Chapter 11

Bimolecular Fluorescence Complementation for Imaging Protein Interactions in Plant Hosts of Microbial Pathogens

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Abstract

Protein–protein interactions mediate many aspects of cellular function. Scientists have developed numerous techniques to investigate these interactions, both in vitro and in vivo. Among these, the peptide complementation assay Bimolecular Fluorescence Complementation (BiFC) allows visualization of the subcellular sites of protein–protein interactions in living cells. BiFC comprises a "split GFP" system: GFP protein (or its derivatives) is split into two fragments, neither of which fluoresces on its own. Interacting proteins linked to these peptide fragments may bring them into proximity, allowing them to refold and restore fluorescence. Although this system was first exploited for use in animal cells, we have developed BiFC for use in plants. Pathogens transfer numerous effector proteins into eukaryotic cells and manipulate host cellular processes through interactions between effector and host proteins. BiFC can therefore facilitate studies of host–bacterial interactions. In this chapter, we describe the numerous BiFC vectors we have constructed, their uses, and their limitations.

Key words Fluorescence imaging, Peptide complementation, Fluorescent proteins, Subcellular localization, Plant vectors

1 Introduction

Protein function is often mediated through formation of transient or stable complexes with other proteins. Some proteins, such as kinases, phosphatases, F-box proteins, ubiquitin ligases, etc., associate transiently with their targets to effect protein modifications. Some enzymes must be activated by posttranslational modifications catalyzed by proteins with which they interact. Many proteins interact with numerous partners to form complexes necessary for function. Finally, many proteins, including translocated bacterial effector proteins, must interact with other proteins for proper intracellular localization. Thus, investigations of protein–protein interactions are essential to understand cellular functions and to gain a better understanding of host–pathogen interactions at the protein level.

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Scientists have developed numerous techniques to investigate protein–protein interactions. Several of these function in vitro using purified proteins or cellular extracts. These include structural studies on co-crystallized proteins, co-immunoprecipitation, "pull-down" assays using tagged proteins, and "far-western" blotting or gel overlay assays. Other "quasi-in vivo" assays use surrogate hosts to investigate protein–protein interactions. These include yeast and bacterial two-hybrid systems. Additional in vivo assays use the natural host in which particular proteins are normally produced. The advantages of these latter assays are that the proteins are made (and modified) in their natural environments, and one can often follow subcellular localization and trafficking of protein complexes. The disadvantage of these techniques is that they are conducted in a complex milieu, and that protein–protein interactions can be indirect, reflecting complexes with "bridging" molecules.

In vivo protein–protein interaction techniques include co-immunoprecipitation, tandem affinity purification of complexes (TAP tagging), Förster resonance energy transfer (FRET), and bioluminescence resonance energy transfer (BRET). Recently, peptide complementation assays using split luciferase, GFP, or RFP molecules, or their derivatives, have become popular [1–8]. Split GFP/RFP assays are relatively easy to conduct and, unlike several other techniques, do not require specialized equipment or computer algorithms other than a fluorescence or confocal microscope.

Bimolecular fluorescence complementation (BiFC) is a peptide complementation assay in which complementing fluorescent protein fragments, neither of which fluoresces on its own, are brought together in such a way that they can fold and restore fluorescence. In practice, this is accomplished by individually placing each of the two partial fluorescent protein fragments, as translational fusions, onto other proteins of interest. If the two proteins of interest interact, they may bring together the complementing fluorescent protein fragments, and fluorescence may be restored (Fig. 1). Scientists first developed BiFC in animal systems [1, 9], but quickly adapted it for use in bacterial [10, 11] and plant species [12–15]. In addition to indicating protein–protein interactions, BiFC can be used to localize the subcellular site of interaction [14, 16, 17]. The inclusion of alternatively colored fluorescent organelle markers helps identify these sites [18]. For reviews of BiFC, *see* [19–23]. Although BiFC can be practiced using proteins derived from DsRed (e.g., 3, 6–8), GFP derivatives form the basis of most BiFC systems. Several of the initially described systems used the enhanced Yellow Fluorescent Protein (EYFP), whereas later systems employed the brighter and more stable yellow derivative Venus [24]. Similarly, the brighter blue fluorescent protein Cerulean augmented the original blue Cyan Fluorescent Protein (CFP) [17, 25–27]. However, there are advantages and disadvantages of using each fluorescent protein. Scientists need to be open-minded in order to

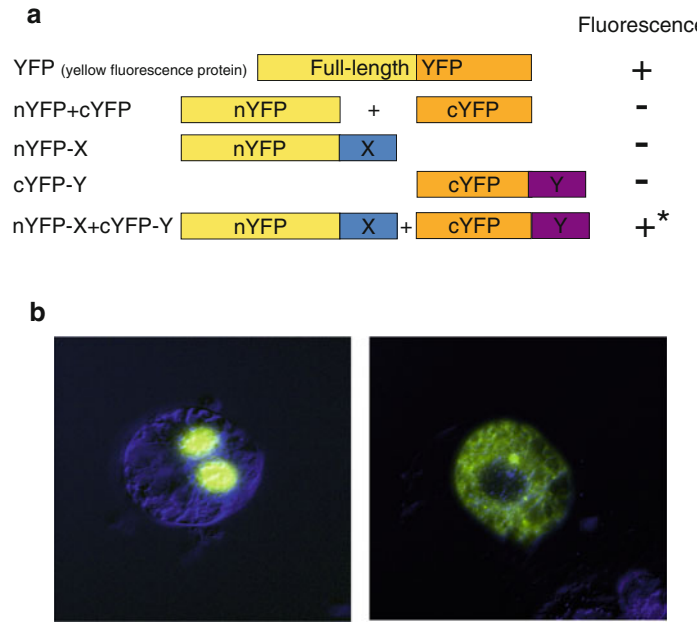

Fig. 1 (**a**) Schematic diagram of the BiFC process. A fluorescent protein (YFP is shown as an example) is split; one part is affixed to protein X, and the other to protein Y. If proteins X and Y interact, they may bring the two portions of YFP together such they will fold correctly and restore fluorescence (*asterisk*). (**b**) Examples of BiFC in tobacco BY-2 protoplasts. These are merged bright field images (pseudocolored in *blue*) with YFP fluorescence images (*yellow*). *Left panel*, interaction of *Agrobacterium* VirD2 protein with the *Arabidopsis* importin-α protein AtImpa-1. Note the *yellow fluorescence* signal in the nuclei; *right panel*, interaction of *Agrobacterium* VirE2 protein with VirE2. Note the *yellow fluorescence* signal in the cytoplasm. nYFP, N-terminal fragment of YFP; cYFP, C-terminal fragment of YFP

choose the best candidate with which to work. Table 1 presents information regarding the fluorescence characteristics and imaging of these proteins. The "split" in these 238 amino acid derivatives can be in one of several places. Initial systems used fragments split between amino acids 154 and 155, whereas more recent systems utilize splits between amino acids 173 and 174. An interesting variant of these systems employs an "overlap" of fluorescent protein fragments: the N-terminal fragment extends to amino acid 173, whereas the C-terminal fragment initiates at amino acid 155. This combination results in brighter fluorescence complementation [17, 25].

