Arabidopsis mesophyll protoplasts: a versatile cell system for transient gene expression analysis

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The transient gene expression system using *Arabidopsis* mesophyll protoplasts has proven an important and versatile tool for conducting cell-based experiments using molecular, cellular, biochemical, genetic, genomic and proteomic approaches to analyze the functions of diverse signaling pathways and cellular machineries. A well-established protocol that has been extensively tested and applied in numerous experiments is presented here. The method includes protoplast isolation, PEG–calcium transfection of plasmid DNA and protoplast culture. Physiological responses and high-throughput capability enable facile and cost-effective explorations as well as hypothesis-driven tests. The protoplast isolation and DNA transfection procedures take 6–8 h, and the results can be obtained in 2–24 h. The cell system offers reliable guidelines for further comprehensive analysis of complex regulatory mechanisms in whole-plant physiology, immunity, growth and development.

INTRODUCTION

Plant protoplasts without cell walls offer a versatile cell-based experimental system. Macromolecules such as DNA, RNA and proteins can be delivered into protoplasts using various methods, e.g., PEG–calcium fusion, electroporation and microinjection. Signal transduction and metabolic pathways as well as transcription and translation machineries can be transiently manipulated to investigate cell-autonomous regulation and responses. The method is especially powerful in functional genomics analysis when combined with rich genetic resources, such as insertion libraries, available in the model plant *Arabidopsis thaliana*. We describe here a protocol for a system of transient expression in *Arabidopsis* mesophyll protoplast (TEAMP) and provide troubleshooting advice and detailed information on useful reporter constructs. Applications, advantages and limitations of the TEAMP assay are also discussed. More general information about plant protoplasts can be found in a review[1] and a live performance of the TEAMP procedure can be seen at the Sheen lab website (http://genetics. mgh.harvard.edu/sheenweb/protocols_reg.html).

Overview

The isolation of plant protoplasts was first reported more than 40 years ago[2]. Protoplasts had been used to observe cellular processes and activities such as cell wall synthesis, cell division, embryogenesis, differentiation during regeneration, photosynthesis activity, calcium signaling and regulation, and the modulation of ion channels by light, stress and hormone responses in various plant species[1]. Protoplasts were also isolated from special cell types to investigate cell type–specific responses. For example, gibberellin and abscisic acid actions on α-amylase synthesis and blue light–induced H^+-ATPase activity have been studied with protoplasts isolated from barley aleurone layer[3] and *Vicia faba* guard cells[4], respectively.

After the development and significant improvement of various DNA transfection methods (e.g., PEG, electroporation and microinjection), diverse cellular functions of genes of interest have been studied using protoplasts generated from several plant species[1]. Since *Arabidopsis* became the central model system in plant biology with the availability of its whole genome sequence, we and several other laboratories have established the TEAMP system as a cell-based functional genomics tool[1,5].

The TEAMP protocol is based on mesophyll protoplasts freshly isolated from leaves of *Arabidopsis* wild-type or mutant plants growing on soil (see **Fig. 1a**). After cell walls are removed using fungal cellulase and macerozyme, protoplasts are released, washed and collected (see **Fig. 1b,c**). Remarkably, these protoplasts maintain many of the same physiological responses and cellular activities as intact plants, despite the enzymatic treatment step[1]. PEG–calcium-mediated transfection is used to deliver DNA into protoplasts, followed by 2–24 h incubation to allow gene expression (see **Fig. 1d**). A sterile technique is not necessary owing to the short incubation time. To allow constitutive expression of transfected
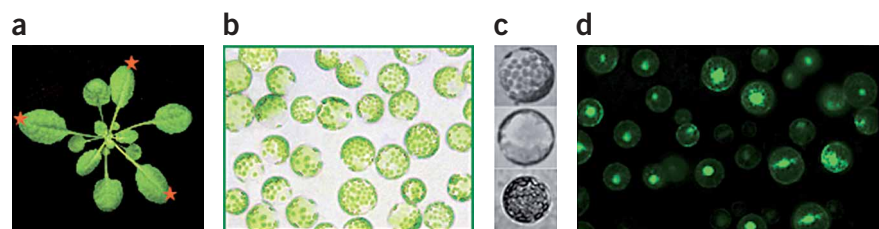

