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Chapter 12

Bimolecular Fluorescence Complementation (BiFC) to Study Protein–Protein Interactions in Living Plant Cells

Katia Schütze, Klaus Harter, and Christina Chaban

Abstract

Dynamic networks of protein–protein interactions regulate numerous cellular processes and determine the ability of cells to respond appropriately to environmental stimuli. However, the study of protein complex formation in living plant cells has remained experimentally difficult and time-consuming and requires sophisticated technical equipment. In this report, we describe a bimolecular fluorescence complementation (BiFC) technique for visualization of protein–protein interactions in plant cells. This approach is based on the formation of a fluorescent complex by two non-fluorescent fragments of the yellow fluorescent protein (YFP) brought together by the association of interacting proteins fused to these fragments. We present the BiFC vectors currently available for the transient and stable transformation of plant cells and provide a detailed protocol for the successful use of BiFC in plants.

Key words: Protein–protein interaction, BiFC, YFP, tobacco infiltration, protoplast transfection, intracellular localization, bZIP transcription factors.

1. Introduction

The identification and characterization of protein–protein interaction provide crucial information for understanding the molecular mechanisms underlying biological processes. Although numerous methods for the detection of interacting proteins have been developed, they often operate under non-native conditions or in non-plant systems. In vitro interaction assays using recombinant proteins, which are not folded properly, may result in the formation of non-specific aggregations or loss of interaction. Furthermore, co-immuno-precipitation experiments on the basis of plant

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extracts bear the risk that proteins that are normally localized in different cell compartments are observed to interact *(1, 2)*. The yeast two-hybrid screen is widely used for the detection of interacting proteins in living cells, while retaining the native state of proteins. However, this heterologous host system suffers from significant false-positive and false-negative rates *(1, 3, 4)*.

The cloning of spontaneously fluorescent protein from the jellyfish *Aequorea victoria* opened a new era in the visualization of proteins in vivo. With the help of this approach several methods have been developed to analyse protein–protein interactions in the homologous host system. By monitoring the fluorescence resonance energy transfer (FRET) between colour variants of GFP fused to given proteins, molecular interactions have been successfully examined (reviewed in **Ref. 5**). Nevertheless, the necessity for the expression of high protein levels and for sophisticated equipment to determine small changes in the fluorescence restricts FRET-based methods to selected applications and experienced laboratories (the advantages and limitations of the method have been recently reviewed by Bhat et al., *6)*. Here, we describe the relatively fast and inexpensive method, bimolecular fluorescence complementation (BIFC), which was originally applied for protein–protein interaction studies in animal cell systems *(7)* and has since been successfully used in the plant field. It is based on the formation of a fluorescent complex by non-fluorescent fragments of the enhanced yellow fluorescent protein (YFP) when brought together by the interaction of two partners fused to these fragments (**Fig. 12.1**, *7–9)*.

Besides the direct visualization of complex formation in living cells, this method also allows the detection of the intracellular location at which the protein association occurs *(9, 10)*. However, it should be mentioned that sometimes very high expression of the YFP fragments may lead to the detection of unspecific fluorescence *(9)*. This might be due to the intrinsic tendency of YFP fragments to form irreversible complexes *(11)*. This phenomenon might also enhance sporadic false-positive signals. By removing the amino acids 153 to 155 in the N-terminal fragment of YFP by the introduction of a pre-mature stop codon

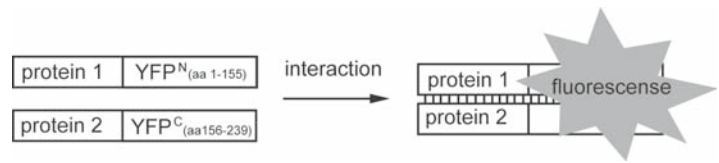

Fig. 12.1. Reconstitution of fluorescent YFP by its two non-fluorescent fragments mediated by protein–protein interaction. YFP fragments (YFP^{N}, N-terminal fragment [amino acids 1–155]; YFP^{C}, C-terminal fragment [amino acids 156–239]) are fused to the proteins of interest. Interaction of these proteins leads to the formation of a fluorescent complex by the fragments of the YFP.

