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In planta analysis of protein–protein interactions related to light signaling by bimolecular fluorescence complementation

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Summary. The determination of protein–protein interactions is becoming more and more important in the molecular analysis of signal transduction chains. To this purpose the application of a manageable and simple assay in an appropriate biological system is of major concern. Bimolecular fluorescence complementation (BiFC) is a novel method to analyze protein–protein interactions in vivo. The assay is based on the observation that N- and C-terminal subfragments of the yellow-fluorescent protein (YFP) can only reconstitute a functional fluorophore when they are brought into tight contact. Thus, proteins can be fused to the YFP subfragments and the interaction of the fusion proteins can be monitored by epifluorescence microscopy. Pairs of interacting proteins were tested after transient cotransfection in etiolated mustard seedlings, which is a well characterized plant model system for light signal transduction. BiFC could be demonstrated with the F-box protein EID1 (empfindlicher im dunkelroten Licht 1) and the Arabidopsis S-phase kinase-related protein 1 (ASK1). The interaction of both proteins was specific and strictly dependent on the presence of an intact F-box domain. Our studies also demonstrate that etiolated mustard seedlings provide a versatile transient assay system to study light-induced subcellular localization events.

Keywords: Bimolecular fluorescence complementation; Protein–protein interaction; Transient transfection; *Sinapis alba*; Phytochrome; SCF complex.

Abbreviations: BiFC bimolecular fluorescence complementation; BRET bioluminescence resonance energy transfer; FRET fluorescence resonance energy transfer; phy phytochrome; YC C-terminal fragment of yellow-fluorescent protein; YN N-terminal fragment of yellow-fluorescent protein.

Introduction

Dynamic interactions between proteins have become a central topic of signaling. Various genetic and biochemical techniques have been used to identify factors which regulate signal transduction chains either by mutual interaction or by the formation of complexes. Once these factors have been identified, the next step will be to elucidate their physical interaction and define the corresponding domains by functional assays.

To analyse protein–protein interactions in plants, several in vitro and in vivo methods have been established. Pull-down techniques using tagged proteins or coimmunoprecipitation with antisera are approaches restricted either to recombinant proteins that are brought into contact in the test tube or to extracts derived from plant material. Therefore, these techniques can give only limited information about potential protein–protein interactions in vivo, mainly because both test situations do not reflect the natural cellular environment of the proteins. Colocalization studies using antibodies or fusion proteins with fluorescent proteins can be conducted quite easily, but colocalization does not necessarily mean that proteins are physically interacting. Common in vivo techniques like yeast two-hybrid assays are restricted to evolutionary distinct systems and do not necessarily reflect correct modifications or subcellular localizations of proteins. Techniques like FRET (fluorescence resonance energy transfer) and BRET (bioluminescence resonance energy transfer) have been successfully applied to study protein–protein interactions in planta, but they are technically laborious and complex, and thus, they need specialized and expensive equipment (for reviews, see Hink et al. 2002, Vermeer et al. 2004, Subramanian et al. 2004).

Bimolecular fluorescence complementation (BiFC) is a novel promising tool to study protein–protein interactions in vivo (Hu et al. 2002). The method relies on the expression of two proteins of interest as translational fusions either to the nonfluorescent N-terminal (YN, amino acids

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1–154) or the C-terminal (YC, amino acids 156–238) fragments of YFP (yellow-fluorescent protein, 238 amino acids). Only when both YFP fragments are brought together by interactions of the fused proteins, they can reconstitute a functional YFP fluorophore. This technique was developed to detect protein–protein interactions in living animal cells by conventional epifluorescence microscopy (Hu et al. 2002, Hu and Kerppola 2003). Recently, the BiFC assay also was applied successfully to plant systems (Bracha-Drori et al. 2004, Walter et al. 2004).

An ideal assay system to study protein–protein interactions during signaling in vivo by BiFC should meet the following criteria: it should be fast, it should be easy to handle and accessible to microscopic observation, and it should be endowed with the ability to respond to the signal of choice. In order to functionally map the domains involved in the interaction of interest, transient expression is sufficient and thus preferred over the time-consuming stable transformation. Transfection of tobacco BY-2 cells, onion bulb epidermis, or Arabidopsis protoplasts have been widely used for transient-expression studies in plants. However, these systems suffer from the drawback that their innate signaling is relatively limited and at least partially unknown. We therefore decided to use etiolated mustard (*Sinapis alba* L.) seedlings that were transfected by particle bombardment. The mustard system offers several advantages. Dark-grown seedlings are very large and can easily be fixed on glass slides for transfection even under dim green safelight. In contrast to Arabidopsis seedlings, mustard seedlings have a very rigid tissue with large cells that can easily be transfected by particle bombardment with very high efficiency. Mustard and *Arabidopsis thaliana* belong to the same plant family (Brassicacae), and thus, homologous genes of both plant species exhibited extraordinarily high similarities (Batschauer et al. 1991, Gauly et al. 1992, Malhotra et al. 1995), which should minimize artificial effects that might be caused by illegitimate interactions of heterologous gene products. Furthermore, mustard seedlings are a well established standard system for studies about light regulation and the analyses of temporal and spatial patterns of gene expression during seedling development (Mohr 1972, Wenng et al. 1989, Frohnmeyer et al. 1992, Nick et al. 1993, Kretsch et al. 1995). Therefore, they are versatile tools to study effects during transition from skoto- to photomorphogenesis.