Figure 1 | *Arabidopsis* protoplast isolation and transient gene expression. (**a**) A healthy 4-week-old *Arabidopsis* plant suitable for protoplast isolation. Asterisks indicate the optimal true leaves (fifth, sixth and seventh) to be used as a mesophyll protoplast source. (**b**) Mesophyll protoplasts (×400). (**c**) Isolated protoplasts retain the characteristics of the genetic backgrounds from which they were obtained; protoplasts isolated from wild-type *Col-0* (top), ethylene-insensitive *etr1-1* mutant (middle) and the constitutive ethylene response *ctr1-1* mutant (bottom) show different cellular morphology and characteristics under light microscopy (×600). (**d**) Protoplasts expressing the nuclear-targeted GFP driven by the constitutive 35S enhancer fused to the maize *C4PPDK* basal promoter are shown under fluorescence microscopy (×400). More than 90% of protoplasts were transfected successfully with the GFP reporter gene construct.

genes, the cauliflower mosaic virus 35S promoter derivative with the maize *C4PPDK* basal promoter and the 5′-untranslated region (5′-UTR) enhancer is used[6] (see **Fig. 2a**). To control the level of gene expression, a chemically inducible gene expression system has also been developed (see Advantages and limitations).

Luciferase[7] (LUC) and β-glucuronidase[8] (GUS) are routinely used as reporters for the quantitative measurement of gene expression in TEAMP assays (**Fig. 2b**, **Table 1**). A set of LUC reporters controlled by diverse promoters that can respond to specific signals (**Table 1**) have been donated by the Sheen lab to the public stock center, the *Arabidopsis* Biological Resource Center (ABRC). These reporters can serve as positive controls to help set up the TEAMP assay in laboratories new to the system. However, the application of TEAMP is not limited to reporter assays. Physiological and cell-autonomous responses can be observed and scored using various analytical methods to investigate protein activity, localization, trafficking, interaction and stability.

Figure 2 | Commonly used plasmid constructs in the transient expression in *Arabidopsis* mesophyll protoplast (TEAMP) system. (**a**) Schematic representation of a regulatory gene construct under the control of the constitutive 35S enhancer fused to the maize (*C4PPDK*) basal promoter. *GOI*, gene of interest; *NOS*, nopaline synthase terminator. (**b**) Schematic representation of a luciferase (*LUC*) reporter gene construct driven by the promoter of interest (*POI*) and terminated by *NOS*. (**c**) Schematic representation of the dexamethasone (DEX)-inducible constructs. The top picture illustrates the artificial transcription activator (ATA), constructed with a bacterial DNA-binding domain (*LexA-DB*), a viral transcription activation domain (*VP16*) and a mammalian (rat) glucocorticoid receptor (*GR*). The ATA is driven under the constitutive 35S enhancer with the maize *C4PPDK* basal promoter. Upon DEX treatment, the ATA (*LexA-VP16-GR*) is released from the cytoplasm HSP90 complex and moves into the nucleus to induce target gene transcription (represented by the black arrow). In the nucleus (bottom picture), the ATA controls the transcription of a DNA construct that is driven by eight copies of the bacterial *LexA* operator (8x*LexA-OP*) and the minimum 35S promoter. In the example presented, the LUC reporter gene is included in the 8x*LexA-OP*-driven construct. (**d**) Time course analysis of the LUC reporter activation in the absence and presence of 10 μM soluble DEX. Values are means of ratio in triplicates. The β-glucuronidase (GUS) activity expressed from the co-transfected *UBQ10-GUS-NOS* serves as an internal control. LUC expression is induced upon DEX treatment and shows linear expression kinetics.

Applications

Physiological and cell-autonomous responses and assays in protoplasts have been widely used to identify key regulators and elucidate molecular mechanisms underlying intracellular signal transduction pathways involved in plant physiology, immunity, growth and development. The application of TEAMP system has been significantly broadened in recent years and will be further expanded by integration with *Arabidopsis* genomics and genetic resources for high-throughput explorations.

Using TEAMP assays, molecular mechanisms underlying plant hormone signaling pathways have been elucidated. In auxin signaling, regulatory functions and responses of AUX/IAA proteins[9], RAC GTPase[10] and auxin response factors[11] have been characterized. The protein stability control of EIN3, a key nuclear transcription factor in ethylene signaling, has been shown to be the main regulatory event involved in an unexpected crosstalk between glucose an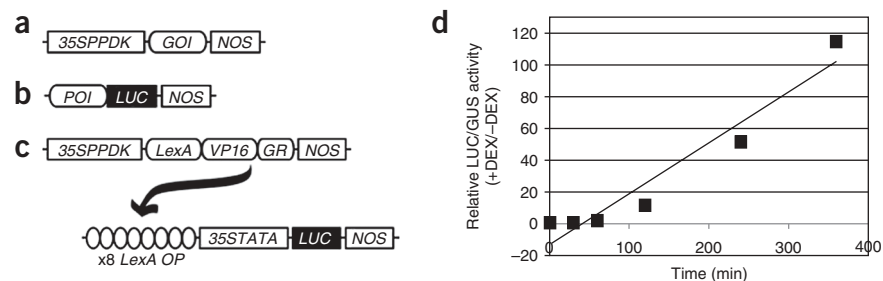d ethylene signaling[12]. Significantly, the phospho-relay via the *Arabidopsis* two-component system has been established as the main cytokinin regulatory circuitry[13], which has been verified by numerous genetic and phenotypic analyses of corresponding *Arabidopsis* mutants[14]. Both abiotic and biotic stress signaling can also be studied in the TEAMP system. For example, the functions of the evolutionarily conserved mitogen-activated protein kinase (MAPK) cascades have been identified and characterized in *Arabidopsis* mesophyll protoplasts for oxidative stress signaling[5] and innate immune responses[15]. Subsequently, the virulence functions of type III effectors, AvrPto and AvrPtoB from the pathogenic bacterium *Pseudomonas syringae*, have been shown to be potent suppressors of the MAPK-dependent plant innate immunity[16].

