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Protein fragment bimolecular fluorescence complementation analyses for the *in vivo* study of protein-protein interactions and cellular protein complex localizations

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Summary

The analyses of protein-protein interactions is crucial for understanding cellular processes including signal transduction, protein trafficking and movement. Protein fragment complementation assays are based on the reconstitution of protein function when non-active protein fragments are brought together by interacting proteins that were genetically fused to these protein fragments. Bimolecular fluorescence complementation (BiFC) relies on the reconstitution of fluorescent proteins and enables both the analysis of protein-protein interactions and the visualization of protein complex formations *in vivo*. Transient expression of proteins is a convenient approach to study protein functions *in planta* or in other organisms, and minimizes the need for time-consuming generation of stably expressing transgenic organisms. Here we describe protocols for BiFC analyses in *Nicotiana benthamiana* and *Arabidopsis thaliana* leaves transiently transformed by *Agrobacterium* infiltration. Further we discuss different BiFC applications and provide examples for proper BiFC analyses *in planta*.

Keywords

Protein fragment complementation; bimolecular fluorescence complementation; protein-protein interactions; *Agrobacterium* infiltration

1. Introduction

The investigation of protein-protein interactions (PPIs) is essential for the understanding of nearly all cell biological processes. PPI analyses can be performed either *in silico*, *in vitro* or *in vivo* using different methods (summarized in 1, 2). *In silico* predictions strongly rely on the quality of prediction programs and their algorithms, and provide only an indication for a possible PPI that needs to be further confirmed. Even literature curated datasets are possibly of low quality and error-prone (3). *In vitro* PPI analyses in particular calorimetric methods

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(4), surface plasmon resonance (5) or atomic force microscopy (6) enable the measurement of thermodynamic constants of certain PPIs. However, the most dramatic disadvantage of *in vitro* PPI analyses is that the biochemical and physiological context within a cell cannot be ideally simulated and that the cellular compartmentation is absent. Loss of compartmentation is the main reason for false positive interactions in *in vitro* PPI analyses (2).

Methods to study PPIs *in vivo* are based on genetically encoded reporters and rely on fluorescence resonance energy transfer (FRET), bioluminescence resonance energy transfer (BRET), Ca^{2+} /Annexin mediated translocation of protein complexes (7) or protein fragment complementation assays (PCA; 8, 9). For FRET/BRET analyses, proteins of interest are fused to donor and acceptor chromophore pairs with distinct spectral properties (FRET: CFP/YFP variants and GFP/RFP variants [10–12]; BRET: RLuc/YFP variants [13, 14]). In case of a PPI a dipole-dipole resonance mediated energy transfer from the donor- to the acceptor-chromophore occurs leading to an enhanced acceptor emission and to a reduced donor emission and lifetime of its excited state (15). An alternative for FRET/BRET assays is the Ca^{2+} dependent translocation of protein complexes where one bait protein is fused to Annexin A4 and a fluorescent protein (FP) and its target proteins are fused to FPs with different spectral properties compared to the bait-FP (7). Elevated intracellular Ca^{2+} levels induce the translocation of Annexin A4 from the cyto- and nucleoplasm to the plasma- and nuclear membrane thereby translocating bait and target proteins. This approach was conducted to visualize the translocation of a four-component protein complex (7).

In PCAs, interaction of protein pairs, which were genetically fused to non active fragments of a reporter gene, reconstitutes the function of this reporter gene. Methods based on protein fragment complementation have either a direct or indirect readout and are reversible or irreversible. PCAs with an indirect readout are the yeast-two-hybrid (Y2H) and split-ubiquitin system (SUS). In Y2H assays PPI pairs are fused to the binding- and activation domain of a transcription factor and positive interaction and translocation of the reconstituted transcription factor to the nucleus induces the activation of a reporter gene (16). The two-hybrid technology has also been applied to *Arabidopsis* protoplasts (17). SUS is used for PPI analyses of membrane proteins. Here, PPI pairs are respectively fused to the N- and C-terminus of ubiquitin, of which the latter is also fused to a transcription factor. Interaction induces the reconstitution of ubiquitin and subsequent proteolysis by an ubiquitin specific protease enables the transcription factor release from the membrane into the nucleus and the induction of reporter gene expression (18, 19). An alternative method that also can be applied to membrane and soluble proteins is based on split tobacco etch virus protease (split TEV; 20).

PCAs with a direct readout use the reconstitution of an enzyme or a chromophore. Split enzyme assays use fragments of β -galactosidase (21), dihydrofolate reductase (22, 23) or β -lactamase (24–26). Positive interactions induce the catalysis of a specific substrate that can be monitored in a colorimetric assay or by antibiotics resistance. Bioluminescence complementation (BiLC) relies on the reconstitution and activity of a luciferase as a readout. The splicing based split luciferase system combines split luciferase with split intein (DnaE), where PPI induces the splicing and ligation of luciferase fragments to recover an irreversible

luciferase activity (27). Reversible split luciferase assays have also been developed for luciferases with different emission properties and from different species (28–32) and have been successfully applied to plants (33–35). Split luciferase assays have two drawbacks, namely the need for coelenteracin/luciferin as an externally applied substrate and a low sub-cellular resolution, due to the low intensity of this bioluminescence reporter, which usually requires integrated signal detection from whole tissues, or groups of cells.

Bimolecular fluorescence complementation (BiFC) is based on the reconstitution of N- and C-terminal fragments of green fluorescent protein (GFP, cut between amino acids Gln157 and Lys158; 36) or of enhanced yellow fluorescent protein (eYFP, cut between amino acids Ala154 and Asp155 or between Glu172 and Asp173; 37, 38, Figure 1a) as a reporter for PPI. Interaction of protein pairs that are fused to the FP fragments results in fluorescence complementation thereby enabling the visualization of complex formation with subcellular resolution. One characteristic of BiFC is its irreversibility (9, 39), which is in some cases considered as drawback because of possible unspecific FP assembly that results in higher background signals. By introducing point mutations (I152L or V150A/V150L) into Venus (40) or by using a YFP N-terminal fragment cut after Ile152, BiFC signal/noise-ratio could be improved (41–44). However, the impossibility of FP fragment dissociation stabilizes weak or transient interactions which are not detectable when reversible methods for PPI analyses are used (9, 39). BiFC has been extensively applied to many living systems (summarized in http://sitemaker.umich.edu/kerppola.lab/kerppola.bifc/bifc_results; 45) including plants (summarized in 15, 46, 47). A whole spectrum of FPs can be used for BiFC analyses. These are CFP and its improved variants Cerulean and SCFP3A (cyan; 38, 48, 49), GFP, super folder GFP (sfGFP) and mKG2 (green; 36, 50–55), YFP, super folder YFP and the YFP variant Venus (37, 38, 48, 49, 56–58), mDsRed, mRFP, mCherry and mLumi (red; 59–63) and finally the reversible photo-switching GFP-like FP Dronpa (64). The simultaneous use of FPs with different spectral properties, e.g. CFP-, GFP- or YFP-BiFC combined with mDsRed-BiFC, enables the visualization of different protein complexes at the same time and in the same cell (62). We call this application coBiFC for co-localization analyses of protein-BiFC complexes (Figure 1b). Besides coBiFC, multicolor BiFC (mcBiFC) visualizes the complex formations of alternative interaction partners (9). In mcBiFC assays protein A is fused to the C-terminal fragment of CFP-(CFP^C) or brighter variants and alternative interaction partners B and C are fused to the N-terminal fragment of CFP-(CFP^N) and YFP-(YFP^N) variants, respectively (38, 48, 49). Interaction of A–B reconstitutes CFP fluorescence (cyan, 474 nm), whereas A–C interaction leads to CFP^C-YFP^N assembly with intermediate (green, 511 nm) emission maximum (Figure 1c). mcBiFC was more efficient when FPs were separated between amino acids Glu172 and Asp173 compared to Asp155 (38). Co-expression of interacting proteins tagged to YFP- or Venus fragments with one interacting protein tagged to *Renilla reniformis* luciferase or the FP Cerulean enables BiFC based BRET or FRET analyses for the analyses of three component protein complexes (65–68, Figure 1d). Even further, combination of BiLC and BiFC based BRET, in which reconstituted *Gaussia princeps* luciferase was used for BRET on reconstituted YFP enabled analyses of four component protein complexes (69, Figure 1e). FRET between two complemented FPs (e.g. Venus and mCherry) may also allow the visualization of such four component protein complexes (70). Other applications of BiFC

