

Research Article

A Universal Method of Protoplast Isolation and Transient Expression in Plants

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Abstract

Protoplasts isolated from plant tissues have been testified as useful and versatile materials for performing cell-based experiments such as protein subcellular localization, *in vivo* protein-protein interaction, determination of vacuolar pH and promoter activity. Protoplast isolation and transient expression methods have already been reported in some plants, which with some limitations for universal application to some extent. In this report, we presented an improved method of protoplast isolation and transient expression, which was verified by using four representative plant materials including *Arabidopsis*, chrysanthemum, tomato and apple. To illustrate the practicability of the method, we transiently expressed the Green Fluorescent Protein (GFP) using chrysanthemum leaf protoplasts, and observed that the fluorescent protein could be successfully delivered into protoplasts under optimal conditions. In all, the results including protoplast isolation and plastid transfection can be obtained in only two days, which efficiently saving much time compared with corresponding protocols. The cell system offers reliable guidelines for further comprehensive analysis of complex regulatory mechanisms in whole-plant physiology, immunity, growth and development.

Keywords: protoplasts; isolation; transient expression; *Arabidopsis*; chrysanthemum; tomato; apple

Introduction

Plant protoplasts, the extremely fragile membrane-bound cells, are protected by the outer cell wall comprised of polysaccharides (e.g. cellulose, hemicelluloses, chitin and lignin) and pectin, which provide basic support for the plant growth and development [1,2]. In general, the plant cell wall can be easily digested and removed by enzymes including cellulose and macerozyme, leading to the release of protoplasts from plant cells [3,4]. The isolated protoplasts still retain their cell identity and differentiated state [5], therefore, it have been proved that fresh protoplasts are physiological and versatile cell systems for studying protein subcellular positioning, protein-protein interaction, as well as various cellular processes and signaling mechanisms in many plant species [6-9]. As for protoplast isolation method, there have been some reports in *Arabidopsis*, [4] cereals, [10] tobacco, [11] strawberry, [12] petunia [13] and soon. However, the current method of protoplast isolation has some limitation in its utility, such as the strict control

of plant growth environment and the requirement of manually experience [14].

A series of common transfection methods such as microinjection bombardment, poly Ethylene Glycol (PEG)-calcium fusion, agro infiltration and electroporation, etc. have been used to deliver recombinant DNA plasmids into the plant protoplasts [13,14]. The transfected protoplasts are subsequently used for studying the functions of diverse signalling pathways and cytoarchitecture [4,16,17]. Therein, the method of microparticle bombardment is widely used, which needs epidermis of onion as the material [18]. Due to lack of well-developed chloroplasts in onion, this method limits the research of chloroplast-targeted proteins [19]. So far, the PEG-mediated transient transfection has been widely used for its high transformation efficiency [20-22].

In *Arabidopsis* research, the methods of protoplast isolation and transient expression have been well-studied [4,16,17]. For example, Yoo et al. described a protocol of transient expression in *Arabidopsis* mesophyll protoplasts (TEAMP), and provide detailed information on isolation of *Arabidopsis* mesophyll protoplasts. These studies provided simple and efficient methods to study many

Arabidopsis mutants and ecotypes, and there is no need to produce suspension cultures. Additionally, these protoplasts maintain normal physiological and signal transduction functions. Therefore, these protocols thus have been widely used in plants investigation in recent years. Moreover, there were some reports on protoplasts isolation and transient expression in horticultural crops such as petunia [13], cucumber [22] and tomato [23]. However, a universal method that was used for protoplast isolation and transient expression in horticultural crops has not been studied.

In current study, we reported an improved method of protoplast isolation and transient expression in four kinds plants, and we transiently expressed a fluorescent protein using chrysanthemum mesophyll protoplasts and to verify its availability of this method. In our method, replacement of the sliced leaves with the torn thin hypodermis film on the leaves effectively enhanced the yield of protoplasts. Meanwhile, the protoplasts isolated from the hypodermis rule out the interference of much chloroplasts in the subsequent fluorescence observation. Moreover, the modified PEG-mediated transient expression increased the efficiency of the transformation. Finally, the potential application of this universal method that was used for studying intracellular signal transduction pathways is discussed.