More recently, protein–protein interactions *in vivo* between the glucose sensor hexokinase 1 (HXK1) and its partners have been demonstrated for HXK1-dependent nuclear glucose signaling[17]. Through application of the yeast two-hybrid concept,

TABLE 1 | List of useful reporter constructs for transient expression in *Arabidopsis* mesophyll protoplast assays.

Name	GenBank locus	*Arabidopsis* Biological Resource Center stock number	Promoter of interest (POI)	Use	References
pHBT-sGFP(S65T)-NOS	EF090408	CD3-911	35S derivative	Transfection control	19
pRD29A-LUC-NOS	EF090409	CD3-912	At5g52310	Abscisic acid response	5,13
pAtGH3-LUC-NOS	EF090410	CD3-913	At2g23710	Auxin response	5,13
pWRKY29-LUC-NOS	EF090411	CD3-914	At4g23550	Bacterial flg22 response	15
pGST6-LUC-NOS	EF090412	CD3-915	At2g47730	General stress response	5
pHSP18.2-LUC-NOS	EF090413	CD3-916	At5g59720	Heat response	13
pARR6-LUC-NOS	EF090414	CD3-917	At5g62920	Cytokinin response	13
pGCC1-LUC-NOS	EF090415	CD3-918	8xGCC box synthetic promoter	Ethylene response	12
pFRK1-LUC-NOS	EF090416	CD3-919	At2g19190	Bacterial flg22 response	16

protein–protein interaction has also been examined in *Arabidopsis* mesophyll protoplasts to establish a heterodimerization map of specific groups of bZIP transcription factors[18].

Single-cell imaging of diverse fluorescence marker proteins, e.g., GFP and its variants, fused to functional proteins have also been extensively utilized to examine protein localizations, protein domain functions in protein targeting and protein transport machinery functions in vesicle trafficking. For instance, subcellular localization of various proteins has been determined using the GFP tag[11,12,17–19]. Novel roles of the transmembrane domain and C-terminal 7 amino acids of *Arabidopsis* small outer envelope membrane protein 7 have been identified for chloroplast targeting[20]. Interesting regulatory roles of dynamin-like ADL6 (ref. 21), small GTP binding protein Rha1 (ref. 22) and actin filaments[23] have been studied in vacuolar trafficking. The protoplast isolation method and fluorescence-assisted cell sorting technique have been further applied to reveal genome-wide cell type–specific transcript profiles in *Arabidopsis* roots[24].

Advantages and limitations

Uniform and abundant mesophyll protoplasts isolated from mature *Arabidopsis* leaves can respond to diverse signals in a physiological manner similar to the responses observed in leaves of whole plants. Importantly, application of the TEAMP system is not limited to processes and activities that occur in leaves. Proteins that are normally expressed in other cell types and tissues can be ectopically expressed and examined in mesophyll protoplasts to learn their molecular and cellular functions. Analysis using mesophyll protoplasts often provides information of greater functional and physiological relevance to plants than data obtained using heterologous cell systems, e.g., bacteria, yeast, insect and mammalian cells. As most of the quantitative and physiological responses can be observed and measured within 2–10 h after DNA transfection, the experiments do not require sterile techniques and complex culture medium, which represents a tremendous saving in time and costs. There is no other biological system for gene expression analysis (including bacterial, yeast and mammalian cell culture) that can be handled with the same minimal laboratory requirement. The requirement for a relatively small number of protoplasts is another advantage of the TEAMP system owing to its high efficiency and sensitivity. In some other protoplast protocols, 10–1,000 times more protoplasts (10^5–10^7) are recommended for each DNA transfection experiment[25].