are ubiquitin-mediated fluorescence complementation (UbFC; 71, 72), the visualization of nucleic acid-protein interactions (73–76), the analysis of protein architecture or folding (77, 78), protein topology (79), protein co-expression (80) and the isolation of BiFC stabilized complexes (iBiSC; 74). Finally the combination of BiFC with fluorescence-activated cell sorting (BiFC-FC) could provide a tool for high throughput screening of weak PPIs (42, 81).

The most common methods to study BiFC *in planta* employ protoplast transfection of tobacco or *Arabidopsis*, particle bombardment of onion or tobacco epidermal cells or *Agrobacterium* infiltration of tobacco, mostly *Nicotiana benthamiana* (summarized in 15, 46, 47). Protocols for such assays are available (82–89). However, to our knowledge, there are only two reports describing BiFC in *Arabidopsis* plants that were transiently transformed by *Agrobacterium* infiltration (56, 90). There is a whole palette of vectors with different FP fragments available to perform BiFC analyses in plants (49, 53, 56, 57, 59, 62, 63, 90–93). However, different BiFC applications may require the use of different FPs and their N- and C-terminal fragments (see Figure 1). Here we present protocols for BiFC assays in *Nicotiana benthamiana* and *Arabidopsis thaliana* using the *Agrobacterium* infiltration method (adapted from 82, 94, 95).

2. Materials

2.1 BiFC vectors and constructs

1. PPI pairs of interest should be cloned into appropriate vectors that enable the intended BiFC application (see Figure 1; Note 1). The use of BiFC vectors that harbor an epitope tag between FP fragment and cloning site, which enables western blot analyses as expression control in case of negative BiFC results is recommended (49, 56, 57, 59, 63, 93). However GFP antibodies are also suitable for detecting FP N-terminal fragments (56, Figure 2).
2. Vectors with reference FP (e.g. CFP or RFP variants) with the same vector backbone than the BiFC constructs (Note 2).
3. Reagents, primers and DNA-Polymerase for PCR-based analyses of BiFC constructs.

¹Before generating BiFC-fusion constructs, use web resources to analyze topology, sub-cellular localization and targeting signals of proteins of interest (e.g. <http://aramemnon.botanik.uni-koeln.de/> [100]; <http://www.expasy.ch/> [101] and links therein). Fusion of BiFC-tags to the wrong terminal end could mask targeting signals and affect protein localization. On the other hand, expression levels of some proteins are drastically reduced when a certain tag orientation is used. It is recommended to generate full-length FP fusion constructs in a vector backbone similar to the BiFC-vectors used and to analyze proper localization and expression of fusion proteins before generating the BiFC constructs. If expression and proper localization could be confirmed by microscopic analyses the use of a similar BiFC-tag orientation is recommended. If no information about the protein localization is available, generate FP fusion constructs with respective N- and C-terminal FP-tag orientations (e.g. CFP-query and query-YFP) and perform co-localization analyses. If both tag-orientations are possible, use C-terminal BiFC-tag fusions (query-BiFC) as N-terminal BiFC-tag fusions may result in higher background signals (Waadt and Kudla, unpublished results).

Proper controls are the key for adequate semiquantitative BiFC analyses as BiFC is irreversible and may stabilize randomly formed protein complexes (9, 39). The best negative control is the use of a different family member of one of the investigated proteins. Point mutations that disrupt interaction or deletion constructs of the interacting domain may also be suitable. It is important that the negative control is localized in the same cellular compartment and exhibits a similar expression level compared to the reference protein. The use of empty-vector controls is not recommended as the expression of FP-fragments alone does not fulfill the requirements mentioned above.

²In order to suppress plant immune responses one might also consider co-expression of the *Pseudomonas syringae* type III effector AvrPto (102).

2.2 *Agrobacterium tumefaciens* strain and media for *Agrobacterium* culture and infiltration

1. Electro-competent *Agrobacterium tumefaciens* GV3101 (pMP90) (96) or other *Agrobacterium* strains.
2. p19 silencing suppressor strain (97).
3. 2 M NaOH.
4. 1 M KOH and 5 M KOH.
5. 1 M MgSO₄ (autoclave and store at 4 °C).
6. 1 M MgCl₂ (autoclave and store at 4 °C).
7. 1 M 2-(N-morpholino) methanesulfonic acid (MES). Adjust to pH 5.6 with 5 M KOH and filter sterilize through a 0.45 µm filter.
8. 3,5-dimethoxy-4-hydroxyacetophenone (Acetosyringone) 200 mM in dimethyl sulfoxide (DMSO). Store 500 µL aliquots at – 20 °C.
9. YEB medium: 5 g/L sucrose, 5 g/L beef extract, 1 g/L yeast extract, 1 g/L peptone, 2 mM MgSO₄ (add from sterile 1 M stock after autoclaving). Adjust to pH 7.4 with 2 M NaOH and autoclave. For solid medium add 20 g/L agar, autoclave and pour into petri dishes.
10. Infiltration medium for *N. benthamiana* infiltration: 10 mM MES pH 5.6, 10 mM MgCl₂, 150 µM Acetosyringone (dilute from stocks).
11. Induction medium for *Arabidopsis* infiltration: 10.5 g/L K₂HPO₄, 4.5 g/L KH₂PO₄, 1 g/L (NH₄)₂SO₄, 0.5 g/L NaCitrate, 1 g/L glucose, 1 g/L fructose, 4 g/L glycerol, 1 mM MgSO₄, 10 mM MES. Adjust pH to 5.6 with 1M KOH and autoclave. Before use add antibiotics (see 2.3) and 100 µM Acetosyringone (dilute from stocks).
12. Infiltration medium for *Arabidopsis* infiltration: 10 mM MES pH 5.6, 10 mM MgSO₄, 200 µM Acetosyringone (dilute from stocks).

2.3 Antibiotics

1. Rifampicin: 50 mg/mL stock in DMSO. Store 1 mL aliquots at – 20 °C, working concentration 100 µg/mL.
2. Kanamycin: 100 mg/mL stock in H₂O. Store 1 mL aliquots at – 20 °C, working concentration 25 µg/mL.
3. Gentamycin: 50 mg/mL stock in H₂O. Store 1 mL aliquots at – 20 °C, working concentration 25 µg/mL.

