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Efficient protoplast isolation and transient gene expression system for *Phalaenopsis* hybrid cultivar 'Ruili Beauty'

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Abstract With the release of the *Phalaenopsis equestris* (Schauer) Rchb.f. genome database, more in-depth studies of *Phalaenopsis* spp. will be carried out in the future. Transient gene expression in protoplasts is a useful system for gene function analysis, which is especially true for *Phalaenopsis*, whose stable genetic transformation is difficult and extremely time-consuming. In this study, juvenile leaves from aseptic *Phalaenopsis* seedlings were used as the starting material for protoplast isolation. After protocol refinement, the highest yield of viable protoplasts [5.94×10^6 protoplasts g^{-1} fresh weight (FW)] was achieved with 1.0% (*w/v*) Cellulase Onozuka R-10, 0.7% (*w/v*) Macerozyme R-10, and 0.4 M D-mannitol, with an enzymolysis duration of 6 h. As indicated by transient expression of green fluorescent protein (GFP), a transformation efficiency of 41.7% was achieved with 20% (*w/v*) polyethylene glycol (PEG-4000), 20 μg plasmid DNA, 2×10^5 mL^{-1} protoplasts, and a transfection duration of 30 min. The protocol established here will be valuable for functional studies of *Phalaenopsis* genes.

Keywords *Phalaenopsis* · Protoplast isolation · Green fluorescent protein · Transient expression

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Introduction

Phalaenopsis spp., also known as moth orchid, is one of the most popular tropical flowers in the world. The recently released genome sequence of *P. equestris* (Schauer) Rchb.f., showed that moth orchids contain 29,431 predicted protein-coding genes (Cai *et al.* 2015). Annotation and functional characterization of these identified genes would help reveal the unique reproductive and ecological adaptations of this orchid species and would facilitate genetic improvement for orchid breeding (Su and Hsu 2003; Hsieh *et al.* 2013; Cai *et al.* 2015). However, stable genetic transformation in orchids is notoriously difficult to achieve and extremely time-consuming (Mishiba *et al.* 2005). For this reason, transient gene expression is an efficient alternative way to analyze gene function (Luehrsen *et al.* 1992; Chen *et al.* 2006; Julkifle *et al.* 2010; Huang *et al.* 2013). Along with the virus-induced gene silencing system developed in *Phalaenopsis* (Hsieh *et al.* 2013), protoplast-based transient gene expression is another useful experimental approach to study gene function, allowing for the elucidation of protein subcellular localization, interactions, and/or catalytic activities *in vivo* (Maddumage *et al.* 2002; Locatelli *et al.* 2003; Yoo *et al.* 2007; Zhang *et al.* 2011; Yao *et al.* 2016). Successful protoplast isolation in Orchidaceae species has been reported in *Cymbidium* (Pindel 2007), *Dendrobium* (Khentry *et al.* 2006), and *Phalaenopsis* (Kobayashi *et al.* 1993; Shrestha *et al.* 2007; Qiao *et al.* 2008). In *Phalaenopsis*, however, protoplast isolation efficiency needed further improvement before a protoplast-based transient gene expression system could be established.

The most commonly used method for protoplast isolation is by enzymatic digestion of the cell wall components (*e.g.*, cellulose, hemicellulose, and pectin) (Rose 1980; Sun *et al.* 1992; Duquenne *et al.* 2007; Zhao *et al.* 2011; Ratanasanobon

and Seaton 2013). During enzymatic digestion, the enzymolysis time and osmotic pressure have to be adjusted; the latter, by applying specific concentrations of osmotic regulators (*e.g.*, sorbitol, sucrose, or D-mannitol) (Zhou *et al.* 2008; Rezazadeh and Niedz 2015). Polyethylene glycol (PEG)-mediated protoplast transfection and transient gene expression is the most widely used system for protoplast transformation, and variables, such as the PEG concentration and incubation time, should be optimized for different plant species (Kao and Michayluk 1974; Maas and Werr 1989).

The objectives of this present study were to develop an efficient protocol for isolating protoplasts derived from mesophyll cells of *Phalaenopsis*, followed by PEG-mediated transfection, and subsequent transient expression of reporter genes. Various key parameters were adjusted in this study and the presented optimum protocol for transient gene expression in *Phalaenopsis* protoplasts was highly reliable with repeatable results.

