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Plant Secondary Metabolism Engineering

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Cover illustration: Fruit-bearing shoots of the Brazilian Atlantic Forest understorey tree *Psychotria brachyceras* (Rubiaceae). This species accumulates an unusual monoterpene indole alkaloid, brachycerine, which is induced in response to wounding, UV-exposure, jasmonate application, drought and osmotic stress. Its function is probably related to its capacity to mitigate reactive oxygen species, acting both as antioxidant and antimutagenic agent.

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Chapter 19

Fusion with Fluorescent Proteins for Subcellular Localization of Enzymes Involved in Plant Alkaloid Biosynthesis

Patrícia Duarte, Johan Memelink, and Mariana Sottomayor

Abstract

To establish the role in alkaloid metabolism of candidate genes identified in silico or by Omics approaches, it may be essential to determine the subcellular localization of the encoded proteins. The fusion with fluorescent proteins (FP) may now be used as a quite effective and reliable tool to investigate this question. The methodology involves the choice of the FP, the design and production of the appropriate FP fusions, and the use of a transient or stable transformation protocol applied to a homologous or heterologous plant system. This chapter describes the application of this methodology to an enzyme involved in indole alkaloid biosynthesis, with general considerations on the development of the approach.

Key words: Fluorescent proteins, GFP, *Catharanthus roseus*, *Arabidopsis*, subcellular localization, vacuole, confocal microscopy.

1. Introduction

Alkaloids are a large and diverse group of secondary metabolites including some of the most remarkable pharmacological products of plant origin, like the muscle relaxants (+)-tubocurarine and papaverine, the anticancer agents vinblastine and vincristine, or the still number one painkiller, morphine (1, 2). In plants, alkaloids are thought to play a role in defense against herbivores, which would explain their strong physiological activity in animals. In plant cells, alkaloids and many other secondary metabolites accumulate in the vacuole, separated from much of the cell physiological activities with which they could interfere. Likewise, a few

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alkaloid biosynthetic enzymes have been shown to be located in the vacuole (1–4).

Due to the complexity of alkaloid biosynthetic pathways and the low concentrations of alkaloid biosynthetic enzymes in plant tissues, a thorough knowledge of alkaloid biosynthetic pathways is still lacking in many cases (2, 5). However, we can now use new research strategies involving the characterization of recombinant proteins produced from candidate genes selected by homology-based screening or from differential transcriptomic/proteomic analyses (5). In this context, determining the subcellular localization of the candidate proteins may be essential to firmly establish their role in cells. In order to approach this question, fusions with fluorescent proteins (FPs) may be used as a quite effective and reliable tool, provided proper care is taken in the design of the fusions, the choice of the FP, and/or the conditions needed to obtain fluorescence in the case of vacuolar or apoplast localization.

1.1. Outline of the Experimental Procedure

We describe the methodology used to determine the vacuolar localization of the main leaf class III peroxidase (CrPrx1) of the medicinal plant *Catharanthus roseus,* which has been implicated in the biosynthesis of the anticancer alkaloids vinblastine and vincristine (6). The procedure employed involved the following steps:

1. Choice of the fluorescent reporter to be used as the sGFP (S65T) (7, 8).
2. Design and construction of the CPrx1-GFP fusions needed to evaluate the subcellular sorting of CrPrx1.
3. Transient and stable transformation of *C. roseus* cells by particle bombardment with the different constructs.
4. Polyethyleneglycol-mediated transfection of protoplasts from *Arabidopsis* cell cultures with the different constructs.
5. Analysis of transformed cells for the subcellular localization of GFP fluorescence using confocal microscopy.

Some theoretical and practical considerations for the choice of the FP and of the transformation system and the design of fusions are discussed below.

1.2. Choice of the FP Reporter

The use of FPs in plant biology has become a powerful and widespread tool and a number of excellent papers reviewing the use and applications of FPs are available (9–13). GFP, as the first FP to be isolated, characterized, and applied, is still among the most used FPs in plant studies, although YFP and the red variants mRFP and mCherry are gaining preference due to higher brightness and stability, particularly under the acidic pH of the vacuole and the apoplast (10, 14). The two main GFP variants

used in plant cells are the mGFP5 developed by Jim Haseloff and colleagues (http://www.plantsci.cam.ac.uk/Haseloff/imaging/GFP.htm), (15, 16) and the sGFP(S65T), similar to EGFP, developed for human cells (17) and first applied to plants by Chiu, Niwa, and colleagues (7, 8).