Several excellent BiFC systems exist for use in plants [8, 12, 13, 15, 27]. Below, we describe a system developed in our laboratory [14, 17] that we have used to show subcellular localization of several *Agrobacterium tumefaciens* virulence effector proteins that are translocated by the bacterial type IV secretion system into host

Table 1
Characteristics of fluorescent proteins used for BiFC

Protein	Excitation (nm)	Emission (nm)	Brightness[a]	Photostability[b]	p*K*a	Oligomerization
EGFP	488	507	34[c]	174	6.0	Weak dimer
EYFP	514	527	51[c]	60	6.9	Weak dimer
Venus	515	528	53[c]	15	6.0	Weak dimer
ECFP	433	475	18[c]	59	5.0	Weak dimer
Cerulean	433	475	27[c]	36	4.7	Weak dimer
DsRed	558	583	41[d]	16 (for monomer)	4.7 (4.7)	Tetramer
				Maturation rate at 37 °C: 9.9 h		
mRFP1	584	607	6[d]	9	4.5	Monomer
				Maturation rate at 37 °C: 0.2 h		
mRFP1-Q66T	549	570	16[d]	>9	7.5	Monomer
				Maturation rate at 37 °C: 0.6 h		
mCherry	587	610	16[c,d]	96	<4.5	Monomer
				Maturation rate at 37 °C: 0.25 h		

[a]Brightness values are the product of the extinction coefficient and quantum yield at pH 7.4 under maturation conditions, in mM^{-1} cm^{-1}. For comparison, free fluorescein at pH 7.4 has a brightness of ~69 $(mM\ cm)^{-1}$
[b]Time for bleaching from an initial emission rate of 1,000 photons/s down to 500 photons/s ($t_{1/2}$; for comparison, fluorescein at pH8.4 has $t_{1/2}$ of 5.2 s). Data for photostability are from [37]. Data for maturation rate are from [3, 38]
[c]Data are from [37]
[d]Data are from [3, 37, 38]

cells, and their interactions with host proteins. We give a step-by-step description of the transfection of tobacco BY2 cells, and the analysis of BiFC interactions using fluorescence microscopy. The Notes section contains much additional information on the methodology that will aid the reader in conducting research using BiFC.

2 Materials

1. pSAT-derived plant BiFC vectors (Table 2 describes the current BiFC vectors from our laboratory (*see* **Note 1**).
2. BY-2 medium: 4.3 g Murashige and Skoog (MS) salts, 1 mg vitamin B1, 370 mg KH_2PO_4, 2 mg 2,4-dichloro-phenoxyacetic acid (2,4-D), and 30 g sucrose in 950 ml water. Adjust pH to

Table 2
Fluorescent protein tagging vectors

Gelvin lab stock number	Plasmid name	Expression cassette flanking sites	Reference
BiFC Vectors contain a CaMV double 35S promoter and TEV translation enhancer, except when indicated, a nopaline synthase (nos) promoter is used			
nVenus-C			
E3228	pSAT1-nVenus-C	*Asc*I	[17]
E3229	pSAT4-nVenus-C	I-*Sce*I	[17]
E3230	pSAT6 -nVenus-C	PI-*Psp*I	[17]
nVenus-N			
E3308	pSAT1-nVenus-N	*Asc*I	[17]
E3231	pSAT1A-nVenus-N	*Asc*I	[17]
E3310	pSAT4-nVenus-N	I-*Sce*I	[17]
E3232	pSAT4A-nVenus-N	I-*Sce*I	[17]
E3233	pSAT6-nVenus-N	PI-*Psp*I	[17]
E3962	pSAT1-nVenus(155)-N	AscI	Unpublished
E3963	pSAT1-nVenus(155)I152L-N	AscI	Unpublished
cCFP-C			
E3242	pSAT1-cCFP-C	*Asc*I	[17]
E3243	pSAT4-cCFP-C	I-*Sce*I	[17]
E3244	pSAT6-cCFP-C	PI-*Psp*I	[17]
cCFP-N			
E3449	pSAT1-cCFP-N	*Asc*I	[17]
E3450	pSAT1A-cCFP-N	*Asc*I	[17]
E3451	pSAT4-cCFP-N	I-*Sce*I	[17]
E3347	pSAT4A-cCFP-N	I-*Sce*I	[17]
E3497	pSAT6-cCFP-N	PI-*Psp*I	[17]
nCerulean-C			
E3415	pSAT1-nCerulean-C	*Asc*I	[17]
E3416	pSAT4-nCerulean-C	I-*Sce*I	[17]
E3417	pSAT6-nCerulean-C	PI-*Psp*I	[17]
nCerulean-N			
E3307	pSAT1-nCerulean-N	*Asc*I	[17]
E3246	pSAT1A-nCerulean-N	*Asc*I	[17]
E3309	pSAT4-nCerulean-N	I-*Sce*I	[17]
E3247	pSAT4A-nCerulean-N	I-*Sce*I	[17]
E3248	pSAT6-nCerulean-N	PI-*Psp*I	[17]
nEYFP-C			
E3075	pSAT1-nEYFP-C1	*Asc*I	[14]
E3081	pSAT4-nEYFP-C1	I-*Sce*I	[14]
E2884	pSAT6-nEYFP-C1	PI-*Psp*I	[14]
E4054	pSAT1-Pnos-nYFP-C	*Asc*I	Unpublished

(continued)

Table 2
(continued)

Gelvin lab stock number	Plasmid name	Expression cassette flanking sites	Reference
nEYFP-N			
E3077	pSAT1-nEYFP-N1	*Asc*I	[14]
E3079	pSAT1A-nEYFP-N1	*Asc*I	[14]
E3083	pSAT4-nEYFP-N1	I-*Sce*I	[14]
E3085	pSAT4A-nEYFP-N1	I-*Sce*I	[14]
E2913	pSAT6-nEYFP-N1	PI-*Psp*I	[14]
E3960	pSAT1-nEYFP(155)-N	AscI	Unpublished
E3961	pSAT1-nEYFP(155)I152L-N	AscI	Unpublished
E4053	pSAT1-Pnos-nYFP-N	*Asc*I	Unpublished
cEYFP-C			
E3076	pSAT1-cEYFP-C1(B)	*Asc*I	[14]
E3082	pSAT4-cEYFP-C1(B)	I-*Sce*I	[14]
E3108	pSAT6-cEYFP-C1	PI-*Psp*I	[14]
E4020	pSAT1-Pnos-cEYFP-C	*AscI*	Unpublished
cEYFP-N			
E3078	pSAT1-cEYFP-N1	*Asc*I	[14]
E3080	pSAT1A-cEYFP-N1	*Asc*I	[14]
E3084	pSAT4-cEYFP-N1	I-*Sce*I	[14]
E3323	pSAT4A-cEYFP-N1	I-*Sce*I	[14]
E2914	pSAT6-cEYFP-N1	PI-*Psp*I	[14]
E3086	pSAT6A-cEYFP-N1	PI-*Psp*I	Unpublished
E4023	pSAT1-Pnos-cEYFP-N	*AscI*	Unpublished
cCFP′-C[a]			
E3596	pSAT1-cCFP′-C	*Asc*I	Unpublished
cCFP′-N[a]			
E3595	pSAT1A-cCFP′-N	*Asc*I	Unpublished
BiFC vectors with nopaline synthase (Pnos) promoter			
E3683	pSAT5-Pnos-cCFP-C	I-*Ceu*I	Unpublished
E3685	pSAT5-Pnos-cCFP-N	I-*Ceu*I	Unpublished
E3684	pSAT5-Pnos-nVenus-C	I-*Ceu*I	Unpublished
E3686	pSAT5-Pnos-nVenus-N	I-*Ceu*I	Unpublished
BiFC vectors with peptide tags			
T7-nVenus-C			
E3454	pSAT1-T7-nVenus-C	*Asc*I	Unpublished
E3455	pSAT4-T7-nVenus-C	I-*Sce*I	Unpublished
E3456	pSAT6-T7-nVenus-C	PI-*Psp*I	Unpublished
nVenus-T7-N			
E3727	pSAT1A-nVenus-T7-N	*Asc*I	Unpublished
E3726	pSAT4A-nVenus-T7-N	I-*Sce*I	Unpublished
E3728	pSAT6-nVenus-T7-N	PI-*Psp*I	Unpublished
T7-nCerulean-C			
E3723	pSAT1-T7-nCerulean-C	*Asc*I	Unpublished
E3724	pSAT4- T7-nCerulean-C	I-*Sce*I	Unpublished
E3725	pSAT6- T7-nCerulean-C	PI-*Psp*I	Unpublished