On the other hand, the conserved functions of molecular domains and regulatory mechanisms among bacteria, yeasts, animals and plants allow us to utilize molecular switches, originally identified in other organisms, to control gene and protein expression levels in plant mesophyll protoplasts. For instance, the mammalian steroid nuclear receptor has been used to provide a ligand inducible gene expression system in mesophyll protoplasts[12]. As depicted in **Figure 2c**, an artificial transcription activator (ATA) is constructed with a bacterial DNA-binding domain (*LexA-DB*), a viral transcription activation domain (*VP16*) and a mammalian (rat) glucocorticoid receptor (*GR*). The ATA is driven under the constitutive 35S enhancer fused to the maize *C4PPDK* basal promoter[6]. Upon dexamethasone (DEX) treatment, the ATA (*LexA-VP16-GR*) is released from the cytoplasm HSP90 complex and moves into the nucleus to induce target gene transcription[26].

In the nucleus, the ATA controls the transcription of a DNA construct that is driven by eight copies of the bacterial *LexA* operator (8x*LexA-OP*) and the minimum 35S promoter (see **Fig. 2c**). In the example presented (in which the LUC reporter gene is included in the 8x*LexA-OP*-driven construct; see **Fig. 2d**), LUC expression is induced upon DEX treatment and shows linear expression kinetics. The expression level of LUC is also controlled in a ligand dosage–dependent manner, indicating that the expression level of desired genes and proteins can be manipulated using this inducible system.

Mesophyll protoplasts can be isolated from fresh leaves of wild-type and most *Arabidopsis* mutant plants and used immediately for experiments without the time-consuming step of cell culture establishment. Wild-type mesophyll protoplasts maintain most leaf features and responses, which are often lost in undifferentiated suspension culture cells. The mutant protoplasts possess the cellular nature that is characteristic of the original mutant plants. For example, mesophyll protoplasts isolated from the ethylene-insensitive *etr1-1* and the constitutive ethylene-response *ctr1-1* mutants show cellular morphology and characteristics distinct from those in the wild type (see **Fig. 1c**). There are many successful examples of using the TEAMP assay with various mutant plants, including the analysis of ARF7 functions in auxin signaling[11], the antagonistic interaction between HXK1-dependent glucose and ethylene signaling[12], the receptor kinase FLS2 function in flg22 signaling[15] and roles of various plant hormone signaling pathways in programmed cell death induced by the fungal toxin fumonisin B1 (ref. 27). Conceptually, the use of protoplasts generated from mutant plants in TEAMP assays to screen for new regulators is similar to performing mutant suppressor or enhancer screens, which are commonly applied in the genetic analysis of specific signaling pathways. In general, it is necessary empirically to optimize the protoplast isolation and DNA transfection conditions for different *Arabidopsis* accessions and mutants. Once conditions are optimized, the use of genetically diverse mesophyll protoplasts makes the TEAMP system even more powerful in functional genomics research.

Unlike plant and animal cell culture lines, mesophyll protoplasts do not divide in the simple incubation buffer described in this protocol. Thus, this TEAMP protocol is not suitable for the study of cell cycle regulation. To use protoplasts to study cell division and dedifferentiation or other long-term plant responses, specialized plant growth conditions and sterile culture media will be required. Such protoplast regeneration protocols have been developed over the past two decades as well[28–30].

For successful experiments, a high level of protoplast transfection efficiency (greater than 50%) is very important to obtain reliable and reproducible data using the TEAMP system. We recommend the use of an improved GFP vital marker donated to the ABRC to visually monitor protoplast transfection efficiency[19] (see **Fig. 1d**). Careful practice, keen observation and accumulated experience contribute to the acquisition of meaningful results. All experiments need to be repeated multiple times to ensure consistency and to avoid pitfalls and false positive results. Even though the protocol appears to be simple and straightforward, the importance of practice and empirical optimization of each experiment for the individual researcher's purpose cannot be over-emphasized.