To validate BiFC, we used the well-established interaction between EID1 (empfindlicher im dunkelroten Licht, hypersensitive in far-red light) and ASK1 (Arabidopsis S-phase kinase-related protein 1). EID1 is an F-box protein that is involved in phytochrome A-specific light signal transduction (Büche et al. 2000, Dieterle et al. 2001). F-box and Skp1-like (S-phase kinase-related protein 1) proteins normally are components of so-called SCF (Skp 1, Cullin 1, F-box protein) ubiquitin ligase complexes. The Cullin protein forms the backbone of these complexes. Its C-terminal half interacts with the small RBX (RING box) protein that mediates the interaction with activated ubiquitin residues. Because Skp1 proteins can bind to the amino-terminal end of the Cullin protein and to F-box domains, they are thought to function as linkers between the core complex and various F-box proteins. F-box proteins are supposed to convey substrate specificity to the SCF ubiquitin ligase complex because they carry a multitude of classical protein–protein interaction domains and because the number of F-box genes is very high in all organisms analyzed so far, including *A. thaliana* (Bai et al. 1996, Craig and Tyers 1999, Willems et al. 1999, Gagne et al. 2002).

Material and methods

Plasmid construction

All vectors used in this study were derived from the pMAV4 plasmid described by Kircher et al. (1999). For the expression of YFP and CFP fusion proteins, the *GFP* coding region was exchanged by the respective *YFP* and *CFP* equivalents. The *PHYA* (Kircher et al. 1999b) and *CPRF2* (Kircher et al. 1999a) fragments were introduced in the modified vectors using *Bam*HI and *Sma*I restriction sites. The amino-terminal *YFP* fragment was amplified by polymerase chain reaction (PCR) using the pBiFC-YN155 plasmid (Hu et al. 2002) together with the oligonucleotides 5′-TCTCCCGGGTCCAGATCCATCGCCACCATG-3′ and 5′-ATCCC TAGGCCATGATATAGACGTTGTG-3′. The carboxy-terminal *YFP* fragment was obtained by PCR using the pBiFC-YC155 vector (Hu et al. 2002) together with the oligonucleotides 5′-TCTCGAGGTCCCGGGGC CGCACGTCC-3′ and 5′-CGCGGGAGCTCACTTGTACAGTTCGTC CATGCC-3′. The *GFP* reading frame in pMAV4 was replaced by the *YN* and *YC* fragments using *Sma*I and *Sst*I restriction sites (Fig. 1). The *EID1* fragment (Dieterle et al. 2001) was cloned into the pMAV-YN/YC vectors using *Bam*HI and *Sma*I restriction sites. The *EID1ΔF* fragment was obtained by PCR using the oligonucleotides 5′-ACCTCGAGCCAT GGCGATCCCCACCGTCATCTCCGATC-3′ and 5′-TTCCGGGAGCG CTAGTGTAGAGAGGTAAAGCAGTCCAAGCACC-3′. The deletion construct was cloned into the already existing *EID1-YN* and *EID1-YC* vectors by replacing a *Xho*I-*Bgl*II fragment of the wild-type cDNA. To delete the stop codon of *ASK1*, a PCR fragment was amplified from the cDNA clone described by Dieterle et al. (2001) using the oligonucleotides 5′-AAGGATCCCCATGTCTGCGAAGAAGATTGTGTTG-3′ and 5′-GACCCGGGTTCAAAAGCCCATTGGTTCTCTCTGC-3′. *Pwo* DNA polymerase (Invitrogen, Karlsruhe, Federal Republic of Germany) was used for all PCR reactions. The integrity of all clones was verified by sequence analyses (GATC, Konstanz, Federal Republic of Germany).

Yeast two-hybrid assays

Yeast two-hybrid assays were performed using *Saccharomyces cerevisiae* haploid strains PJ69-4a and PJ69-4 alpha (*MAT***a**/α, *trp1-901*, *leu2-3,112*, *ura3-52*, *his3-200*, *gal4*Δ, *gal80*Δ, *LYS*::*GAL1-HIS3*, *GAL2-ADE2*, *met2*::*GAL7-lacZ*) (James et al. 1996). ASK1 was expressed as a GAL4 activation domain (AD) fusion protein using the yeast expression vector

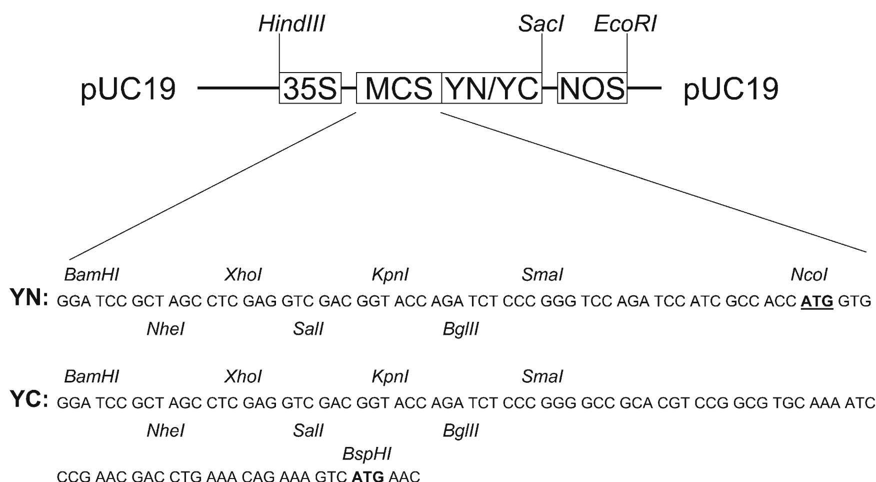


Fig. 1. Schematic representation of the composition of the pMAV-YN and pMAV-YC vectors. The relative positions of the 35S promoter (*35S*), the multiple cloning site (*MCS*), the N-terminal (*YN*) or C-terminal (*YC*) YFP fragments, and the *nos* terminator (*NOS*) are indicated. The construct was cloned into a pUC19 vector (Amersham, Freiburg, Federal Republic of Germany). The sequences of the MCS in front of the ATG start codon of the YN and YC coding regions are given in the lower part of the figure