In spite of the reported difficulty on observing GFP fluorescence in the vacuole (10, 18), both mGFP5 and sGFP(S65T) were used successfully in vacuoles (4, 6, 18–23), with no special difficulty mentioned in those reports. However, according to Tamura and colleagues (18) and to our own experience (6), at least sGFP(S65T) shows fluorescence in the vacuole only if the transgenic plants or cells are maintained in the dark for 48 h prior to observation. Tamura and colleagues (18) show that this is due to a light-induced conformational change of GFP that makes it susceptible to degradation by vacuolar papain-type cysteine proteinase(s) under acidic pH. Our experience with sGFP(S65T) fusions showed us that vacuolar fluorescence was only observed in either *C. roseus* cells or *Arabidopsis* protoplasts if the cells were previously incubated in the dark. It was also quite evident that the fluorescence observed in the vacuoles of *C. roseus* cells was much fainter than in vacuoles of *Arabidopsis* protoplasts for the same constructs. Although this may result from differences in the expression levels in the two systems, it is also possible that this is due to different proteolytic or pH properties of the vacuoles from the two cell types. In fact, it has been recently reported that similar mGFP5-derived fusions presented high vacuolar fluorescence in *Arabidopsis* transgenic plants, but very low vacuolar fluorescence in *Nicotiana tabacum* or *Nicotiana benthamiana*, a fact that was attributed to differences in the vacuolar accumulation of light-dependent proteases (21).

As stated above, mRFP has been suggested to be more stable in the vacuole (10, 14) and has also been successfully used as a vacuolar marker (14). Moreover, the red variant mCherry seems to have superior photostability, rendering mRFP obsolete (*see* **Note 1**) (10, 11). The problem with the RFPs is that, although their fluorescence is easily distinguished from the red autofluorescence of chloroplasts during computer-mediated observation at the confocal microscope, this is not the case when using an epifluorescence microscope. In any case, for the purpose of investigating the subcellular localization of uncharacterized proteins, the use of sGFP (S65T) seems to us perfectly suitable, provided a 48 h dark incubation is applied before observation.

1.3. Choice of the Transformation System

The ideal transformation system to use when investigating the subcellular localization of uncharacterized proteins by fusion with FPs is not only the homologous species, but even the tissue/cell type where the protein is usually expressed, since differences in

subcellular accumulation have been reported for the same FP fusions in different tissues or cells of the same plant (21, 23). The easiest way to do this is probably through a transient transformation protocol using either *Agrobacterium* infiltration, particle bombardment, or polyethyleneglycol-mediated transfection of protoplasts. Such protocols are available for a number of plant species and can be optimized for the plant species of interest. In parallel, transient expression in an heterologous system such as *Arabidopsis* can be performed using the protocol below.

These transient transformation protocols may not always yield sufficient material for biochemical characterization of the fusion proteins, namely for the characterization of their size using GFP antibodies, in order to determine if a putative or confirmed sorting signal is excised and constitutes a propeptide. For such characterization, stable transformed cells or plants may be necessary, as described below for the transformation of *C. roseus* cells.

1.4. Design of Fusions

The position of the protein under investigation in relation to the FP is crucial in determining the correct final destination of the fusion protein, since N-terminal or C-terminal sorting signals will not be recognized if they are sandwiched between the two sequences of the fusion. Therefore, the rules of thumb for the design of GFP fusions capable of indicating reliably the subcellular localization of the protein of interest are the following: (i) perform an exhaustive in silico analysis of the full-length deduced protein sequence in order to determine the putative presence of subcellular sorting signals or propeptides and (ii) design carefully the fusions, locating correctly the putative sorting/propeptide sequences and the putative mature protein sequence at the N-terminus or C-terminus of GFP (or both in case of doubt).