(153STOP), the stability of the fluorophore complex could be reduced, thus lowering the false-positive rate and probably making it possible to follow the dynamics of protein–protein interactions in vivo (C. Oecking, personal communication).

In this report we present sets of plant-compatible BiFC vectors, which allow the examination of protein–protein interactions using either the N- or C-terminal fusion of YFP fragments in transiently or stably transformed plant systems. Vectors have been generated containing Gateway-compatible or classic multiple cloning sites. Using these vectors, we investigated dimerization between the subfamily C members of the *Arabidopsis thaliana* basic region-leucine zipper (bZIP) transcription factors *(9)*. The detailed protocols for the transformation of *Agrobacterium tumefaciens*, the infiltration of *Nicotiana benthamiana* leaves and the transfection of protoplasts are described here. Depending on particular bZIP proteins studied, the interacting complexes were detected either in the nucleus or in the cytoplasm of the plant systems (**Fig. 12.2**).

The differential intracellular location of bZIP factors was confirmed by an alternative method *(12)* and may contribute to the distinctive mechanisms of the regulation of bZIP factor-dependent transcription *(13)*. Our study indicates that the BiFC technique represents a fast, efficient and convenient tool to investigate protein–protein interactions in living plant cells.

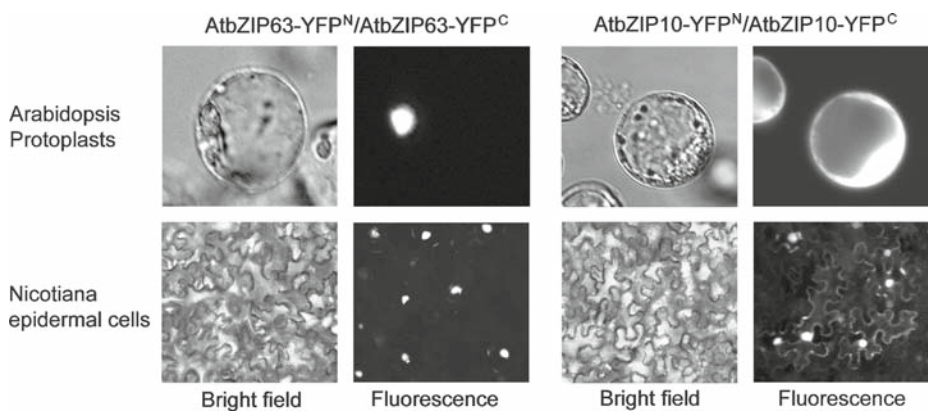

Fig. 12.2. BiFC visualization of AtbZIP63 and AtbZIP10 homodimerization in *Arabidopsis* protoplasts and *Agrobacterium* infiltrated tobacco leaves. (I) Epifluorescence and (II) bright field images of *Arabidopsis* protoplasts (*upper row*) and epidermal leaf cells (*lower row*) infiltrated with a mixture of *Agrobacterium* suspensions harbouring the constructs encoding the indicated fusion proteins. In both cell systems AtbZIP63 homodimers accumulate exclusively in the nucleus, whereas AtbZIP10 homodimers are found in the nucleus and the cytoplasm.

Table 12.1
Vectors for transient protoplast transfection[1]

YFP-Fusion	Cloning	Vector	Type	Reference
C-terminal	Gateway	pUC-SPYNE GW	High copy	*(9)*
		pUC-SPYCE GW	High copy	*(9)*
	MCS	pUC-SPYNE^{153STOPGW}	High copy	Oecking, unpublished
		pUC-SPYNE	High copy	*(9)*
		pUC-SPYCE	High copy	*(9)*
		pUC-SPYNE^{153STOP}	High copy	Oecking, unpublished
N-terminal	Gateway	pE SPYNE	High copy, binary	*(18)*
		pE SPYCE	High copy, binary	*(18)*

[1]A schematic sketch of vectors is shown in **Fig. 12.3**.