pGADT7 (Clontech). EID1 and EID1ΔF were expressed as GAL4 DNA binding-domain (BD) fusion proteins using the yeast expression vector pGBKT7 (Clontech). Yeast transformations were performed by the polyethylene glycol–lithium acetate method according to Gietz and Woods (2002). PJ69-4a strain was transformed with AD fusions constructs and PJ69-4 alpha was transformed with BD fusions constructs. The yeast two-hybrid analysis was performed using the cross-mating assay described in Kolonin et al. (2000). Diploid yeast cells and colonies expressing the interacting-protein pairs were selected on SD/−Leu/−Trp and SD/−Leu/−Trp/−Ade media, respectively.

Plant growth and transfection

Mustard (*Sinapis alba* L.) seeds were sown on four layers of moist filter paper in Plexiglass boxes and cultivated for four days in darkness at 25 °C. For each transfection and protein–protein interaction experiment eight etiolated seedlings were fixed to a standard microscopic glass slides with surgical adhesive (B-400 Secure Adhesive; Factor II Inc., Lakeside, Ariz., U.S.A.). The split YFP and the CFP constructs (4 μg of each plasmid) were introduced into mustard hypocotyl cells by gold particles as described previously (Holweg et al. 2004). After transfection, seedlings were kept vertically in sterilized water at 25 °C in darkness for 4–8 h prior to microscopic analysis. Routinely, 10–20 transfected cells were obtained per transfection. Transfection and microscopic analysis of parsley protoplasts derived from a dark-grown suspension culture of *Petroselinum crispum* was performed as described by Kircher et al. (1999a). All manipulations were performed under dim green safelight.

Microscopic techniques

For epifluorescence and light microscopy, mustard seedlings and parsley protoplasts were handled under dim green safelight until analysis with an Axioskop II microscope (Zeiss, Oberkochen, Federal Republic of Germany). Excitation and detection of the fluorophores were performed with specific CFP (exciter, D 436/20; beam splitter, 455 DCLP; emitter, D 480/40) and YFP (exciter, HQ 500/20; beam splitter, Q 515 LP; emitter, HQ 535/30) filter sets (AHF Analysentechnik, Tübingen, Federal Republic of Germany). Representative cells were recorded with a digital Axiocam camera system controlled by the Axiovision software (Zeiss). Photographs were processed for optimal presentation by Photoshop 5.5 (Adobe Systems Europe, Edinburgh, U.K.) and MS Office 97 (Microsoft, Redmond, Wash., U.S.A.) software packages.

Results

Transiently transfected etiolated mustard seedling exhibited characteristic subcellular distribution patterns of phyA-YFP upon light treatments

The plant photoreceptor phytochrome A (phyA) responds to light by characteristic intracellular rearrangements. Whereas in the dark phyA remains evenly distributed in the cytoplasm, it forms cytoplasmic sequestered areas of phytochrome (SAP) almost concomitantly with the onset of irradiation. At the same time phyA enters the nucleus, where nuclear speckles (NUS) start to aggregate. Both forms of speckles (SAP and NUS) disappear when the photoreceptor becomes degraded upon continuous irradiation (Kircher et al. 1999b, 2002; Kim et al. 2000). To verify this pattern of redistribution in the mustard system, etiolated seedlings were transfected with *A. thaliana* phyA-YFP. In darkness, fluorescence was evenly distributed in the cytosol (Fig. 2A). Immediately after the onset of irradiation, the introduced phyA-YFP fusion protein forms SAP and NUS that are very similar to the structures known from the respective transgenic *A. thaliana* lines (Fig. 2B, C). Both types of aggregates disappeared under continuous white light irradiation within 4 h, reflecting the degradation of light-activated phyA (data not shown). Thus, the localization pattern observed in transiently transfected mustard seedlings perfectly matches the results obtained with transgenic *A. thaliana* lines.

Protein–protein interactions in transiently transfected mustard seedlings studied by bimolecular fluorescence complementation

The interaction between ASK1 and EID1 has already been demonstrated in yeast two-hybrid and GST pull-down assays.

