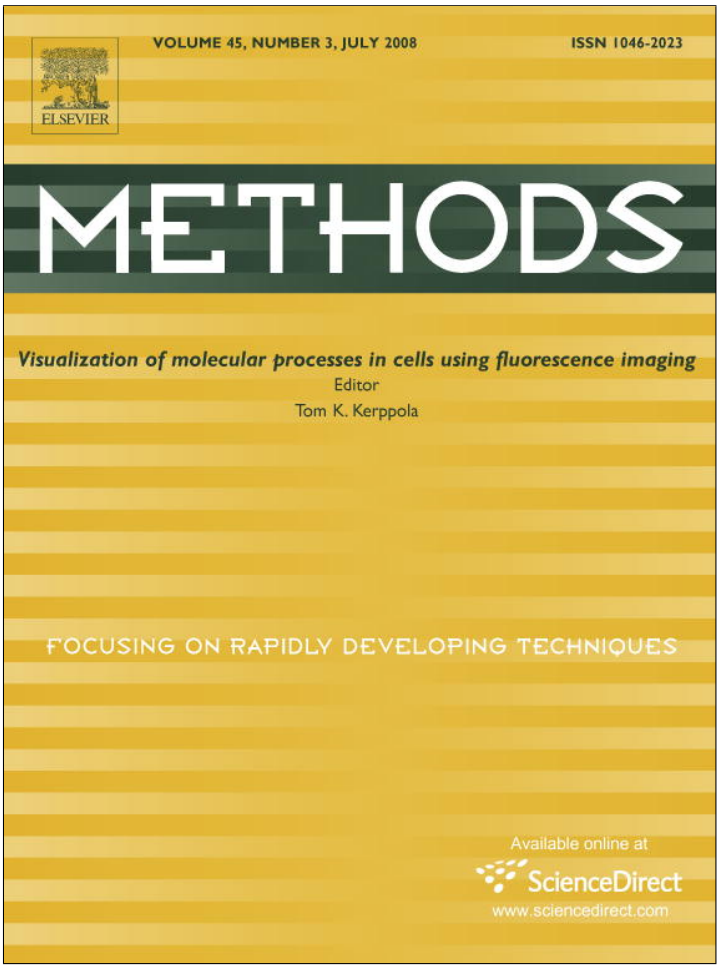

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# Localizing protein–protein interactions by bimolecular fluorescence complementation *in planta*

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ABSTRACT

The application of novel assays for basic cell research is tightly linked to the development of easy-to-use and versatile tools and protocols for implementing such technologies for a wide range of applications and model species. The bimolecular fluorescence complementation (BiFC) assay is one such novel method for which tools and protocols for its application in plant cell research are still being developed. BiFC is a powerful tool which enables not only detection, but also visualization and subcellular localization of protein–protein interactions in living cells. Here we describe the application of BiFC in plant cells while focusing on the use of our versatile set of vectors which were specifically designed to facilitate the transformation, expression and imaging of protein–protein interactions in various plant species. We discuss the considerations of using our system in various plant model systems, the use of single versus multiple expression cassettes, the application of our vectors using various transformation methods and the use of internal fluorescent markers which can assist in signal localization and easy data acquisition in living cells.


## 1. Introduction

The study of protein–protein interactions is fundamental for cell biology research. A wide range of methods have been developed to facilitate the identification and characterization of protein–protein interactions both *in vitro* and *in vivo*. The co-immunoprecipitation [co-IP, reviewed in Ref. 1] and yeast two-hybrid systems [2] are just two examples of the most commonly used methods for the detection and analysis of protein–protein interactions *in vitro* and *in vivo*, respectively. Each of these methods and many others (e.g. *in vivo* protein crosslinking, co-fractionation, gel-blot overlay assays and the two-hybrid Sos recruitment system) possess unique features which make them most suitable for specific applications. For example, the ability to raise specific antibodies against interacting proteins and/or to molecularly tag them with small epitopes makes co-IP the method of choice for the detection of protein–protein interactions in cell lysates. Alternatively, the ability to fuse a library of coding sequences to one domain of a fragmented transcription factor makes the yeast two-hybrid system an excellent tool for large-scale screening of unknown proteins which may interact with a known bait protein, fused to the second domain of this transcription factor. While proven useful for unveiling novel interactions and for analysis of known interactions among various proteins in different biological systems and a variety of organisms, the abovementioned methods and many others do not permit direct visualization of protein complexes and thus hinder our ability to study such interactions under native cellular conditions.

Realizing the importance of analyzing protein complexes under native conditions, several methods have been developed to facilitate the detection and imaging of protein–protein interactions in living cells, including those of plant species. One such pioneering method was based on the fusion of interacting proteins to inactive β-galactosidase mutants and *trans* complementation of an active enzyme upon their interaction in living cells [3,4]. While found useful for monitoring protein–protein interactions in living mammalian cells [3,4], the method's reliance on enzymatic detection did not allow accurate subcellular localization of the interactions in this model system. Furthermore, the method was found difficult to implement in plant cells, most likely due to low permeation of the substrate into the plant cell cytoplasm and organelles, and to the best of our knowledge, its use was limited to plant protoplasts [5]. In a more direct approach, protein interactions in living cells have been measured by changes in fluorescence emissions from fluorescent proteins fused to interacting proteins. Methods such as the fluorescence resonance energy transfer (FRET) [6,7] and bioluminescence resonance energy transfer (BRET) [8] assays have been successfully used to monitor protein interactions in living cells [9,10]. Both FRET and BRET allow not only detection, but also

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subcellular localization of the interacting proteins in plant cells [e.g. Refs. 11,12–18]. Nevertheless, use of these methods is rather challenging due to various technical and biological limitations, including autofluorescence, photobleaching, the application of luciferin and the need for specialized equipment and software for imaging and data analysis [reviewed in Refs. 6,19].

A more direct approach to visualizing protein–protein interactions in living cells is bimolecular fluorescence complementation (BiFC). Originally developed to monitor protein–protein interactions in mammalian cells [20], the method, which relies on the ability to reconstruct, in living cells, a functional yellow fluorescent protein (YFP) signal from two non-fluorescent fragments of YFP via their fusion with two interacting partners, was quickly adapted for studying protein–protein interactions in various organisms [e.g. Refs. 21,22–25], including plants [e.g. Refs. 26,27–33] (Table 1). BiFC offers several advantages over the use of FRET or BRET for monitoring protein–protein interactions in living cells. These include higher sensitivity, relative technical simplicity and the ability to use epifluorescence microscopy as a low-cost alternative to confocal microscopy. Although a growing number of publications describe the use of BiFC for the analysis of protein–protein interactions in different organisms [for recent reviews see Refs. 34,35], its application still requires overcoming several technical difficulties, some of which are unique to plant cells.

Here we discuss the use of BiFC for the detection of protein–protein interactions in living plant cells. We describe systems which have been specifically designed to facilitate the use of BiFC for plant research, while focusing on our own series of vectors which have been designed with an emphasis on modularity and flexibility. We demonstrate the use of our system for the detection of protein–protein interactions in diverse plant species, tissues and cell types, and we provide guidelines for using our modular pSAT-BiFC set of vectors for the analysis of protein–protein interactions in living plant cells.

