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Transient Gene Expression for the Characteristic Signal Sequences and the Estimation of the Localization of Target Protein in Plant Cell

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[Abstract] We have proposed and tested a method for characterization of the signal sequences and determinations of target protein localization in a plant cell. This method, called the AgI-PrI, implies extraction of protoplasts from plant tissues after agroinfiltration. The suggested approach combines the advantages of two widely used methods for transient gene expression in plants–agroinfiltration and transfection of isolated protoplasts. The AgI-PrI technic can be applied to other plant species.
Keywords: Agroinfiltration, Protoplast isolation, Tobacco, Transient expression, Signal sequences, Subcellular localization

[Background] To date, the following techniques for transient expression of genes in plants have been developed and widely used: agroinfiltration, biolistics of plant explants and transfection of protoplasts using polyethylene glycol or electroporation. The effectiveness of these approaches has been clearly demonstrated. Each strategy for transient expression of genes in plants, along with benefits, has its limitations and disadvantages, such as the difficulties in the fine imaging of recombinant reporter proteins in plant cell compartments owing to intricate shapes of plant epidermal cells (agroinfiltration), a low efficiency of transformation and the necessity of specialized equipment and auxiliary material (for biolistics), as well as complex preparatory procedures required for a high yield of viable protoplasts and their effective transfection. This is the reason for developing and testing new methods for transient expression of genes in a plant cell, preferably by improving the experimental protocols and preserving the physiological significance of the results of the studies. Since the cellular localization of proteins in living organisms, including plants, is closely interrelated with their functions, a fine visualization of proteins in living cells becomes an important tool for assessing the functions of the proteins.

Materials and Reagents

1. Pipettes (Corning, Costar®, catalog number: 4101)
2. Inoculation loop
3. Petri dishes (Thermo Fisher Scientific, Thermo Scientific™, catalog number: 101IRR)
4. Syringe without a needle (B. Braun Melsungen, catalog number: 4645103C)

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5. Scalpel
6. Nylon mesh, pore size, 40 µm (Sterile Cell Strainers, Corning, catalog number: 431750)
7. 10-ml tubes (Corning, Axygen®, catalog number: SCT-10ML)
8. *Agrobacterium tumefaciens* strain GV3101 (Mohamed *et al.*, 2004; strain is available in the collection of the Institute of Plant Physiology and can be provided to researchers for experiments)
9. *Nicotiana benthamiana* (Sheludko *et al.*, 2007; seeds are available in the collection of the Institute of Plant Physiology and can be provided to researchers for experiments)
10. LB medium (MP Biomedicals, catalog number: 113002042)
11. Rifampicin (Fisher Scientific, catalog number: BP26791)
12. Gentamicin (Thermo Fisher Scientific, Gibco™, catalog number: 15750060)
13. Kanamycin (Thermo Fisher Scientific, Gibco™, catalog number: 11815024)
14. Sodium chloride (NaCl) (Sigma-Aldrich, catalog number: S9888)
15. Tris-HCl, pH 7.0 (Roche Diagnostics, catalog number: 10812846001)
16. Calcium chloride ($CaCl_2$) (Sigma-Aldrich, catalog number: C1016)
17. Acetosyringone (Abcam, catalog number: ab146533)
18. MES (Sigma-Aldrich, catalog number: 69892)
19. Magnesium sulfate heptahydrate ($MgSO_4·7H_2O$) (Merck, catalog number: 1058860500)
20. Calcium chloride dihydrate ($CaCl_2·2H_2O$) (AMRESCO, catalog number: 0556-500G)
21. Ammonium phosphate monobasic ($NH_4H_2PO_4$) (Sigma-Aldrich, catalog number: A3048)
22. Sorbitol (Sigma-Aldrich, catalog number: S1876)
23. Potassium hydroxide (KOH) (AppliChem, catalog number: 211514)
24. Cellulase Onozuka R10 (Kinki Yakult)
25. Pectinase Macerozyme R10 (Kinki Yakult)
26. Driselase (Sigma-Aldrich, catalog number: D9515)
27. Calcium nitrate tetrahydrate ($Ca(NO_3)_2·4H_2O$) (Sigma-Aldrich, catalog number: C2786)
28. Potassium phosphate monobasic (KH_2PO_4) (Fisher Scientific, catalog number: P285)
29. Magnesium sulfate ($MgSO_4$) (Acros Organics, catalog number: AC413485000)
30. Potassium chloride (KCl) (Sigma-Aldrich, catalog number: P9333)
31. Ferric chloride ($FeCl_3$) (Sigma-Aldrich, catalog number: 157740)
32. MS medium (Sigma-Aldrich, catalog number: M5519)
33. Sucrose (MP Biomedicals, catalog number: 04802536)
34. Solution 1 (see Recipes)
35. Solution 2 (see Recipes)
36. Solution 3 (see Recipes)
37. Solution 4 (see Recipes)
38. Solution 5 (see Recipes)
39. Knop’s solution (see Recipes)
40. Agroinfiltration buffer (see Recipes)