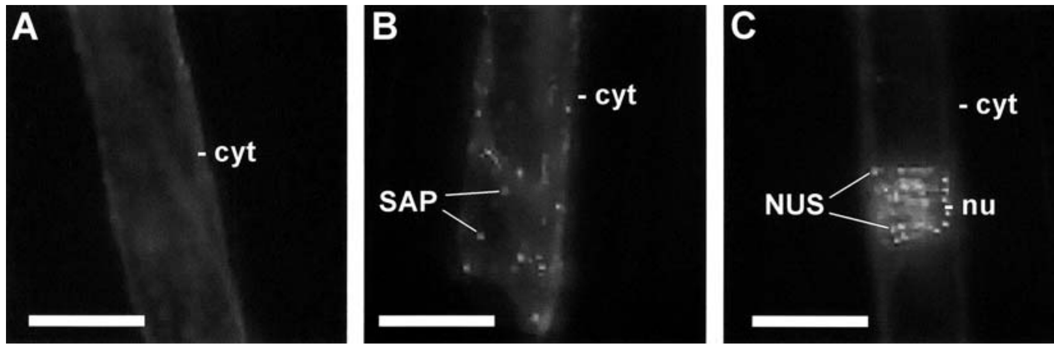


Fig. 2 A–C. Light-dependent redistribution of phytochrome A in transiently transfected mustard seedlings. Seedlings were raised for four days in the dark and then transfected by particle bombardment with an *A. thaliana phyA-YFP* construct under the control of the 35S promoter. Images of cells expressing phyA-YFP were recorded with a YFP-specific filter set. *cyt* Cytoplasm; *nu* nucleus; *SAP* sequestered areas of phytochrome A; *NUS* nuclear speckles. Bars: 40 µm. **A** Cytosolic region of an etiolated cell immediately after transfer from the dark to the microscope stage. **B** Cytosolic region of an etiolated cell 3 min after irradiation with the actinic light source of the microscope. **C** Nuclear region of an etiolated cell 3 min after irradiation with the actinic light source of the microscope

Furthermore, it has been shown that this interaction strictly depends on the presence of an intact F-box domain (Dieterle et al. 2001). In a pilot experiment, we confirmed these results by a corresponding two-hybrid experiment, shown in Fig. 3A. Thus, an F-box deletion construct can be used to test for the specificity of the interaction in the BiFC system.

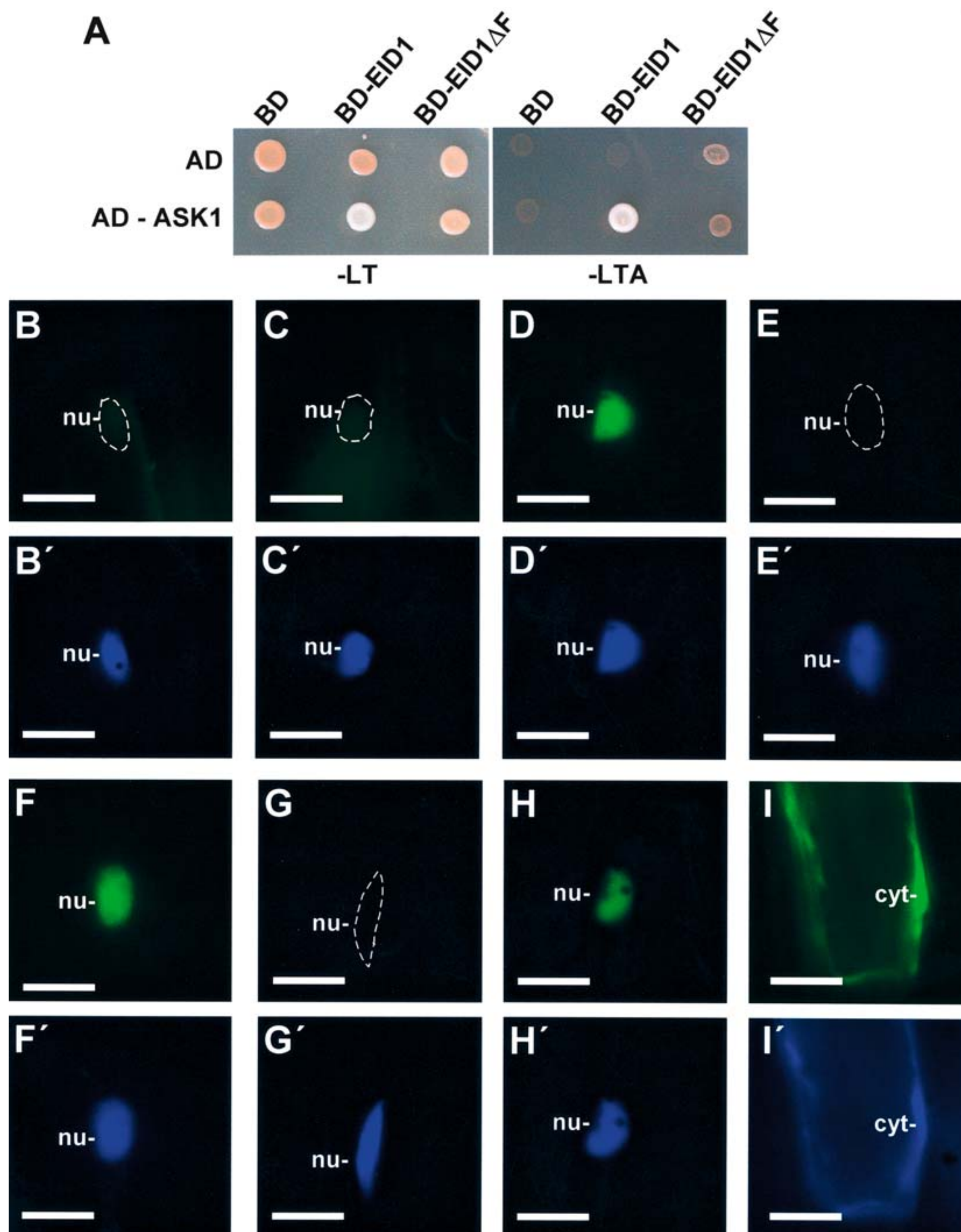
A construct consisting of CPRF2 (common plant regulatory factor 2) fused to CFP was always cobombarded as a transfection control. CPRF2 is a basic leucine zipper transcription factor from parsley, which can form homodimers and accumulates in the nucleus (Armstrong et al. 1992, Kircher et al. 1998). Thus, the CPRF2-CFP fusion protein could also serve as a nuclear marker in transfected cells. Analyses in the yeast two-hybrid system demonstrated that CPRF2 does not interact with EID1 (data not shown).