## 2. Principles of the BiFC assay

In the BiFC assay, two proteins are fused to two non-fluorescent fragments of a fluorescent protein. The interaction of these proteins brings together the non-fluorescent fragments, which may result in the reconstruction of an active fluorescent signal (Fig. 1). Since both fused proteins may be directed to distinct subcellular organelles or compartments, the reconstructed signal is confined to specific subcellular structures, allowing not only for the detection, but also the localization of the interacting proteins in living cells. The BiFC assay was originally developed using the yellow spectral variant (YFP) of the green fluorescent protein (GFP) [20]; however, several other fluorescent proteins, e.g. cyan fluorescent protein (CFP) [21], blue fluorescent protein (BFP) [21], Venus and cerulean fluorescent proteins [36], citrine fluorescent protein [37] and even red fluorescent protein (RFP) [38], have been reported useful for the reconstruction of BiFC assay signals. The expansion of the BiFC assay for use with a collection of fluorescent proteins also led to the development of multi-color BiFC as a tool for the simultaneous visualization of multiple protein interactions in a single cell [21]. In addition, it resulted in the discovery of favorable combinations of fluorescent protein fragments for either brighter or more efficient complementation [21,34,36]. For example, due to their high complementation efficiency and low fluorescence background, the use of YFP fragments is recommended for most applications. The use of Venus fragments, on the other hand, may produce a brighter fluorescent signal but has also been suggested to produce a background signal [reviewed in Ref. 34]. Interestingly, the splitting point of the fluorescent protein can also affect the efficiency of the reconstructed BiFC signal and truncating YFP, the most commonly used BiFC fluorescent protein, between amino acids 154 and 155 or between amino acids 173 and 174 has proven useful for the construction of efficient BiFC signals [reviewed in Ref. 34].

Application of the BiFC assay requires the fusion of two interacting partner proteins to truncated fluorescent proteins. Data from a variety of reports have demonstrated that various combinations of N- or C-terminal fusion of target proteins to the N- or C-terminal ends of the fluorescent protein fragments can function in reconstructing the BiFC signal [reviewed in Ref. 34]. It should be noted, however, that while it was previously suggested that all eight possible basic combinations should be experimentally tested [34,35], certain fusions may not be possible if one wishes to maintain proper biological function of the fused proteins. For example, proteins that are targeted to chloroplasts in plant cells may carry a specific signal peptide at their N terminus, limiting their fusion to the C-terminus of the fluorescent protein fragments. This naturally limits the number of possible combinations to be tested but may also lower the possibility of detecting these interactions using the BiFC assay. Another possible alternative to the traditional N- and C-terminal fusions is to internally tag the interacting proteins with the N and C fragments of the fluorescent proteins. This option, which has been successfully implemented in an analysis of integral membrane topology in plant cells [39], increases the number of possible combinations that can be tested to 18 (Fig. 2). It should be noted, however, that there are still no dedicated BiFC-specific vectors which would facilitate internal tagging and users need to rely on self-assembly of such fusions for analysis of their proteins.

Additional considerations surrounding application of the BiFC assay are the choice and length of the peptide linkers between the fused proteins and the fluorescent protein fragments, the level of expression, the use of various controls, proper quantifications of the signal reconstruction and proper interpretation of the results from BiFC experiments. These topics are beyond the scope of the current paper, which focuses on the methodology of applying the BiFC assay in plant cells, and the reader is therefore referred to an excellent review by Kerppola [see Ref. 34] which discusses many of these experimental issues.

## 3. BiFC in plants

Application of the BiFC assay in plant cells is not fundamentally different from its use in other organisms, and this assay has been successfully used to analyze protein–protein interactions in various plant species [reviewed in Ref. 35]. Table 1 exemplifies how the BiFC assay has been instrumental in unveiling various structures and mechanisms in plant cell research, including determination of molecular structures during host–pathogen interactions [26,28,37,40], determination of transcription factor interactions during hormonal responses [41], analysis of homomeric channel formation in plant vacuoles [42], among others. Furthermore, BiFC has been used to image protein–protein interactions in various subcellular compartments, such as the ER [39], chloroplasts [37], plasmodesmata [40], the vacuole [42], the nucleus [30] and nuclear dicing bodies [43]. Table 1 also demonstrates the wide range of plant species, e.g. tobacco [39], parsley [30], mustard [30], leek [44] and *Arabidopsis* [42], and tissue and cell types, e.g. intact leaves, a single layer of epidermal cells [26], seedlings [30] and protoplasts [38] used in the BiFC assay. Finally, Table 1 demonstrates the different types of transformation methods (i.e. *A. tumefaciens*-mediated, co-infiltration, polyethylene glycol (PEG)-mediated and particle bombardment) and the different fluorescent protein fragments (i.e. mRFP, citrine and YFP) used for BiFC in plant species. Together with many other studies, Table 1 clearly illustrates the power and versatility of the BiFC assay for studying the involvement of protein–protein interactions in a wide range of biological processes and plant species.

**Table 1**
Selected examples of BiFC analysis of protein–protein interactions in plant cells (not all of the interactions and plant systems described in each paper are presented)

| Interacting proteins[a] | Fluorescent fragments[b] | Biological context | Transformation method | Localization | Plant species | Localization marker | Microscope [c] | Reference |
|---|---|---|---|---|---|---|---|---|
| hrGFP dimer | mRFP-Q66T | Development of RFP as BiFC marker | PEG | Cytoplasm and nucleus | BY2 tobacco protoplasts | — | CLSM | [38] |
| CPC and GL3, transcription factors | mRFP-Q66T | Interaction between MYB and BHLH transcription factors | *A. tumefaciens*, co-infiltration | Nucleus | Intact leaves of *N. benthamiana* | DAPI | FM | [38] |
| CPC and GL3, transcription factors | mRFP-Q66T | Interaction between MYB and BHLH transcription factors | Particle bombardment | Nucleus | Onion leaves | DAPI | CLSM | [38] |
| VIP1 and H2A | YFP163 | Functions of VIP1 in plant transformation by *Agrobacterium* | Particle bombardment | Nucleus | Intact leaves of *N. tabacum* | DsRed2 | CLSM | [28] |
| ASK1 and EID1 | YFP155 | Light signal transduction | Particle bombardment | Nucleus | Etiolated mustard seedlings | CPRF2-CFP | FM | [30] |
| CPRF2 dimer | YFP155 | Dimerization of CPRF2 bZIP-like transcription factor | PEG | Nucleus | Parsley protoplasts | EID1-CFP | FM | [30] |
| VirE2 and VirE3 | YFP173 | Function of bacterial VirE3 in plant cells | Particle bombardment | Nucleus | Onion glades | — | CLSM | [26] |
| ER tags | YFP155 | Analyzing integral membrane topology | Particle bombardment and *A. tumefaciens*, co-infiltration | Endoplasmic reticulum | Intact leaves of *N. benthamiana* | — | CLSM | [39] |
| ER tag and TGBp2 | YFP155 | Analyzing integral membrane topology | Particle bombardment and *A. tumefaciens*, co-infiltration | Endoplasmic reticulum | Intact leaves of *N. benthamiana* | — | CLSM | [39] |
| ChrD dimer | YFP174 | Dimerization of ChrD | Particle bombardment | Chloroplast | Intact leaves of *N. benthamiana* | mRFP-ChrD | CLSM | [40] |
| GID1s and DELLA | YFP155 | GA-dependent interaction | *A. tumefaciens*, co-infiltration | Nucleus | Intact leaves of *N. benthamiana* | DAPI | CLSM | [71] |
| SE, DCL1 and HYL1 | YFP155 | Identification of nuclear dicing bodies | *A. tumefaciens*, co-infiltration | Subnuclear | Intact leaves of *N. tabacum* | Hoechst 33342 | CLSM | [43] |
| NRIP and p50 | Citrin155 | Interaction between host and viral protein | *A. tumefaciens*, co-infiltration | Cytoplasm, nucleus and chloroplast, p50 can interact and alter the localization of NRIP1 | Intact leaves of *N. benthamiana* | cerulean | CLSM | [37] |
| NRIP1 and N | Citrin155 | P50-dependent interaction of NRIP with N | *A. tumefaciens*, co-infiltration | Cytoplasm, nucleus and chloroplast | Intact leaves of *N. benthamiana* | cerulean | CLSM | [37] |
| WRKY51 and WRKY71 | YFP155 | Transcription factors-interactions | Particle bombardment | Nucleus | Barley half-seeds | SYT017 | CLSM | [41] |
| STM and BLH | YFP155 | Nuclear targeting of STM via interactions with BLH | Particle bombardment | Cytoplasm and nucleus | Leek | — | FM | [44] |
| TPK1 dimer | YFP155 | Formation of homomeric vacuolar channels | PEG | Vacuole | *Arabidopsis* protoplasts | — | CLSM | [42] |
| SAD and GAMYB | GFP155 | Transcription factors-interactions | Particle bombardment | Nucleus | Onion cells | DAPI, bright-field | CLSM | [29] |
| TYLCV CP self-interaction | YFP174 | Self-association of viral protein | Particle bombardment | Nucleus | Tomato trichomes | brightfield | FM | [40] |