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Equipment

1. Incubator Shaker (Biosan, model: ES-20, catalog number: BS-010111-AAA)
2. Centrifuge (Eppendorf)
3. Transilluminator (Vilber, model: ETX-F26.M, catalog number: Vilber Lourmat 2131 2600 1)
4. Microscope Axio Imager Z2 (ZEISS, model: Axio Imager Z2) equipped with digital camera (ZEISS, model: AxioCam MRc5), filter set No. 38 (38 Endow GFP shift free (EX BP 470 nm/40 nm, BS FT 495 nm, EM BP 525 nm/50 nm), ZEISS, catalog number: 000000-1031-346) and module ApoTome (ZEISS, model: ApoTome)

Software

1. ZEN, AxioVision 4.8 (ZEISS)

Procedure

For this protocol, we used plant expression vectors, with leader signal sequences providing their localization in various compartments of plant cells. The vectors were obtained according to the procedure described earlier (Tyurin *et al*., 2017).

A. Cultivation, transformation and selection of *A. tumefaciens*
   1. Seed cells of the *A. tumefaciens* strain GV3101 strain using an inoculation loop in 5 ml of LB medium containing rifampicin (50 µg/ml), gentamicin (25 µg/ml), and carbenicillin (50 µg/ml), (antibiotics do not affect the efficiency of plant transformation) and grow overnight at 28 °C in a shaker incubator (~140 pm).
   2. Transfer the bacterial culture to 95 ml of LB medium without antibiotics and grow to an optical density of OD_{600} = 0.5-0.6, at 28 °C in a shaker incubator (~140 pm).
   3. Centrifuge cells at 3,500 *x g* for 5 min, at room temperature. Resuspend the pellet in 20 ml of cold (0 °C) solution 1 (see Recipes).
   4. Centrifuge cells at 3,500 *x g* for 5 min, at room temperature. Resuspend the pellet in 2 ml of solution 2 (see Recipes).
   5. Competent cells should be used for transformation immediately.
   6. Add 1 µg of an expression vector (with the kanamycin resistance gene) to the competent cells. Incubate cells on ice for 20 min. Heat the solution for 5 min at 37 °C in the shaker incubator. Place cells for 5 min on ice, add 2 ml of LB medium without antibiotics and incubate at 28 °C in the shaker incubator (~140 rpm) for 2 h.
   7. Plate transformed cells on Petri dishes in the medium containing 50 µg/ml of the selective antibiotic kanamycin (use the appropriate selective agent at concentrations recommended by the manufacturer). Isolate colonies and resuspend the cells in 100-150 µl of LB medium without antibiotics, and then freeze them.

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B. Plant material

Grow *Nicotiana benthamiana* at 20 ± 2 °C, a photoperiod of 8 h, and an illumination of 100 µmol quanta/(m^2 sec) in soil or hydroponics using Knop's solution (see Recipes) as a nutrient medium for 6 weeks. Frequency of use: 2 times per week.

C. Agroinfiltration of the *N. benthamiana* plants

1. Thaw frozen *A. tumefaciens* cells and grow them for 48 h at 27 °C in LB medium containing rifampicin (50 µg/ml), gentamicin (25 µg/ml), and carbenicillin (50 µg/ml) in the shaker incubator (~140 pm). Replace the medium with LB medium containing the same antibiotics and solution 3 (see Recipes). Grow the cells to an optical density of OD_{600} = 0.8.
2. Centrifuge the cells at 3,000 *x g* for 5 min, at room temperature. Resuspend the pellet in agroinfiltration buffer (see Recipes) to an optical density of OD_{600} = 2.4.
3. Infiltrate the cells into abaxial epiderm of 6-week-old *N. benthamiana* leaves using a syringe without a needle (Figure 1).
4. Estimate the quality of transformation on the 4th day after agroinfiltration by imaging of the zones with an expression of the desC-egfp hybrid gene in tobacco leaves at 312 nm, using an ETX transilluminator (Vilber Lourmat). Cut the high fluorescence regions of tobacco leaves out for protoplast extraction (Figure 2).