Materials and Methods

Plant materials and expression vectors The *Phalaenopsis* hybrid cultivar 'Ruili Beauty' was used in this study. Aseptic seedlings were derived from flower stalk explants of 'Ruili Beauty' when the stalks were sterilized for 20 min with 1% (*w/v*) sodium hypochlorite (NaClO) solution diluted from a solution of sodium hypochlorite containing 5.2% active chlorine (Shanghai Lingfeng Chemical Reagent Co., Ltd., Shanghai, China), then cultured in the induction and proliferation medium, consisting of MS salts (Murashige and Skoog 1962), 3% (*w/v*) sucrose, 100 ml L^{-1} coconut water, 5.6 g L^{-1} agar (plant micropropagation grade, gelling strength 900 g cm^{-2}), 7.0 mg L^{-1} N^6-benzyladenine (6-BA), and 0.5 mg L^{-1} α-naphthaleneacetic acid (NAA), adjusted with NaOH to a pH of 5.6 prior to autoclaving at 122°C for 20 min. *In vitro* plantlets were cultured in an illumination incubator (GXZ, Ningbo Jiangnan Instrument Factory, Ningbo, China) at 25°C with a 12-h photoperiod of 80 μmol m^{-2} s^{-1} supplied by fluorescent lamp.

The green fluorescent protein (GFP) expression vector pGreen-*GFP* and the GFP fusion-protein cucumber transport inhibitor response 1 (TIR1; Accession number: JX901282.1) vector pGreen-*CsTIR1-GFP* were used in this study; the 35S promoter was used to drive the GFP reporter or target-GFP fusion genes (Xu *et al.* 2017). The plasmids were extracted using the MiniBEST Plasmid Purification Kit (TaKaRa, Dalian, China).

The isolation, purification, and viability of 'Ruili Beauty' mesophyll protoplasts The improved method of protoplast isolation was based on a report by Huang *et al.* (2013). Only young leaves of aseptically grown plantlets were chosen for protoplast isolation, cut into 0.5–1.0-mm thin strips (Fig. 1*A*,