Materials and Methods

Plant Material and Growth Conditions

Arabidopsis thaliana ecotype Columbia (Col-0) plants were grown in a mixture of vermiculite, perlite and peat moss at a 1:1:1 ratio in an environmentally-controlled chamber with a 16-h light photoperiod (200 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) at 21°C. Tomato (*Lycopersicon esculentum* cv Micro-Tom) plants were grown in an indoor growth room at 25°C under a 14hr light/10hr dark photoperiod at approximately 600 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ as previously described. [23].

The apple seedlings (*Malus domestica* cv 'Gala') were sub cultured on Murashige and Skoog (MS) medium supplemented with 0.8 mg L⁻¹ 6-BA and 0.2 mg L⁻¹ IAA under long-day conditions (16 h light /8 h dark) with 25°C/20°C day/night cycle according to previous study [24]. The tissue-cultured chrysanthemum (*Chrysanthemum morifolium* cv Jinba) plantlets were kindly provided by China Agricultural University (Beijing). The plantlets were propagated under sterile condition on MS agar medium. The growth condition (23 \pm 1°C, 16/8 h light/dark, 100-120 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) was set according to previous study [25].

Construction of the Expression Vectors

The full length cDNAs of CmANR1 and LeHSP21 were cloned into PRI plant transformation vector with a GFP tag downstream CaMV 35S promoter. Subsequently, the plastids containing the recombinant expression vectors CmANR1-GFP and LeHSP21-GFP were used for genetic transformation. The full-length of CmANR1

was amplified with primers: CmANR1-F: 5'-AATGGGGAGAG-GTAAGATCGTG-3'; CmANR1-R: 5'-TGTAGTTGTAATCCCA-AATTTGTAG-3'. Meanwhile, the encoding region of LeHSP21 was amplified with primers: LeHSP21-F: 5'-AATGAGTGTGTCAT-CAGCAAATTAAC-3'; LeHSP21-R: 5'-AAGCTCTTCTCTAA-CAGAGGACTC-3'.

Protoplast Isolation

The tender leaves were collected from *Arabidopsis*, tomato, apple and *chrysanthemum* plants grown under optimal growth conditions, and were rinsed with sterile water. After being briefly dried, the washed leaves were tore off the lower epidermis with a sharp-pointed tweezer, and then the lower epidermis were quickly transferred into 10 ml of enzymolysis solution of cell wall containing [1.5% cellulase 'Onozuka' R10 (Yakult, Tokyo, Japan), 0.4% macerozyme 'Onozuka' R10 (Yakult, Tokyo, Japan), 0.05% pectolase Y-23 (Yakult, Tokyo, Japan), 0.4 M mannitol, 10 mM CaCl₂, 20 mM KCl, 20 mM MES and 2% sucrose, pH 5.8], and were placed at room temperature in the dark for 12-14 hours.

Subsequently, the enzyme solution including leaf lower epidermis were gently shaken (30 rpm on a platform shaker) in light for 30 minutes so that the protoplasts were completely released into the solution. Add the same volume of pre-chilled modified W5 solution (154 mM NaCl, 125 mM CaCl₂, 5 mM KCl, and 2 mM MES, pH 5.8), and then filtered the mixture with 75 μm nylon fabric into 50 ml centrifuge tube. The protoplasts were centrifuged at 500 rpm for 10 minutes, and then gently removed the supernatant. The protoplasts at the bottom pipe were repeatedly washed twice with 10-15 ml of prechilled modified W5 solution, and incubated on ice for 30 minutes. During the incubation period, the protoplasts were counted using a hemocytometer under a light microscope. The protoplasts were then centrifuged and resuspended in prechilled modified MMg solution (0.4 M mannitol, 15 mM MgCl₂, and 4 mM MES, pH 5.8) to a final concentration of 1 to 5 $\times 10^4$ cells/mL.

Protoplast Transfection Assays

Protoplasts were transfected by a PEG-mediated method as described by previous study [4] with some modifications. Approximately 5 $\times 10^4$ protoplasts in 1 mL of MMg solution were mixed with about 30 μg of plasmid DNA in 10 ml centrifuge tube at room temperature. 1 mL of a freshly-prepared 40% PEG4000 (w/v) solution including 0.1 M CaCl₂ and 0.2 M mannitol was added, and the mixture was incubated for 5 min at room temperature. After incubation, 6 mL of W5 solution was added and mixed slowly. The mixture was then centrifuged at 400 rpm for 5 min, and removed the supernatant. The transfected protoplasts were washed twice with W5 solution. Subsequently, the protoplasts were gently resuspended in 1 mL of WI solution (0.5 M mannitol, 20 mM KCl, 4 mM MES, pH 5.8), and were transferred in 6-well plates. These protoplasts were expressed in dim light for no more than 9 h.