(continued)

Table 2
(continued)

Gelvin lab stock number	Plasmid name	Expression cassette flanking sites	Reference
myc-nCerulean-N			
E3734	pSAT1A-nCerulean-myc-N	*Asc*I	Unpublished
E3453	pSAT4A-nCerulean-myc-N	I-*Sce*I	Unpublished
Rare cloning sites (RCS) on high copy number plasmid			
E3074	pBluescript, ampicillin resistant	–	Unpublished
E3414	pUC119, ampicillin resistant	–	[17]
E3610	pRCIII, kanamycin resistant		Unpublished
Rare cloning sites (RCS) on T-DNA binary vectors			
E3185	*hpt*II for plant selection	–	Unpublished
E3184	*npt*II for plant selection	–	Unpublished
E3407	*bar* for plant selection	–	Unpublished
E3055	*bar* for plant selection	–	[17]
E3519	*bar* for plant selection	–	Unpublished
E4082	*hpt*II for plant selection	–	Unpublished
E4085	*npt*II for plant selection	–	Unpublished
E4145	*bar* for plant selection	–	Unpublished
Full-length fluorescent protein tagging vectors			
Cerulean-C			
E3528	pSAT1-Cerulean-C	*Asc*I	Unpublished
E3529	pSAT4-Cerulean-C	I-*Sce*I	Unpublished
E3530	pSAT6-Cerulean-C	PI-*Psp*I	Unpublished
Cerulean-N			
E3534	pSAT4A-Cerulean-N	I-*Sce*I	Unpublished
Venus-C			
E3542	pSAT1-Venus-C	*Asc*I	Unpublished
E3543	pSAT4-Venus-C	I-*Sce*I	Unpublished
E3544	pSAT6-Venus-C	PI-*Psp*I	Unpublished
E4041	pSAT1-Pnos-Venus-C	*Asc*I	Unpublished
Venus-N			
E3758	pSAT1-Venus-N	*Asc*I	Unpublished
E3533	pSAT4A-Venus-N	I-*Sce*I	Unpublished
E4042	pSAT1-Pnos-Venus-N	*Asc*I	Unpublished
EYFP-C			
E3150	pSAT6-EYFP-C1	PI-*Psp*I	Unpublished
EYFP-N			
E3225	pSAT6-EYFP-N1	PI-*Psp*I	Unpublished
mRFP-C			
E3026	pSAT6-mRFP-C1	PI-*Psp*I	[17]
mRFP-N			
E3025	pSAT6-mRFP-N1	PI-*Psp*I	[17]
mCherry-C			
E3275	pSAT6-mCherry-C	PI-*Psp*I	Unpublished
mCherry-N			
E3279	pSAT4A-mCherry-N	I-*Sce*I	Unpublished

[a]Mutant cCFP to diminish dimerization

5.7 using 1 N KOH. Top up with water to 1 L. Aliquot 50 ml into each of 20 250-ml flasks. Seal flasks with aluminum foil and autoclave the medium at 250 °F for 20 min.

3. Protoplast isolation solution: 7.4 g $CaCl_2 \cdot 2H_2O$, 1.6 g $NaOAc \cdot 3H_2O$, and 45 g mannitol in 950 ml water. Adjust pH to 5.7 using 1 N KOH. Top up with water to 1 L. Sterilize by autoclaving at 250 °F for 20 min.
4. Protoplast enzyme digestion solution: 0.48 g of Cellulase R10 (Onazuka, 1.2 %) and 0.24 g of Macerozyme (0.6 %) in 35 ml water. Stir until most of the powder is dissolved, adjust pH to 5.7 using 0.1 N HCl, and add water to make up to 40 ml. This solution needs to be prepared fresh immediately before use. Transfer the solution into a 50-ml conical tube, centrifuge the solution at $1600 \times g$ for 10 min to pellet the insoluble substances. Sterilize the clear supernatant fluid by passing the solution through a 0.2 μm syringe filter into a sterile tube.
5. Protoplast floating solution: 99 mg myo-inositol, 2.88 g L-proline, 100 mg enzymatic casein hydrolysate, 102.6 g sucrose, 97.6 mg MES, 4.3 g MS salts, 1 mg vitamin B1, 370 mg KH_2PO_4 in 950 ml water. Adjust the pH to 5.7 using 1 N KOH. Top up with water to 1 L. Filter-sterilize the solution using a 0.2 μm sterile filter unit (e.g., Nalgene or Millipore).
6. 40 % PEG solution (1 ml): 0.4 g of PEG4000 (Fluka), 0.5 ml of 0.8 M mannitol, 0.1 ml of 1 M $CaCl_2$, and 0.05 ml water. Warm the tube in a 55 °C water bath and vortex the solution from time to time to help the PEG dissolve completely. Always prepare this solution fresh immediately before use. Depending on the number of samples in your experiment, you may need to scale up the quantity of this solution by increasing all components proportionally.
7. W5 solution: 154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl, 2 mM MES, pH 5.7. The solution can be sterilized by autoclaving. Store the solution refrigerated (*see* **Note 2**).
8. MMg solution: 0.4 M mannitol, 15 mM $MgCl_2$, 4 mM MES, pH 5.7. Filter-sterilize using a 0.2 μm filter. Keep the solution refrigerated all the time (*see* **Note 2**).
9. Incubation solution: Same as preparation of BY-2 medium except add 72.9 g mannitol when making 1 L of BY-2 medium.
10. 1 % BSA (bovine serum albumin).
11. Shaker with clips for 250-ml flask, operate at room temperature.
12. Tabletop low-speed centrifuge with swing-bucket rotor.
13. Sterile 50 ml conical centrifuge.
14. 12 × 75 mm polypropylene tube.
15. 20 × 100 mm sterile plastic petri dishes.

16. Parafilm.
17. Aluminum foil.
18. Rotary shaker, operate at room temperature.
19. Inverted light microscope.
20. Glass slides and coverslips.
21. Hemocytometer.
22. BY-2 cells.
23. Epi-fluorescence microscope or confocal microscope equipped with filters for YFP, CFP, and RFP and 20× and 40× objective lens.
24. 24-well tissue culture plates.