Table 12.2
Vectors for either tobacco leaf infiltration or stable transformation of plants[1]

YFP-Fusion	Cloning	Vector	Type	Reference
C-terminal	Gateway	pSPYNE-35S GW	Binary	Lahaye, unpublished
		pSPYCE-35S GW	Binary	Lahaye, unpublished
	MCS	pSPYNE-35S	Binary	*(9)*
		pSPYCE-35S	Binary	*(9)*
N-terminal	Gateway	pE SPYNE	High copy, binary	*(18)*
		pE SPYCE	High copy, binary	*(18)*

[1]A schematic sketch of vectors is shown in **Fig. 12.3**.

2. Materials

2.1. Plant BiFC Vectors

2.2. Protoplast Transfection

1. Wall digestion solution without enzymes: 8 mM $CaCl_2$, 0.4 M mannitol, pH 5.5, filter sterile.
2. Wall digestion solution: 1% cellulase, 0.25% macerozym, 8 mM $CaCl_2$, 0.4 M mannitol, pH 5.5, filter sterile.

3. W5 solution: 154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl, 5 mM glucose, pH 5.8–6.0, autoclave.
4. MMM solution: 15 mM $MgCl_2$, 0.1% MES, 0.5 M mannitol, pH 5.8, autoclave.
5. PEG solution: 40% PEG 4000, 0.4 M mannitol, 0.1 M $Ca(NO_3)_2$, pH 8–9 (the pH needs 1–2 h to stabilize), autoclave.
6. Macro stock (1000 ml): 1.5 g $NaH_2PO_4 \bullet H_2O$, 9.0 g $CaCl_2 \bullet 2H_2O$, 25 g KNO_3, 2.5 g NH_4NO_3, 1.34 g $(NH_4)_2SO_4$, 2.5 g $MgSO_4 \bullet 7H_2O$, add H_2O up to 1 l, autoclave.
7. Micro stock (100 ml): 75 mg KI, 300 mg H_3BO_3, 1 g $MnSO_4$ $7H_2O$ (0.6 g $MnSO_4 * H_2O$), 200 mg $ZnSO_4 \bullet 7H_2O$, 25 mg $Na_2MoO_4 \bullet 2H_2O$, 2.5 mg $CuSO_4 \bullet 5\ H_2O$, 2.5 mg $CoCl_2 \bullet 6H_2O$, add H_2O up to 100 ml, filter sterile and freeze.
8. K3 solution (100 ml): 10 ml macro stock, 0.1 ml micro stock, 0.1 ml vitamin stock, 0.5 ml EDTA stock, 1 ml Ca-phosphate stock, 10 mg myo-inositol, 25 mg d(+)-xylose, 13.7 g sucrose, pH 5.6, filter sterile and freeze in 10 ml aliquots.
9. Vitamin stock (100 ml): 100 mg nicotinacid, 100 mg pyridoxin HCl, 1 g thiamin $\bullet$ HCl, add H_2O up to 100 ml, filter sterile and freeze.
10. EDTA stock (1000 ml): 7.46 g EDTA dissolve in 300 ml H_2O and cook, 5.56 g $Fe*SO_4$ $7H_2O$ dissolve in 300 ml H_2O and cook, mix and add H_2O up to 1 l, autoclave and keep in the dark.
11. Ca-phosphate stock (200 ml): 1.26 g $CaHPO_4 \bullet 2H_2O$ dissolve in H_2O, add H_2O up to 200 ml, pH 3 with 25% HCl, autoclave and keep in the dark.

2.3. Transformation of Agrobacterium cells

Agrobacterium tumefaciens GV3101/pMP90, a strain based on the C58 and pTiC58 genotypes *(14–16)* that carries genes for resistance to gentamycin and rifampicin, was used in this study. An *Agrobacterium* strain containing the p19 protein of tomato bushy stunt virus was used to suppress gene silencing in transformed tobacco leaves *(17)*.

1. YEB-medium (1000 ml): 5 g bactopeptone, 5 g beef extract, 1 g yeast extract, 5 g saccharose, 0,5 g $MgSO_4 \bullet 7H_2O$ and add 15 g agar for solid medium, autoclave.
2. TE buffer: 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, autoclave.

3. The antibiotics for the strain and for the plasmids were used at the following concentrations: rifampicin 100 µg/ml, gentamycin 40 µg/ml, kanamycin 25 µg/ml, carbenicillin 50 µg/ml.