H₂DCFDA Staining for ROS Accumulation

ROS accumulation in protoplasts was determined by staining with the fluorescent dye C-H₂DCFDA (Sigma-Aldrich) as described [26]. For C-H₂DCFDA staining, protoplasts were treated with 20 mM C-H₂DCFDA (Invitrogen) for 30 min and subsequently imaged using an inverted laser scanning confocal microscope (LSM780; Zeiss) with an excitation at 488 nm. The imageJ was used for quantifying ROS intensity.

Confocal laser Scanning Microscopy

Protoplasts were observed with an inverted laser scanning confocal microscope (LSM780; Zeiss) with a Plan-Neofluar ×40/1.3 oil differential interference contrast objective or ×63/1.45 oil differential interference contrast objective. Fluorescence was detected using a 505- 550 nm band-pass filter for GFP, and 488 nm/band-pass 650-710 nm for chloroplast auto-fluorescence. Post acquisition image processing was performed with the LSM image-processing software (Zeiss).

Immunoblotting Assays

The extraction of proteins obtained from protoplast cells and immunoblotting assays were performed as described by Hu et al. [27]. The anti-GFP antibody were prepared by the Abmart Company (Shanghai, China).

Results

Isolation of protoplasts from plant leaves.

Four types of plant species including *Arabidopsis*, tomato, apple and chrysanthemum were used for protoplast isolation in this study (Figure 1).

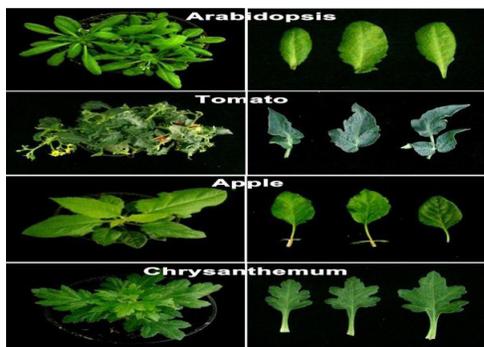


Figure 1: Four types of healthy plant materials including *Arabidopsis*, tomato, apple and chrysanthemum that were used for protoplasts isolation.

The washed plant leaves were briefly dried, tore off the lower epidermis with a sharp-pointed tweezers, and then quickly transferred these leaf lower epidermis to enzymolysis solution of cell wall (Figure 2)

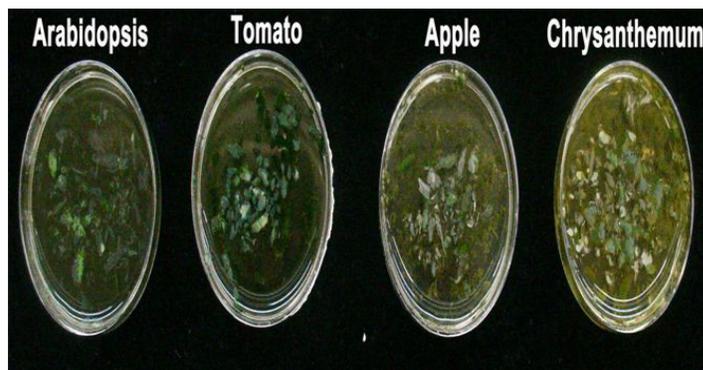


Figure 2: Leaf lower epidermis cells were immersed in enzyme solution to allow the release of protoplasts.

The leaf lower epidermal cells were reserved and used for follow-up experiments in our study. This was distinct with mostly other methods which were mainly peeled away the lower epidermal cell layer and retained the upper epidermal cells. The enzyme mixture only digested the cell walls from lower epidermal cells. Microscopy confirmed that the protoplasts were released from lower epidermal cells into solution, as shown in (Figure 3).