3 Methods

3.1 Cloning Using pSAT-Derived Plant BiFC Vectors

1. Choose the appropriate vector (*see* **Notes 3–6**, Table 1 and Fig. 2).
2. Generate fusions of proteins of interest with fluorescent protein fragments using standard recombinant DNA techniques (*see* **Notes 7** and **8**).
3. Optional: Combine multiple BiFC expression cassettes into one vector using standard recombinant DNA techniques (*see* **Notes 9–12**) [17, 28]. Figure 3 shows maps of these vectors.

3.2 Tobacco BY-2 Protoplast Preparation

1. Tobacco BY-2 suspension cultures are maintained in BY-2 medium. Every 7 days, transfer 1.2 ml of BY-2 cells into 50 ml of fresh BY-2 medium in a 250-ml flask. Shake the cells on a shaker at 140 rpm at room temperature for 4–5 days before use (*see* **Note 13**).
2. Using sterile technique, transfer 20 ml of cells into a 50 ml sterile conical centrifuge tube, cap the tube, and centrifuge the cells at 190 × *g* for 5 min at room temperature.
3. With a sterile pipette, gently remove the supernatant solution (being careful not to disturb the rather loose pellet), and suspend the cells in 40 ml of Protoplast Enzyme Digestion Solution.
4. Transfer the suspended cells into two 20 × 100 mm sterile plastic petri dishes, tape the dishes with Parafilm, cover the dishes with aluminum foil and place them on a rotary shaker with gentle shaking (60 rpm) for about 4 h at room temperature (*see* **Note 14**).
5. To purify protoplasts away from non-protoplasted cells and cellular debris, collect digested cells in a sterile 50 ml conical tube. Cap the tube and centrifuge at 190 × *g* for 5 min.

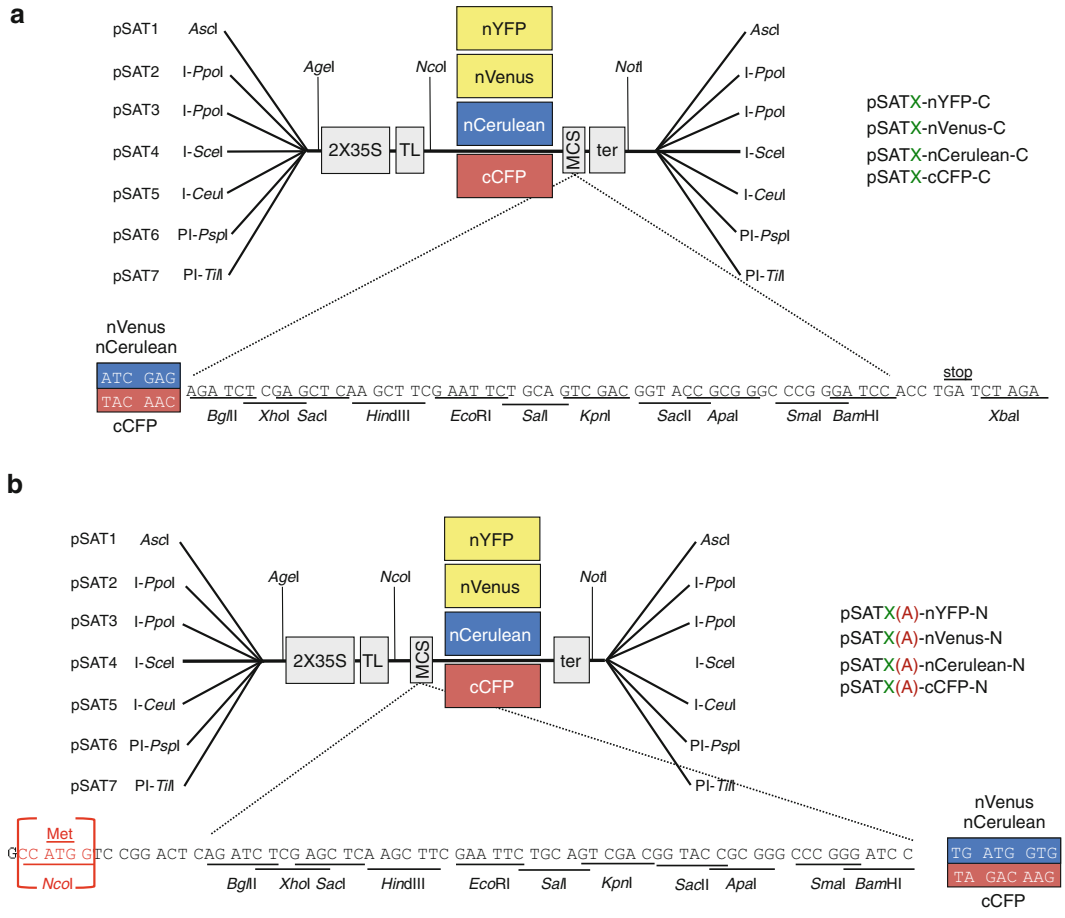

Fig. 2 Maps of the various vectors used for tagging proteins with split GFP derivatives. Protein reading frames are indicated by the indicated three-nucleotide codons. (**a**) Vectors for use in tagging proteins at their N-termini (i.e., the protein of interest has an N-terminal tag). (**b**) Vectors for use in tagging proteins at their C-termini (i.e., the protein of interest has a C-terminal tag). Note that in vectors denoted by pSATX(A), the *Nco*I site (depicted in brackets) upstream of the multiple cloning site has been deleted, resulting in loss of the upstream methionine codon

Fig. 3 (continued) resistance upon bacteria. *Arrows* indicate the known direction of transcription. The direction of transcription of the plant selection markers in E3184 and E3185 is not yet determined. LB/RB, T-DNA left/right border sequences; P, plant-active promoter; Term, polyA addition signal sequence; *ocs*, octopine synthase; *nptII*, neomycin phosphotransferase II gene conferring resistance to kanamycin; *hptII*, hygromycin phosphotransferase II gene conferring resistance to hygromycin; *bar*, gene conferring resistance to the herbicides Basta/bialophos/phosphinothricin. "E" numbers indicate strain numbers in the Gelvin laboratory *E. coli* stock collection. Sites marked in *black* are unique. Unique rare-cutting sites are marked in *red*. Sites that are present but are not unique are marked in *blue*