[a] ASK1, S-phase kinase-related protein 1; BLH, Bel1-like homeodomain; ChrD, cucumber chromoplast D protein; DCL1, Dicer-like1; CP, coat protein; CPC, MYB transcription factor; CPRF2, common plant regulatory factor 2; EID1, Empfindlicher im Dunkelroten Licht 1 F-box protein; ER tags, the N-terminal signal peptide sequence of *Arabidopsis thaliana* basic chitinase and the C-terminal ER-retention signal HDEL were used as interacting domains; GAMYB, GA responsive MYB-like transcription factor; GID1s, gibberellin receptor; GL3, BHLH transcription factor; H2A, histone H2A; hrGFP, humanized Renilla green fluorescent protein; HYL1, double-stranded RNA binding-domain protein Hyponastic Leaves1; N, *Arabidopsis* immune receptor; NRIP1, N receptor interacting protein; p50, 50 kDA helicase of tobacco mosaic virus; SAD, a DOF-like transcription factor; SE, zinc-finger-domain protein Serrate; STM, shoot meristemless; TGBp2, potato mop-top virus membrane-associated movement protein; TPK1, tandem-pore $K^+$ channels; TYLCV, tomato yellow leaf curl virus; VIP1, VirE2 interacting protein 1.

[b] GFP, green fluorescent protein; mRFP, monomeric red fluorescent protein; YFP, yellow fluorescent protein. The type of split is indicated by numbers.

[c] CLSM, confocal laser scanning microscope; FM, fluorescent microscope.

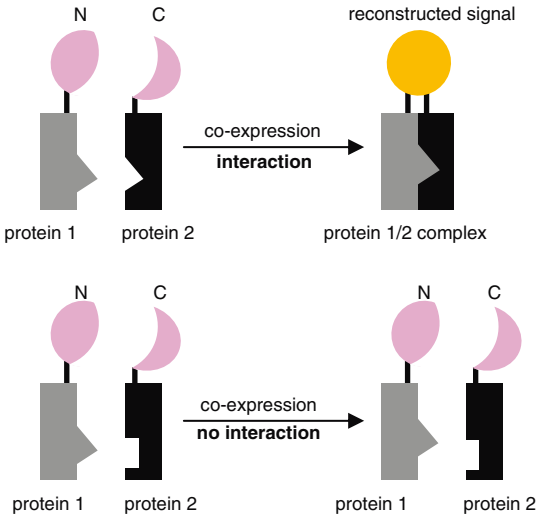


**Fig. 1.** Principles of the BiFC assay. In the BiFC assay, a fluorescent protein reporter (e.g. EYFP, mRFP, citrine or BFP) is split into two, N- and C-terminal, non-fluorescent fragments. Bringing both fragments together, via fusion to interacting proteins (proteins 1 and 2) at either their N- or C-terminus, results in reconstruction of a fluorescent signal, typically in a specific subcellular compartment or area where both interacting proteins reside or interact. Adapted from Citovsky et al. [40] with modifications.

### 3.1. BiFC plasmid systems for plant research

Realizing the importance of the BiFC assay for plant research, three different research groups [31,32,40] developed BiFC-dedicated sets of plasmids which could potentially facilitate the application of this assay to plants. All of these vector systems have been shown useful for monitoring protein–protein interactions in various plant species and cell types, and the choice of system needs to be carefully examined in light of the different advantages that each has to offer. We focus here on the pSAT-BiFC vector system (see below) and only briefly describe the features of the two other vector systems [31,32]. For further details on these, the reader is referred to the original papers by Walter et al. [31] and Bracha-Drori et al. [32], where they will find an in-depth discussion of the technical details of their systems.

The system described by Bracha-Drori et al. [32] uses the N-terminal (designated YN, amino acids 1–154) and C-terminal (designated YC, amino acids 155–238) fragments of YFP. The authors constructed a set of vectors in which the YN constructs have a combination of EE tag and TAISR linker fused either upstream or downstream of YN and in which the YC constructs have a combination of HA tag and HNMVKQKLDNPIKCAPK linker fused either upstream or downstream of YC. The authors also constructed a multiple-cloning site (MCS) upstream or downstream of the EE or HA tag, enabling the fusion of genes of interest at either the 5′ or 3′ ends of the YN and YC fragments. Expression of the BiFC fragments was under the control of the cauliflower mosaic virus (CaMV) 35S constitutive promoter and all four different expression cassettes were cloned into *Agrobacterium* binary vectors. One of the important advantages of this BiFC system is the presence of the EE and HA tags, which allow for immunoblot and co-IP analyses of the interacting proteins [32]. These tags can potentially be used for further visualization of the interacting proteins by immunofluorescence analysis as described, for example, during the analysis of HMGN homodimeric complex formation in human cells by BiFC [45]. Another clear advantage of this system is that only a single cloning step is required for fusion of the interacting proteins to the YN and YC fragments in the *Agrobacterium* binary vectors. However, it should be noted that the flexibility of cloning into these vectors was rather limited due to their relatively narrow MCS [32].

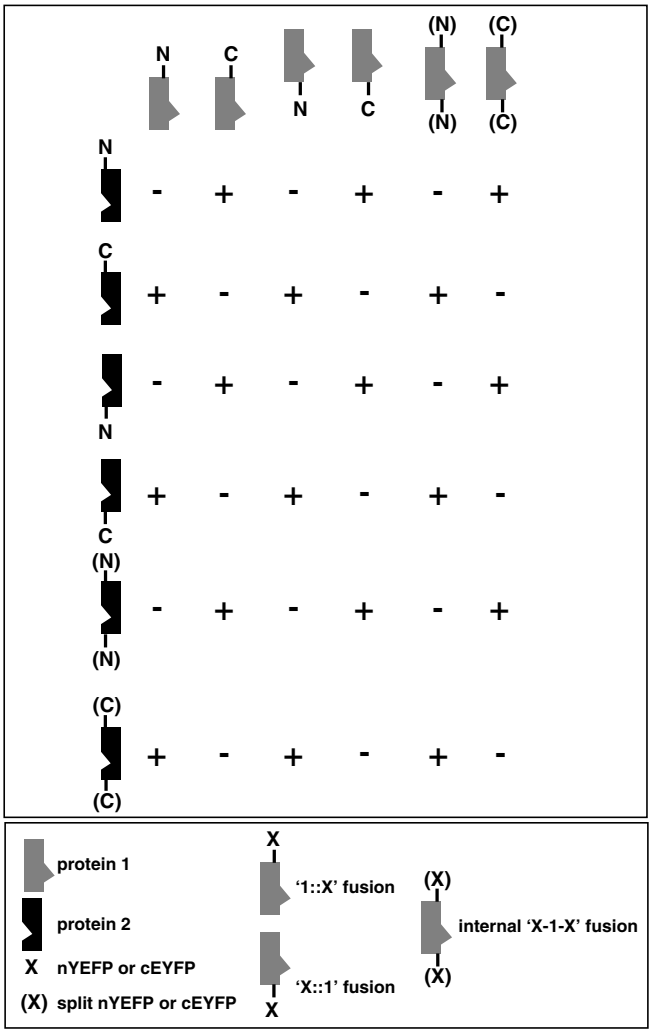


**Fig. 2.** Possible combinations of the BiFC assay. There are 18 possible configurations for analyzing protein–protein interactions using the BiFC assay. Each target protein (1 or 2) can be tagged at its N or C end to the N- or C-terminal fragment of the fluorescent marker protein. Alternatively, the target proteins can also be tagged, internally, by the N or C fragments of the fluorescent marker protein. The plus and minus symbols indicate potential positive and negative reconstruction, respectively, of a fluorescence signal upon interaction of proteins 1 and 2 and reconstruction of a functional fluorescent marker protein. Adapted from Ohad et al. [35] with modifications.