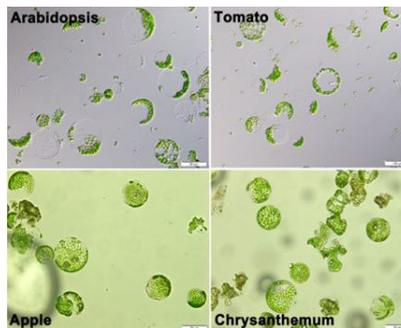


Figure 3: Protoplasts derived from leaf lower epidermis cells of Arabidopsis, tomato, apple and chrysanthemum.

Transient expression in protoplast system for gene expression.

To determine the availability of protoplasts for transient expression of plant genes, a chrysanthemum MADS-box transcription factor gene CmANR1 driven by CaMV 35S promoter-GFP vector was constructed and transiently transformed into chrysanthemum leaf protoplasts. Consequently, the recombinant protein 35S:: CmANR1-GFP from the transfected protoplasts were detected by immunoblotting assay using anti-GFP antibody. As a result, we found that the recombinant proteins 35S:: CmANR1-GFP were successfully expressed in the transfected chrysanthemum leaf protoplasts (Figure 4) indicating that the extracted chrysanthemum leaf protoplasts using this method contribute to useful plant material for transient expression.

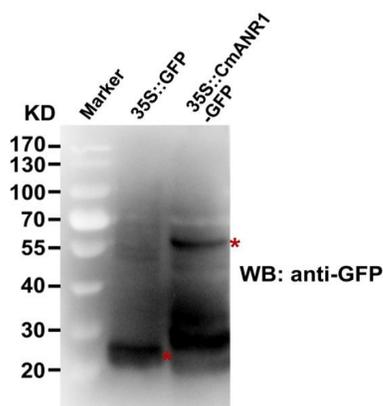


Figure 4: Transfected chrysanthemum protoplast protein was isolated for Western blot assay. Chrysanthemum protoplasts were transfected with 35S::CmANR1-GFP plasmid (MW: 59.2 kDa). Western blotting assay was performed with an anti-GFP antibody. 35S::GFP (MW: 29.6 kDa) vector was used as a negative control.

Transient expression in protoplast system for protein subcellular localization

To further identify the feasibility of protoplasts for study of protein subcellular localization, a previously reported chloroplast-localized tomato heat shock protein LeHSP21 driven by CaMV 35S promoter-GFP construction was used, [28] and then transiently transformed into the tomato leaf protoplasts. The results showed that the green fluorescence of 35S:: LeHSP21-GFP overlapped with the auto-fluorescence of chlorophyll (Figure 5) suggesting that LeHSP21 is localized at chloroplasts. This result demonstrated that the availability of protoplast system for studying protein subcellular localization.

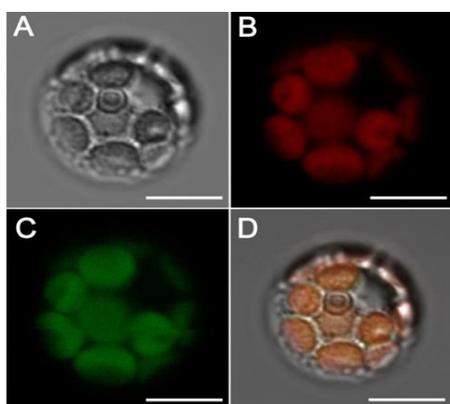


Figure 5: Plasmids encoding chloroplast-localized tomato heat shock protein LeHSP21 driven by the constitutive 35S enhancer may be successfully delivered to tomato protoplasts. (A) The bright-field images from LeHSP21-transfected tomato protoplasts. (B) The images showing the red auto fluorescence of chloroplast. (C) The images showing the green GFP signal. (D) The overlapped images of red auto-fluorescence of chloroplast and green GFP signal. In (A-D), bars = 100 μm.

Availability of protoplast system for study of Reactive Oxygen Species (ROS) accumulation.

Furthermore, our previously reported MdsOS2L1 encoding a CIPK family protein kinase, decreases ROS accumulation to enhance salt tolerance in transgenic apple [29]. To testify whether protoplast system is suitable for study ROS accumulation, we isolated protoplasts from MdsOS2L1-overexpressed apple leaves. Thereafter, carboxylated 2',7'-dichlorodihydrofluorescein diacetate [C-H2DCFDA] staining were performed to visualize ROS accumulation in the protoplasts isolated from wild-type (WT) and MdsOS2L1 transgenic apple leaves. The results indicated that the MdsOS2L1 transgenic apple protoplasts produced less ROS than the WT controls (Figure 6).