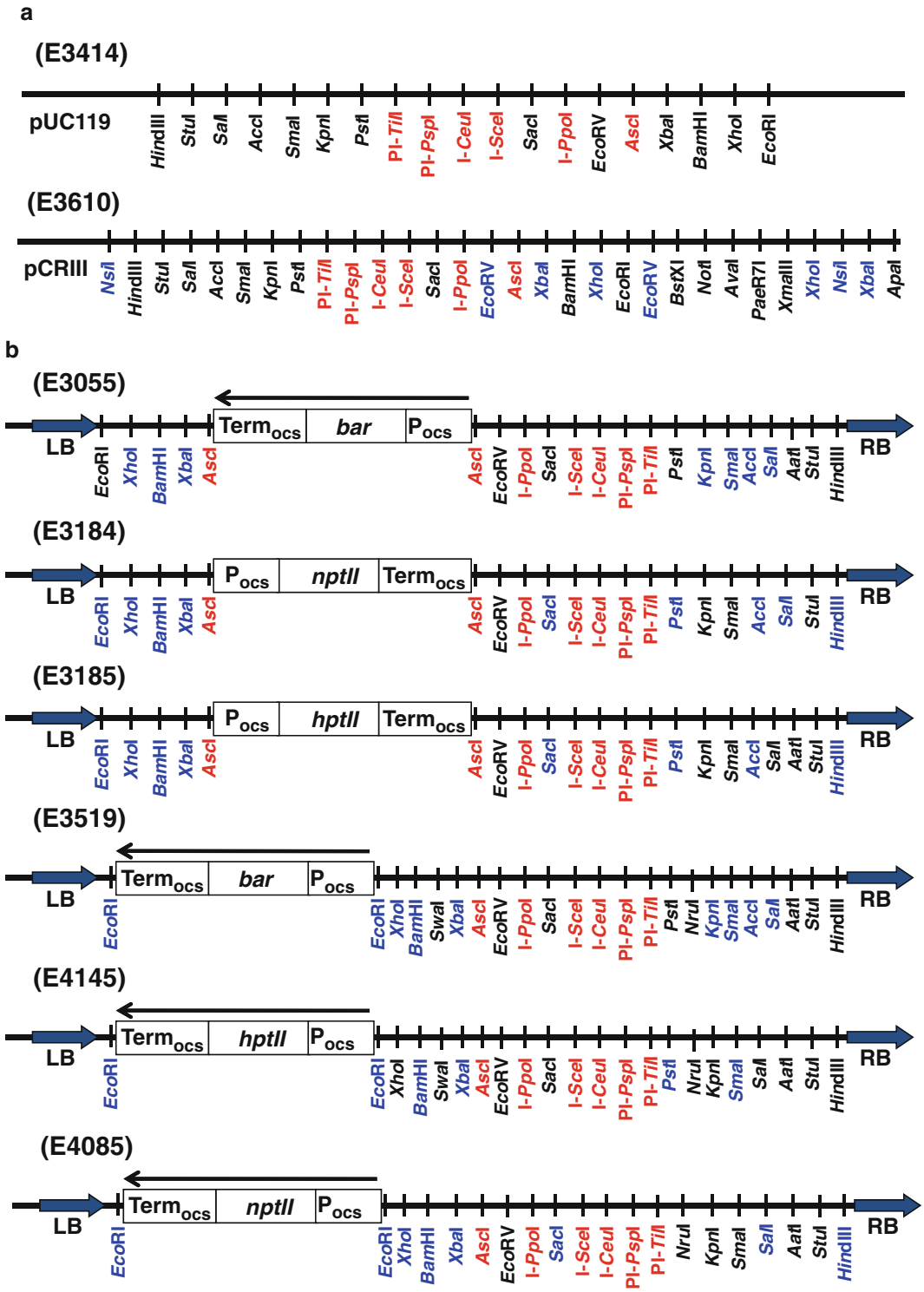

Fig. 3 Restriction/homing endonuclease maps of the "final vectors" used to clone multiple BiFC expression cassettes. (**a**) High copy number vectors based on pUC119 (ampicillin-resistant) and pCRIII (kanamycin-resistant) plasmids. (**b**) T-DNA binary vectors. These binary vectors contain an *aadA* gene conferring spectinomycin

After centrifugation, gently remove the supernatant solution with a sterile pipet.

6. Add 40 ml Protoplast Isolation Solution, cap the tube, and suspend the protoplasts by "rocking" the tube (*see* **Note 15**) gently. Centrifuge the cells at 190 × *g* for 5 min. Again, gently remove the supernatant solution and suspend the protoplasts *gently* in 40 ml of Protoplast Isolation Solution. Cap the tube and centrifuge again 190 × *g* for 5 min. Discard the supernatant solution after centrifugation.
7. Suspend the protoplasts in 45 ml of Protoplast Floating Solution. Cap the tube and centrifuge the cells at 190 × *g* for 10 min. In this solution, the protoplasts will float to the top of the tube, while any non-protoplasted cells will pellet.
8. Gently remove the floating protoplasts with a sterile, cut-end Pipetman P1000 tip and transfer to a sterile 50 ml conical tube.
9. Add 30 ml of W5 Solution and suspend the cells gently. Cap the tube and centrifuge at 190 × *g* for 5 min. The protoplasts will pellet in this solution. Gently remove the supernatant solution.
10. Gently resuspend the protoplasts in 40 ml of W5 Solution. Centrifuge at 190 × *g* for 5 min. Gently remove all *except* 10 ml of the supernatant solution. Gently resuspend the protoplasts in this 10 ml of solution.
11. Make 1:10 and 1:100 dilutions of a small sample of protoplasts in W5 solution, and count the cells using a hemocytometer. Keep protoplasts on ice for at least 30 min before using them.

3.3 PEG-Mediated Transfection of BY-2 Protoplasts

1. Coat 24-well tissue culture plates by adding 0.8 ml of 1 % BSA in each well to avoid protoplast attachment to the bottom of the wells. Leave the plate at room temperature for 30 min.
2. Prepare the 40 % PEG solution.
3. Centrifuge the protoplast suspension at 190 × *g* for 3 min. Remove the supernatant solution and add an appropriate volume of cold MMg solution to adjust the cell density to 1×10^6 cells/ml. Keep the tube on ice.
4. Mix all DNA samples (10 μg of each DNA sample, *see* **Note 16**) in a volume of 10 μl in a 12 × 75 mm polypropylene tube. Gently add 100 μl of protoplasts to the tube and mix well by gently tapping the tube with your fingers.
5. Add 110 μl of 40 % PEG solution to the tube and mix with the cells gently but thoroughly by tapping the tube with your fingers. Leave the transfection reaction at room temperature for 5 min.
6. After 5 min, add 2 ml of W5 solution to the tube and mix thoroughly but gently to make sure the PEG is well mixed

with the W5 solution. Centrifuge the protoplast suspension at 190 × *g* for 3 min.

7. Repeat **step 6** one more time.
8. Remove the BSA solution from the 24-well plate.
9. Remove the supernatant solution from the tubes and add 0.7 ml of incubation solution to each tube. Suspend the cells gently by tapping the tube with fingers. Transfer all cells in each tube into individual wells of the plate. Incubate the plate overnight at room temperature in the dark (*see* **Note 17**).
10. The protoplasts can be used for 24 h after preparation. Just maintain the cells in W5 solution at 4 °C.

3.4 Acquire Fluorescence Images

1. Take out 20 μl of cells from each sample using a cut-end plastic tip and apply on a glass slide.
2. Place a coverslip gently on the slide and make sure there is no bubble trapped under the coverslip (*see* **Note 18**). Attach the coverslip to the slide at the four corners using nail polish.
3. Place the slide on the microscope stage to view fluorescence. Either an epi-fluorescence microscope or a confocal microscope with the proper lens, fluorescent filters, and imaging software can be used (*see* **Note 19**).