MATERIALS

REAGENTS

- 0.2 M 4-morpholineethanesulfonic acid (MES, pH 5.7; Sigma, cat. no. M8250), sterilize using a 0.45-µm filter
- 0.8 M mannitol (Sigma, cat. no.M4125), sterilize using a 0.45-µm filter
- 1 M CaCl$_2$ (Sigma, cat. no. C7902), sterilize using a 0.45-µm filter
- 2 M KCl (Sigma, cat. no. P3911), sterilize using a 0.45-µm filter
- 2 M MgCl$_2$ (Sigma, cat. no. M9272), sterilize using a 0.45-µm filter
- β-Mercaptoethanol (Sigma, cat. no. M6250)
- 10% (wt/vol) BSA (Sigma, cat. no. A-6793), sterilize using a 0.45-µm filter
- Cellulase R10 (Yakult Pharmaceutical Ind. Co., Ltd., Japan)
- Macerozyme R10 (Yakult Pharmaceutical Ind. Co., Ltd., Japan) ▲ CRITICAL We have obtained the best results with this enzyme provider.
- PEG4000 (Fluka, cat. no. 81240) ▲ CRITICAL We found that the PEG source is very critical to achieve high transfection efficiency.
- 1 M Tris–phosphate (pH 7.8), sterilize using a 0.45-µm filter
- 100 mM *trans*-1,2-diaminocyclo-hexane-*N*,*N*,*N'*,*N'*-tetraacetic acid (DACTAA; Sigma, cat. no. D-1383)
- 50% (vol/vol) glycerol (Fisher, cat. no. 15892), sterilize using a 0.45-µm filter
- 20% (vol/vol) Triton X-100 (Sigma, cat. no. T-8787)
- 1 M DTT (Sigma, cat. no. D-9779)
- LUC assay system (Promega, cat. no. E1501)
- 1 M Tris–HCl (pH 8.0) (US Biological, cat. no. T8650), sterilize using a 0.45-µm filter
- 0.1 M 4-methylumbelliferyl glucuronide (MUG; Gold BioTechnology, Inc., cat. no. MUG-1G)
- 0.2 M Na$_2$CO$_3$ (Sigma, cat. no. S7795)
- 1 M methylumbelliferone (MU; Fluka, cat. no. 69580)
- Metro-Mix 360 (Sun Gro Horticulture, Inc.)
- Jiffy7 (Jiffy Products Ltd., Canada)
- *Arabidopsis* accessions: Col-0 and L*er* (ABRC)
- Enzyme solution (see REAGENT SETUP)
- Washing and incubation (WI) solution (see REAGENT SETUP)
- W5 solution (see REAGENT SETUP)
- MMG solution (see REAGENT SETUP)
- PEG–calcium transfection solution (see REAGENT SETUP)
- Protoplast lysis buffer (see REAGENT SETUP)
- MUG substrate mix for GUS assay (see REAGENT SETUP)
- Ampicillin

EQUIPMENT

- Fluorescence microscope (Leica DM5000; Leica)
- Luminometer (Monolite3010; Pharmigen)
- Fluorometer (TK100; Hoefer)
- Bench-top centrifuge (IEC Centra CL2; International Equipment Company)
- 0.45-µm syringe sterilization filter (Whatman, cat. no. 6870-2504)
- Generic razor blade (single-edged blade; VWR Scientific, cat. no. 55411-055)
- Petri dish (100 × 25 mm^2 for 10 ml enzyme solution; VWR Scientific, cat. no. 25389-000)
- Nylon mesh (75 µm, laboratory sifters, Carolina Biological Supplies, cat. no. 65-2222N)
- Improved Neubauer 0.1-mm-deep Reichert hemacytometer (Hausser Scientific, cat. no. 1483)
- 30-ml round-bottomed tube (Sarstedt, cat. no. 55.517)
- 2-ml round-bottomed natural microcentrifuge tube (SealRite; USA Scientific, cat. no. 1620-2700)
- 6-well culture dish (Falcon, cat. no. 3046)

REAGENT SETUP

Enzyme solution Prepare 20 mM MES (pH 5.7) containing 1.5% (wt/vol) cellulase R10, 0.4% (wt/vol) macerozyme R10, 0.4 M mannitol and 20 mM KCl. Warm the solution at 55 °C for 10 min to inactivate DNAse and proteases and enhance enzyme solubility. Cool it to room temperature (25 °C) and add 10 mM CaCl$_2$, 1–5 mM β-mercaptoethanol (optional) and 0.1% BSA. ▲ CRITICAL Addition of 1–5 mM β-mercaptoethanol is optional, and its use should be determined according to the experimental purpose. ▲ CRITICAL Before the enzyme powder is added, the MES solution is preheated at 70 °C for 3–5 min. The final enzyme solution should be clear light brown. Filter the final enzyme solution through a 0.45-µm syringe filter device into a Petri dish (100 × 25 mm^2 for 10 ml enzyme solution). ▲ CRITICAL The enzyme solution should be prepared fresh.

WI solution Prepare 4 mM MES (pH 5.7) containing 0.5 M mannitol and 20 mM KCl. The prepared WI solution can be stored at room temperature (22–25 °C).

W5 solution Prepare 2 mM MES (pH 5.7) containing 154 mM NaCl, 125 mM CaCl$_2$ and 5 mM KCl. The prepared W5 solution can be stored at room temperature.

MMG solution Prepare 4 mM MES (pH 5.7) containing 0.4 M mannitol and 15 mM MgCl$_2$. The prepared MMG solution can be stored at room temperature.