In our case, to investigate the subcellular sorting of CrPrx1 and the respective sorting signal, we considered the presence in the full-length protein sequence of a signal peptide directing the nascent polypeptide to the ER (SP), and the presence of a C-terminal extension (CTE), compared to all the mature Prx proteins sequenced. Accordingly, we designed fusion constructs with GFP, attaching the SP to its N-terminus and the CTE to its C-terminus (**Fig. 19.1**). We also designed constructs including the mature protein sequence with and without the CTE, attached to the C-terminus of SP-GFP.

2. Materials

2.1. Plasmids, Constructs, and Cloning

1. Plasmid pTH-2 corresponding to pUC18 carrying the 35SΩ-sGFP(S65T)-nos construct and an ampicillin/carbenicillin-resistance marker (7, 8). May

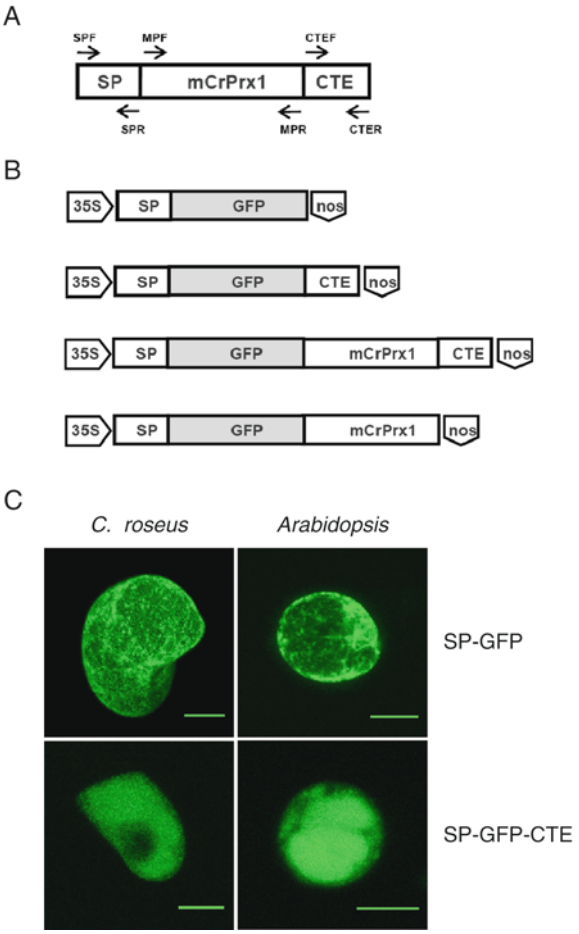

Fig. 19.1. **a** Structure of the full-length CrPrx1 protein with localization of the primers used for the amplification of the signal peptide (SP), the mature protein (mCrPrx1), and the C-terminal extension (CTE). **b** Scheme of the GFP-CrPrx1 fusions. **c** Typical GFP fluorescence pattern observed for transformation with two of the fusions.

be requested from Yasuo Niwa (niwa@fns1.u-shizuoka-ken.ac.jp).

2. Plasmid pTH-2BN lacks the stop codon and is used for fusions at the GFP C-terminus (24). May be requested from Johan Memelink (j.memelink@biology.leidenuniv.nl).
3. Plasmid pGL2 harbouring the hygromycin B-resistance gene (25). May be requested from Jerzy Paszkowski (jerzy.paszkowski@bioveg.unige.ch).
4. Primers used for amplification of CrPrx1 sequences are shown in **Table 19.1**.
5. Luria-Bertrani (LB) medium: dissolve 10 g of bacto-tryptone, 5 g of yeast extract, and 10 g of NaCl in water and adjust the volume to 1 L. Sterilize by autoclaving. Solid