3.5 Advanced BiFC and Troubleshooting

1. Multicolor BiFC (*see* **Note 20**, and Fig. 4), BiFC combined with FRET (*see* **Note 21**), bridge-BiFC (*see* **Note 22**), interactions with peptide aptamers (*see* **Note 23**), and screening of cDNA libraries (*see* **Note 24**) are recent adaptations of the protocol.
2. **Notes 25–29** describe several limitations and problems that can arise during BiFC experiments and analysis of interactions.

4 Notes

1. Tzfira's laboratory first described the pSAT system for expressing multiple proteins in plants [28, 29]. The pSAT system consists of numerous "satellite" vectors containing expression cassettes which can be combined into one of several pUC- or T-DNA binary vector-based final vectors. These final vectors contain a "polylinker" containing multiple rare-cutting sites (RCS) described in Goderis et al. [30]. These different rare-cutting restriction or homing endonuclease sites flank the expression cassettes in each of the pSAT vector series (pSAT1-6).

   Table 2 describes the current BiFC vectors from our laboratory. The pSAT vectors are built in modular form (see Fig. 2). Promoters and terminators flank a "standard" multiple cloning

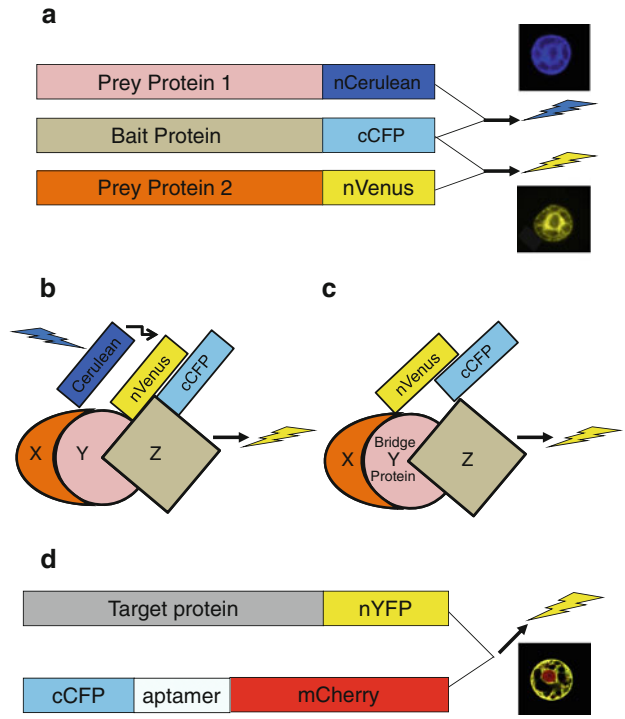

Fig. 4 Schematic diagrams depicting "advanced" uses of BiFC. (**a**) Multicolor BiFC. The "bait" protein is tagged with cCFP, and two different "prey" proteins are individually tagged with either nVenus or nCerulean. Interaction of the nVenus tagged protein with the bait protein may result in *yellow fluorescence*, whereas interaction of the nCerulean tagged protein with the bait protein results in *blue fluorescence*. (**b**) BiFC-FRET to indicate interaction of three proteins. Protein X is tagged with full-length Cerulean, and the two other proteins (Y and Z) are individually tagged with nVenus and cCFP, respectively. Excitation with *blue light* may result in Cerulean fluorescence, which may in turn excite *yellow fluorescence* from interacting proteins tagged with nVenus and cCFP. For this to occur, the three proteins must be closely aligned in a complex. (**c**) Bridge-BiFC. Two proteins, individually tagged with nVenus and cCFP, cannot interact without a "bridging" protein, which simultaneously interacts with both of them, forming a three-protein complex. Only under these conditions may fluorescence complementation occur. (**d**) Interaction of a target "bait" protein, tagged with nYFP, with a peptide aptamer tagged at the N-terminus with cCFP and at the C-terminus with full-length mCherry. If the peptide aptamer and target protein interact, this may permit folding of nYFP with cCFP, generating *yellow fluorescence*. Expression of the aptamer can be monitored by visualizing mCherry *red fluorescence*

site (mcs) composed of numerous six-base recognition restriction endonuclease sites. The promoters and terminators are, in turn, flanked by restriction endonuclease sites, allowing "switching" of these elements with other promoters or terminators. Some of the pSAT vectors additionally incorporate

full-length fluorescent protein genes, either preceding the polylinker (for N-terminal tagging of proteins) or following the polylinker (for C-terminal tagging of proteins). Users can employ these vectors to make fusion proteins and check the subcellular localization of proteins of interest. BiFC pSAT vectors contain fluorescent protein gene fragments for N- or C-terminal tagging of proteins (Fig. 2). These gene fragments encode N-terminal fragments of EYFP, Venus, or Cerulean (nEYFP, nVenus, nCerulean), or C-terminal fragments of EYFP or CFP (cEYFP, cCFP). Venus is a variant of EYFP, generated from multiple amino acid residue mutations of EYFP. This fluorescent protein has rapid and efficient maturation kinetics and is relatively less sensitive to acid and quenching by chloride ion [24] than is EYFP. Therefore, Venus enables the visualization of fluorescent fusion proteins in an acidic environment. Furthermore, the fluorescence intensity of Venus-based BiFC is about ten times higher than that of EYFP-based BiFC [25]. Thus, when using Venus in BiFC assays, less quantity of DNA is needed to ensure fluorescence visualization. On the other hand, when the high intensity fluorescent protein Venus is used in a BiFC assay, the signal-to-noise ratio will be lowered due to possible spontaneous self-assembly of the two split fluorescent protein fragments [31]. Users should be aware of this possibility and may wish to choose a lower intensity variant, such as EYFP, instead.

Although most pSAT-based BiFC vectors utilize a Cauliflower Mosaic Virus (CaMV) double 35S promoter, we have also generated a set of vectors incorporating the nopaline synthase (*nos*) promoter. This weaker promoter mitigates background fluorescence, as described below. In addition, other promoters or terminators, including those of the native gene, can replace the CaMV 35S promoter and terminator using the unique restriction sites (*Age*I and *Nco*I for promoters, *Xba*I and *Not*I for terminators) flanking these regions.

We have also added sequences encoding T7 or myc tags to a subgroup of our pSAT BiFC tagging vectors to facilitate the confirmation of protein expression.

The plasmids described in this chapter have been deposited in the ABRC stock center at The Ohio State University. The authors request that interested parties obtain them from this source.

2. For W5 solution and MMg solution, the user can make various sterile stock solutions first and combine individual components before use based on the proper compositions. These stock solutions include 0.8 M mannitol, 3 M NaCl, 0.2 M KCl, 0.1 M MES, pH 5.7, 1 M $CaCl_2$, and 1 M $MgCl_2$.

3. The pSAT vectors are distinguished by a code in the following order [14, 17]:

   The number (X) of the vector after "pSAT" identifies the rare cutting site flanking the expression cassette (Table 1 and Fig. 2); If "A" follows the vector number, this indicates that the *Nco*I site has been removed from the position preceding the mcs (in vectors used for C-terminal tagging of proteins only; see Fig. 2b).

   The fluorescent protein fragment tag; N indicates that the protein of interest is placed at the N-terminus of the fusion (i.e., the protein is tagged at its C-terminus). C indicates that the protein of interest is placed at the C-terminus of the fusion (i.e., the protein is tagged at its N-terminus).