2.4 Plants and materials for plant culture

1. *Nicotiana benthamiana* seeds.
2. *Arabidopsis thaliana* seeds (Note 3).

3. Plant pots with 5 – 6 cm diameter for *Arabidopsis* and with 8 – 9 cm diameter for *N. benthamiana*, trays and plastic covers.
4. Soil.
5. Fertilizer.
6. Fungicide (optional).
7. Insecticide (optional).
8. Ultra pure H₂O (e.g. Milli Q) autoclaved.
9. 100 % ethanol.
10. 10 % sodium dodecyl sulfate (SDS, sterilized through a 0.45 µm syringe filter).
11. Seed sterilization solution: 70 % ethanol, 0.04 % SDS.
12. 1 M KOH
13. 0.5 MS agar plates: half concentrated Murashige and Skoog basal medium (98) with Gamborg B5 vitamins adjusted to pH 5.8 with 1 M KOH and supplemented with 0.8 % agar. Autoclave, pour into petri dishes and store at 4 °C.

2.5 Media for protein extraction, SDS-PAGE and western blotting (after 99)

1. Ultra pure H₂O.
2. Protein extraction buffer: 100 mM Tris (hydroxymethyl) aminomethane (Tris)/HCl pH 8 (1 M stock), 100 mM NaCl (1 M stock), 5 mM EDTA (0.5 M stock, adjust to pH 8 with NaOH tablets), 5 mM EGTA (0.5 M stock, adjust to pH 8 with NaOH tablets), 20 mM DTT (1–2 M stock, – 20 °C), 1× protease inhibitor mix (Roche 11873580001, 25× stock, – 20 °C), 0.5 % Triton X-100 (10 % stock). Prepare fresh before use.
3. 1× SDS sample buffer: 62.5 mM Tris/HCl pH 6.8, 2 % SDS, 5 % β-mercaptoethanol, 10 % glycerol, 0.002 % bromophenol blue. Store 1 mL aliquots at – 20 °C.
4. 4× SDS sample buffer: 250 mM Tris/HCl pH 6.8, 8 % SDS, 20 % β-mercaptoethanol, 40 % glycerol, 0.008 % bromophenol blue. Store 1 mL aliquots at – 20 °C.
5. Amidoblack staining solution: 10 % acetic acid, 90 % methanol, 0.1 % Amidoblack filtered through a flute filter and stored at 4 °C.
6. Amidoblack de-staining solution: 10 % acetic acid, 90 % methanol (store at 4 °C).
7. 0.2 M NaOH.
8. Bovine serum albumin.

³Additional to the infiltration of wild type plants, BiFC experiments could also be conducted in mutant plants, in GVG-AvrPto plants (102) that enable dexametasone inducible immune response suppression or in the *rdr6-11* mutant (103) that is deficient in post translational gene silencing.

9. 1.5 M Tris/HCl pH 8.8 (degas and store at 4 °C).
10. 0.5 M Tris/HCl pH 6.8 (degas and store at 4 °C).
11. 20 % SDS.
12. 40 % Acrylamide/Bisacrylamide solution (37.5:1, store at 4 °C).
13. *N,N,N',N'*-tetramethyl-ethane-1,2-diamine (TEMED, store at 4 °C).
14. 10 % ammonium persulfate (store 1 mL aliquots at – 20 °C).
15. 70 % ethanol.
16. Acetone.
17. Isopropanol.
18. Methanol.
19. SDS running buffer: 5g/L Tris, 14.4 g/L glycine, 1g/L SDS. Prepare 10× solution and dilute before use.
20. Blotting buffer: 24 mM Tris, 192 mM glycine, 20 % ethanol. Prepare 5× solution without ethanol and filter sterilize through a 0.45 µm filter. Dilute before use with ultra pure H₂O and 100 % ethanol to final concentration.
21. 10× Tris buffered saline (TBS): 100 mM Tris/HCl pH 7.4, 1.5 M NaCl. Filter sterilize through a 0.45 µm filter.
22. Western blot blocking buffer: 1× TBS, 0.05 % Tween 20, 5 % non fat dry milk powder.
23. Western blot washing buffer (TBS-T): 1× TBS, 0.05 % Tween 20.
24. Coomassie brilliant blue (CBB) staining solution: 50 % methanol, 10 % acetic acid, 0.25 % Coomassie brilliant blue R-250. Filtrate through a fluted filter and store at 4 °C. Alternatively to CBB staining PageBlue (<http://www.fishersci.com>) could be used for SDS-gel staining.
25. Coomassie de-staining solution: 50 % methanol, 10 % acetic acid (store at 4 °C).
26. Enhanced chemo luminescence (ECL) solution A: 0.1 M Tris/HCl pH 8.6, 0.25 mg/mL luminol (store at 4 °C).
27. ECL solution B: 1.1 mg/mL para-hydroxycoumaric acid in DMSO.
28. 30 % (8.8 M) H₂O₂ (store at 4 °C).
29. ECL developing solution: use for one 7 cm × 9 cm PVDF membrane 2 mL ECL solution A, 200 µL ECL solution B and 0.6 µL 30 % H₂O₂ (prepare fresh before use).
30. Precision plus dual-color protein standard (<http://www.bio-rad.com/>).
31. Monoclonal HA antibody (<http://www.covance.com/>). Prepare 15 mL of 1:4000 dilution in TBS-T, 0.05 % NaAcide (reusable, store at 4 °C).

32. Monoclonal c-myc antibody (<http://www.cellsignal.com/>; <http://www.invitrogen.com/>). Prepare 15 mL of 1:4000 dilution in TBS-T, 0.05 % NaAcide (reusable, store at 4 °C).
33. Monoclonal FLAG M2 antibody (<http://www.sigmaaldrich.com/>). Prepare 15 mL of 1:4000 dilution in TBS-T, 0.05 % NaAcide (reusable, store at 4 °C).
34. Monoclonal GFP antibody (Zymed; <http://www.invitrogen.com/>). Prepare 15 mL of 1:4000 dilution in TBS-T, 0.05 % NaAcide (reusable, store at 4 °C).
35. Secondary goat anti mouse IgG (H + L) horseradish peroxidase-conjugate antibody (<http://www.bio-rad.com/>). Prepare 15 mL of 1:10000 dilution and discard after use.

2.6 Consumables

1. 10 µL, 200 µL, 1 mL and 5 mL pipette tips.
2. Plastic transfer pipettes.
3. PCR tubes.
4. 1.5 mL and 2 mL reaction tubes.
5. Sterile 15 mL and 50 mL Falcon tubes.
6. 24 well plates.
7. 1 mL plastic cuvettes.
8. Electroporation cuvettes.
9. Petri dishes.
10. Razor blades.
11. Microscope slides and cover-glass.
12. Kimwipes.
13. Sterile paper covered in aluminum-foil and autoclaved.
14. Sterile toothpicks stored in 100 mL glass beaker covered with aluminum-foil and autoclaved.
15. Micropore surgical tape.
16. Examination gloves.
17. Plastic syringes without needle (1 and 60 mL).
18. Fluted filters.
19. Sterile 0.45 µm syringe filters.
20. Sterile 0.45 µm filters with bottles (0.5 or 1 L).
21. Polyvinylidene fluoride (PVDF) Immobilon-Transfer Membrane, 0.2 µm pore size.
22. Whatman chromatography paper.