Table 19.1
Primers used for the amplification of CrPrx1 sequences

Amplification product	Forward primer	Reverse primer
SP	**SPF**-*ACGCGTCGACAAAA*ATGGCT TTTTCTTCTTCAACTTCTCTGC	**SPR**-*CATGCCATGGG*CCGAGTT GTTTGAGCTACAATATGG
mCrPrx1-CTE	**MPF**-*GAAGATCTTA*CCACCA ACAGTGAGTGGACTTTC	**CTER**-*CCGCTCGAG*TTAAAACAT AGACAAGCCAATTTCAGC
mCrPrx1	**MPF**-*GAAGATCTTA*CCACCAAC AGTGAGTGGACTTTC	**MPR**-*CCGCTCGAG*TTAATTTCG AAGTGAACAATTGGATC
CTE	**CTEF**-*GAAGATCTTA*GCTGCTG CCATGGGACGTTCTTC	**CTER**-*CCGCTCGAG*TTAAAACAT AGACAAGCCAATTTCAGC

SP, signal peptide; mCrPrx1, mature CrPrx1 protein; CTE, C-terminal extension. Bold, primer designation; italic, oligonucleotide extension introducing the new restriction site; underlined, start codon and stop codon. See position of primers in **Fig. 19.1a**

medium is supplemented with 15 g/L of Bacto-agar (Becton Dickinson).

6. *Escherichia coli* XL1-blue competent cells.
7. Carbenicillin (Duchefa): 200 mg/mL in water (toxic, avoid contact with skin, or breathing dust). Filter-sterilize through a 0.22 μm membrane (Millipore, Bedford, MA, USA). Store at –20°C and add to the LB medium, prior to use, to the concentration of 200 μg/mL.
8. Qiagen CompactPrep Plasmid Midi Kit (Qiagen GmbH, Düsseldorf, Germany).

2.2. C. roseus *Cell Cultures*

1. MP183L cell line (26). May be requested from Johan Memelink (J.Memelink@biology.leidenuniv.nl).
2. Linsmaier-Skoog-13 (LS-13) medium: standard LS including vitamins (27) (Duchefa) with 30 g/L sucrose, 2 mg/L 1-naphthalene acetic acid (NAA), and 0.2 mg/L kinetin (Kin). Adjust pH to 5.8 with KOH, autoclave 20 min at 120°C, and store at 4°C (stable for several months).
3. Kin and NAA (Duchefa): 100x stock solutions with a concentration of 20 and 200 mg/L, respectively. For a stock of 100 mL, dissolve the powder in 1 mL of KOH 1 M, add water to the final volume, and store at 4°C (stable for several months).

2.3. Preparation of C. roseus *Cells for Particle Bombardment*

1. Solid LS-13 medium: LS-13 supplemented with 0.7% of plant tissue culture agar (Imperial laboratories). Autoclave 20 min at 120°C and store at 4°C (stable for several months).

2. Whatman filter paper No. 4, 42.5 mm diameter (Schleicher & Schuell).
3. Sterile porcelain Büchner funnel maximum content 25 mL, for 40 mm diameter filters (Haldenwanger 127C/0 Technische Keramik GmbH, Berlin, Germany).
4. Small Petri dishes (60 × 15 mm), vacuum pump, and Erlenmeyer vacuum flask.

2.4. Pre-treatment and Coating of Tungsten Particles

1. Tungsten particles Pioneer Hi-Bred, 1.8 μm.
2. 0.1 M HNO_3.
3. Ethanol.
4. Sonicator 1: VibraCell VCX130 equipped with a SM1004 microtip (Sonics and Materials, Newtown, USA).
5. Sonicator 2: Vibracell VC300 equipped with a cup horn model 07-88 (Sonics and Materials, Newtown, USA).

2.5. Coating of Tungsten Particles

1. 2.5 M $CaCl_2$: dissolve 3.67 g of $CaCl_2.2H_20$ in 10 mL of sterile water. Filter-sterilize with a 0.22 μm membrane (Millipore, Bedford, MA, USA) and store at –20°C indefinitely.
2. Spermidine (free base): 0.1 M in sterile water. Filter-sterilize as above and store at –20°C. This solution must be prepared fresh every month.