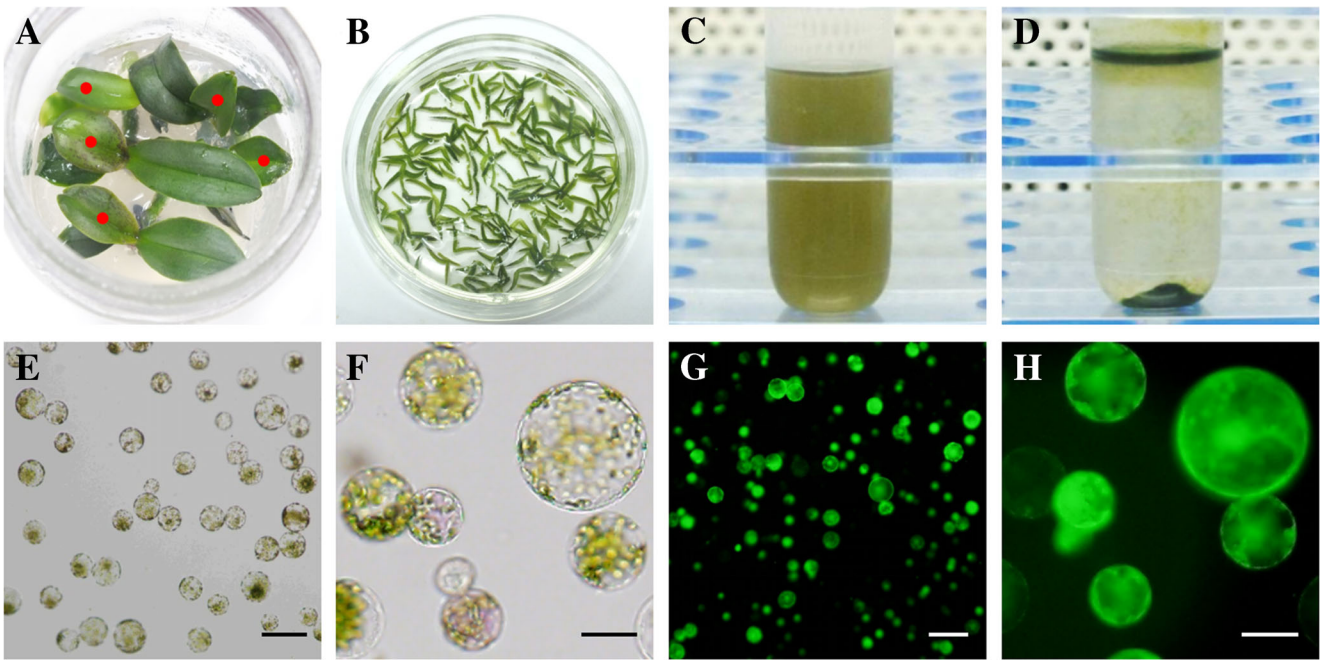

Figure 1. Isolation of *Phalaenopsis* hybrid cultivar 'Ruili Beauty' mesophyll protoplasts. (*A*) Aseptic 'Ruili Beauty' seedlings; *red circles* mark young leaves used in this study. (*B*) 'Ruili Beauty' leaves were cut into thin strips. (*C*) Protoplast suspension; (*D*) purified protoplast *layer* after density gradient centrifugation. (*E*), (*F*) Visualization of 'Ruili Beauty' protoplasts under bright-field microscopy; (*G*), (*H*) 'Ruili Beauty' protoplasts under fluorescence microscopy, visualizing fluorescein in live cells with excitation/emission wavelengths 470-490 and 510-530 nm. (*E*, *G*) *Bars*: 50 μm; (*F*, *H*) *bars*: 20 μm.

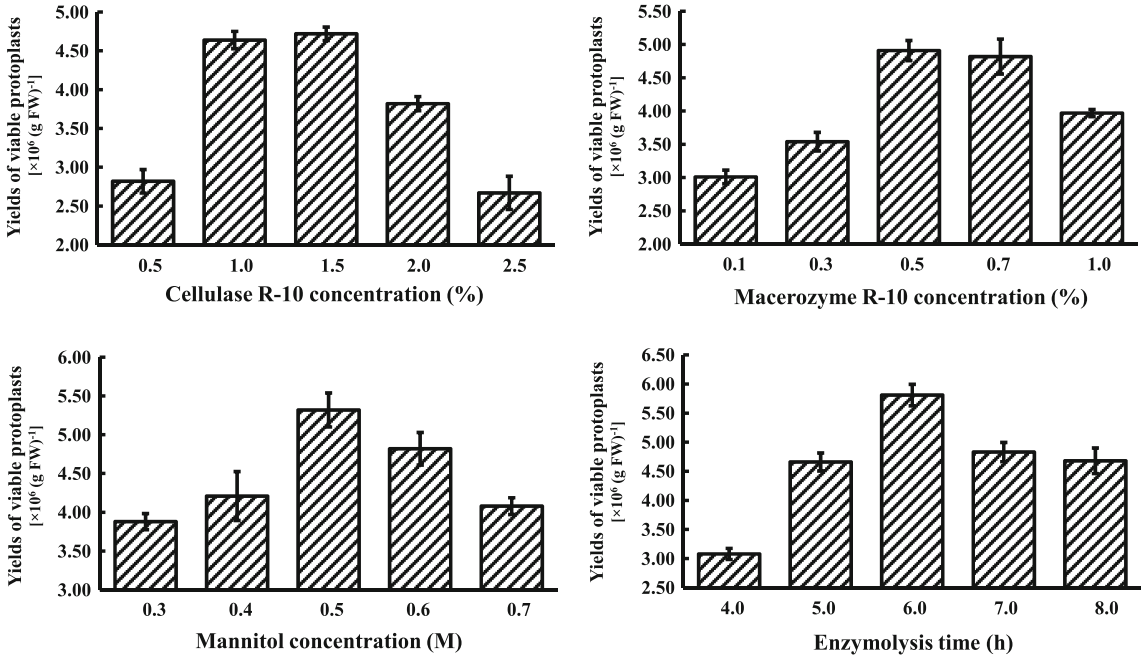

Figure 2. Effects of single factors on the yield of viable *Phalaenopsis* hybrid cultivar 'Ruili Beauty' protoplasts. The effects of *Cellulase R-10 (%)*, *Macerozyme R-10 (%)*, *D-mannitol (M) concentrations,* and *enzymolysis time (h),* on the *yield of viable* 'Ruili Beauty' *protoplasts*. *Bars* represent standard errors (SE).

B), and pretreated with 0.4–0.6 M D-mannitol (Solarbio, Beijing, China) for 1 h [1 g fresh weight (FW) leaf material was added to 10 mL of D-mannitol]. Then 1.0 g (10 mL)$^{-1}$ leaf material was transferred into an Erlenmeyer flask with mixed enzymes and osmotic regulator [1.0–2.0% (*w/v*) Cellulase Onozuka R-10(Yakult Honsha Co., Ltd., Tokyo, Japan), 0.3–0.7% (*w/v*) Macerozyme R-10 (Yakult Honsha Co.), and 0.4–0.6 M D-mannitol] at pH 5.6, and incubated at 25°C in darkness with rotation of 0.1 *g* for 5, 6, or 7 h.