A more flexible BiFC vector system was developed by Walter et al. [31], who constructed several pairs of vectors useful for traditional and Gateway-mediated cloning of target proteins to the N-terminal (designated $YFP^N$, amino acids 1–155) and C-terminal (designated $YFP^C$, amino acids 155–239) fragments of YFP. As with the system of Bracha-Drori et al. [32], the authors chose to include epitope tags (c-myc and HA) upstream of the YFP fragments, and demonstrated their use for immunodetection of the interacting partners. The flexibility of this vector system was evidenced not only by the user's ability to choose between traditional or recombinase-mediated cloning, but also by the presence of a wide MCS that contained 14 unique restriction recognition sites on most of the vectors. Furthermore, the authors constructed high-copy, pUC-based vectors which are suitable for bombardment experiments, as well as low-copy binary vectors which are useful for *Agrobacterium*-mediated genetic transformation experiments. The authors also constructed promoterless vectors which can be useful for cloning genomic $YFP^C$- and $YFP^N$-tagged fragments driven by native promoters. Binary vectors

also carried different plant selection markers, allowing the user to apply these vectors for the production of transgenic plants using a single co-transformation and co-selection experiment. It should be noted, however, that the cloning of target proteins was limited to the C termini of $YFP^C$ and $YFP^N$ and that the authors did not report on the construction of vectors that would allow fusion of target proteins to the YFP fragments' N termini.

The above-described vector systems provide the user with excellent choices of vectors, cloning flexibility and the ability to use different transformation methods. These systems also provide the user with epitope tagging, useful for confirmation and further biochemical analysis of the interacting partners. Nevertheless, these vector systems suffer from several limitations, including limited MCSs on some of the vectors, the need to use dual-selection markers for the production of transgenic plants and the inability to deliver the interacting partners, as well as additional proteins and/or reporters, into plant cells from a single plasmid. We thus designed and constructed a series of vectors suitable for BiFC in plants which provides a flexible and versatile alternative to the above-described systems. The fluorescent fragments used in our vector were derived from the enhanced yellow fluorescent protein (EYFP) and the structure, features, uses and possible modifications of our pSAT-BiFC vector system for the application of BiFC assays in plant cells are discussed in the following sections.

### 3.2. The pSAT-BiFC vector system

We designed the pSAT-BiFC vector systems for maximum flexibility, versatility and simplicity. We aimed to provide the users with several important features that were either not found or were limited in other BiFC vector systems. These include the ability to (i) clone the target interacting proteins to both 5′ and 3′ ends of the N-terminal (designated nEYFP, amino acids 1–174) and C-terminal (designated cEYFP, amino acids 175–238) fragments of the enhanced YFP, (ii) express the nEYFP/cEYFP-tagged interacting partners under the control of a strong constitutive promoter, (iii) provide the user with a wide MCS, (iv) mount two BiFC expression cassettes onto a single binary vector, (iv) have several choices of plant transformation selectable markers, (v) add the expression of an internal reporter marker and/or other proteins and (vi) allow further modification of the basic BiFC vectors, by replacing their regulation elements and/or the type of fluorescent protein fragments.

#### 3.2.1. The pSAT vector: basic features

The pSAT vector's basic design is illustrated in Fig. 3. It is comprised of a complete expression cassette containing four distinct parts: the promoter (e.g. tandem 35S and its adjunct translational enhancer leader), the terminator (e.g. the 35T), the fluorescent protein fragments (i.e. nEYFP and cEYFP) and the MCS. The pSAT-BiFC vectors [as well as other pSAT-based vectors, such as pSAT-AFP, pSAT-MCS and pSAT-RNAi, Refs. 46,55,72] are based on the pUC plasmid, which is a high-copy plasmid with a broad-range origin of replication. This feature not only allows the user to propagate its plasmid in its preferred bacterial strain, but also assists in recovering the large quantities of plasmid DNA that are typically needed for PEG-, electroporation- and particle bombardment-mediated transformation experiments in plant species.

Several fusion configurations between interacting proteins and the fluorescent protein fragments should potentially be tested when using the BiFC assay [34,35] (Fig. 2). A minimal number of four BiFC vectors are thus required to allow the fusion of interacting proteins to the N- or C-terminal ends of both fluorescent protein fragments. Our system provides three basic types of pSAT-BiFC vectors: (i) the pSATN-nEYFP-C1 and pSATN-cEYFP-C1 pair type, which allows fusion of interacting proteins to the C-terminus of the EYFP fragments; (ii) the pSATN-nEYFP-N1 and pSATN-cEYFP-N1 pair type, which allows fusion of interacting proteins to the N terminus of the EYFP fragments and carries an ATG at the beginning of the MCS and (iii) the pSATNA-nEYFP-N1 and pSATNA-cEYFP-N1 pair type that allows fusion of interacting proteins to the N terminus of EYFP fragments but does not carry an ATG at the MCS. This expanded collection of plasmids with wide MCSs (carrying over 10 unique restriction recognition sites) allows for high cloning flexibility while fusing target proteins to the EYFP fragments.

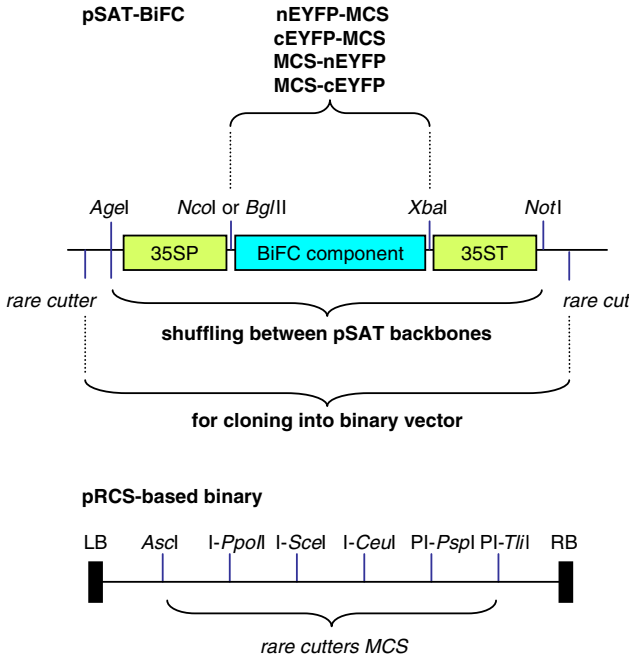


**Fig. 3.** The pSAT-BiFC series of vectors. The pSAT-BiFC vector's main structural features are the cEYFP and nEYFP fragments and their adjacent MCS which are controlled by the tandem CaMV 35S promoter and 35S terminator. Plant expression cassettes can be shuffled between different pSAT backbones, and several expression cassettes can be mounted onto an acceptor binary vector using sets of unique rare-cutting restriction enzymes. Key restriction enzymes which can be used for further promoter and terminator region replacements, as well as for shuffling and mounting of whole expression cassettes, are indicated.