2.6. Bombardment of Plant Cells with a Helium-Powered Particle Gun

1. Home-built particle gun according to (28).
2. Support screens 13 mm SS (Poretics Corporation, Livermore, CA, USA).
3. Dispersal screens: stainless steel screens, mesh 100 μm (Poretics Corporation, Livermore, CA, USA).
4. Rings: Teflon gasket 13 mm (Poretics Corporation, Livermore, CA, USA).
5. Sonicator: Vibracell VC300 equipped with a cup-horn model 07-88 (Sonics and Materials, Danbury, Newtown, USA).
6. LS-13 medium supplemented with 50 μg/mL hygromycin B.
7. Hygromycin B (concentrated solution from Calbiochem-Novabiochem): dilute in water to 50 mg/mL and adjust pH to 7 (very toxic, avoid contact with skin or eyes, causes severe burns). Filter-sterilize as above and store at 4°C for several months. Add to the liquefied solid culture medium prior to plating.

2.7. Subculturing of Resistant Calli

1. Solid selection medium: solid LS-13 medium supplemented with 50 μg/mL hygromycin B.

2. Walk-in chamber at 25°C, 16 h light photoperiod, 2000 lux light intensity.

2.8. Conversion of Calli into Cell Suspensions

1. Selection medium: LS-13 medium supplemented with 50 μg/mL hygromycin B.
2. Orbital shaker.
3. Walk-in chamber at 25°C, 16 h light photoperiod, 2000 lux light intensity.

2.9. Fluorescence or Confocal Laser Scanning Microscopy

1. Filter set for an excitation wavelength of 488 nm and an emission wavelength of 522 nm ± 16 nm.

2.10.* Arabidopsis *Cell Cultures

1. Cell suspension culture of *Arabidopsis thaliana* L. (Heynh.) ecotype Columbia – AtCol (29).
2. Culture medium: 30 g/L sucrose, 3.2 g/L Gamborg's B5 basal medium with mineral organics (Sigma G5893), and 0.2 mg/L NAA. Adjust pH to 5.8 with KOH and autoclave 20 min at 120°C.

2.11. Preparation of* Arabidopsis *Protoplasts

1. Medium A: 0.4 % Macerozyme R-10 (Yakult), 2 % Cellulase "Onozuka" R-10 (Yakult), 12% sorbitol. Dissolve sorbitol first, add enzymes, heat in microwave for 15–20 s to 50–60°C, stir for 1 h, adjust to pH 5.8 with HCl. Filter-sterilize and store at –20°C in 20 mL aliquots.
2. Plastic disposable 70 μm cell sieves (BD Biosciences #352350).
3. Medium B: culture medium in which sucrose is replaced by 0.1 M glucose plus 0.25 M mannitol. Adjust pH to 5.8 with KOH or HCl and autoclave 20 min at 120°C.
4. Counting chamber: Bürcker, depth 0.1 mm.

2.12. Transformation of* Arabidopsis *Protoplasts by Polyethyleneglycol-Mediated Transfection

1. PEG solution: 25% PEG 4000 (Fluka), 0.2 M mannitol, and 0.1 M $CaCl_2.2H_2O$. Filter-sterilize and store at –20°C indefinitely.
2. Sterile 24-well cell culture plates.

3. Methods

3.1. Constructs, Cloning, and Plasmid Midi-Preps

Steps 1–3 must be customized according to the objectives of each researcher. Here, in Steps 1–3, we describe the procedure used for CrPrx1.

1. The constructs designed to study the subcellular sorting of CrPrx1 are shown in **Fig. 19.1**. The CrPrx1 sequences used

in fusions were amplified by PCR with primers extended to include the adequate restriction sites, using the CrPrx1 cDNA as template (**Table 19.1**).

2. The amplified CrPrx1 signal peptide (SP) was cloned in frame in the plasmid pTH-2 using a SalI/NcoI ligation to generate the construct 35S-SP-GFP.
3. The same was done for the plasmid pTH2^{BN}, resulting in a 35S-SP-GFP construct without stop codon, which was then used to further insert the CTE or the sequence of mature CrPrx1 with and without the CTE at the C-terminus of GFP. A BglII/XhoI ligation generated the constructs 35S-SP-GFP-CTE, 35S-SP-GFP-mCrPrx1, and 35S-SP-GFP-mCrPrx1-CTE (**Fig. 19.1**).
4. Use all construct plasmids to transform *E. coli* XL1-blue competent cells as follows: mix 1 μg of plasmid DNA with 100 μL of cells on ice, heat shock at 42°C for 30 s, put the tubes back on ice.
5. Plate 5–10 μL on LB solid medium supplemented with carbenicillin 200 μg/mL.
6. Obtain pure plasmid preps using 50 mL of overnight *E. coli* cultures and the Qiagen CompactPrep Plasmid Midi Kit according to the manufacturer's instructions.