23. X-ray Films.
24. Parafilm.
25. Tape.
26. Cling film.
27. Cellophane.
28. Paper towels.

2.7 Equipment

1. Pipettes (2 μ L, 20 μ L, 100 μ L, 200 μ L, 1000 μ L and 5000 μ L).
2. Stirring bars.
3. Forceps.
4. Scalpel.
5. Scissors.
6. Cork borer.
7. Dewar flask.
8. Styropore box.
9. Grinding balls for Retsch Mill.
10. Autoradiography cassette.
11. Systems for protein electrophoresis, blotting and SDS-gel drying.
12. Electrophoresis power supply.
13. Electroporator.
14. Heat block for 1.5 and 2 mL tubes adjustable to 95 °C.
15. 28 °C incubator with shaking device.
16. Platform shaker.
17. Vortex.
18. Stirrer/Heater combination.
19. Microwave.
20. Sonicator.
21. PCR machine.
22. Centrifuge for 1.5 and 2 mL tubes $\pm$ cooling device.
23. Centrifuge with swinging bucket rotor and adaptors for 15 mL tubes.
24. Retsch Mill with buckets resistant to liquid N₂ (<http://www.retsch.com/>).

25. Spectrophotometer.
26. X-ray film developing machine.
27. Scanner.
28. Ice machine.
29. Inverted fluorescence microscope or spinning disc confocal microscope equipped with appropriate excitation and emission filters or laser units to image CFP, YFP and RFP, with 20×, 40× and 60× objectives and a CCD camera.
30. Sterile hood.
31. Greenhouse and/or plant growth device to maintain stable growth conditions (e.g. growth room or growth chamber) to enable light intensities of 50 – 120 $\mu\text{E m}^{-2} \text{s}^{-1}$ under long day (16 h light/8 h dark) or short day (8 h light/16 h dark) conditions and 22 – 27 °C.
32. Freezer (– 20 °C and – 80 °C).
33. Fridge and/or cold room (4 °C).
34. Dark room.

2.8 Software

1. ImageJ (<http://rsbweb.nih.gov/ij/>) or Fiji (<http://fiji.sc/wiki/index.php/Fiji>).
2. Microsoft Excel or other software for data analyses and calculations.

3. Methods

3.1 Transformation and culture of *Agrobacteria*

1. Prepare for each transformation one 1.5 mL tube with 100 ng plasmid DNA (BiFC-constructs etc.), one electroporation cuvette and one 2 mL tube containing 1 mL YEB medium without antibiotics and place on ice.
2. Thaw for each transformation one 30–50 μL aliquot of electro-competent *Agrobacteria*, stored at – 80 °C, on ice.
3. Mix plasmid DNA and electro-competent *Agrobacteria* and transfer into electroporation cuvette.
4. Transfer cuvette into electroporator, apply a 5 ms electric pulse of 1.8 – 2.5 kV, immediately resuspend *Agrobacteria* in 1 mL YEB medium without antibiotics and transfer into 2 mL tube.
5. Shake *Agrobacteria* for 2 h at 28 °C and 180 – 220 rpm.
6. Plate 30 – 200 μL of *Agrobacteria* transformation on YEB agar plates containing appropriate antibiotics and incubate 2 days at 28 °C.
7. Re-strike four colonies of each transformation to fresh YEB agar plate containing appropriate antibiotics and incubate one additional day at 28 °C.

8. Re-confirm positive transformation of *Agrobacteria* by colony-PCR.
9. Inoculate positively transformed *Agrobacteria* in 3 mL YEB liquid medium containing appropriate antibiotics and shake at 28 °C and 180 – 220 rpm over night.
10. Mix 930 µL *Agrobacteria* culture with 70 µL DMSO in 2 mL tube, incubate on ice for 30 min, flash freeze in liquid N₂ and store at – 80 °C. Alternatively, *Agrobacteria* could be stored on YEB agar plates with appropriate antibiotics at 4 °C if transferred monthly to a fresh plate.

3.2 Growing of *Nicotiana benthamiana*

1. Prepare six pots with watered soil containing fertilizer, fungicide and insecticide. Pots should be prepared one day before plant sowing.
2. Sow nine *N. benthamiana* seeds per pot using a toothpick and dip seeds gently into the soil. Cover pots with plastic cover to ensure high humidity and transfer to greenhouse or growth device (Note 4).
3. After seed germination, usually four days, remove plastic cover.
4. As soon as the first true leaves emerge (12 – 14 days), transfer seedlings to single pots and cover again for four days with plastic cover.
5. Grow *N. benthamiana* plants for 5 – 6 weeks and water every day or every second day. Plants should not flower at time point of infiltration.

3.3 Infiltration of *Nicotiana benthamiana*

1. Inoculate *Agrobacteria* harboring BiFC-constructs, a reference FP control (e.g. CFP or RFP variant) and the p19 strain in sterile 50 mL Falcon tubes containing 15 mL YEB medium and appropriate antibiotics and shake over night at 28 °C and 180 – 220 rpm. Do not close Falcon tube lid but prevent lid falling off by taping (Note 5).
2. Prepare for each *Agrobacteria* culture one 1 mL plastic cuvette with 900 µL YEB medium. Add 100 µL *Agrobacteria* culture to the respective cuvette and measure the optical density at 600 nm (OD₆₀₀) with the spectrophotometer using a cuvette containing 1 mL YEB medium as blank.
3. Calculate the amount of *Agrobacteria* needed for a final 4 mL suspension per leaf using the formula $V_{\text{culture}} = V_{\text{final}} \times \text{OD}_{600 \text{ final}} / \text{OD}_{600}$. For *Agrobacteria* harboring BiFC and reference FP constructs standard OD_{600 final} is 0.5 (Note 6). Standard OD_{600 final} for the p19 strain is 0.3.

⁴*N. benthamiana* plants can be either grown in the greenhouse or in a growth room with long day conditions, 90 – 120 µE m⁻² s⁻¹ light intensity and 22 – 27 °C.

Optimal growth conditions for *Arabidopsis* plants are short day conditions (8 h light/16 h dark) with 90 µE m⁻² s⁻¹ light intensity at 23 °C (94, 95). However, transient expression is also achieved in long day conditions (16 h light/8 h dark) with 50 – 80 µE m⁻² s⁻¹ light intensity at 27 °C. Most important for achieving high protein expression levels, is the use of young leaves of plants in the pre-bolting stage. Two to three days prior to infiltration *Arabidopsis* plants should be stopped watering and the evening prior infiltration plants should be kept under constant darkness until the infiltration procedure. This minimizes the syringe pressure needed for the infiltration procedure. High syringe pressure during infiltration could damage the leaves. After infiltration *Arabidopsis* plants need to be rewatered and kept under high humidity by water spraying and covering with a plastic cover for at least 24 h.