The ATG codon on the pSATN-nEYFP-N1 and pSATN-cEYFP-N1 vectors is part of a preferred ATGG translation–initiation site and we recommend using this pair of plasmids for fusion of target proteins to the N terminus of EYFP fragments. Such fusion will ensure high expression levels of the tagged proteins; it may also result in the addition of a few amino acids to the fused protein's N terminus, depending on the type of enzymes used for the cloning. It should be noted that such an addition may interfere with proper processing of certain N-terminal signal peptides. If this is a concern, we recommend using the pSATNA-nEYFP-N1 and pSATNA-cEYFP-N1 vectors which were designed to rely on the native protein's own translation–initiation site.

The entire expression cassette of each of the basic types of pSAT-BiFC vectors (and, in fact, of any pSAT vector) can be mobilized as AgeI–NotI fragments (Fig 3) among seven different pUC-based backbones, designated pSAT1-7 [46]. These backbone plasmids are virtually identical, except that each plasmid carries a distinct pair of rare-cutting restriction recognition sites flanking the AgeI–NotI sites (Fig. 3). This design allows the user to clone one or two BiFC expression cassettes onto a single plasmid, as well as add selectable markers and internal reporter genes [40,46]. It should be noted, however, that since the construction of binary vectors using the pSAT-BiFC system requires additional cloning steps, it is better to select the type of tissue and transformation

method for the BiFC analysis prior to assembling complex vector structures.

*3.2.2. BiFC analysis in onion cells and use of the basic pSAT-BiFC vectors*

Onion epidermal cells are an excellent experimental system for analyzing the activity of protein fusion and for protein-localization studies [47–49]. Onion epidermal cells are large, relatively uniform in size and shape, transparent and typically organized in a single cell layer which can be easily peeled off of mature scales. Onion cells exhibit low autofluorescence and can be easily visualized by not only confocal, but also light and epifluorescence microscopy. The preferred method for onion cell transformation is particle bombardment [48,50] and it has been successfully used for protein subcellular localization studies using various reporter genes, including EGFP, DsRed2 (variant of the red fluorescent protein DsRed from *Discosoma*), and EYFP [e.g. Refs. 26,27,47–49]. High-quality onion bulbs can be purchased year-round from nearly every local market and we have successfully used different onion and scallion varieties for protein-localization and BiFC analysis. Although the use of fresh onions is not critical, the selection of inner onion scales with an intact epidermis layer is important for efficient gene expression and high-quality imaging. We normally prepare fresh batches of 4–5 × 2–3 cm onion scales and place them on a wet filter paper inside deep Petri dishes, keeping them in a moist environment throughout the experiment. We use DNA-coated gold particles and the portable Helios gene gun system (model PDS-1000/He, Bio-Rad) for microbombardment of compatible pairs of BiFC plasmids. It is very important to thoroughly mix the pair of BiFC plasmids during the preparation of the gold particles in order to ensure efficient delivery of both plasmids into the same transformed cells. A pressure of 150–200 psi and a distance of 1–2 cm between the gun barrel and the tissue are sufficient for delivery of the particles to the epidermal cell layer. We typically analyze the transformed tissues 16–24 h post-bombardment, but shorter incubation periods [e.g. 6–8 h, Ref. 51] have also been reported. The epidermal cell layer should be carefully peeled off and placed on a microscope slide for direct observation by epifluorescence or confocal microscopy. While subcellular reconstruction of a BiFC signal can be analyzed by colocalizing the fluorescence signal with brightfield imaging of the entire cell, the use of fluorescent dyes [e.g. DAPI, Ref. 29] or an additional fluorescent marker [e.g. mRFP, Ref. 51] has also been demonstrated. It should be noted that adding an internal reference protein that is cloned and expressed from a third vector will usually add to the complexity of the transformation since three vectors need to be delivered into a single cell. We thus recommend the construction of a multi-gene vector using the pSAT-BiFC system (Fig. 3). Note, however, that the physical delivery of large vectors may be somewhat inefficient and the use of *Agobacterium*-mediated genetic transformation, which has been recently shown to be viable for transient gene expression in onion cells [49], should thus be considered.

*3.2.3. BiFC analysis in tobacco leaves and the use of multi-gene binary BiFC vectors*

Tobacco species, *Nicotiana benthamiana* in particular, are a popular model for plant biology research and imaging analysis [52,53]. Most tobacco species are highly susceptible to *Agrobacterium* infection, easy to transform and regenerate, grow rapidly and are easy to maintain in greenhouses, growth chambers and tissue culture. In contrast to epidermal peels of onion glades, detaching a single cell layer from tobacco leaves is next to impossible and imaging is thus preformed on whole leaf tissues. This, of course, raises the problem of transforming and imaging internally residing cells and for most applications, it is recommended that the abaxial side of the leaf be used, as it is characterized by relatively large and oddly shaped mesophyll cells. These cells are not only easy to observe by low-magnification epifluorescence or confocal microscopy they are also highly transformable by *Agrobacterium* and by particle bombardment.

We typically use the older leaves of 1- to 2-week-old plants grown in the greenhouse or growth chamber for BiFC experiments. For particle bombardment, we use detached leaves which are kept with their abaxial side up on a wet tissue paper in a humidified chamber. We also place a small water-soaked cotton ball at the tip of the petiole so that the leaves will not dry out during the transformation procedure and incubation period. Similar to bombardment of onion cells, we deliver DNA-coated gold particles using a portable Helios gene gun system; however, we use a lower pressure (50–100 psi) and a wider distance (3–4 cm) between the barrel and the tissue. Bombarding with higher pressure is not recommended because it not only reduces DNA delivery to the abaxial mesophyll cell layers, it can also lead to extensive damage to the transformed tissue, thereby reducing the overall transformation efficiency.

While particle bombardment is considered simple and efficient for the delivery of foreign genes into tobacco leaf disks, *Agrobacterium*-mediated genetic transformation is perhaps the most commonly used method for transient and stable transformation of tobacco tissues. The pSAT-BiFC vector system supports the construction of *Agrobacterium* binary vectors by transferring the EYFP-fragment-tagged interacting protein-expression cassettes onto the T-DNA region of pPZP-based vectors [54,55]. Since *Agrobacterium*-mediated transient transformation of tobacco leaf tissues is rather efficient and often results in a high number of transformed cells [52,56,57], it is possible to simply launch the EYFP-fragment-tagged interacting proteins from two different *Agrobacterium* strains. Nevertheless, the pSAT-BiFC also allows the construction of multi-gene plasmids, enabling the assembly of both EYFP-fragment-tagged interacting proteins and even a third cassette for the expression of an internal reference marker (see below) onto a single binary vector.

The choice of using a single *Agrobacterium* strain or a mixture of several strains depends on the type of proteins being analyzed, their localization, and the anticipated transformation and interaction efficiencies. Detection of protein–protein interactions in large subcellular compartments (e.g. the cytoplasm and the nucleus), for example, is expected to be simpler than imaging protein–protein interactions in small organelles (e.g. peroxisomes, mitochondria or chloroplasts). The latter, for example, require efficient transfer of the interacting partners into the same organelle (a process which is affected by the number of organelles in a particular cell and by the expression levels of both proteins in that cell) and proper processing of both fused proteins in the target organelles. We recommend using a multi-gene vector to facilitate the analysis of protein–protein interactions in small organelles. We also recommend adding an internal reference marker which can assist with not only detecting the transformed cells, but also estimating the overall transformation efficiency and localizing the interacting proteins. We exemplified these features during the detection of protein–protein interactions in chloroplasts of tobacco and *Arabidopsis* leaves. We analyzed the interactions between subunits of a cucumber chromoplast D (ChrD) protein, which is known to localize and dimerize in plastids [58]. Co-delivery of EYFP-fragment-tagged ChrD into tobacco by microbombardment or *Agrobacterium* was inefficient for the detection of BiFC signal since the co-transformation efficiency was very low and from a technical standpoint, the detection of doubly transformed cells with a clear BiFC signal was next to impossible. We therefore constructed a triple expression cassette vector carrying not only both EYFP-fragment-tagged ChrD proteins but also an internal mRFP-tagged reference gene that facilitated the detection of transformed cells (Fig. 4) (see also below).