3.2. C. roseus *Cell Cultures*

1. Sub-culture *C. roseus* cell cultures weekly by pipetting 7.5 mL of culture into 50 mL of fresh LS-13 medium, in 250 mL Erlenmeyer flasks with foam stoppers.
2. Grow in an orbital shaker at 125 rpm, under a 16 h light photoperiod, at 25°C.

3.3. Preparation of C. roseus *Cells for Particle Bombardmentss(30)*

Perform all the steps in a laminar flow hood.

1. Prepare small Petri dishes with 6–8 mL of solid LS-13 medium.
2. Dilute 4-day-old cell suspension cultures of *C. roseus* 1:1 with fresh LS-13 medium.
3. Resuspend the cells by swirling the flask, pipette 3–8 mL, pour onto a pre-wetted sterile filter paper in a sterile Büchner funnel, and apply vacuum (*see* **Note 2**). Cells must form an even thin layer and should not be too wet (*see* **Note 3**).
4. Place the filter with the cells onto the solid medium and put the lid on the Petri dish until bombardment.

3.4. Pre-treatment of Tungsten Particles

1. Pre-treat the tungsten particles by adding 2 mL of 0.1 M HNO_3 to 375 mg of tungsten particles in a sterile 10 mL tube.

2. Sonicate with the sonicator 1 microtip for 20 min in an ice/water bath at maximum amplitude.
3. Centrifuge for 1 min at 2000 rpm, remove the HNO_3, resuspend the particles in 2 mL of sterile water, and repeat centrifugation.
4. Remove the water, add 2 mL of ethanol, and sonicate 2 s in a sonication cup (sonicator 2) using the settings duty cycle 50% and output control 10.
5. Centrifuge as above and remove the ethanol.
6. Add again 2 mL of ethanol, sonicate as in Step 4, and aliquot the particle suspension in 25 μL portions in sterile 1.5 mL tubes, vortexing vigorously each time before taking an aliquot.
7. Leave the tubes opened in a sterile flow cabinet overnight, allowing the particles to air dry.
8. Close the tubes and store at room temperature indefinitely.

3.5. Coating of Tungsten Particles

Perform all the steps in a laminar flow hood.

1. For transient transformation, add 10 μg of plasmid DNA (obtained in **Section 3.1**, step 6), in a total volume of 10 μL of sterile water to the dry particles and mix well by flicking the tube.
2. For stable transformation (*see* **Note 4**), mix 8 μg of test plasmid DNA with 2 μg of plasmid pGL2 (hygromycin B resistance) in a final volume of 10 μL of sterile water, add to the dry particles, and mix well by flicking the tube.
3. Add 25 μL of 2.5 M $CaCl_2$ and mix by flicking.
4. Add 10 μL of 0.1 M spermidine and mix by flicking.
5. Leave the particles to sediment for at least 15 min and remove 15 μL of supernatant.

3.6. Bombardment of Plant Cells with a Helium-Powered Particle Gun

1. Mount the particle gun inside a laminar flow hood and clean with ethanol prior to use.
2. Sterilize by submersion in ethanol all the equipment to be used during the bombardment procedure, namely loaders, screens, rings, and Petri dish base, and allow to air dry inside the laminar flow hood.
3. Sterilize with ethanol and under flame the tweezers needed to handle and assemble the bombardment apparatus.
4. Sonicate the coated particles for 2 s in a sonication cup using duty cycle 50% and output control 10, and immediately pipette 2 μL of the particle suspension onto the loader screen. You may wish to avoid sonication at this step, which may lead to DNA fragmentation. Instead you may sonicate the dry particles in a small volume of sterile water in

Section 3.5, step 1, previous to the addition of DNA. And just vortex at this stage.