4. Using the calculated culture volumes (V_{culture}) and according to the intended BiFC application (see Figure 1) mix the respective *Agrobacteria* suspensions (except of the p19 strain) in a 15 mL Falcon tube. Transfer the total V_{culture} of the p19 strain, calculated for all samples, to additional 15 mL Falcon tubes. At this stage do not mix p19 culture with BiFC and FP cultures.
5. Spin cultures in swinging bucket rotor for 15 min at 4000 g.
6. Discard supernatant, resuspend BiFC and FP *Agrobacteria* culture mixes in 2 mL infiltration medium by gentle pipetting and add additional infiltration medium to reach $0.5 V_{\text{final}}$. Resuspend p19 cultures by gentle pipetting in 2 mL infiltration medium and add additional infiltration medium to reach $0.5 V_{\text{final}}$.
7. Mix BiFC and FP culture mix 1:1 (v/v) together with the p19 culture and incubate for 2 h at room temperature.
8. Select *N. benthamiana* plants of the same age and size and label for each construct combination the same leaf of individual plants for infiltration (Note 7).
9. Carefully cut infiltration points on abaxial leaf side by touching with a razor blade at different leaf regions.
10. Infiltrate *Agrobacteria* suspensions at infiltration points into the abaxial leaf side using a 1 mL syringe without needle and ensure that the complete leaf is infiltrated.
11. Return infiltrated *N. benthamiana* plants to growth device, water them and incubate for 2 – 5 days. Semiquantitative BiFC analyses should be performed on day 3 or 4 post infiltration.

⁵To investigate PPIs of proteins A and B, typical construct combinations for single BiFC analyses (see Figure 1a) with possible negative controls are listed below:

- a. A-YFP^N / B-YFP^C / CFP or RFP (sample)
- b. C-YFP^N / B-YFP^C / CFP or RFP (negative control)
- c. A-YFP^N / D-YFP^C / CFP or RFP (negative control)
- d. A_{mutation}-YFP^N / B-YFP^C / CFP or RFP (negative control)
- e. A-YFP^N / B_{mutation}-YFP^C / CFP or RFP (negative control)
- f. A_{deletion}-YFP^N / B-YFP^C / CFP or RFP (negative control)
- g. A-YFP^N / B_{deletion}-YFP^C / CFP or RFP (negative control)

Here, C and D belong to the respective protein family of A and B and mutation or deletion indicates possible modifications in the respective interaction domains. The BiFC-tag orientation should be determined according to Note 1. For infiltration of *N. benthamiana*, co-infiltration of the p19 silencing suppressor strain is recommended (97).

⁶For infiltration of *N. benthamiana* leaves the OD₆₀₀ of *Agrobacteria* could be adjusted between 0.05 and 2, where higher OD₆₀₀ typically leads to higher expression levels.

For infiltration of *Arabidopsis* leaves the OD₆₀₀ of *Agrobacteria* could be adjusted between 0.4 and 0.9 to obtain an appropriate expression level but should not exceed a total OD₆₀₀ of 1.2 as this induces strong necrosis formations. OD₆₀₀ of 0.3 and lower on the other hand drastically reduces transient expression efficiencies (95).

⁷The choice of leaves for infiltration is critical for performing adequate semiquantitative BiFC analyses in *N. benthamiana*. *N. benthamiana* leaves of different age vary in their transient expression efficiencies. To compare BiFC efficiencies of different construct combinations, it is recommended infiltrating each construct combination into leaves of different plants but of the same age, size and physiological status. The use of different leaves of the same plant is not recommended. Alternatively, two different BiFC combinations could be infiltrated into one leaf on both sides of the leaf mid-vein. More than two different infiltrations per leaf are not recommended as for semiquantitative BiFC analyses samples from the same leaf area are needed (see Note 8).

3.4 Growing and preparation of *Arabidopsis* plants

1. Prepare 2 mL tubes with 20 – 50 μ L *Arabidopsis* seeds, add 1 mL sterilization solution and shake or rotate gently for 10 min.
2. Wash seeds three times with 100 % ethanol and discard final ethanol.
3. Dry seeds in sterile hood.
4. Transfer sterile seeds in sterile hood to 0.5 MS agar plates using sterile toothpicks, close plates with micropore surgical tape and incubate for 3 – 4 days at 4 °C for stratification. Alternatively, seeds can be directly sowed on soil, covered with plastic cover and stratified for 3 – 4 days at 4 °C.
5. Transfer sown seeds to growth device and incubate for 5 – 7 days (Note 4).
6. Prepare pots with watered soil containing fertilizer, fungicide and insecticide. Pots should be prepared one day before plant transfer.
7. Transfer *Arabidopsis* seedlings to single pots and cover the plants for 4 days with plastic cover to maintain high humidity. Water plants if needed.
8. Grow *Arabidopsis* plants for a total of 3 – 4 weeks. *Arabidopsis* infiltration should be performed before flowering.
9. Stop watering plants 2 – 3 days prior to infiltration.
10. Keep plants under constant dark and high humidity conditions the evening before and until the actual infiltration procedure by covering them with a light impermeable tray.

3.5 *Arabidopsis* infiltration

1. Depending on the amount of *Agrobacteria* culture needed, inoculate *Agrobacteria* harboring BiFC-constructs and a reference FP control (e.g. CFP or RFP variant) either in sterile 50 mL Falcon tubes containing 15 mL YEB medium and appropriate antibiotics or in sterile 15 mL Falcon tubes containing 3 – 5 mL YEB medium and appropriate antibiotics and shake over night at 28 °C and 180 – 220 rpm. Do not close Falcon tube lid but prevent lid falling off by taping (Note 5).
2. Transfer 2 – 3 mL of *Agrobacteria* culture to sterile 15 mL Falcon tube and spin in swinging bucket rotor for 15 min at 4000 g.
3. Discard supernatant and resuspend *Agrobacteria* by gentle pipetting in 2 mL induction medium. Add additional 3 mL of induction medium and shake at 28 °C and 180 – 220 rpm for 5 – 6 h.
4. Prepare for each *Agrobacteria* culture one 1 mL plastic cuvette with 900 μ L H₂O. Add 100 μ L *Agrobacteria* culture to the respective cuvette and measure OD₆₀₀ with the spectrophotometer using a cuvette containing 1 mL H₂O as blank.
5. Calculate the amount of *Agrobacteria* needed for a final 2 mL suspension using the formula $V_{\text{culture}} = V_{\text{final}} \times \text{OD}_{600 \text{ final}} / \text{OD}_{600}$. A 2 mL *Agrobacteria* suspension is

sufficient for infiltration of 16 – 20 *Arabidopsis* leaves. OD_{600 final} of 0.4 should be used as standard but could be adjusted (Note 6).

6. Using the calculated culture volumes (V_{culture}) and according to the intended BiFC application (see Figure 1) mix the respective *Agrobacteria* suspensions by transferring them into a 15 mL Falcon tube and spin in swinging bucket rotor for 15 min at 4000 g.
7. Discard supernatant, resuspend *Agrobacteria* by gentle pipetting in 2 mL infiltration medium and transfer to 2 mL tube.
8. Infiltrate *Agrobacteria* into the abaxial side of *Arabidopsis* leaves (4th – 6th leaf) using a 1 mL syringe without needle. Use the leaf tip as infiltration point as this enables the infiltration of the entire leaf at once. Infiltrate each construct combination into a total of eight *Arabidopsis* plants.
9. Transfer infiltrated *Arabidopsis* plants to growth device, water plants, additionally spray with water and use a plastic cover to maintain high humidity. Incubate plants for seven days and remove plastic cover after 24 h.