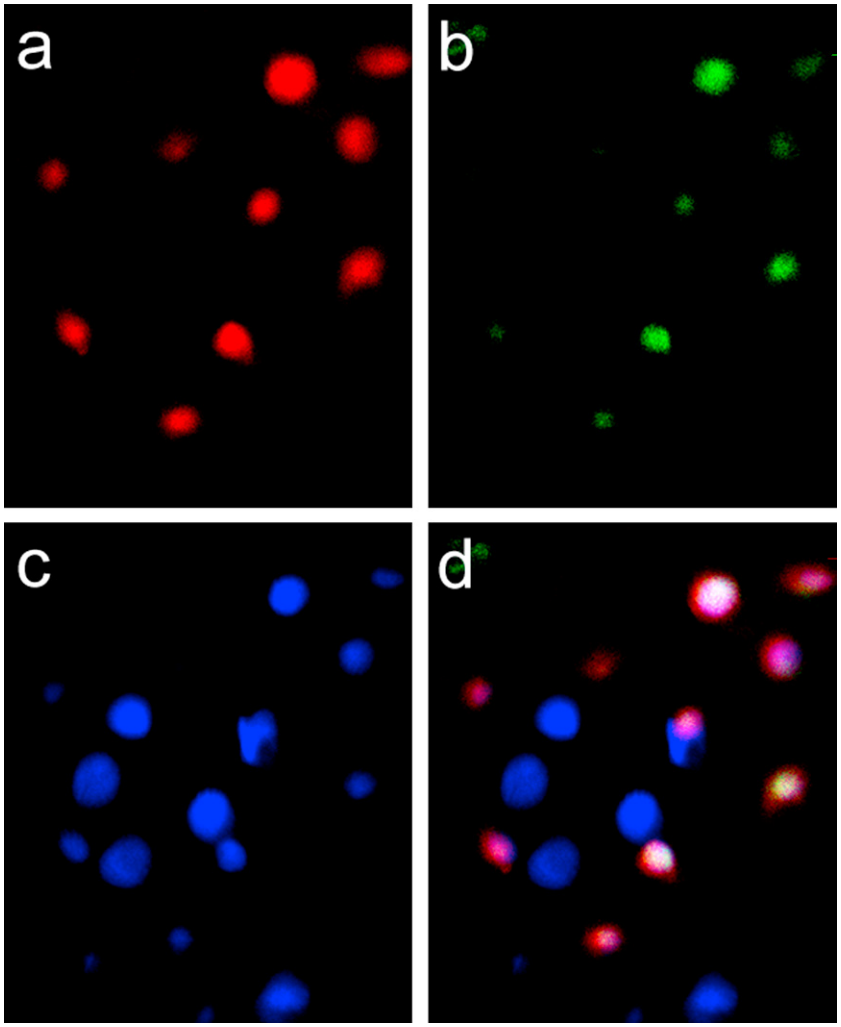


**Fig. 4.** Use of an internal reference marker and autofluorescence for subcellular localization of ChrD dimerization in plant chloroplasts. Plant cells were transformed with mRFP-tagged ChrD and nEYFP- and cEYFP-tagged ChrD. Images of the (a) mRFP-tagged ChrD, (b) reconstructed EYFP signal and plastid autofluorescence (c) were superimposed (d). Reproduced from Citovsky et al. [40] with modifications.

We recommend using intact leaves for *Agrobacterium*-mediated transient delivery of BiFC and other proteins into tobacco tissues. We suggest using small (10–20 cm tall) greenhouse- or growth-chamber-grown plants and to avoid using *in vitro*-grown plants since the latter tend to be fragile and difficult to handle. Fresh *Agrobacterium* cultures should be grown overnight and induced by 200 mM acetosyringone (3′,5′-dimethoxy-4-hydroxy-acetophenone) prior to their use for the infection experiments [59]. If a mixture of *Agrobacterium* strains is to be used for specific infection, these strains should be grown and induced separately, and then thoroughly mixed just before the actual infection, to avoid overgrowth and one strain taking over. We harvest the cells by centrifugation, resuspend them in infiltration medium (5 mg/ml D-glucose, 50 mM MES, 2 mM $Na_3PO_4$) to an $OD_{600}$ of 0.1–0.2 and gently deliver the cells to the leaves' abaxial side using a 5-ml syringe.

Regardless of the transformation method, we recommend incubating the transformed tissues for a period of 16–48 h in the dark (small plants can simply be placed in a big cardboard box). Transformed tissues can be analyzed by either epifluorescence or confocal microscopy, depending on the type and localization of the interacting proteins, and the level of resolution required. It should be noted, however, that the presence of chloroplasts in tobacco leaves (and any other green tissue, for that matter) and their autofluorescence can sometimes interfere with detection of the target fluorescence signal, and caution should therefore be exercised when analyzing the reconstruction of BiFC signal in these tissues. Naturally, the use of confocal microscopy equipped with suitable laser lines and filter sets will allow for higher resolution and more detailed imaging of the BiFC reconstructed signal than that obtained by epifluorescence microscopy, but nevertheless, the latter should not necessarily be avoided. The combination of a bandpass 480/20 excitation filter with a bandpass 527/30 suppression filter for example, can be a good choice for reducing chlorophyll autofluorescence while maintaining good excitation and visibility of YFP signal using epifluorescence microscopy.

The main drawback of using our pSAT-BiFC vector system for *Agrobacterium*-mediated genetic transformation is the significant addition of cloning steps and the need to transform the vectors into *Agrobacterium* cells. Cloning of the EYFP fragment-tagged proteins, internal reference marker and other protein-expression cassettes requires successive mounting of each cassette onto the MCS of pPZP-based binary vectors [46,55,60] using a collection of rare-cutting restriction enzymes. While BiFC assay expression cassettes can easily be shuffled between different pSAT backbones (Fig. 3), we recommend using a combination of *Asc*I, I-SceI and PI-PspI as the enzymes of choice due to their high digestibility and specificity relative to the rest of the rare-cutting enzymes.

*3.2.4. BiFC analysis in protoplasts and the use of electroporation and PEG transformation*

One efficient way to study foreign genes' expression and their protein products' interaction with other proteins is to transiently express them in plant leaf protoplasts. We typically use tobacco (*Nicotiana tabacum*) protoplasts for BiFC assay and protein-localization studies, though tomato protoplasts have also been used. The advantage of tobacco protoplasts is their higher yield when preparing them from leaves, as well as the higher efficiency of their transformation. Mesophyll protoplasts are isolated from leaves of plants grown *in vitro* under sterile conditions in magenta containers on MS-agar medium [61]. Transformation can be achieved *via* several methods, of which electroporation and PEG have been found to be the most reliable in our hands. Electroporation has been used to promote the cellular uptake of exogenous molecules and macromolecules, including nucleotides, dyes, RNA, DNA and even small proteins [62,63]. Useful attributes of this method are its simplicity and general effectiveness with a wide range of cell types. We utilize high-voltage electric fields for cell permeabilization and electroporation of pSAT plasmids (as well as other plant expression plasmids) into our target protoplasts. In our experiments, electroporation of $5 \times 10^5$ protoplasts is carried out in pre-chilled electroporation medium [64]. We usually adjust the DNA concentration to a final 10 μg plasmid DNA and 30 μg calf thymus DNA per ml of electroporation solution. Alternatively, PEG offers a simpler alternative for protoplast transformation. PEG has been used extensively to induce DNA uptake into protoplasts [65] and we typically use PEG 6000 for transformation of tobacco and tomato protoplasts. While the transformation efficiency is usually lower than that with electroporation, it is still a valid method where equipment is unavailable or unaffordable.