5. Place the loader and dispersal screens into the gun and place an opened Petri dish with the cells 15 cm below.
6. Bombard the cells using 2.5 Bars of helium pressure under partial vacuum (≤50 mBar) (*see* **Note 5**).
7. Release vacuum, cover the Petri dish, and seal with two rounds of parafilm.
8. For each construct, bombard three to five Petri dishes.
9. If transient expression is the goal, incubate cells at 25°C, in the dark, for 1–2 d prior to observation under the confocal laser scanning microscope (go directly to **Section 3.9**) (*see* **Note 6**).
10. If the goal is to obtain stable transgenic cell lines, incubate cells for 24 h at 25°C, under a 16 h light photoperiod, covering the Petri dishes with a piece of filter paper to lower the light level.
11. After 24 h, transfer the filters into new Petri dishes containing solid LS-13 medium supplemented with 50 μg/mL hygromycin (*see* **Note 7**) and incubate at 25°C under a 16 h light photoperiod until calli appears – usually it takes about 4–5 weeks (*see* **Note 8**).

3.7. Subculturing of Resistant Calli

1. In order to avoid chimeric transformants, transfer single hygromycin-resistant calli of 1–2 mm diameter to solid selection medium and allow to grow for several weeks. Transfer about 30 calli for each construct (*see* **Note 9**).
2. After 2–4 weeks, transfer a small piece of each callus to fresh solid selection medium to increase the probability that homogeneous clones are obtained.
3. Analyse calli for the presence and expression of the construct of interest (GFP fluorescence or PCR and RT-PCR). Analyse positive lines under the confocal microscope (**Section 3.9**). Calli can be converted into cell suspensions for further studies.

3.8. Conversion of Calli into Cell Suspensions

In order to obtain a stable cell suspension culture a critical cell density needs to be maintained. Suspension cells can be easily observed in the confocal microscope, or can be used for protoplast or vacuole isolation for characterization of the fusion proteins using anti-GFP antibodies.

1. For each callus line, transfer an area of ca. 2–4 cm^2 of callus tissue (*see* **Note 10**) to 5 mL of liquid selection medium in a 100 mL Erlenmeyer flask. Disperse cell clumps with forceps, this will immediately create a cell suspension like mixture.
2. Incubate the flasks at 25°C, under a 16 h light photoperiod, on an orbital shaker at 125 rpm.

3. Keep adding 5 mL of fresh selection medium whenever cell density becomes high, so that gradually the culture volume is increased up to 20 mL (*see* **Note 11**).
4. When a volume of 20 mL of dense cell suspension is reached (after about 2–3 weeks), transfer the whole suspension into a 250 mL Erlenmeyer flask containing 50 mL of selection medium.
5. Sub-culture weekly, starting with 20 mL of inoculum in the first week, reducing gradually to 10 mL over the weeks, as the cells start to grow as a regular cell suspension. This takes about 4–5 weeks.

3.9. Fluorescence or Confocal Laser Scanning Microscopy

1. Place cells in a drop of water on a glass microscope slide.
2. Visualize using an excitation wavelength of 488 nm and an emission wavelength of 522 nm ± 16 nm (*see* **Notes 12 and 13**). In our case, we used an Axioplan upright microscope (Zeiss) equipped with a Bio-Rad MRC1024ES scanhead with a krypton/argon laser.

3.10. Arabidopsis Cell Cultures

1. Sub-culture *Arabidopsis* cell cultures weekly by pipetting 5 mL of culture into 50 mL of fresh medium in 250 mL Erlenmeyer flasks with foam stoppers.
2. Grow on an orbital shaker at 125 rpm, under a 16 h light photoperiod, at 25°C.