In a first round of experiments, it was tested whether EID1-YC, EID1-YN, ASK1-YC, and ASK1-YN can interact with the corresponding YN and YC fragments to form a functional YFP. Representative cells coexpressing EID1-YC with YN and ASK1-YN with YC are shown in Fig. 3B, B′ and C, C′, respectively. Even though more than 60 transfected cells expressing CPRF2-CFP were analysed in at least 4 independent experiments, no or only very faint YFP signals were detectable. The same results were obtained using the F-box deletion construct of EID1 (EID1ΔF) fused to YN or YC (data not shown). This data demonstrates that the YC or

Fig. 3 A–I. Characterization of the EID1–ASK1 interaction by yeast two-hybrid assay and by BiFC assays in transiently transfected mustard seedlings. **A** Yeast two-hybrid assays were performed using haploid yeast strains after a cross-mating assay. ASK1 was expressed as GAL4 activation domain fusion protein (AD-ASK1) using the yeast expression vector pGADT7. EID1 and EID1ΔF were expressed as GAL4 DNA binding-domain fusion proteins (BD-EID1 and BD-EID1ΔF) using the yeast expression vector pGBKT7. Diploid yeast and clones expressing interacting pairs of proteins were selected on SD/−Leu/−Trp (−*LT*) and SD/−Leu/−Trp/−Ade (−*LTA*) media, respectively. Haploid yeast strains transformed with the empty pGADT7 (*AD*) and pGBKT7 (*BD*) vectors were used as negative controls. **B–I** and **B′–I′** BiFC in transiently transfected mustard seedlings. The expression of all fusion proteins was driven by a 35S promoter. Images of the nuclear or cytosolic regions were recorded immediately after the transfer of the cell from the dark to the microscope stage using YFP-specific (**B–I**) and CFP-specific (**B′–I′**) filter sets. For details refer to the legend of Fig. 1. The CFP-specific filter set shows the signal from the CPRF2-CFP transfection control (**B′–I′**) that is mainly localized in the nucleus of transfected mustard cells. *nu* Nucleus, *cyt* cytosol. Bars: 40 µm. **B** and **B′** Representative cell that was cobombarded with the empty pMAV-YN vector, the *EID1-YC* construct, and the CPRF2-CFP control plasmid. Exposure time: B, 30 s; B′, 0.5 s. **C** and **C′** Representative cell that was cobombarded with the *ASK1-YN* construct, the empty pMAV-YC vector, and the CPRF2-CFP control plasmid. Exposure time: C, 30 s; C′, 0.3 s. **D** and **D′** Representative cell that was cobombarded with the *ASK1-YN* construct, the *EID1-YC* construct, and the CPRF2-CFP control plasmid. Exposure time: D, 3.2 s; D′, 0.4 s. **E** and **E′** Representative cell that was cobombarded with the *ASK1-YN* construct, the *EID1ΔF-YC* construct, and the CPRF2-CFP control plasmid. Exposure time: E, 30 s; E′, 0.4 s. **F** and **F′** Representative cell that was cobombarded with a construct carrying *EID1ΔF* fused to full-length *YFP* and the CPRF2-CFP control plasmid. Exposure time: F, 2.4 s; F′, 1.2 s. **G** and **G′** Representative cell that was cobombarded with the *CPRF2-YN* construct, the *EID1-YC* construct, and the CPRF2-CFP control plasmid. Exposure time: G, 30 s; G′, 0.2 s. **H** and **H′** Nuclear plane of a representative cell that was cobombarded with the *CPRF2-YN* construct, the *CPRF2-YC* construct and the CPRF2-CFP control plasmid. Exposure time: H, 4.1 s; H′, 0.9 s. **I** and **I′** Cytosolic plane of a representative cell that was cobombarded with the *CPRF2-YN* construct, the *CPRF2-YC* construct and the CPRF2-CFP control plasmid. Exposure time: I, 9 s; I′, 2.5 s

YN fusion proteins cannot be complemented to functional YFP fluorophores with the respective YC and YN protein fragments alone. They also show that optical contamination of YFP signals by filter leakage from the CFP signal was not detectable in these experiments.

Cotransfection of the *EID1-YC* with the *ASK1-YN* construct resulted in strong YFP signals in the nucleus. This result clearly indicates that a successful BiFC takes place when the two interacting proteins can come into close contact (Fig. 3D, D′). As expected, the YFP signal remains restricted to the nucleus, because colocalization of ASK1 and EID1 should be limited to this cellular compartment (Dieterle et al. 2001, Farrás et al. 2001). Furthermore, and very important for the control experiments, it could be seen that all cells which expressed the CPRF2-CFP transfection control also exhibited a YFP fluorescence signal. This observation is based on the analysis of more than 150 cells in 6 independent experiments. The same results were obtained when *ASK1-YC* and *EID1-*



YN constructs were used (data not shown). As pointed out above, we can exclude that the strong YFP is caused by optical bleed-through from the CPRF2-CFP signal.