Following electroporation or PEG-mediated transformation, protoplasts are transferred to growth medium and incubated in the dark at 27 °C for 24 h before imaging. We recommend first looking at the protoplasts under a light microscope to confirm their viability: healthy protoplasts are round and smooth, whereas damaged ones look distorted. We do not recommend continuing the analysis if over 15% of the protoplasts are damaged. Since protoplasts can be maintained as single cells throughout the infection, incubation and imaging processes, they can be easily imaged using epifluorescence instead of confocal microscopy. We demonstrated the use of our pSAT-BiFC vector system for analyzing the interaction of plant virus coat protein (CP), known to be karyophilic, with its cytoplasmic receptor that imports it into the nucleus in tobacco protoplasts. *Tomato yellow leaf curl virus* (TYLCV) is a begomovirus [66] in which the nuclear import of its genome is mediated by a single viral protein, the CP [67,68]. The interaction of TYLCV CP with importin α was imaged in transiently transformed protoplasts by a combination of confocal and brightfield microscopy. Fig. 5 shows that TYLCV CP directly binds the tomato homolog of importinα, LeKAPα1 [69], in a living protoplast cell, and that the interacting proteins accumulate within the plant cell nucleus. It should be noted that while protoplasts may offer a simple method for imaging protein–protein interactions in a single cell, their use may not be possible for the detection of certain organelles and subcellular compartments (e.g. cell wall and plasmodesmata), and other cell types and tissues should be considered.

*3.2.5. BiFC analysis in other tissues, cell types and plant species*

The BiFC assay for the detection and imaging of protein–protein interactions can potentially be implemented in every plant tissue and/or cell type which can be efficiently genetically transformed

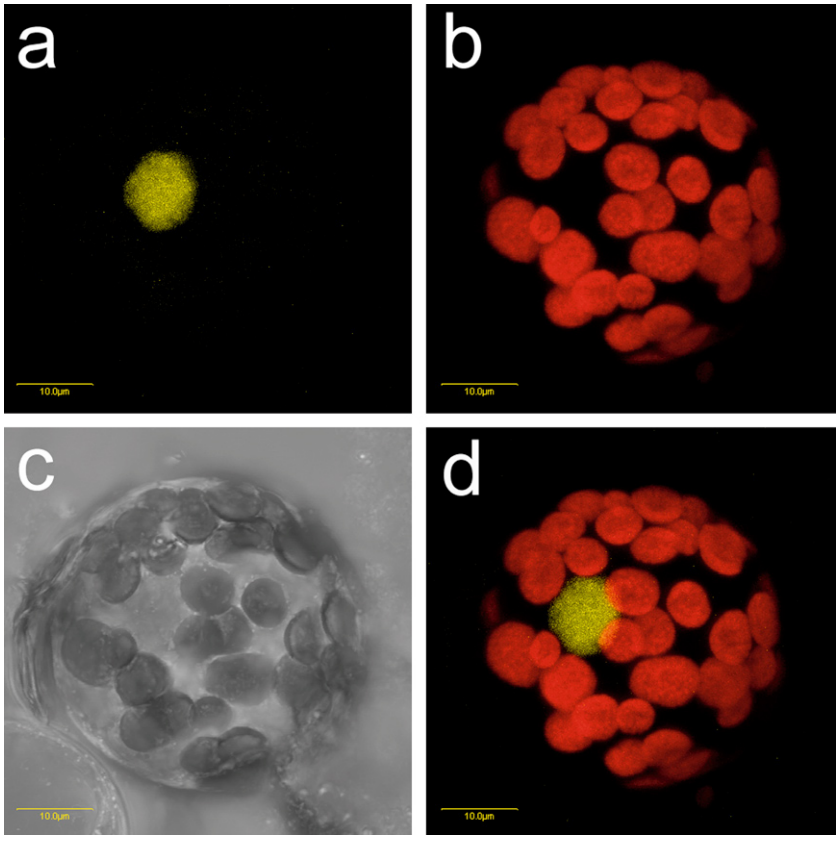


**Fig. 5.** TYLCV CP interacts with tomato importin α (LeKAPα1) in the nucleus of tobacco protoplasts. protein–protein interaction and subcellular localization were assayed by BiFC between cEYFP-tagged CP and nEYFP-tagged LeKAPα1. (a) EYFP signal, (b) plastid autofluorescence, (c) phase image, (d) merged YFP and plastid autofluorescence signals. All fluorescence images are projections of several confocal sections. Scale bar = 10 μm.

by one method or another and can be imaged by confocal or epifluorescence microscopy. Indeed, a wide range of other tissue and cell types (e.g. BY2 cell lines, trichomes and even whole seedling tissues) and other plant species (e.g. barley, mustard and parsley) have been used for BiFC analysis of various protein interactions (Table 1). The choice of tissue and cell type should be made according to the type of interactions, their expected localization and their biological context. For example, an aggregated subnuclear reconstructed BiFC signal was observed during the analysis of TYLCV CP's self-association in tomato trichome cells [40]. While the precise biological relevance of this aggregation is still unknown, it was not observed in a BiFC assay of TYLCV CP self-association in tomato mesophyll cells or tobacco protoplasts [40]. We thus suggest that while initial analysis of protein–protein interactions by BiFC assay can be performed using onion, tobacco leaves or tobacco protoplasts, additional cell lines and tissues should be adopted to allow for more accurate and biologically relevant results. We should also note that the BiFC assay has been found instrumental for analyzing protein–protein interactions in *Arabidopsis* (Table 1) and that the pSAT-BiFC vector system is useful for both transient and stable transformation of this important model organism.

## 4. Reference markers for subcellular localization

One of the clear advantages of using the BiFC assay over other methods for the detection of protein–protein interactions in living cells is the ability it affords to localize the interacting proteins to specific subcellular structures and organelles. While certain organelles and subcellular compartments are clearly visible under natural conditions using light and/or confocal microscopy, it is sometimes useful to have an internal reference marker which can assist in accurately localizing the BiFC reconstructed signal. Furthermore, because transformation efficiency of many plant models and tissues (including onion and tobacco leaves) is still relatively low in comparison to other experimental models and systems (e.g. mammalian, yeast and *Escherichia coli* cells), internal reference markers can also be useful in the identification of transfected cells when searching for BiFC-derived signals. For example, mounting the expression cassette for a RFP-tagged reference gene (Fig. 4) together with EYFP-fragment-tagged ChrD proteins on a single plasmid was found instrumental not only for the proper localization of the interacting partners, but also during the initial screening of the transformed cells [40]. Realizing the importance of reference markers as a tool for enhancing the BiFC assay in plant species, Lee and Gelvin [Ref. 40 and personal communication] developed a series of pSAT-compatible plasmids which express cDNAs and genes encoding various marker proteins fused to mRFP (e.g. mitochondrion, plasma membrane, nuclear and trans-golgi markers). The vectors were based on the pSAT6 backbone expressing the reference markers under the 35S promoter and are compatible with the pSAT-BiFC vector system; as such, they are expected to facilitate the use of the pSAT-BiFC system for the detection and localization of protein–protein interactions in various subcellular compartments in a variety of plant species, tissues and cell types. In addition, we successfully used untagged DsRed2 [e.g. Ref. 27] and CFP [e.g. Ref. 40] to localize a nuclear-specific BiFC signal in live onion, tobacco and *Arabidopsis* plants. Both markers are available as part of the extended pSAT family of plasmids [46,55], can be used for N- or C-tag fusion of target marker genes and can potentially extend the number and type of markers available for analyzing protein–protein interactions using the pSAT-BiFC vector system.