3.11. Preparation of Arabidopsis Protoplasts(31)

1. The day before protoplast preparation, transfer 10 mL of a 5-day-old culture into 40 mL of fresh medium and maintain at the normal growth conditions.
2. Pour the cells into one 50 mL Falcon tube, centrifuge for 5 min at 80×*g* without brake, and remove all the supernatant with a pipette, removing the last drops with a P1000 micropipette with the tip touching the bottom of the tube.
3. Resuspend the cells in 20 mL of medium A (enzyme mixture) and incubate the tube at 28°C, in the dark, for 2–3 h with gentle shaking (e.g. 60 rpm).
4. Separate protoplasts from undigested cells by filtration through a plastic disposable 70 μm cell sieve and add 30 mL of medium B.
5. Pellet protoplasts at 80×*g* for 5 min without brake, remove most of the supernatant by aspiration with a pipette and resuspend carefully the protoplasts in 50 mL of medium B.
6. Pellet protoplasts again as before, remove the supernatant, and resuspend carefully the protoplasts in 15 mL of medium B.

7. Determine the number of protoplasts per mL using a counting chamber.
8. Dilute again to 50 mL with medium B, pellet as above, and resuspend the protoplasts to a concentration of 4×10^6 per mL.

3.12. Transformation of Arabidopsis *Protoplasts by Polyethyleneglycol-Mediated Transfection(31)*

1. Before preparing the protoplasts, aliquot the DNAs to be transfected into 2 mL sterile tubes. The ideal amount of plasmid DNA may vary for each construct and needs to be optimized. A good starting point is 10 μg.
2. Prepare the plate wells putting 4.5 mL of medium B in the wells to be used.
3. Add 250 μL of protoplast suspension (10^6 cells) to each of the 2 mL tubes containing the DNA (pipette cells using a 1 mL plastic tip).
4. Add one volume of PEG solution, drop by drop, and flick the tube every five drops until the solution is mixed. Leave the tubes standing for 15 min at RT.
5. Transfer the cells to the plate wells containing medium B and incubate for 48 h at 25°C, without agitation, in the dark. Analyse under the confocal microscope.

4. Notes

1. FP mCherry can be obtained for example at http://www.bio.purdue.edu/people/faculty/gelvin/nsf/protocols_vectors.htm. With this FP the infiltration of tobacco leaf epidermis with *Agrobacterium* (10, 32) can be used as a transformation system, since mCherry shows high fluorescence even in this biological material (33).
2. The cell suspension has to evenly and completely cover the filter. For transient expression purposes a thin layer of cells on the filter is required corresponding to ca. 4 mL of an average grown diluted culture. Conversely, when the goal is to obtain stably transformed cells, a thicker layer of cells on the filter paper is required corresponding to ca. 8 mL of an average grown diluted culture.
3. Cells should become lighter coloured/whitish.
4. In stable transformation, one important control experiment is the bombardment of cells with particles coated with pGL2 plus an empty vector and with one of the GFP plasmids alone. Transformation with the first will yield control calli. Transformation with the latter will confirm that the

conditions used are selective for calli transformed with the hygromycin B resistance gene.

5. To increase the transformation efficiency or the transient expression levels, cells can be bombarded two or three times.
6. A 24–48 h dark treatment is essential for observation of GFP fluorescence in the vacuole (**Section 1.2**) (18).
7. The Petri dishes with medium are always prepared fresh and must not have any condensation. The plates should not be turned upside down.
8. Routinely 20–50 hygromycin B-resistant calli are obtained per bombarded Petri dish.
9. The standard co-transformation frequency is 50–75%, about 10–20 hygromycin-resistant calli should be obtained containing the construct of interest and expressing it to some level. High-expression calli appear at about 5–10%.
10. A portion of each callus must be transferred onto a separate plate with solid medium as a back-up. This back-up callus culture should be sub-cultured onto fresh medium every 4 weeks.
11. A relatively high cell density is important for rapid cell division. Therefore, at this stage, each cell suspension should be evaluated independently for dilution rate.
12. Under the confocal microscope, GFP-labelled cells appear as bright green. Yellowish fluorescence may occur and it does not reflect GFP expression.
13. When the transformation efficiency is low, a pre-scan of the filters with the bombarded cells may be performed using a low-magnification epifluorescence microscope, in order to spot the fluorescent cells and use these cells to prepare the microscope slide.

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