PEG–calcium transfection solution Prepare 20–40% (wt/vol) PEG4000 in ddH$_2$O containing 0.2 M mannitol and 100 mM CaCl$_2$. ▲ CRITICAL Prepare PEG solution at least 1 h before transfection to completely dissolve PEG. The PEG solution can be stored at room temperature and used within 5 d. However, freshly prepared PEG solution gives relatively better protoplast transfection efficiency. Do not autoclave PEG solution.

Protoplast lysis buffer Prepare 2.5 mM Tris–phosphate (pH 7.8) containing 1 mM DTT, 2 mM DACTAA, 10% (vol/vol) glycerol and 1% (vol/vol) Triton X-100. The lysis buffer should be prepared fresh.

MUG substrate mix for GUS assay Prepare 10 mM Tris–HCl (pH 8) containing 1 mM MUG and 2 mM MgCl$_2$. The prepared GUS assay substrate can be stored at –20 °C.

PROCEDURE

Plant growth ● TIMING 3–4 weeks

1| Grow *Arabidopsis* plants on either Metro-Mix 360 or Jiffy7 soil in a greenhouse or an environment-controlled chamber with a relatively short photoperiod (10–13 h light at 23 °C/11–14 h dark at 20 °C) under low light (50–75 µE m^{-2} s^{-1}) and 40–65% relative humidity.

▲ CRITICAL STEP Col-0 and L*er* have been extensively tested in our lab. In general, *Arabidopsis* plants are very sensitive to all kinds of environmental changes (e.g., drought, flooding, extreme temperature and constant mechanical perturbation). Try to maintain a constant environment as much as possible.

Protoplast isolation ● TIMING 4–5 h

2| Choose well-expanded leaves from 3–4-week-old plants (usually true leaf numbers five to seven) before flowering (see **Fig. 1a**).

▲ CRITICAL STEP The selection of healthy leaves at the proper developmental stage is a very important factor in protoplast experiments. Protoplasts prepared from leaves recovered from stress conditions (e.g., drought, flooding, extreme temperature and constant mechanical perturbation) may look similar to those from healthy leaves. However, we have often experienced low transfection efficiency with the protoplasts from stressed leaves. High stress–induced cellular status can also be a problem in the study of stress, defense and hormonal signaling pathways.

3| Cut 0.5–1-mm leaf strips from the middle part of a leaf using a fresh sharp razor blade without tissue crushing at the cutting site. A good preparation yields approximately 10^7 protoplasts per gram fresh weight (approximately 100–150 leaves

digested in 40–60 ml of enzyme solution). For routine experiments, 10–20 leaves digested in 5–10 ml enzyme solution will give 0.5–1 $\times$ 10^6 protoplasts, enough for more than 25–100 samples (1–2 $\times$ 10^4 protoplasts per sample).
▲ CRITICAL STEP Change the blade after cutting four to five leaves. We routinely cut leaves on a piece of clean white paper (8" $\times$ 11") on top of the solid and clean laboratory bench, which provides for good support and easy inspection of wounded/crushed tissue (juicy and dark green stain).

4| Transfer leaf strips quickly and gently into the prepared enzyme solution (10–20 leaves in 5–10 ml) by dipping both sides of the strips (completely submerged) using a pair of flat-tip forceps.
▲ CRITICAL STEP Immediate dipping and submerging of leaf strips is very critical for protoplast yield. When leaf strips are dried out on the paper during cutting, the enzyme solution cannot penetrate and protoplast yield is decreased significantly.

5| Vacuum infiltrate leaf strips for 30 min in the dark using a desiccator.

6| Continue the digestion, without shaking, in the dark for at least 3 h at room temperature. The enzyme solution should turn green after a gentle swirling motion, which indicates the release of protoplasts.
▲ CRITICAL STEP Digestion time depends on the experimental goals, desirable responses and materials used, and it needs to be optimized empirically. After 3 h digestion, most protoplasts are released from leaf strips in case of Col-0. However, the protoplast isolation efficiency differs significantly for different ecotypes and genotypes. The digesting time has to be optimized for each ecotype and genotype. Prolonged incubation of leaves (16–18 h) in the dark is stressful and might eliminate physiological responses of leaf cells. However, the stress might be important for the dedifferentiation and regeneration processes recommended in other protoplast protocols.

7| Check for the release of protoplasts in the solution under the microscope; the size of *Arabidopsis* mesophyll protoplasts is approximately 30–50 μm.
▲ CRITICAL STEP It is not necessary to release all the protoplasts from leaf strips. Be gentle with protoplasts, but you can handle them with regular pipettes and pipette tips.

8| Dilute the enzyme/protoplast solution with an equal volume of W5 solution before filtration to remove undigested leaf tissues.