   The properties of the target protein need to be considered when choosing the optimal BiFC tagging vector.

4. As examples:

   (a) pSAT1-nVenus-N indicates that *Asc*I sites flank the expression cassette, and that the mcs is placed such that the protein of interest will be at the N-terminus of the fusion, tagged at its C-terminus with the N-terminal fragment of Venus (nVenus). A *Nco*I site precedes the mcs; thus, because this site contains an ATG sequence, the user must be careful that this ATG is in-frame with the ATG of the introduced gene.

   (b) pSAT1A-nVenus-N indicates that *Asc*I sites flank the expression cassette, and that the mcs is placed such that the protein of interest will be at the N-terminus of the fusion, tagged at its C-terminus with the N-terminal fragment of Venus. Because there is no *Nco*I site in this vector, the user needs not worry that a "false" ATG will place the protein of interest out of frame.

   (c) pSAT1-nVenus-C indicates that *Asc*I sites flank the expression cassette, and that the mcs is placed such that the protein of interest will be at the C-terminus of the fusion, tagged at its N-terminus with the N-terminal fragment of Venus.

   (d) pSAT1-cCFP-C indicates that *Asc*I sites flank the expression cassette, and that the mcs is placed such that the protein of interest will be at the C-terminus of the fusion, tagged at its N-terminus with the C-terminal fragment of CFP.

5. For each pair of proteins to test for interaction, one must be tagged with a N-terminal fragment of a fluorescent protein, and the other protein must be tagged with a C-terminal fragment of a fluorescent protein.

6. In some situations, existing data may indicate which end of a protein can be tagged and not destroy function. The BiFC tag should thus be placed on this end of the protein. If such data are not available, both ends of the protein should individually

be tagged and tested for interaction with the second protein. In this case, the user should also test whether the tagged fusion protein still possesses its normal function.

7. To generate translational fusions of proteins of interest with fluorescent protein fragments, the user needs to add restriction sites to flank the DNA sequence encoding the open reading frame (ORF) of the protein of interest, then clone the DNA fragment into the mcs of the pSAT BiFC vector.
8. Care must be taken to make sure that the ORF of interest is in-frame with the ORF encoding the fluorescent protein fragment. To tag a protein of interest at its C-terminus (using pSAT-N vectors), the stop codon of the protein of interest must be removed to allow fusion with the ORF encoding the fluorescent protein fragment.
9. When performing BiFC analyses using transient expression in plant cells or protoplasts, one can introduce multiple separate BiFC expression cassettes on different pSAT plasmids, or one can clone all BiFC expression cassettes onto the same vector (the "final vector"). If the user eventually wishes to clone more than one expression cassette into the same final vector, each expression cassette should be flanked by different rare cutting sites. Thus, for example, one cassette should be flanked by *Asc*I sites (pSAT1/1A vectors), one with I-*Sce*I sites (pSAT4/4A vectors), and one with PI-*Psp*I sites (pSAT6 vectors), etc.
10. The user needs to ensure that the first ATG following the promoter region is in-frame with both the gene of interest and the fluorescent protein gene (Fig. 2a, b).
11. Whereas combining all BiFC expression cassettes onto the same vector guarantees that each cell will receive all expression cassettes, this entails additional cloning steps. In our experience, if multiple individual plasmids are co-introduced into protoplasts, there is a >80 % probability that a protoplast competent to take up one plasmid will also take up all additional plasmids.
12. To clone all BiFC expression cassettes into a single plasmid, cDNAs encoding each protein of interest must first be cloned into a pSAT BiFC vector containing an expression cassette surrounded by different rare cutting sites (*see* Fig. 2 and **Note 5**). Each expression cassette is subsequently released from the initial pSAT BiFC vector using the appropriate rare cutting enzyme and ligated into the corresponding site of the final vector [17]. We have constructed several pUC119-derived vectors containing the RCS polylinker (Fig. 3a). These vectors replicate to high copy number in *E. coli*, and are therefore useful for generating large quantities of plasmids for direct DNA delivery into protoplasts or plant cells by electroporation, Ca_2PO_4/PEG-mediated transformation, or particle bombardment.

Alternatively, we have constructed several T-DNA binary vectors containing the RCS polylinker (Fig. 3b). These vectors can be used in *Agrobacterium-mediated* transformation processes, such as the generation of transgenic lines or transient agroinfiltration experiments.

13. When the passage of BY-2 cells becomes a routine, and the cells grow normally, cells should reach the log phase of growth 4–5 days after transfer to fresh medium.
14. Check the cells every hour for the production of protoplasts after placing them in the protoplast enzyme digestion solution. The protoplasts can easily be seen as perfectly spherical cells using an inverted light microscope. BY-2 cells with walls have unusual shapes, but will not be perfectly spherical. Do not "over-digest" the cells. Protoplasts will lose viability if they stay too long in the protoplast enzyme digestion solution.
15. Remember, protoplasts no longer have cell walls. Therefore, they are very fragile and can lyse easily. Extreme caution is necessary during all washing and resuspension steps.
16. Because the transfection efficiency of protoplasts is highly dependent on the quality of plasmid DNA used, the user may consider using CsCl quality DNA or DNA purified using a commercially available plasmid extraction kit (e.g., Qiagen) to guarantee the quality and quantity of DNA for transfection or bombardment into plant cells.
17. The plate can be either wrapped in aluminum foil or kept in a closed chamber without light. Fluorescence signals can be detected as early as 4 h after transfection using fluorescence microcopy. We often finish the transfection in the late afternoon and incubate the cells overnight before viewing the cells.
18. Transfected protoplasts are very fragile, the cells could lyse if the coverslip is forced on top of them. Therefore, gentle handling is necessary. The authors normally place a self-sticking "Hole reinforcing ring" purchased from a stationary supply store on top of slide to form a chamber with depth, then place the protoplasts inside the ring and cover the ring with a coverslip.
19. The authors normally use a Nikon Eclipse 600 epifluorescence microscope or a Nikon A1R confocal microscope to view the samples. For the Nikon Eclipse 600 microscope, the authors use Yellow GFP HYQ41028 as a YFP filter; HcRED#41043 as a RFP filter; 96188 m (Chroma) as a CFP filter. The software Metamorph is used to capture and analyze the fluorescence images. For the Nikon A1R confocal microscope, the authors use either a 20× water/oil lens (Plan Fluor 20×/0.75 Mimm DIC N2) or a 40× water lens (ApoLWD 40×/1.15 W1XS). Nikon Elements ND2 is used for image processing and data management.