3.6 Microscopic imaging and semiquantitative BiFC analyses

1. Prepare 24 well plate for sample harvesting. Label wells and add 1 mL tap water to each well.
2. Punch out *N. benthamiana* leaf discs (without veins) of 14 mm diameter or cut off *Arabidopsis* leaves and transfer to 24 well plate. Harvest maximum four samples simultaneously. For sample comparison punch out leaf discs from the same leaf area of *N. benthamiana* leaves or harvest always the same leaf (e.g. leaf 5) from different *Arabidopsis* plants.
3. Place a drop of water on a microscope slide and transfer a leaf disc or leaf (sample) with the abaxial side up on water drop. If needed, cut off the mid vein of *Arabidopsis* leaves with a scalpel and place a second drop of water on the sample. Cover the sample with a cover-glass and ensure that there are no air-bubbles between sample and cover-glass by gently pushing with a forceps.
4. Mount the microscope slide onto an inverted fluorescence microscope or a spinning disc confocal microscope with the abaxial side of the sample towards the objective.
5. For semiquantitative fluorescence intensity measurements analyze samples with respective FP imaging settings using a 20× objective. Adjust exposure time, gain, etc. with a reference sample to obtain optimal imaging conditions (e.g. sample (a) in Note 5). Image all samples using identical settings for each FP channel.
6. Take minimum 10 images from different areas of each sample using both FP reference and BiFC settings. For background subtraction take also images of *N. benthamiana* samples infiltrated with p19 alone or from non-infiltrated *Arabidopsis* leaves. For *N. benthamiana* samples use bright field settings to select different leaf regions with a flat focal plane. In *Arabidopsis* select regions with flat focal plane and high reference FP expression.

7. Measure mean fluorescence intensities of entire images for each fluorescence channel using the microscope software or Fiji.
8. Calculate for every sample and FP channel the average mean intensities and standard errors. After background fluorescence subtraction, normalize BiFC fluorescence relative to reference sample (e.g. sample (a) in Note 5). In addition and after background subtraction calculate BiFC/reference FP-ratios for each image individually. Calculate average and standard error of resulting BiFC/reference FP-ratios and normalize values relative to reference sample (Note 8; Figure 2).
9. For qualitative high resolution BiFC analyses use a 60× objective and adjust exposure time, gain, etc. for each sample individually. As standard procedure record overview images and z-stacks of representative cells. Also convert z-stacks into .avi file and re-constitute 3D maximum projections (Note 9; Figure 2).

3.7 Protein extraction and protein quantification

1. Harvest two *N. benthamiana* leaf discs of 14 mm diameter or one *Arabidopsis* leaf from infiltrated plants and background controls into 2 mL tubes containing one

⁸Semiquantitative BiFC analyses are critical for the evaluation of positive BiFC results. The best system to perform BiFC quantification analyses is *N. benthamiana* epidermal cells, as these cells are easy to image and exhibit, depending on the proteins to be expressed, a remarkable high expression efficiency when BiFC samples are co-expressed with the silencing suppressor p19. It is recommended to infiltrate samples to be compared into leaves of similar physiological status. Samples for microscopic or western-blot analyses also need to be harvested from similar leaf areas. Expression levels in infiltrated *N. benthamiana* leaves vary dependent on the time point after infiltration. Usually strongly expressed protein constructs are already detectable at day two after infiltration and expression increases until day five after infiltration. However, it is strongly recommended to perform semiquantitative BiFC analyses only on days 3 – 4 after infiltration because of a maximal signal/noise-ratio during that time phase.

As an example for BiFC analyses in *N. benthamiana* the well known interaction of the calcineurin B-like (CBL) calcium sensor CBL10 and the CBL interacting protein kinase CIPK24 was investigated (Figure 2, 49, 104). As negative control the CIPK24_{NAF} construct was used, in which the NAF-domain, that is necessary and sufficient for CBL calcium sensor interaction (105), is deleted. Imaging was conducted using an inverted spinning disc confocal microscope. Higher resolution overview images and maximum projections of a z-stack including 32 focal planes were taken with a 60× objective (Figure 2a) and indicate CIPK24/CBL10 complex formations (yellow) at the vacuolar membrane that is clearly distinguishable from SCFP3A (cyan) localization in the cytoplasm and nucleus. For semiquantitative analyses images were taken with a 20× objective and quantified 3 days after infiltration (Figure 2b). After p19 background subtraction BiFC intensities were normalized to CIPK24/CBL10 interaction. Here, the use of the NAF domain deletion construct (CIPK24_{NAF}) resulted in reduced BiFC intensities of 41 % (BiFC constructs only) and 44 % (co-expressed with SCFP3A). Normalization of the BiFC/SCFP3A (Y/C)-ratios resulted in a reduced BiFC efficiency for CIPK24_{NAF}/CBL10 of 57 %. These data indicate that compared to CIPK24 wild type protein CIPK24_{NAF} interacts less efficient with CBL10 (49, 106). For western analyses, proteins were extracted using 1× SDS sample buffer, separated in 10 % Acrylamide/Bisacrylamide SDS gels and blotted onto PVDF membranes. Immuno detection of the different BiFC and SCFP3A construct combinations resulted in comparable expression levels of the respective fusion proteins (Figure 2c). Samples infiltrated only with the p19 strain were used as background controls and did not exhibit a respective signal in the western blots. PageBlue staining of the large RuBisCO subunit was used as loading control.

Taken together, co-expression of a reference FP, here SCFP3A (107), is not necessarily needed for BiFC quantification in *N. benthamiana* but it could minimize differences in BiFC signals due to differences in expression efficiencies between different leaves or leaf areas. Semiquantitative BiFC analyses in *N. benthamiana* as presented in Figure 2 are only applicable for PPIs that are localized in the plasma membrane, the cytoplasm including the nucleus, the endoplasmic reticulum and the tonoplast. Such BiFC quantifications are not applicable for smaller compartments or organelles like the nucleus, peroxisomes, chloroplasts, mitochondria and the endosomal compartments as subtraction of the background signals from entire images most likely would lead to undetectable signals. Here, signal intensities of individual organelles from different samples need to be compared.

As transient expression in *Arabidopsis* is less robust than expression in *N. benthamiana*, co-expression of a reference FP is recommended for semiquantitative BiFC analyses. In addition, prior to BiFC analyses, it is recommended to infiltrate single FP-fusions of proteins, which should be investigated, to verify proper expression. Infiltration of YN-CIPK24/CBL10-YC into *Arabidopsis* did not result in detectable BiFC signals, possibly due to silencing or inefficient expression of YN-CIPK24 (data not shown). Type 2C protein phosphatases interact with SnRK2 protein kinases (108–111). Interaction of the SnRK2 kinase OST1 (112) and the PP2C phosphatase ABI1 (108, 110) was exemplarily investigated in infiltrated *N. benthamiana* and *Arabidopsis* leaves. YFP^{N173} was fused to the N-terminus of OST1 (YN-OST1) and YFP^{C155} was fused to the N-terminus of ABI1 (YC-ABI1), infiltrated into *N. benthamiana* and *Arabidopsis* leaves and analyzed four and seven days after infiltration, respectively (Figure 2d). BiFC analyses in both plant species indicate OST1/ABI1 complex formations with similar cytoplasmic and nuclear localization patterns.

grinding ball (sample) and freeze in liquid N₂. Samples could be stored at – 80 °C for few days but immediate protein extraction is recommended.