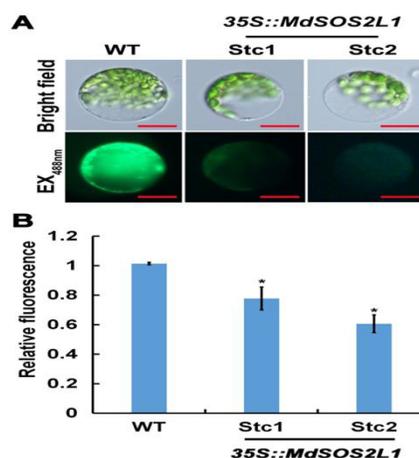


Figure 6: ROS production in apple protoplasts. (A) Protoplasts isolated from wild-type (WT) and MdsOS2L1-overexpressed apple leaves were incubated in C-H2DCFDA for 90 s. H2DCFDA is a ROS indicator that becomes fluorescent when oxidized by ROS within the cell. Note: Stc1 and Stc2 represent two lines of MdsOS2L1-overexpressed apple plants. (B) The fluorescence of protoplasts from WT and MdsOS2L1-overexpressed apple leaves was quantified by pixel intensity. Data points represent means \pm SD of 12 to 15 individual protoplasts. PIV, pixel intensity value and this data was in line with our previous findings. This result demonstrated that the availability of protoplast system for studying ROS accumulation.

Discussion

Transient expression and physiological assays in protoplast systems have been used as an effective biochemical tool to identify key regulators and clarify molecular mechanisms involved in plant growth, development and signaling [8]. To date, there have been several reports of protoplasts extraction and application in plants including *Arabidopsis* [13-15], tobacco [10], strawberry [11], *Petunia* [12], cucumber [21], and rice [30,31]. Yoo et al. established a transient gene expression system including protoplast isolation, PEG-calcium transfection of plasmid DNA and protoplast culture using *Arabidopsis* mesophyll protoplasts (TEAMP). Where after, Wu et al. developed a simple and fast *Arabidopsis* protoplast isola-

tion method called the Tape-Arabidopsis sandwich. In this study, we further modified previous methods and developed a universal method of protoplast isolation and transient expression in plants. To prove this, we randomly selected four types of plant species including *Arabidopsis*, tomato, apple and chrysanthemum as the materials and verified protein expression (Figure 4), protein subcellular localization (Figure 5) and ROS accumulation (Figure 6) by our improved method. The satisfying results provide evidences for the potential application of our method to many other plants.

Most of previous studies obtained the plant mesophyll protoplasts and were allowed to removal of the lower epidermal cells. [4,15,16] As is well known, Mesophyll is a photosynthetically active tissue of the leaf, which contains a very high density of chloroplasts [32] However, the chloroplast-rich mesophyll protoplasts may sometimes interfere with the subsequent experiment results. For example, mesophyll protoplast is not very suitable for the subcellular localization of the nucleus-localized protein, due to their auto-fluorescence interferences from chloroplasts. By contrast, the protoplasts isolated from lower epidermal cells had relatively small amount of chloroplasts in our study, which was more suitable for more experiments than mesophyll protoplasts. Therefore, our improved method provided a more widely available for further protoplast-mediated transient transfection.

Remarkably, although protoplast system was an effective tool to study many cell-based experiments, we must pay attention to some details during the process of transfection and staining. For instance, we should keep the balance of interior and exterior osmotic pressure of protoplasts in order to avoid protoplasts bursting during the course of its operation. In addition, it mentioned that protoplasts were isolated from lower epidermis of the leaves with a sharp-pointed tweezer-tearing method in present study. However, we felt that this process was relatively time-consuming. Therefore, we try to find a similar “Tape-Arabidopsis Sandwich” method to replace the “sharp-pointed tweezer-tearing” method in future.

In summary, our modified protoplast isolation and transient expression method presented here allows us to study cell-based experiments more conveniently, such as protein subcellular localization, *in vivo* protein-protein interaction, determination of vacuolar pH, ROS accumulation and promoter activity. However, some steps in the method may be time-consuming and require more skill experience for researchers, which need further modification in the future. In conclusion, we believe that this protoplast isolation and transient expression method could have more widely applications and be adopted by more researchers in future.

Acknowledgements

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Competing Interest

The authors declare that they have no conflict of interest.

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