2.4. Infiltration of Nicotiana benthamiana

1. AS-medium (100 ml): 1 ml 1 M MES-KOH, pH 5.6, 333 µl 3 M $MgCl_2$, 100 µl 150 mM acetosyringon (in DMSO, stored in aliquots at –20°C); prepare fresh from stock solutions.
2. Sterile single-use syringes (1 ml)

2.5. SDS-PAGE and Western Analysis

1. Sample buffer: 8 M urea, 2% SDS, 0.1 M DTT, 20% glycerol, 0.1 M Tris-HCl (pH 6.8), 0.004% bromophenol blue (store frozen in aliquots).
2. Blocking buffer: TBS (50 mM Tris-HCl pH 7.4, 150 mM NaCl) containing 0.1% Tween-20 and 4% milk powder.
3. Primary antibodies: rat monoclonal anti-HA and mouse monoclonal anti-c-myc (Roche).
4. Secondary antibodies: anti-rat IgG alkaline phosphatase conjugate developed in goat (Sigma) and anti-mouse IgG alkaline phosphatase conjugate developed in goat (BIO-RAD)
5. AP-buffer: 100 mM Tris-HCl pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$
6. NBT-Solution: 50 mg/ml nitro-blue tetrazolium chloride (NBT) in 70% dimethylformamide, store in aliquots at –20°C.
7. BCIP-Solution: 50 mg/ml 5-bromo-4-chloro-3-indolyl-phosphate, disodium salt (BCIP) in H_2O, store in aliquots at –20°C.
8. AP-staining solution: mix 66 µl of NBT-solution and 33 µl of BCIP solution in 10 ml AP-buffer.

3. Methods

3.1. Cloning into BiFC vectors

Different sets of vectors are available for N-terminal or C-terminal YFP fusions. Because the fusion can interfere with the interaction domains of proteins of interest, it is important to decide whether the N-terminal or C-terminal YFP fusion is more suitable for the study (*see* **Note 1**). For example, with some S-group bZIP transcription factors from *Arabidopsis*, the interaction could only be shown when the YFP fragments were fused to the N-terminus of the proteins *(18)*. Depending on the cloning strategy (classic or Gateway) the cDNA can be cloned through restriction and ligation or through LR-recombination from the entry clone into the

destination/expression clone. The presence and correct orientation of the insert should be confirmed with appropriate restriction enzymes and sequencing (compare **Tables 12.1** and **12.2**, *see* **Fig. 12.3**).

3.2. BiFC in Protoplasts

3.2.1. PEG Mediated Transfection of Arabidopsis thaliana Cell Suspension Protoplasts

1. Use cells 3 days after sub-cultivation.
2. Collect the cells (2 × 10 ml culture) by centrifugation at 400× g for 5 min.
3. Wash once with 10 ml of wall digestion solution without enzymes, centrifuge at 100 × g for 5 min.
4. Resuspend each pellet in 7 ml of digestion solution and dispense each into a Petri dish.
5. Incubate at 26°C in the dark for 6 h in a shaker at 50 rpm.
6. Collect protoplasts in 2 tubes by centrifugation at 100× g for 5 min.

