence) are illustrated in normal letters. Restriction sites marked with * are not unique in pGPTVII.Bar.

**Figure S2.** (a) Schematic depiction of the series of pGPTVII.GFP vectors (kan, bar, hyg). Unique restriction sites are given in bold letters, others in italics.

(b) Multiple cloning site of pGPTVII. GFP vectors. Below the nucleotide sequence the amino acid sequence of the multiple cloning is illustrated, which is in frame to the first methionine of GFP (underlined). Unique restriction sites (above the nucleotide sequence) are illustrated in normal letters, others in italics. Restriction sites marked with * are not unique in pGPTVII.GFP.Bar. In pGPTVII.CFP and pGPTVII.YFP the GFP was replaced by the respective fluorescent marker proteins.
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