As a control, *EID1ΔF-YN* and *ASK1-YC* or *ASK1-YN* and *EID1ΔF-YC* constructs were coexpressed in mustard seedlings. In contrast to the full-length *EID1* fragments, no or only a very faint YFP fluorescence was detectable in cells that expressed the CPRF2-CFP control after cotransfection (Fig. 3E, E′). To exclude that the loss of BiFC with the EID1ΔF fusion proteins is caused by a mislocalization or the loss of expression of the truncated protein, the mutated cDNA construct was cloned in front of a full-length *YFP* coding region. All cells expressing the CPRF2-CFP control also exhibited EID1ΔF-YFP fluorescence signals

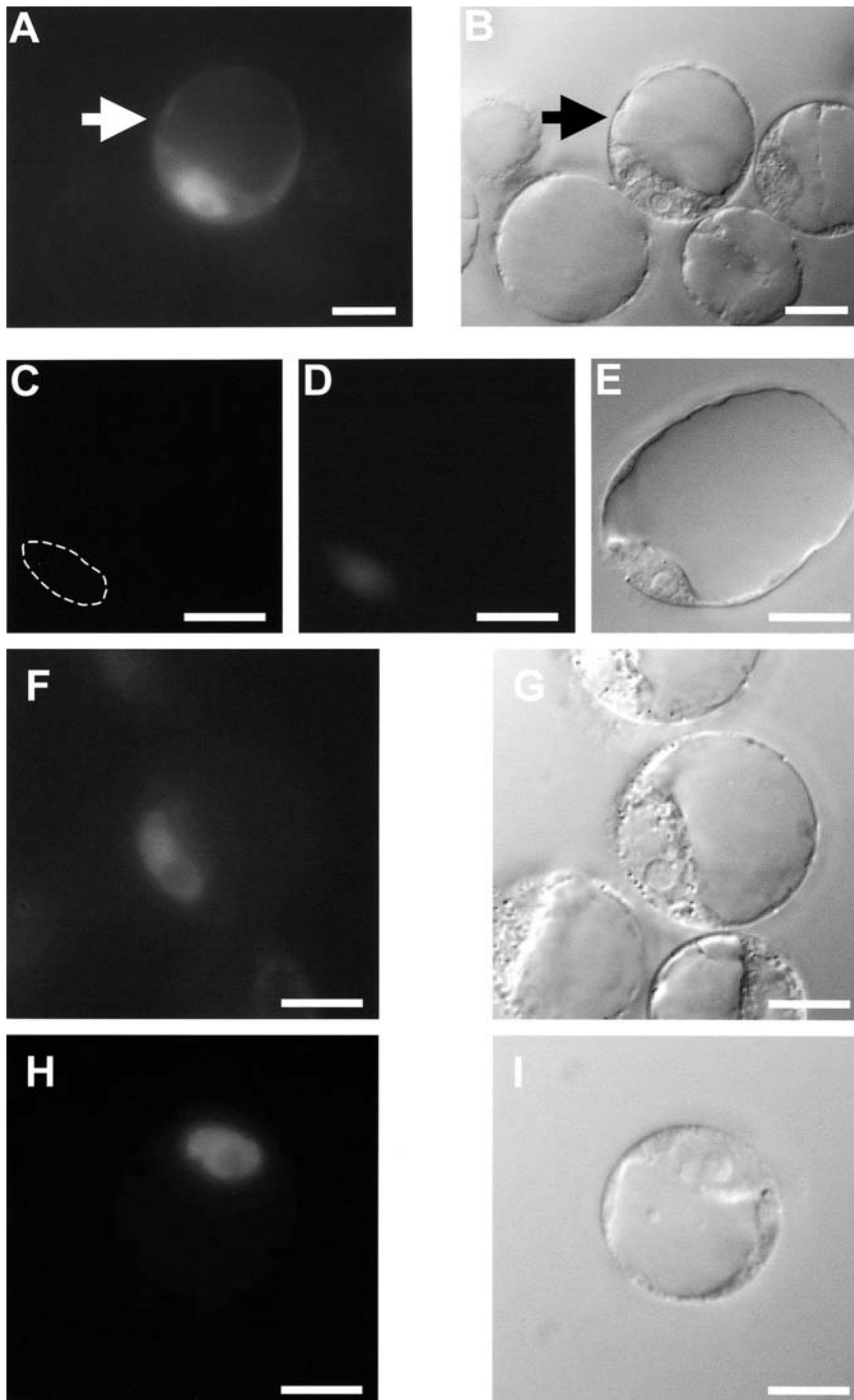


Fig. 4 A–I. Bimolecular fluorescence complementation in transiently transfected parsley protoplasts. Parsley protoplasts were transfected by electroporation. The expression of all fusion proteins was driven by the 35S promoter. Photographs of nuclear regions were recorded immediately after the transfer of the sample from the dark to the microscope stage using YFP-specific and CFP-specific filter sets. Bars: 10 μm. **A** YFP signals obtained from a protoplast that was cotransfected with the *CPRF2-YN* construct, the *CPRF2-YC* construct, and an EID1-CFP control vector. **B** DIC (differential interference contrast) image of the sample shown in **A**. The transfected protoplast is indicated by an arrow. **C** YFP signals obtained from a protoplast that was cotransfected with the *CPRF2-YN* construct, the *EID1-YC* construct, and the EID1-CFP control vector. **D** CFP signals obtained from the protoplast shown in **C**. **E** DIC image of the protoplast shown in **C**. **F** YFP signals obtained from a protoplast that was cotransfected with the *CPRF1-YN* construct, the *CPRF1-YC* construct, and an EID1-CFP control vector. **G** DIC image of the protoplast shown in **F**. **H** YFP signals obtained from a protoplast that was cotransfected with the *CPRF1-YC* construct, the *CPRF2-YN* construct, and an EID1-CFP control vector. **I** DIC image of the protoplast shown in **H**