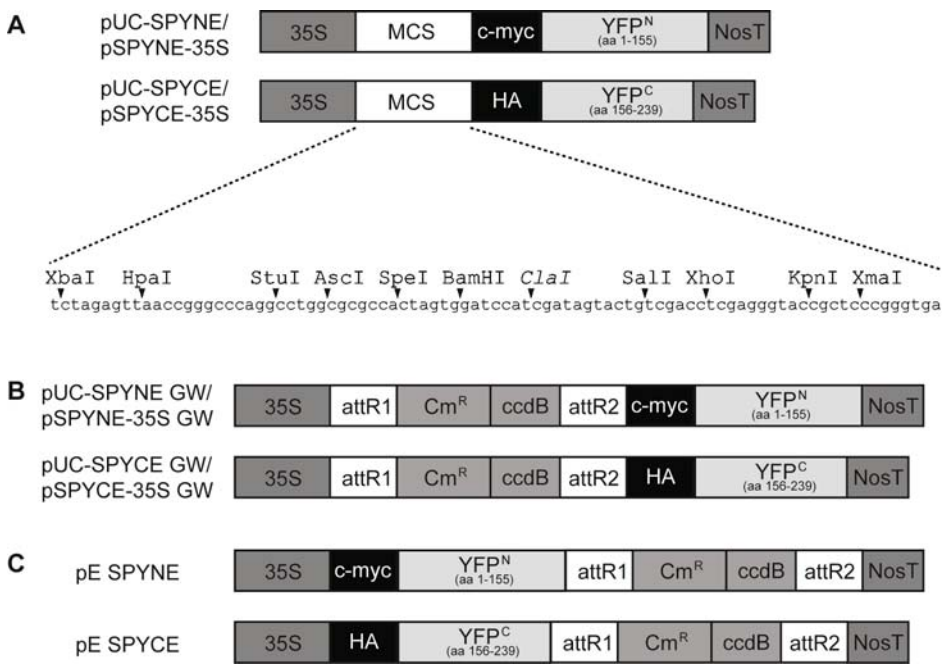

Fig. 12.3. Schematic representation of plant-compatible BiFC vectors. (**a**) Classic BiFC vectors with C-terminal fusion of the YPF fragments: pUC-SPYNE/ 35S-pSPYNE and pUC-SPYCE/35S-pSPYCE *(9)*. 35S, 35S promoter of the cauliflower mosaic virus; MCS, multiple-cloning site. Unique restriction sites are illustrated with normal letters, others with italic letters. c-myc, c-myc affinity tag; HA, hemagglutinin affinity tag; YFP^N, N-terminal fragment of YFP (amino acid 1–155); YFP^C, C-terminal fragment of YFP (amino acid 156–239); NosT, terminator of the nos-gene. (**b**) Gateway compatible BiFC vectors with C-terminal fusion of the YPF fragments: pUC-SPYNE GW/35S-pSPYNE GW and pUC-SPYCE GW/35S-pSPYCE GW. attR1-CmR-ccdB-attR2, Gateway conversion cassette (Invitrogen). (**c**) Gateway compatible BiFC vectors with N-terminal fusion of the YPF fragments: pE SPYNE and pE SPYCE *(18)*.

7. Wash once with 10 ml of wall digestion solution without enzymes.
8. Centrifuge at 100× g for 5 min.
9. Remove the supernatant and resuspend cells in the remaining solution, slowly add 10 ml of W5 solution, mix gently and centrifuge at 100× g for 5 min.
10. Resuspend pellets in 10 ml of W5 solution, take an aliquot for counting, store 20 min in the dark at 4°C and count protoplasts during this time, for example in a Fuchs-Rosenthal chamber.
11. Centrifuge at 100× g for 5 min, remove all the W5 solution and resuspend pellet in MMM solution to a density of 2 × 10^6 protoplasts/ml.
12. Redistribute samples of 250 µl into tubes, add 30 µg of plasmid DNA, (high DNA purity is required) very slowly add 250 µl PEG solution, mix gently and incubate 15–20 min.
13. Gradually add 10 ml of W5 solution; this needs to be done very slowly, to not disrupt the protoplasts.
14. Centrifuge at 100× g for 5 min, and remove the supernatant.
15. Resuspend the protoplasts in 2 ml of K3 solution, and incubate at 26°C in the dark.

3.2.2. Microscopy

Usually the expression of the fusion proteins is efficient 12–18 h after the transfection. Fluorescence was assayed with the Nikon eclipse 90*i* microscope and quantification of the fluorescence intensity was performed using the Metamorph software (Universal Imaging Corporation Downington, PA, USA). The optimal excitation wavelengths for YFP are in the range of 490–515 nm; the maximal emission intensity is observed in the range of 520–560 nm. Therefore, the YFP fluorescence might be easily visualized with a fluorescence microscope using an appropriate commercially available filter set.

3.3. BiFC in Nicotiana benthamiana

Two alternative protocols can be used for successful transformation of *Agrobacterium tumefaciens* cells.