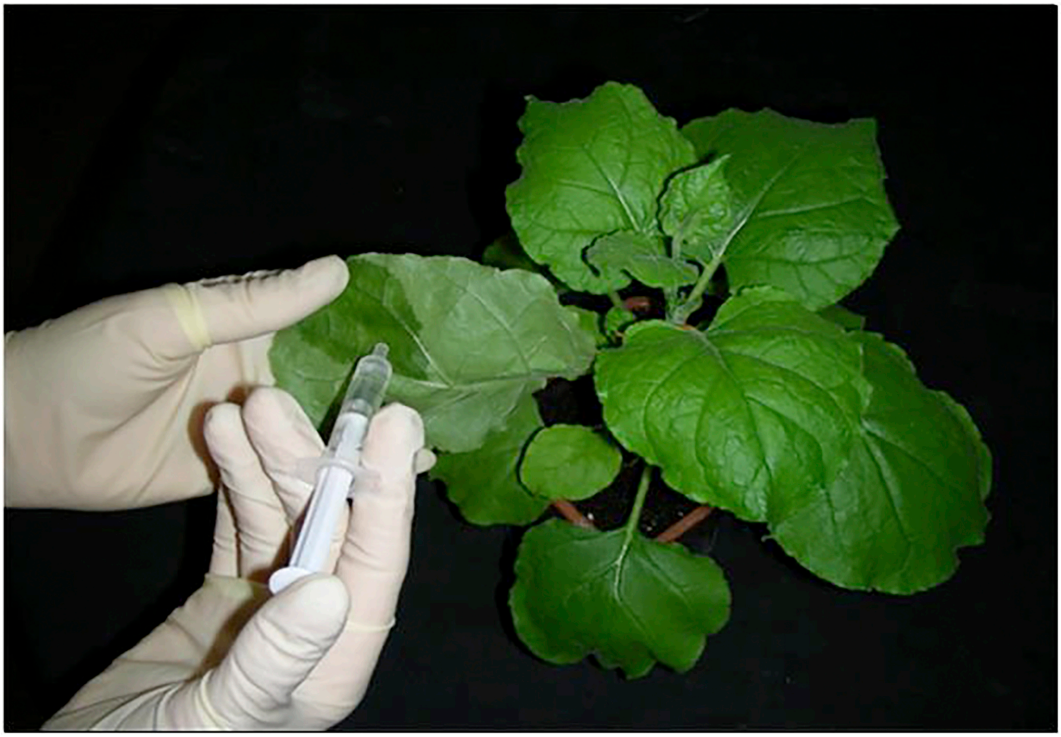

Figure 1. Procedure of agroinfiltration into abaxial epiderm of *N. benthamiana* leaves using a syringe without a needle

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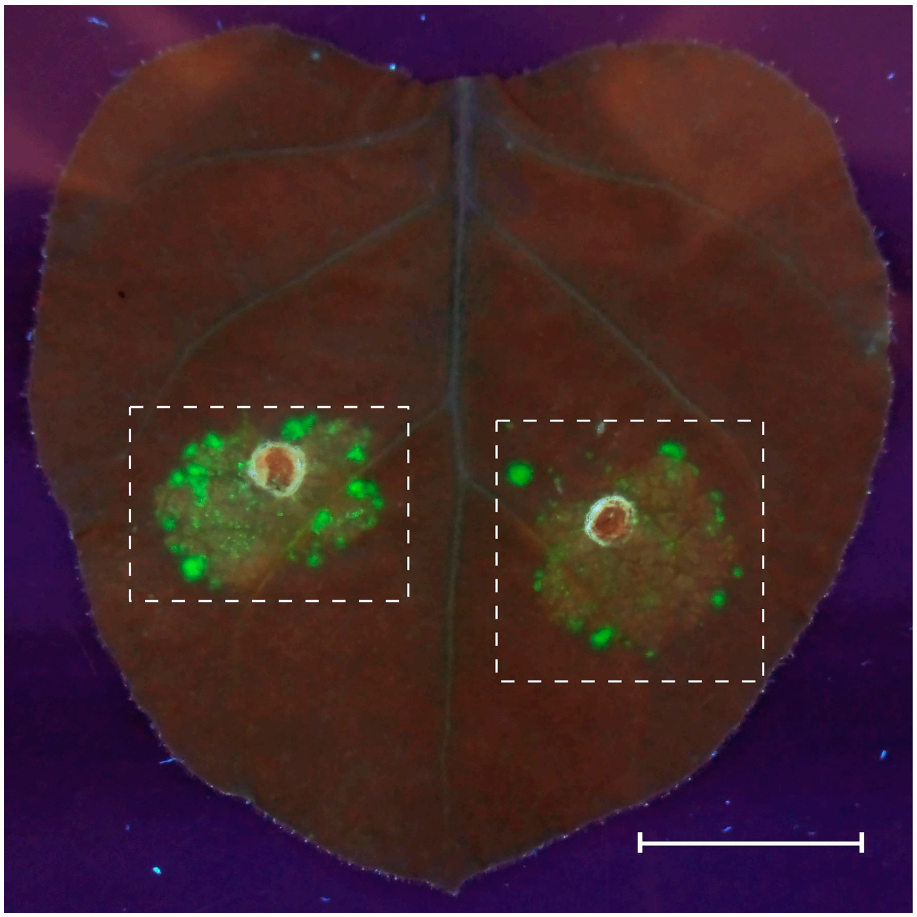