9| Wash a clean 75-μm nylon mesh with water to remove ethanol (the mesh is normally kept in 95% ethanol) then remove excess water before protoplast filtration. Filter the enzyme solution containing protoplasts after wetting the 75-μm nylon mesh with W5 solution.

10| Centrifuge the flow-through at 100*g* to pellet the protoplasts in a 30-ml round-bottomed tube for 1–2 min. Remove as much supernatant as possible and re-suspend the protoplast pellet by gentle swirling.
▲ CRITICAL STEP A higher speed (200*g*) of centrifugation may help to increase protoplast recovery.
? TROUBLESHOOTING

11| Re-suspend protoplasts at 2 $\times$ 10^5 ml^{-1} in W5 solution after counting cells under the microscope ($\times$100) using a hemacytometer. Rest the protoplasts by keeping on ice for 30 min.
▲ CRITICAL STEP If cold response is desired, keep protoplasts at room temperature. Although the protoplasts can be kept on ice for at least 24 h, freshly prepared protoplasts should be used for the study of gene expression regulation, signal transduction and protein trafficking, processing and localization.

12| Protoplasts should begin to settle at the bottom of the tube by gravity after 15 min. Remove the W5 solution as much as possible without touching the protoplast pellet. Re-suspend protoplasts at 2 $\times$ 10^5 ml^{-1} in MMG solution kept at room temperature.

DNA–PEG–calcium transfection ● TIMING Less than 1 h for 20 samples
13| Add 10 μl DNA (10–20 μg of plasmid DNA of 5–10 kb in size) to a 2-ml microfuge tube.
▲ CRITICAL STEP To study the role of a candidate regulator, three plasmids expressing a regulatory effector, a specific reporter and a transfection control reporter are used at the ratio of 5:4:1. However, it is highly recommended to optimize the plasmid ratio depending on each reporter's sensitivity. For example, a highly sensitive reporter needs only half the amount. Whenever the plasmid amount is changed, the same total plasmid DNA amount is compensated with a control plasmid that does not express any effector or reporter. The plasmid DNA has to be purified appropriately to support high transfection efficiency. Additional information on the preparation of plasmid DNA using the economical CsCl gradient is provided on the Sheen lab website (http://genetics.mgh.harvard.edu/sheenweb/protocols.html).

14| Add 100 μl protoplasts (2 $\times$ 10^4 protoplasts) and mix gently.

15| Add 110 μl of PEG solution, and then mix completely by gently tapping the tube (handle six to ten samples for each transfection).

16| Incubate the transfection mixture at room temperature for up to 15 min (5 min is sufficient).
▲ **CRITICAL STEP** Transfection time should be optimized empirically.

17| Dilute the transfection mixture with 400–440 μl W5 solution at room temperature and mix well by gently rocking or inverting the tube to stop the transfection process.

18| Centrifuge at 100*g* for 2 min at room temperature using a bench-top centrifuge and remove supernatant.

19| Re-suspend protoplasts gently with 1 ml WI in each well of a 6-well tissue culture plate.
▲ **CRITICAL STEP** We also use 12-well (0.5 ml WI) or 24-well (0.25 ml WI) plates for larger-scale experiments (50–100 samples). The culture plate is normally coated with 5% (vol/vol) sterile calf serum for a short time (1–2 s) to prevent sticking of the protoplasts to the plastic surface. The depth of the WI solution is approximately 0.1 mm to prevent hypoxia stress during protoplast incubation.
▲ **CRITICAL STEP** The use of carrier DNA is unnecessary for this type of DNA delivery.
▲ **CRITICAL STEP** High transfection efficiency can be achieved using 10–20% PEG final concentration. The optimal PEG concentration should be determined empirically for each experimental purpose. Visual reporters such as GFP can be used to determine optimal DNA transfection conditions (**Fig. 1d**, **Table 1**). If protoplasts are derived from healthy leaf materials, most protoplasts should remain intact throughout the isolation, transfection, culture and harvesting procedures.
▲ **CRITICAL STEP** Protoplast transfection can be scaled up or down depending on the experimental needs. Approximately 10^{3-4} protoplasts are sufficient for reporter enzyme assays, 10^{4-5} protoplasts are required for protein labeling and immunoprecipitation or western blot analysis and 10^6 protoplasts for RNA extraction and microarray analysis. If the protoplast transfection efficiency is low (less than 20%), the quality of plasmid DNA or the ratio of plasmid DNA and protoplast number should be systematically examined to find the optimal condition.
? TROUBLESHOOTING