Electroporation (according to the method described by Mersereau et al. *19)*:

3.3.1. Transformation of Agrobacterium *Cells*

1. Grow *Agrobacterium* cells at 28°C to a OD_{600} of 1–1.5 and harvest the cells by centrifugation at 3,000× g for 5 min.
2. Wash the cells five times in sterile, cold water, centrifuge at 3,000× g for 5 min and remove the supernatant.
3. Resuspend the cells in 10% glycerol to a density of about 10^{10} cells/ml and store them frozen at –70°C.
4. Thaw the frozen cells in ice and add 0.5–1 µl of plasmid DNA to 50 µl of recipient cells and transfer the cells to a cold electroporation cuvette.

5. Carry out the electroporation by applying a single electrical pulse of 2.5 kV and 400 Ω (e.g., Bio-Rad Gene Pulser).
6. After applying the pulse immediately suspend the cells in 1 ml of cold YEB medium and incubate for 1–2 h at 28°C.
7. Recover the cells by short centrifugation, resuspend in 0.1 ml YEB medium and spread on appropriate selective media. Let colonies grow for 2–3 days at 28°C.

Chemical transformation (freeze–thaw method, according to Ref. *20)*:

This method is simple, rapid and robust, although the cell transformation frequency is comparatively low.

1. Grow a single *Agrobacterium* colony in 5 ml of YEB (with antibiotics) medium overnight at 28°C.
2. Add 2 ml of the overnight culture to 50 ml YEB medium and let the culture grow to an OD_{600} of 0.5–0.7 at 28°C.
3. Centrifuge the cell suspension at 3,000× g for 15 min at 4°C, and discard the supernatant.
4. Wash the cells once in 5 ml pre-cooled TE buffer and finally resuspend the pellet in 5 ml of ice-cold fresh YEB medium. Use aliquots of 0.2 ml directly for transformation or freeze in liquid nitrogen and store at –70°C.
5. Add about 1–5 μg of plasmid DNA to the competent cells, incubate 5 min on ice and freeze the cells in liquid nitrogen (5 min).
6. Thaw the cells by incubating for 5 min at 37°C.
7. Add 1 ml of YEB medium and incubate for 2–4 h at 28°C with shaking.
8. Centrifuge for 30 s at 3,000× g, discard the supernatant, resuspend the cells in 0.1 ml YEB medium and spread the cells on a YEB agar plate containing an appropriate antibiotic selection.
9. Incubate the plate at 28°C. Transformed colonies should appear in 2–3 days. Colonies used for tobacco infiltration should be checked by colony PCR.

3.3.2. Infiltration of Nicotiana benthamiana (according to Ref. 21)

1. Distribute a single colony on a fresh plate and let it grow for 1–2 days at 28°C.
2. Inoculate the cells in 5 ml of YEB medium and incubate the culture overnight under shaking at 28°C.
3. Centrifuge the culture for 15 min at 4,000× g and resuspend pelleted cells in 1 ml of AS medium. Dilute the cells with AS medium to OD_{600} 0.7–0.8 (about 1 ml per leaf).
4. Prepare working suspensions by mixing appropriate clones containing the BiFC constructs and the p19 plasmid at a 1:1:1 ratio (total volume of 3 ml per leaf); let them stand for 2–4 h.

5. Give the excess of water to tobacco plants (*see* **Note 2**). It is convenient to use one plant for each variant. Infiltration of two or three leaves per plant with the same sample might be advisable especially when protein expression level is a problem.
6. Co-infiltrate mixed *Agrobacterium* strains into the abaxial air space of tobacco leaves (*see* **Fig. 12.4**). It is important that the total leaf air space is infiltrated. In the case of incomplete infiltration mark the remaining area with a pen because after a while the infiltrated area will not be distinguishable from that not infiltrated.

3.3.3. Microscopy

Observe the fluorescence in the epidermal cell layer of the lower leaf surface expressing the fusion proteins 1–3 days after infiltration (*see* **Notes 3** and **4**).

3.4. SDS-PAGE and Western Analysis

It is necessary to verify the expression of the BiFC fusion proteins in transfected protoplasts and *Agrobacterium* infiltrated tobacco leaf discs (*see* **Note 5** and **Fig. 12.5**).