Chemical dyes, brightfield imaging and the cell's own autofluorescence can also be used during the analysis of protein–protein interactions in certain subcellular compartments and organelles. For example, cytoplasmic and/or nuclear interactions can be visualized relatively easily by epifluorescence microscopy in leaves, protoplasts, BY2 cells and other tissues and cell types without the addition of special dyes and/or internal reference markers. This was demonstrated by using a combination of confocal and brightfield microscopy during the detection of TYLCV CP interactions with LeKAP$\alpha$1 in tobacco protoplasts (Fig. 5) or during the detection of TYLCV CP self-association in tomato trichome cells [40]. Earlier, we noted that the cell's autofluorescence may interfere with acquisition of a BiFC-specific signal; however, the chloroplast's autofluorescence can also serve as an excellent marker for colocalizing a chloroplast's specific BiFC reconstructed signal. For example, Fig. 4 shows that the BiFC reconstructed signal, which was derived from ChrD dimerization, could be specifically localized to the chloroplasts with the assistance of not only the mRFP-tagged internal reference gene but also the chloroplast's autofluorescent signal.

Other staining methods, which typically include the use of DNA-specific dyes (e.g. DAPI and Hoechst 33342), have been successfully used for localizing protein–protein interactions in the host cell nucleus (Table 1). We typically use freshly prepared 1 $\mu$g/$\mu$l DAPI solution (diluted in PBS) for nuclear staining in tobacco leaves and onion epidermal peels. We place a small drop of DAPI solution directly on the observed tissue, let it soak for about 10 min, blot the excess and place the dyed tissue on a microscope slide. The use of a coverslip is optional and depends on the type of objective mounted on the microscope. We found water-immersion objectives to be most useful for imaging both leaf and tobacco epidermal cells as they allow for simple and fast acquisition of the image in confocal mode. Naturally, the microscope should be equipped with the proper laser line for DAPI (or Hoechst 33342) excitation and/or compatible emission filters to allow proper imaging of both the reconstructed YFP signal and the DAPI staining signal.

## 5. Special considerations for the use of BiFC analysis

The simplicity of the BiFC assay and the availability of various plasmid collections for use in plant cells [e.g. Refs. 31,32,40] have led to wide application of this system for the monitoring of protein–protein interactions in plant cells (Table 1). Nevertheless, certain aspects should be considered when planning and performing BiFC analysis in plants (or in any other organism, for that matter). Many of the requirements, advantages and possible potential pitfalls of BiFC are mentioned above and have been the subject of several excellent reviews [e.g. Refs. 34,35]. Thus, for example, the BiFC reconstructed signal may vary not only between different interacting proteins, but also for a given pair of proteins when different fusion schemes are used. The use of different fusion combinations (Fig. 2) is therefore suggested in order to achieve optimal signal levels [34,35]. In another example, Bracha-Drori et al. [32] suggested that insufficient flexibility between the split YFP fragments and the fused proteins was the reason for the low fluorescence observed during the analysis of protein–protein interactions in their BiFC vector system. Indeed, it has been suggested that the split fluorescent protein fragments must have sufficient freedom to enable reconstruction of the three-dimensional structure needed for an active fluorescent signal [34]. While the lack of detectable levels of BiFC signal can potentially be overcome by empirical analysis using various fusion combinations and different lengths and types of linkers [34], the occurrence of a non-specific signal poses a greater challenge for the application of the BiFC assay in living cells. More specifically, it has been suggested that high-level expression of fusion proteins tagged with fluorescent protein fragments may associate with each other and produce a fluorescence signal independent of their interaction characteristics [reviewed in Ref. 34]. Indeed, non-specific association of YFP fragments with non-interacting fusion partners was reported to lead to background fluorescence [31] and in their analysis, Caplan et al. [37] mentioned that expressing an interacting partner from a strong, constitutive promoter can result

in a non-specific BiFC signal. One possible solution to determining the specificity of BiFC is mutational analysis of the interacting proteins [34]. In this approach, the intensities produced by interacting partners are compared with those produced by mutated partners, with the assistance of an internal reference marker (e.g. CFP). This approach requires that the expression of both wild-type and mutated proteins be at the same levels. While this method may be difficult to implement in plant systems by co-transformation of three independent plasmids, the ability to mount BiFC and reference marker expression cassettes onto a single transformation vector [40] may facilitate its use for plant research. It should be noted that the use of low expression levels (i.e. the use of promoters other than the constitutive, high-expressing CaMV 35S promoter) has also been suggested as a tool to lessen the problem of non-specific interactions [31,35]. Indeed, Caplan et al. [37], for example, avoided the use of the 35S promoter by replacing it with a relatively weak one to drive the expression of one of the citrine-fragment-tagged interacting partners, while performing BiFC analysis in *N. benthamiana* leaves. While the pSAT-BiFC vector system was originally constructed with the tandem 35S promoter on each EYFP fragment-expressing cassette, its design allows for the simple replacement of promoter and terminator regions (Fig. 3). Such replacements are not likely to affect the vector's main features (i.e. the ability to construct a multi-gene transformation vector) and thus make the pSAT-BiFC an excellent choice for those who wish to modify this system for BiFC analysis of their selected proteins under low levels of expression.

## 6. Future prospects and further development of the pSAT-BiFC vector system

Herein we describe the concepts and features of our modular pSAT-BiFC vector system while emphasizing its strength in providing the user with a versatile collection of small, high-copy, basic plasmids. We also demonstrate the uniqueness of the pSAT-BiFC vector system compared to other such systems in the way that it enables mounting several expression cassettes onto a single plasmid and coupling the BiFC analysis with the expression of internal reference genes. We should note, however, that expression of the EYFP-fragment-tagged proteins and the internal reference genes are all under the control of the same high-expression-level tandem 35S promoter which is coupled with the TL enhancer element from tobacco etch virus. In certain cases, tighter regulation and lower expression levels may be needed for clearer interpretation of the reconstructed BiFC signal [e.g. Ref. 37]. Thus, while the basic design of the pSAT-BiFC vector system [and any other pSAT vector, for that matter, Ref. 70] allows for simple replacement of the promoter and terminator regions, it needs to be further developed to support low-level, tissue-specific and inducible expression patterns in order to increase its versatility and flexibility for plant cell research.

The ability to construct *Agrobacterium* binary plasmids for BiFC analysis is an important feature of our and other BiFC vector systems [e.g. Ref. 31,32]. Nevertheless, the low-copy number of pPZP-based binary plasmids [54] makes them less efficient for use in bombardment, electroporation and PEG-mediated genetic transformation experiments, where large quantities of plasmid are typically required for each experiment. We have already constructed modified binary vector backbones in which a second, high-copy origin of replication is introduced into their T-DNA region. These vectors, designated pPZP-RCS1-HC and pPZP-RCS2-HC, allow for high DNA yield from *E. coli* cells and their use is expected to simplify the application of multi-gene vectors for BiFC analysis using physical transformation methods.

An important modification that is missing from the pSAT-BiFC vectors is their conversion to the Gateway universal cloning system of Invitrogen. Such a conversion would facilitate the assembly of pSAT-BiFC-based cDNA libraries and could potentially be used for large-scale screening of protein–protein interactions in protoplasts and using automated fluorescent cell-sorting machines. Work towards conversion of the entire collection of pSAT-BiFC vectors is already underway using the single-step conversion method of Chakrabarty et al. [53] which they used for the conversion of the pSAT-AFP vector system to the pSITE, Gateway-compatible vector system.

Finally, we are currently expanding the pSAT-BiFC vector system to include additional fluorescent fragments which have proven useful for the detection of protein–protein interactions in living cells (e.g. citrine, ECFP and mRFP). We are also investigating the application of a multi-color BiFC assay [21] for the visualization of multi-protein complexes in plant cells and plan to provide additional pSAT-BiFC-compatible vectors for the plant research community in the near future.

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