Protoplast culture and harvest ● TIMING 2–24 h
20| Incubate protoplasts at room temperature (20–25 °C) for the desired period of time.
▲ **CRITICAL STEP** A stimulus can be applied during the incubation according to the experimental purposes. For example, the synthetic flg22 peptide derived from bacteria can be added for 10 min or 1 h for the analysis of protein kinase activity or early transcription responses, respectively. For the inducible gene expression system (**Fig. 2c,d**), DEX can be added to the protoplasts 15–30 min after DNA transfection.
▲ **CRITICAL STEP** In general, the protoplast incubation time is 2–6 h for RNA analysis and 2–16 h for enzyme activity analysis and protein labeling. The TEAMP system is most suitable for the study of early events in signal transduction pathways, gene regulation and cell death.
▲ **CRITICAL STEP** The protoplast incubation temperature has to be optimized according to experimental needs.
▲ **CRITICAL STEP** It is not necessary to perform experiments under sterile conditions in general. Ampicillin (50 μg ml^{-1}) can be added during the protoplast incubation after DNA transfection if bacterial growth is a concern.

21| Re-suspend and harvest protoplasts by centrifugation at 100*g* for 2 min.

22| Remove the supernatant and freeze samples on dry ice.
■ **PAUSE POINT** Samples can be stored at –80 °C until further analysis.

Reporter assays ● TIMING 1–2 h
23| Several assays can be carried out using option A (LUC assay), B (GUS assay) or C (GFP assay).
(A) LUC assay
  (i) Add 100 μl of protoplast lysis buffer to the frozen protoplasts and mix vigorously by vortexing for 2 s to rupture the protoplasts.
  (ii) After 5 min incubation on ice, centrifuge at 1,000*g* for 2 min.
  (iii) Use 5–20 μl of the lysate and 100 μl LUC mix (Promega) to measure LUC activity with a luminometer.
(B) GUS assay
  (i) Add 10 μl of the protoplast lysate from Step 23A(ii) into 100 μl of MUG substrate mix and incubate for 30–180 min at 37 °C.
  (ii) Add 0.9 ml 0.2 M Na$_2$CO$_3$ to stop the reaction.
  (iii) Measure the fluorescence of MU using a fluorometer.
     ▲ **CRITICAL STEP** Further dilution with 0.2 M Na$_2$CO$_3$ for GUS assay should be determined empirically.
(C) GFP assay
  (i) View live cellular image of GFP or GFP fusion proteins (using protoplasts obtained in Step 22) under fluorescence microscopy (×400–600) without any additional sample preparation steps.

● TIMING
Step 1: 3–4 weeks
Steps 2–4: approximately 1 h (depending on numbers of leaves being collected)
Steps 5–12: approximately 3–4 h (depending on *Arabidopsis* accession and mutants)
Steps 13–19: approximately 1 h (depending on numbers of samples being transfected)
Steps 20–22: approximately 2–24 h
Step 23: approximately 1–2 h

? TROUBLESHOOTING
Troubleshooting advice can be found in **Table 2**.

TABLE 2 | Troubleshooting table.

Problem	Possible reason	Solution
Low protoplast yield	Accession	*Arabidopsis* growth condition is dependent on the accession used. As leaves before bolting are used, Col and L*er* plants grown under a relatively shorter photoperiod (10–13 h) perform better in protoplast isolation
	Poor plant growth	Check the quality of seeds (growth and storage conditions) and soil. We have had problems with different types of soil occasionally. The quality of soil may vary from batch to batch
		Check and optimize growth conditions: temperature, humidity, light intensity, photoperiod, watering and nutrients
	Poor leaf condition	Check leaf age, development and morphology (**Fig. 1a**)
	Inefficient enzyme digestion	Check activity of enzyme solution (enzyme quality and heat treatment)
		Optimize leaf digestion time for each accession and mutant
Low transfection efficiency	PEG solution problem	PEG quality depends on sources and storage. Do not autoclave PEG solution
	Low-quality DNA	We use the economical CsCl gradient method for plasmid DNA purification. The detailed protocol is available on the Sheen lab website
	Protoplast/DNA ratio	Ratio should be determined empirically
	Poor protoplast quality	Optimize conditions and the time for protoplast resting, response and incubation
	Stimuli application	We always test and optimize the response timing and analyze broad concentrations of signal dosage to cover a wide range when a new signal is applied for the first time

ANTICIPATED RESULTS
Protoplast transfection efficiency is approximately 60–90% with this protocol in general (**Fig. 1d**). It is necessary to have higher than 50% transfection efficiency for a reliable analysis. A typical use of the TEAMP assay is to determine the regulatory function of an effector using the readout of a specific reporter in cell-autonomous responses. The effect of regulatory candidates that are transfected with a specific reporter (**Table 1**) can be quantified within 2–12 h after transfection of mesophyll protoplasts. The specific reporter readout value should be normalized with that of a co-transfected control reporter driven by a constitutive promoter, e.g., *UBQ10-GUS-NOS*[8].

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