3.4.1. Sample Preparation from Protoplasts

1. Dilute 0.5 ml of protoplast suspension with 0.5 ml water in an Eppendorf tube and centrifuge at 5,000× g for 10 min to collect the protoplasts.
2. Resuspend the protoplasts in 50–100 µl hot SDS-sample buffer and denature for 5 min at 95°C. Shortly spin down cell debris and separate 15–20 µl of the supernatant by SDS-PAGE. Run two parallel gels for subsequent detection of fusion proteins by both anti-HA and anti-c-myc antibodies.

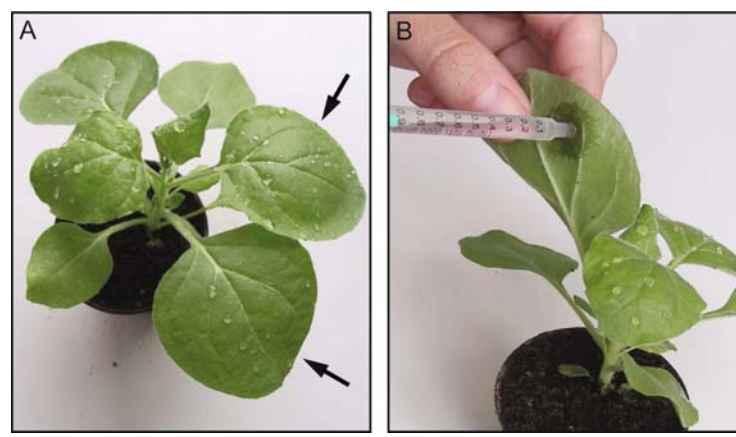

Fig. 12.4. *Agrobacterium* infiltration of *Nicotiana benthamiana* leaves. (**a**) A 5-week-old *N. benthamiana* plant that is appropriate to be infiltrated. The leaves at the optimal developmental stage for infiltration are indicated by arrows. (**b**) Infiltration of the *Agrobacterium* suspension into the abaxial air space of a tobacco leaf (*see Color Plates*).

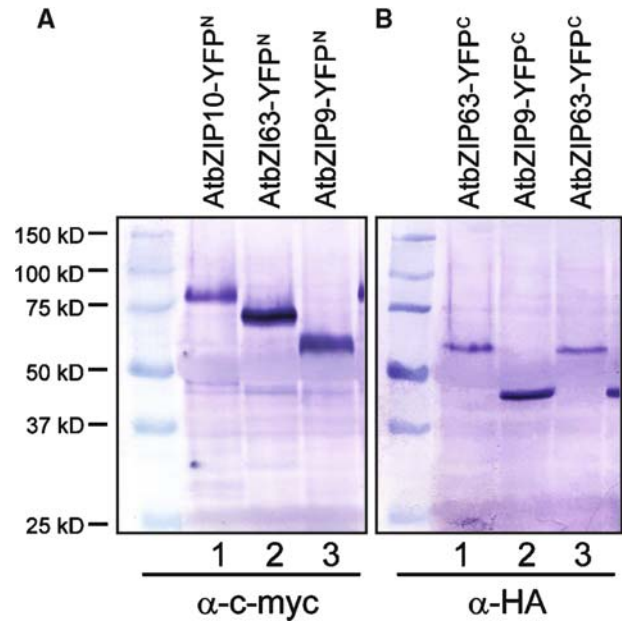

Fig. 12.5. Western blot analysis of overexpressed proteins in *Agrobacterium*-infiltrated tobacco leaves. The expression of the proteins is demonstrated by immunodetection using alkaline phosphatase with (**a**) anti-c-myc antibodies for YFP^N-fusion proteins and (**b**) anti-HA antibodies for YPF^C-fusions. Total protein extracts from tobacco leaves infiltrated with an *Agrobacterium* suspension containing constructs encoding the following fusion proteins (1) AtbZIP10-YFP^N/AtbZIP63-YFP^C, (2) AtbZIP63-YFP^N/AtbZIP9-YFP^C, (3) AtbZIP9-YFP^N/AtbZIP63-YFP^C were separated on a 12.5% SDS-PAGE gel. The size of the marker proteins is shown on the left (*see Color Plates*).