20. Recently, several laboratories have developed a number of "advanced" uses for BiFC (Fig. 4). Multicolor BiFC allows one to present simultaneously a given "bait" protein with the choice of several "prey" proteins to determine whether the bait prefers one or the other, or whether the choice of prey directs the subcellular compartment to which the bait–prey protein complex localizes (Fig. 4a) [17, 18, 27, 32]. For example, a given bait protein can be tagged with cCFP, and two prey proteins individually tagged with either nVenus or nCerulean. Because the chromophore of the fluorescent proteins depends on the amino acid sequence of its N-terminus, folding of cCFP with nVenus generates yellow fluorescence, whereas folding with nCerulean generates blue fluorescence. We have used this system to show that *Agrobacterium* VirE2 protein can interact in plants with multiple importin α isoforms. When VirE2 interacts with the isoform AtImpa-4, the complex localizes to the nucleus. However, when VirE2 interacts with all other tested importin α isoforms, the complex remains cytoplasmic or perinuclear [17].
21. BiFC combined with FRET can visualize interaction of three proteins in a complex (Fig. 4b) [33]. In this system, full-length Cerulean or CFP tags one protein, whereas two other proteins contain N- and C-terminal YFP (or its derivatives) tags. Interaction of the two proteins tagged with YFP fragments restores yellow fluorescence, which is visualized by FRET when the third Cerulean/CFP-tagged protein in the complex is excited by blue light. Kwaaitaal et al. [34] recently used BiFC-FRET to identify three proteins in a SNARE complex in barley leaf epidermal cells.
22. Bridge-BiFC can identify three proteins in a complex (Fig. 4c). In this system, expression of two proteins tagged with N- and C-terminal fragments of YFP does not result in fluorescence complementation unless a third, untagged protein is also expressed. The untagged protein serves as a "bridge" to bring together the tagged proteins. Zaltsman et al. [35] used this assay to identify proteins in a SCF complex important for directing proteolytic degradation of target proteins.
23. Our laboratory recently adapted BiFC to identify interactions between target proteins and small peptide aptamers (Fig. 4d) (L.-Y. Lee, S. Park, Y. Wang, H. Iwakawa, Z. Zhang, and S.B. Gelvin, unpublished). We inserted 20 amino acid long peptides between cCFP and mCherry (a highly fluorescent and photostable derivative of DsRed) to make aptamer fusion proteins. Interaction of the peptide aptamer with a target protein tagged with nYFP results in yellow fluorescence and, in some situations, inactivation of target protein function (aptamer "mutagenesis"). Red mCherry fluorescence marks

cells expressing the aptamer, and also indicates the subcellular position of the peptide aptamer. Yellow fluorescence identifies the subcellular location of target protein–aptamer interaction.

24. Our laboratory has also used BiFC to screen a cDNA library with a "bait" protein for protein–protein interactions directly in planta [36]. In this system, proteins encoded by a library of cDNAs are tagged at their N-termini with cYFP. Co-transfection of the cDNA library with a construction expressing the bait protein tagged with nVenus or nYFP resulted in a small number of fluorescent plant cells. The identity of the cDNA(s) encoding interacting protein(s) was established by successive break-down of pools of cDNA clones. Thus, we were able to develop a plant two-hybrid cDNA library screening system.
25. As with any technique, BiFC has limitations:
    (a) The underlying principle of BiFC is that the two portions of the split fluorescent protein must be brought together to fold correctly. Because it is not usually known in what structural conformation two proteins of interest interact, it may be difficult to predict which end of these proteins to tag. Thus, lack of a fluorescence signal does not necessarily indicate that two proteins do not interact. It may merely indicate that they do not interact in a way necessary to bring together the two portions of the split fluorescent protein. Users of BiFC should consider separately tagging both ends of each protein partner.
    (b) Interacting proteins frequently dissociate from each other (i.e., protein complexes can be transient). Folding of the two portions of the split fluorescent protein may irreversibly hold together two proteins, which would normally dissociate. Thus, BiFC can be used to investigate the formation of protein complexes, but cannot easily be used to explore downstream dissociation of these complexes.
    (c) Over-expression of the two peptides of a split fluorescent protein may result in the restoration of fluorescence independent of interaction of the affixed proteins of interest due to the self-assembly of two nonfluorescent fragments from any fluorescent protein. This likely occurs by "mass action." Because BiFC is a relatively sensitive technique, when a fluorescent protein with higher intensity is used, this background noise may be significant. Scientists have frequently observed fluorescence resulting from interaction of a tagged protein with a peptide generated by an "empty vector" construction. Therefore, it is necessary to have good controls. The best control for this situation is to delete or mutate the known interacting domains of the two proteins in question and demonstrate that one cannot obtain a fluorescence BiFC signal using the mutated proteins

for interaction [1, 27]. Unfortunately, many times the investigator does not know the interacting domains in question. Recently, Kodama and Hu [31] described a new variant of nVenus (nVenusI152L). Use of this fragment increases the signal-to-noise ratio of BiFC in animal cells. However, this variant does not similarly increase the signal-to-noise ratio in plants (communication with C-D Hu and results from our laboratory). We have mitigated the problem of background BiFC signal by lowering the expression of at least one of the interacting partners. We have done this by exchanging the strong CaMV double 35S promoter used in our vectors for a weaker promoter such as the nopaline synthase (*nos*) promoter. In addition, use of the less bright fluorescent protein EYFP, rather than Venus, also decreases background fluorescence [36]. Table 2 lists several vectors we have constructed for this purpose.

26. If you do not generate a fluorescence signal, how do you know that all the tagged genes have been introduced into the cells?

    We frequently include a red fluorescent protein (mRFP or mCherry) expression cassette on the same plasmid harboring the nYFP-tagged and cYFP-tagged protein expression cassettes. Generation of a red fluorescence signal indicates that the cells have received the incoming plasmid and expressed the mCherry protein, and can be used to determine the transfection efficiency. In addition, fusion of mCherry to "marker" proteins or organelle targeting sequences can assist in the identification of specific subcellular compartments or organelles. For example, mRFP fused to a nuclear localization signal (NLS) sequence can be utilized to identify nuclei as an alternative to staining cells with DNA-interacting fluorescent dyes such as DAPI. Several of our vectors contain, in addition to the split YFP tag, a T7 or a myc peptide tag. Expression of the fusion proteins can be detected using antibodies directed against these tags.

27. Problems with over-expression: "Forcing" interactions that may not normally occur

    When interpreting BiFC experiments, one must realize that these experiments will identify protein–protein interactions that *CAN* occur, but not necessarily those that *DO* occur. Over-expressing proteins can "force" interactions of proteins that, at best, would normally interact weakly when expressed at their native levels. To mitigate over-expression artifacts, genes encoding the putative interacting proteins can be expressed from relatively weak promoters, such as those from the nopaline synthase (*nos*), octopine synthase (*ocs*), or mannopine synthase (*mas*) genes. In addition, although it may require additional cloning effort, it is best to express the genes from their native promoters.

In addition, it is possible that in their native organism, these proteins would not normally interact because they would not have the opportunity to contact each other due to different temporal or spatial patterns of expression. For example, the proteins may normally be expressed in different tissue or cell types, or at different developmental stages. Alternatively, the proteins may localize to different subcellular compartments and therefore not normally have the opportunity to interact.

28. Problems with over-expression "mis-localizing" the interacting protein pairs

    One of the advantages of BiFC over other methods to detect protein–protein interactions is that the site of interaction can be visualized in living cells. However, it is possible that over-expression of the interacting proteins may "overload" a routing pathway, resulting in mis-localization of the site of interaction (*see* **Note 10**).

29. Lack of generation of a BiFC signal

    Lack of a BiFC fluorescence signal does not necessarily indicate that two candidate proteins do not interact. It is possible that the proteins do interact, but not in such a way as to allow the two complementary fragments of the split fluorophore to fold correctly. BiFC results should be verified by using a different technique, such as co-immunoprecipitation, TAP-tagging, or interaction in yeast, to detect protein–protein interactions.

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