(Fig. 3F, F′). EID1ΔF-YFP was predominantly localized in the nucleus similar to results obtained with the EID1-YFP full-length protein (Fig. 3F, F′) (Dieterle et al. 2001; data not shown). These results clearly indicate that the deletion of the F-box domain does not alter the expression and localization pattern of the mutated EID1 protein. Taken together, these observations clearly demonstrate that BiFC between ASK1 and EID1 fusion proteins strictly depends on the presence of a functional F-box interaction domain and is not caused by unspecific interactions.

As additional control for the specificity of the BiFC system, *EID1-YC* was cobombarded together with a *CPRF2-YN* construct. No YFP fluorescence was seen, even though again a high number of transfected cells were analyzed (Fig. 3G, G′). In contrast, coexpression of CPRF2-YC together with CPRF2-YN gives a strong and clear YFP signal (Fig. 3H, H′) due to the homodimerization of the bZIP protein (Armstrong et al. 1992, Kircher et al. 1998). Longer exposure times also show that CPRF2 is abundant to a lesser extent in the cytoplasm, pointing out that the BIFC assay is not restricted to the nuclear compartment (Fig. 3I, I′).

Bimolecular fluorescence complementation also obtained in transiently transfected parsley protoplasts

To check whether BiFC can be confirmed in a different expression system, we also tested transiently transfected parsley protoplasts. As expected, clear nuclear and cytosolic YFP fluorescence signals became detectable in cells that were cotransfected with *CPRF2-YN* and *CPRF2-YC* (Fig. 4A, B). In contrast, no BiFC was detectable in control experiments using a combination of *CPRF2-YN* and *EID1-YC* similar to the results observed with the mustard system (Fig. 4C–E). By coexpressing *CPRF1-YN* and *CPRF1-YC*, the BiFC pairs of a constitutive nuclear bZIP from parsley (Kircher et al. 1999a), only nuclear YFP signals have been obtained (Fig. 4F, G). When *CPRF2-YN* was cotransfected with *CPRF1-YC*, YFP fluorescence derived by CPRF1/CPRF2 heterodimers only formed in the nucleus. As expected from the overlapping localization in the nuclear compartment of both CPRFs, no cytosolic signals are observable. These results clearly indicate that the BiFC approach can also be applied in transiently transfected protoplasts.

Discussion

We have demonstrated that mustard seedlings are a versatile tool to study signal-triggered dynamic localization and protein–protein interaction by means of biolistic transfection. The efficiency of transfection is high, the seedlings are large and therefore easy to handle, the cells are large and therefore easily accessible to microscopic observation. Furthermore, they are endowed with a functional light signaling that can be specifically triggered through the phytochrome system that has been thoroughly characterized over the last decades. Moreover, due to the close evolutionary relationship, mustard can be used as a quasi-homologous system for signaling compounds that have been isolated from *A. thaliana*.

Our data clearly demonstrates that transfected cells from etiolated mustard seedlings show the same light-regulated cellular distribution pattern of phyA-YFP as seen with transgenic *A. thaliana* seedlings (Kim et al. 2000, Kircher et al. 2002). Furthermore, transfected mustard cells also exhibited a light-dependent degradation of the introduced phyA-YFP fusion protein that was comparable to the results obtained for *A. thaliana* and mustard (Schäfer et al. 1976, Hennig et al. 1999). On the basis of these observations and because mustard seedlings are very well analyzed with respect to development and photobiology (Mohr et al. 1972, Nick et al. 1993, Kretsch et al. 1995), they provide an ideal system to study intracellular protein distribution and protein–protein interactions related to light signal transduction.

In contrast, clear differences in light-dependent localization patterns have been observed with other test systems. In transiently transfected parsley protoplasts, phyA-GFP always remained evenly distributed in the cytosol in darkness and under all tested light conditions. The fusion protein did neither enter the nucleus nor form SAP or NUS. Additionally, no light-driven degradation of the photoreceptor has been observed (Kircher et al. 1999a; unpubl. results). This effect might be caused by the profound loss of intracellular architecture occurring during the isolation of protoplasts. In addition, unpredictable physiological changes which are due to somatoclonal variation and can affect light responses are often observed during the prolonged propagation of plant cells in tissue culture (Ohl et al. 1989). Differences in light-dependent localization patterns have also been observed between *A. thaliana* seedlings and onion epidermis cells, for example, in the case of FHY1/PAT3 (far-red long hypocotyl 1 and phytochrome A signal transduction 3), a positively acting factor in phyA light signal transduction. Whereas the fusion protein was detected in the nucleus and the cytosol of transiently transfected onion epidermal cells irrespective of the light treatment (Zeidler et al. 2001), FHY1-GFP fusion proteins in transgenic *A. thaliana* lines disappeared upon prolonged irradiation with continuous far-red and white light (Desnos et al. 2001). While these differences potentially could be explained by the different promoters used, again the localization and abundance of phytochrome A–GFP fusion proteins show an aberrant pat-

tern in transiently transfected onion epidermal cells. The photoreceptor appears to be much more stable and does not form the characteristic light-induced cytosolic and nuclear structures known from *A. thaliana* (Seo et al. 2004).