2. Prepare protein extraction buffer and keep on ice.
3. Pre-chill Retsch Mill buckets in liquid N₂, place liquid N₂ frozen samples inside buckets and grind three times for 30 sec with a frequency of 30 s⁻¹. Re-chill buckets with samples in liquid N₂ between grinding steps.
4. Keep samples in cold buckets, until 100 µL protein extraction buffer was added for *N. benthamiana* or 75 µL for *Arabidopsis* samples and rotate samples for 10 min at 4 °C. (Note 10).
5. Sonicate twice for 10 sec (0.5 sec on/off) on ice.
6. Spin samples in a microfuge for 10 min at 14000 rpm and 4 °C.
7. Transfer supernatant to 1.5 mL tube and spin samples for 10 min at 14000 rpm and 4 °C.
8. Transfer supernatant to 1.5 mL tube, flash freeze in liquid N₂ and store at – 80 °C.
9. Prepare one 1.5 mL tube with 195 µL H₂O for each sample, add 5 µL of protein extract and 800 µL Amidoblack staining solution. Vortex vigorously and spin for 20 min at 14000 rpm.
10. Discard supernatant, add 1 mL Amidoblack de-staining solution, vortex and spin for 10 min at 14000 rpm. Repeat de-staining step.
11. Discard supernatant and air dry samples.

⁹A representative cell ideally harbors the nucleus in the center of the cell when looking on the z-axis. The nucleus is the best orientation point in a cell because it allows to distinguish between certain localization patterns. Plasma membrane localization of fluorescent proteins or protein complexes is characterized by a fluorescence signal only at the cell border. Here, plasmolysis may allow the appearance of Hechtian strands. Endoplasmatic reticulum localization displays a ring like structure around the nucleus and a net/mesh-like architecture. The vacuolar membrane is characterized by forming a pocket around the nucleus and most likely pushing the nucleus at the cell border. Here also membraneous invaginations and cytoplasmic strands are visible, forming a tunnel system through the large central vacuole (CIPK24/CBL10 complexes in Figure 2a). In some cases also ring like structures are visible. Cytoplasmic localization is visible by fluorescence of cytoplasmic strands. In addition to that the cytoplasm is forced in close proximity to the cell borders and the nucleus as the central vacuole occupies more than 90 % of the cell volume. Cytoplasmic proteins or protein complexes are often dually localized and also found in the nucleus (SCFP3A in Figure 2a, OST1/ABI1 complexes in Figure 2d), except of those that have a nuclear export signal or are too large to pass the nuclear pores passively. To distinguish between plasma membrane, vacuolar membrane, cytoplasm and endoplasmatic reticulum it is recommended to search for cytoplasmic strands, fluorescence around the nucleus and to carefully analyze the architecture of the fluorescence signal at the cell border in a top or bottom cell view or in a maximum projection (see Figure 2a). The nucleus is the largest organelle in the cell and fluorescence is often excluded from the nucleolus. The second largest organelles are chloroplasts. However compared to mesophyll cells, in epidermal cells chloroplasts are smaller and in lower quantity. Chloroplasts exhibit an elliptical structure and are often localized at the cell borders or around the nucleus. Chloroplasts can be easily visualized by chlorophyll auto-fluorescence. However, chlorophyll auto-fluorescence may also appear if too high excitation energy or exposure times are used, especially when FP or BiFC signals are weak. It is more difficult to identify other small cellular organelles like peroxisomes, the golgi apparatus or other endosomes. Here co-localization studies using fluorescent markers are recommended.

For representative images it is recommended displaying one overview image with the focal plane through the nucleus and the cell center. In addition a maximum projection of a z-stack is the first choice to visualize an entire cell, as here multiple focal planes through the cell are merged in one image (see Figure 2).

¹⁰The use or modification of the protein extraction buffer enables also additional biochemical analyses like co-affinity purification (e.g. iBiSC), which could be conducted using GFP antibodies or antibodies recognizing additional tags in the BiFC constructs. Alternatively to the use of protein extraction buffer, proteins could be extracted directly in 1× SDS sample buffer. In that case, keep samples after grinding in frozen Retsch Mill buckets, add 100 µL (for *N. benthamiana* samples) or 75 µL (for *Arabidopsis* samples) 1× SDS sample buffer and directly boil samples for 5 min at 95 °C. Spin samples for 20 min at 14000 rpm and 4 °C, transfer supernatant to 1.5 mL tube and store at – 20 or – 80 °C. Protein quantification using Amidoblack staining is compatible with SDS sample buffer.

12. Resuspend by vortexing in 1 mL 0.2 M NaOH, transfer to 1 mL plastic cuvette and measure OD_{615 nm} in spectrophotometer.
13. Calculate protein concentrations of each sample according to a BSA standard calibration.

3.8 SDS-gel preparation (prepare one day before use)

1. Rinse glass plates of gel-pouring system with water and acetone and dry with kimwipes. Clean spacers and combs with water and 70 % ethanol and dry with kimwipes.
2. Mount gel-pouring system.
3. Boil 0.375 M Tris/HCl pH 8.8 supplemented with 1 % Agarose in microwave and pour on large glass plate. Immediately place gel-pouring system on polymerizing solution to seal lower part of the gel-pouring system.
4. Prepare separating-gel solution in 50 mL Falcon tube. For one 8 mL SDS-gel with 10 % Acrylamide/Bisacrylamide use: 3.920 mL ultra pure H₂O, 2 mL 1.5 M Tris/HCl pH 8.8, 2 mL 40 % Acrylamide/Bisacrylamide, 40 µL 20 % SDS.
5. To induce polymerization, add 4 µL TEMED and 40 µL 10 % APS, mix gently and pour into gel-pouring system. Leave space for the stacking-gel and comb. Carefully add 1 mL isopropanol to poured gel and incubate until the SDS separating-gel is polymerized, usually 45 – 60 min.
6. Prepare stacking-gel solution. For 2.75 mL stacking-gel use: 1.6 mL ultra pure H₂O, 625 µL 0.5 M Tris/HCl pH 6.8, 500 µL 40 % Acrylamide/Bisacrylamide, 25 µL 20 % SDS.
7. Before pouring the stacking-gel, decant isopropanol from separating-gel and wipe off residual isopropanol with kimwipes.
8. Add 5 µL TEMED and 50 µL 10 % APS to stacking-gel solution, mix by gentle pipetting and pour over separating-gel. Immediately insert comb into gel-pouring system. Stacking-gel will polymerize within 2 min.
9. Wrap polymerized SDS-gels into wet paper towels and cling film and store at 4 °C.

3.9 SDS-PAGE and western-blotting

1. Calculate the volume of extracted protein for each sample to reach 15 µg total protein.
2. Thaw protein extracts on ice and transfer calculated protein amount to 1.5 mL tube. Add 4× SDS sample buffer to reach a final 1× concentration.
3. Label wells of SDS-gel(s) with a waterproof pen, mount gel(s) into electrophoresis system, add 1× SDS running buffer, remove combs of SDS-gels and wash wells with 1× SDS running buffer using a 200 µL pipette.
4. Boil protein samples 3 – 5 min in 95 °C heat-block, spin briefly and place on ice.