Figure 2. GFP fluorescence in agroinfiltrated *Nicotiana benthamiana* leaves. The agrobacteria carrying the expression cassettes of GFP fusion protein were syringe-infiltrated into an *N. benthamiana* leaf, and GFP fluorescence was observed 4 days after infiltration (dpi) in UV light. Fluorescence fields are marked with dashed lines. Scale bar = 20 mm.

D. Protoplast isolation

1. Prepare solution 4 (see Recipes) for the cell wall maceration immediately before use and clarify it by centrifugation.
2. Use the leaf fragments with fluorescence for protoplast isolation according to Nosov *et al*. (2014). Mince the leaves (about 500 mg) with a scalpel. Add 5 ml of solution 4.
3. Isolate the protoplasts at 15 °C in a shaker (50 rpm) for 12 h.
4. Filtrate the protoplast suspension through nylon mesh (pore size, 40 μm). Transfer the protoplast suspension into 10-ml centrifuge tubes and centrifuge at 100 *x g* for 5 min at room temperature.
5. Resuspend the pellet of protoplasts in 10 ml of solution 5 and incubate for 5 min at room temperature, and then centrifuge at 100 *x g* for 5 min, repeat the procedure two times. Resuspend the pellet of protoplasts in 1.5 ml of solution 5 (see Recipes).

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E. Protoplast imaging

Perform the imaging of zones with target protein in freshly isolated protoplasts with an Axio Imager Z2 microscope (ZEISS) equipped with an AxioCam MR digital camera and filter units (Figure 3).