Applying the BiFC assay, we were able to show unequivocally that in mustard seedlings the F-box motif of EID1 is essential for the interaction with the Skp1-like protein ASK1. These results confirm previous results from yeast two-hybrid studies (Dieterle et al. 2001, this study) and are in perfect agreement with the current model of the composition and molecular structure of SCF complexes (Bai et al. 1996, Craig and Tyers 1999, Willems et al. 1999, Zheng et al. 2002). Control experiments for which unrelated proteins or complementary YC or YN fragments were expressed or the interactive F-box domain had been deleted did not lead to functional complementation of the YFP fluorophore. Our data shows that specific protein–protein interaction results in an at least 8 times higher YFP/CFP signal ratio compared to unspecific background activities or combinations of noninteracting proteins. These observations clearly demonstrate that the BiFC method is a powerful tool to specifically address protein–protein interactions in living plant cells.

So far the study of physical interactions in planta could be conducted only by FRET (for reviews, see Hink et al. 2002, Vermeer et al. 2004) or the recently described BRET (Subramanian et al. 2004). For FRET, pairs of proteins have to be tagged with photophysically suited fluorophores. Spatial proximity is analyzed by the detection of resonance energy transfer from a light-excited donor to an acceptor (e.g., CFP donor to YFP acceptor). This means that small overlaps in excitation and emission spectra of fluorophores, limitations of optical filters, and background activities can cause severe artifacts that will lead to false-positive signals. Complicated experimentation and extensive data processing are necessary to safeguard this approach against misinterpretation (Gordon et al. 1998). Several methods have been developed to improve FRET measurements. The widely used acceptor bleaching technique is based on the calculation of a potential increase of donor fluorescence after photobleaching of the acceptor fluorophore (Karpova et al. 2003, Vermeer et al. 2004). But photobleaching approaches have to cope with the temporal gap between the situation before and after bleaching, for instance, movements of structures or diffusion and mixing of bleached and nonbleached protein pools, which could lead to artifacts or at least imprecise results. Moreover, the efficiency of photobleaching is dependent on chromophore concentrations and limited by undesired absorbance by cellular structures (Vermeer et al. 2004). To overcome this problem, fluorescence lifetimes have been measured (Bastiaens and Squire 1999), a photophysical property of fluorescent molecules that is affected by FRET. But the technical equipment needed for this approach is certainly beyond the standards of the average user (Gadella 1999, Sytsma et al. 1998). In addition, it is again time consuming and also has limitations in dynamic systems (Vermeer et al. 2004).

As an alternative to FRET, in a recent study BRET has been proposed as an assay system to analyze protein–protein interactions in planta (Subramanian et al. 2004). In this system, BRET depends on energy transfer from bioluminescing luciferase to YFP after addition of the substrate coelenterazine. Detection of specific photons derived from a potential interaction has to be performed in darkness and must not be disturbed by other light sources under proper assay conditions. Due to the short half-life of luciferase and its slow regeneration during the luminescence reaction, each pair of interacting proteins is expected to be detected only once (van Leeuwen et al. 2000). These properties of luciferase are ideal for a reporter system to measure promoter activity. However, it is less suited to study protein–protein interactions because signal intensities can be low, signal amplification is hardly possible, and dynamic protein pools cannot be monitored continuously.

The BiFC assay has several advantages over the techniques outlined above. First of all, standard epifluorescence microscopy equipment is sufficient to perform this assay, which helps to overcome limitations from a technical and financial point of view. In agreement with earlier studies (Hu et al. 2002, Hu and Kerppola 2003), the method is very robust because of its low sensitivity against disturbing background fluorescence and the high efficiency of photon emission of the YFP fluorophore. Specifically for plants, the YFP marker is superior over other fluorophores because of the relatively low level of chlorophyll fluorescence elicited by green light excitation. The BiFC assay is not sensitive to protein movement during the measurements and allows to track subpopulations of proteins under noninvasive conditions. Furthermore, it requires only standard filter sets to monitor specific signals and does not need extensive calculations to overcome background fluorescence or nonstoichiometric abundance of labeled proteins. Due to these advantages, the method has been successfully applied to address diverse scientific questions in animal cells and bacteria (Hu et al. 2002, von der Lehr et al. 2003, Atmakuri et al. 2003). Limitations of the BiFC assay could derive from two aspects. One limitation it shares with other techniques relying on the expression of translational fusions. While expression of tagged versions of proteins may interfere with their function, also the orientation of the tags, here YN and YC, and the used linker sequences could be

essential to obtain YFP complementation by potential interactions of the proteins of interest. Therefore, N-terminal as well as C-terminal fusions should be considered in the BiFC assay. The other limitation is due to the association kinetics of the YN and YC fragments (half-life, about 60 s) and the low reversibility of YFP fluorophore formation (half-life, >24 h) (Hu et al. 2002). Dependent on the kinetics of potential transient protein–protein interactions, they may be overseen or artificially stabilized.

Here we unequivocally show that the BiFC technique is a versatile tool to study protein–protein interactions in the cytosolic and nuclear compartments of plant systems. By this, our results are in perfect agreement with two parallel studies in plant systems published recently (Bracha-Drori et al. 2004, Walter et al. 2004).

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