5. Load protein samples together with dual-color protein marker on SDS-gel(s), and run at maximum voltage and 20 mA/SDS-gel constant current until the bromophenol blue band reaches the separating gel, then run at constant current of 30 mA/SDS-gel until the bromophenol blue band reaches the bottom end of the SDS-gel(s).
6. While SDS-PAGE is running, prepare an appropriate volume of blotting buffer, PVDF-membranes and Whatman papers with appropriate SDS-gel size.
7. After run unmount SDS-gel(s), remove stacking-gel(s) and incubate separating-gel(s) 10 min in blotting buffer.
8. Activate PVDF-membranes 30 sec in methanol, wash briefly in ultra pure H₂O and incubate in blotting buffer.
9. Mount western-blot(s) according to manufacturer's instructions, usually in a sandwich of Whatman paper(s), SDS-gel, PVDF-membrane and Whatman paper(s). SDS-gel should be placed on the cathode side and PVDF-membrane on the anode side.
10. Run western blot(s) 2 h at constant current of 50 mA.
11. Prepare blocking-buffer and heat up while stirring to solubilize the fat free milk powder. Allow cooling down before use. Up to three PVDF-membranes can be blocked in 100 mL blocking buffer.
12. After run unmount western blot(s) and check successful blotting by visualization of the dual-color protein marker on the PVDF-membrane(s).
13. Block PVDF-membrane(s) with gentle shaking in blocking buffer for 1 h at room temperature or over night at 4 °C.
14. Wash SDS-gel(s) with gentle shaking for 10 min in ultra pure H₂O, discard H₂O and stain SDS-gel(s) in Coomassie staining-solution or PageBlue for 3 – 4 h or overnight (**for Coomassie staining move to 25.**).
15. After blocking, wash PVDF-membrane(s) with gentle shaking three times for 5 min in TBS-T.
16. Discard washing solution and bind primary antibodies (monoclonal HA-, c-myc-, FLAG- or GFP-antibody diluted 1:4000) to membrane(s) by gentle shaking for 2 h at room temperature. Binding of HA-, c-myc- and GFP-antibodies can be extended over night at 4 °C.
17. Recycle primary antibodies and wash PVDF-membrane(s) three times for 5 min in TBS-T with gentle shaking.
18. Discard washing solution and bind secondary antibody (goat anti mouse horseradish peroxidase-conjugate diluted 1:10000) by gentle shaking for 45 min at room temperature.
19. Discard secondary antibody and wash PVDF-membrane(s) three times for 5 min in TBS-T with gentle shaking.

20. While washing the membranes prepare ECL developing-solution and keep in 15 mL Falcon tube wrapped in aluminum foil.
21. Move to the dark room and briefly dry PVDF-membrane(s) from washing buffer by placing them on kimwipes. Place membrane(s) on cling film and add 2 mL ECL developing solution to each membrane. Embed membrane(s) in cling film to ensure even distribution of ECL developing solution and incubate 2 min in the dark.
22. Briefly dry PVDF-membrane(s) from ECL developing solution by placing them on kimwipes. Transfer membrane(s) to autoradiography cassette and in the dark place one x-ray film into cassette, close cassette tightly and incubate for 5 min. Incubation time can be adjusted depending on the strength of the detection signal.
23. Develop x-ray film in the dark using the developing machine.
24. After developing, label protein marker bands with waterproof pen on developed film. Scan developed x-ray film and save as .tiff file.
25. Recycle Coomassie staining or PageBlue solution and wash SDS-gel(s) twice with respective de-staining solution until desired contrast is reached. Dry SDS-gel(s) with gel drying system.
26. Scan SDS-gel(s) and save scans as .tiff file. Dependent on the manufacturer, PVDF-membrane(s) can be Coomassie stained after western blot development by shaking for 1 – 2 h in Coomassie staining-solution. After staining wash membranes two times in de-staining solution, dry on kimwipes, scan and save scan as .tiff file.

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Abbreviations

BiFC	bimolecular fluorescence complementation
BiLC	bimolecular luminescence complementation
BRET	bioluminescence resonance energy transfer
CBB	Coomassie brilliant blue
CFP	cyan FP
coBiFC	co-localization of BiFC complexes
ECL	enhanced chemo luminescence
FP	fluorescent protein
FP^C	FP C-terminal fragment
FP^N	FP N-terminal fragment

FRET	fluorescence resonance energy transfer
GFP	green FP
iBiSC	isolation of BiFC stabilized complexes
mcBiFC	multicolor BiFC
OD₆₀₀	optical density at 600 nm
PCA	protein fragment complementation assay
PPI	protein-protein interaction
PVDF	polyvinylidene fluoride
RFP	red FP
RLuc	<i>Renilla reniformis</i> luciferase
SUS	split-ubiquitin system
TEV	tobacco etch virus protease
UbFC	ubiquitin-mediated fluorescence complementation
Y2H	yeast-two-hybrid
YFP	yellow FP

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Figure 1.

Different examples for BiFC applications. BiFC can be applied to investigate (a) interactions of single PPI pairs (BiFC), (b) co-localization of different PPI pairs (coBiFC), (c) competition of two protein complex formations (mcBiFC), (d) analyses of ternary protein complex formations (BiFC based FRET or BRET) or (e) the analysis of quaternary protein complexes (by combining BiLC and BiFC). The protein fragment and/or donor/acceptor combinations recommended for each application and the resulting excitation and emission maxima or substrate catalysis of the respective (complemented) proteins are indicated. The proteins to be investigated for PPI are labeled from A – D. For more information refer to main text.

Figure 2.

BiFC analyses in *Nicotiana benthamiana* and *Arabidopsis*. (a) Representative images of YN-CIPK24/CBL10-YC BiFC (yellow, tonoplast) co-expressed with SCFP3A (cyan, cytoplasm and nucleus) in *N. benthamiana*. Left panel, overview images and right panel, maximum projections of a z-stack including 32 focal planes of the same cell (60× objective, scale = 20 μm). (b) Normalized YFP fluorescence (BiFC) or BiFC/SCFP3A (Y/C)-ratios from randomly taken images of epidermal cells infiltrated with the indicated BiFC construct combinations using a 20× objective 3 days after infiltration into *N. benthamiana*. Given are averages ± standard errors (n = 10). Compared to CIPK24/CBL10, expression of CIPK24_{NAF}/CBL10 exhibited only 41 – 57 % BiFC. (c) Western blot analyses of BiFC construct/SCFP3A combinations from the same experiments as in (a and b). From top to bottom, CBL10-YC detected with α-HA antibody, YN-CIPK24(NAFΔ) detected with α-GFP antibody, SCFP3A detected with α-GFP antibody and PageBlue staining of the large RuBisCO subunit (rbcL) in representative SDS-gel as loading control. Loaded were 10 μg total protein for CBL10 and rbcL and 15 μg for CIPK24(NAFΔ) and SCFP3A blots. In the p19 lane protein extracts of only with p19 infiltrated leaves were loaded. Western blots confirm expression of BiFC and SCFP3A constructs in indicated construct combinations. (d) BiFC analyses of YN-OST1/YC-ABI1 in *N. benthamiana* (N.b.; left panel) and *Arabidopsis* (A.t.; right panel). Depicted are overview images (left) and maximum projections of 32 focal

plane z-stacks (right). Images were taken with a 60× objective 4 (*N.b.*) and 7 days (*A.t.*) after infiltration. Scalebars = 20 μm . BiFC analyses indicate OST1/ABI1 complex formations in the cytoplasm and the nucleus.