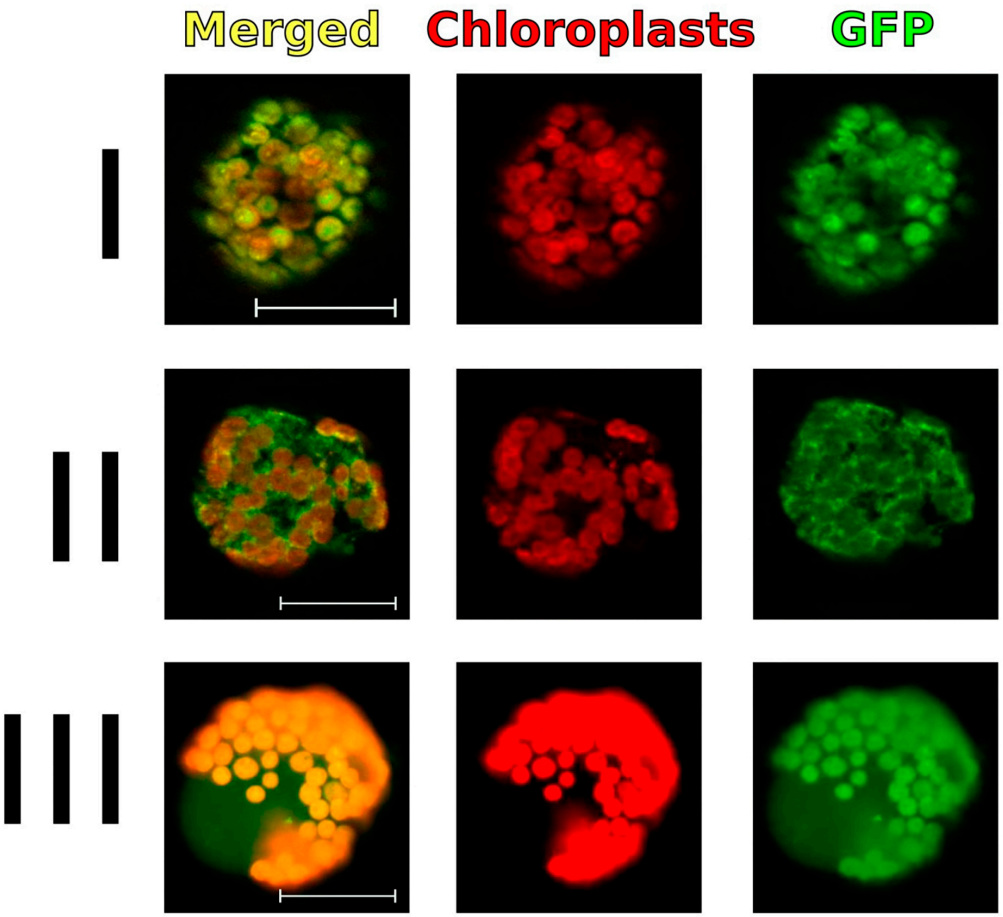

Figure 3. Results of analysis of subcellular localization of GFP fusion proteins in tobacco protoplasts isolated from the cells of an agroinfiltrated leaf area. Transient gene expression of plant expression vectors, with leader signal sequences providing their localization in various compartments of plant cells, demonstrates that GFP fusion protein is localized to the chloroplasts (**I**), endoplasmic reticulum (**II**), and cytoplasm (**III**). The merged images (first column) include the green channel (last column) and the chloroplast autofluorescence channel (middle column). Scale bars = 10 μm.

Data analysis

All experiments were performed in at least three independent replicates, each replicate being analyzed at least three times. To determine how well transformation was done we checked the level of GFP fluorescence in agroinfiltrated leaves of *N. benthamiana* in UV light. For estimation of subcellular localization of GFP fusion proteins in the tobacco protoplasts, we analyzed about 100

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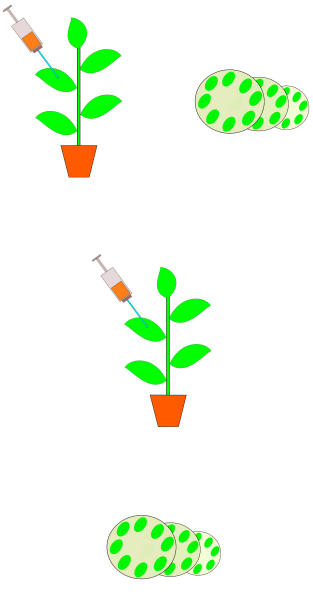
protoplasts and visually determined positive transient gene expression in at least 80% of protoplasts.

Notes

This protocol can be applied to other plant species (Figure 4).



Arabidopsis (Arabidopsis thaliana)	[1, 2]
Tobacco (Nicotiana sp.)	[3, 4]
Aspen (Populus tremula)	[5, 6]
Lettuce (Latucca sativa)	[7, 8]
Switchgrass (Panicum virgatum)	[9, 10]
Soybean (Glycine max)	[11, 12]
Medicago (Medicago sp.)	[13, 14]
Potato (Solanum tuberosum)	[4, 15]
Sunflower (Heliantus annuus)	[16, 17]
Citrus (Citrus sp.)	[18, 19]
Pumpkin (Cucurbita pepo)	[20]
Onion (Allium cepa)	[21]
Carot (Daucus carota)	[22]
Grape (Vitis vinifera)	[23]
Corn (Zea mays)	[24]
African oil palm (Elaies guineensis)	[25]

Figure 4. Potential application of the AgI–PrI technique to different plant species (Tyurin *et al*., 2017, supplementary data)

Recipes

1. Solution 1
   150 mM NaCl
   10 mM Tris-HCl (pH 7.0)
2. Solution 2
   20 mM $CaCl_2$
   10 mM Tris-HCl (pH 7.0)
3. Solution 3
   20 µM acetosyringone
   10 mM MES
4. Solution 4
   200 mg/L $MgSO_4 \cdot 7H_2O$
   100 mg/L $CaCl_2 \cdot 2H_2O$
   150 mg/L $NH_4H_2PO_4$

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0.4 M sorbitol
4 mM $CaCl_2$
12.5 mM MES-KOH (pH 5.7)
1% Cellulase Onozuka R10
0.15% Pectinase Macerozyme R10
0.4% Driselase

5. Solution 5
   0.5 M sorbitol
   2.5 mM $CaCl_2$
6. Knop's solution 1 L
   1 g $Ca(NO_3)_2$
   0.25 g KH_2PO_4
   0.25 g $MgSO_4$
   0.125 g KCl
   0.0125 g $FeCl_3$
7. Agroinfiltration buffer
   1x MS medium
   10 mM MES-KOH (pH 5.6)
   2% sucrose
   200 μM acetosyringone

Acknowledgments

This protocol has been adapted from an earlier study (Tyurin *et al*., 2017). The authors confirm that there are no known conflicts of interest associated with this publication.

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