3.4.2. Sample Preparation from Infiltrated Tobacco Leaves

1. Excise a leaf disc of about 40–50 mg, homogenize it in liquid nitrogen in an Eppendorf tube, add 150–200 µl of hot SDS-sample buffer and vortex. Incubate 5 min at 95°C.
2. Centrifuge 5 min at maximal speed to remove cell fragments.
3. Separate 15–20 µl of the supernatant by SDS-PAGE. Run two parallel gels for subsequent detection of fusion proteins by both anti-HA and anti-c-myc antibodies.
   SDS-PAGE and Western blot transfer are to be carried out using standard protocols.

3.4.3. Immunodetection of the Fusion Proteins

1. Incubate the membranes for 2–3 h at room temperature or overnight at 4°C in blocking buffer with continuous shaking.
2. Discard the blocking buffer and incubate the membranes for 2 h at room temperature with the primary anti-HA (1:800) or anti-c-myc (1:1000) antibodies in TBS containing 0.1% Tween-20.
3. Remove the primary antibody (can be reused several times) and wash 3 × 5 min with TBS-Tween.
4. Incubate the membrane for 1–2 h at room temperature with the secondary antibody (anti-rat-AP 1:7000 for anti-HA and anti-mouse-AP 1:3000 for anti-c-myc).

5. Remove the secondary antibody (can be reused several times) and wash 3 × 10 min with TBS-Tween.
6. Equilibrate the membrane in AP-buffer for a short time.
7. Stain with fresh AP staining solution until the signal is clearly visible (15 min to overnight) – do not shake! To reduce background, keep the membranes at 4°C when a longer reaction time is necessary.
8. Stop the reaction by rinsing the membranes twice with water. Dry the membranes.

4. Concluding remarks

Although the BiFC approach was launched not so long ago, it is becoming more and more widely used in the field of protein–protein interaction research. This can be attributed to its main advantages, which include the possibility to assay protein interaction directly *in planta* with comparative simplicity. Up to now, this assay has been primarily used to verify the interaction of particular proteins, although it is not limited to this. Other possible applications such as multiple protein–protein interaction studies by using multicolor BiFC *(22)* and identification of novel interactions by *in planta* screening are presently being developed and tested. The latter would be achievable by co-transfection of protoplasts with the constructs, one of which bears the protein of interest fused with N-terminus of YFP and the second a cDNA-library fused with C-terminus of YFP, and by subsequent sorting and analysis of positive, i.e., fluorescent protoplasts (K. Berendzen and K. Harter, unpublished).

5. Notes

1. The presence of signal peptides or transmembrane domains determines the protein terminus at which the YFP fragment must be fused. However, in the case of BiFC, additional protein features, namely their allosteric configuration and orientation during interaction, also play an important role. As these features are usually difficult to predict, the testing of all possible protein combinations is crucial. Moreover, the testing of the fusion proteins for their functional activity is recommended when feasible (e.g. by complementation of a mutant phenotype).
2. The developmental stage of the *Nicotiana benthamiana* plants is important for the success of this method. Good

results were obtained with 4- to 6-week-old plants and a leaf size of about 5 cm^2 (*see* **Fig. 12.4**). Older plants already developing flowers are less appropriate.

3. As mentioned in the introduction, the expression levels of investigated proteins are quite important. Usually, the highest rate of protein accumulation is observed on the second day after infiltration. Therefore, when the expression of the BiFC fusion proteins is low or the interaction study is performed with the shortened N-terminal YFP fragment ($SPYNE^{153STOP}$), which by itself decreases the intensity of the fluorescence, the microscopic analysis of the tobacco leaves should be carried out on this day. In contrast, if too high expression of the fusion proteins leads to non-specific complex formation, it might be helpful to survey the leaves 24 h or 5 days after infiltration. Fluorescence is usually detectable for 2–3 days and may persist for up to 6 days.
4. It is not necessary to peel off the epidermis to monitor the fluorescence; however, it might improve the quality of the images.
5. In order to correctly interpret the obtained pictures it is absolutely necessary to analyse the protein expression level by means of western analysis as shown in Fig. 12.5. This is possible due to the presence of c-myc and HA epitopes in the expressed proteins fused with N-terminal and C-terminal YFP fragments, respectively.

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