After enzymolysis, the solution was filtered using a 200-μm mesh sieve, centrifuged (100*g*, 8 min) at room temperature (23 ± 1°C), then resuspended with 5 mL of cell and protoplast washing solution, CPW-9 M [CPW salts consisted of KH_2PO_4 27.2 mg L^{-1}, KNO_3 101 mg L^{-1}, $CaCl_2{\cdot}2H_2O$ 1480 mg L^{-1}, $MgSO_4{\cdot}7H_2O$ 246 mg L^{-1}, KI 0.16 mg L^{-1}, $CuSO_4{\cdot}5H_2O$ 0.025 mg L^{-1}; CPW-9 M was CPW salts with 9% (*w/v*) D-mannitol]. The centrifugation wash step was repeated twice, and the resultant protoplast pellet was resuspended in 1.0 mL of CPW-9 M, floated on the surface of a 15–25% (*w/v*) sucrose gradient, and centrifuged (100*g*, 5 min) at room temperature. The protoplast layer was collected and resuspended in 1.0 mL of CPW-9 M (Fig. 1*C*, *D*).

The collected protoplasts were diluted with CPW-9M (1:3, *v/v*), and yield was calculated using a hemocytometer to count

Table 1. Orthogonal L9 (3^4) array and results of *Phalaenopsis* hybrid cultivar 'Ruili Beauty' protoplast isolation

Treatment combination	Factors				Yield	Yield of viable protoplasts	Viability
	A	B	C	D	× 10^6 (g FW)$^{-1}$	× 10^6 (g FW)$^{-1}$	(%)
T1	*1* (1.0%)	*1* (0.3%)	*1* (0.4 M)	*1* (5 h)	4.14 ± 0.08	3.05 ± 0.06	73.80 ± 1.44
T2	*1* (1.0%)	*2* (0.5%)	*2* (0.5 M)	*2* (6 h)	5.73 ± 0.26	4.83 ± 0.22	84.38 ± 1.21
T3	*1* (1.0%)	*3* (0.7%)	*3* (0.6 M)	*3* (7 h)	4.80 ± 0.08	3.83 ± 0.06	79.76 ± 0.79
T4	*2* (1.5%)	*1* (0.3%)	*2* (0.5 M)	*3* (7 h)	4.33 ± 0.54	2.41 ± 0.30	55.73 ± 1.20
T5	*2* (1.5%)	*2* (0.5%)	*3* (0.6 M)	*1* (5 h)	2.76 ± 0.23	1.65 ± 0.14	59.65 ± 2.18
T6	*2* (1.5%)	*3* (0.7%)	*1* (0.4 M)	*2* (6 h)	4.52 ± 0.19	3.65 ± 0.15	80.78 ± 1.47
T7	*3* (2.0%)	*1* (0.3%)	*3* (0.6 M)	*2* (6 h)	3.58 ± 0.13	2.28 ± 0.08	63.61 ± 1.05
T8	*3* (2.0%)	*2* (0.5%)	*1* (0.4 M)	*3* (7 h)	4.51 ± 0.23	3.08 ± 0.16	68.25 ± 1.01
T9	*3* (2.0%)	*3* (0.7%)	*2* (0.5 M)	*1* (5 h)	3.44 ± 0.06	2.71 ± 0.05	78.79 ± 0.67

A: Cellulase Onozuka R-10 (*w/v*) concentration; *B*: Macerozyme R-10(*w/v*) concentration; *C*: D-mannitol concentration; *D*: enzymolysis time. *FW*: Fresh weight. *T1–T9*: different treatment combination; *1*, *2*, *3*: three levels of factors

Table 2. Range analysis of the orthogonal test

	A	B	C	D
k1	3.9056a	2.5818c	3.2607a	2.4701c
k2	2.5702b	3.1848b	3.3181a	3.5877a
k3	2.6890b	3.3982a	2.5860b	3.1070b
Range	1.3354	0.8164	0.7321	1.1176
Primary and secondary order of factors	ADBC			
Excellent level	A1	B3	C1; C2	D2
Optimal combination	A1B3C1D2; A1B3C2D2			

A: Cellulase Onozuka R-10 concentration; *B*: Macerozyme R-10 concentration; *C*: D-mannitol concentration; *D*: enzymolysis time. *k1*, *k2*, *k3* stand for the average value of viable protoplasts ($\times 10^6$ (g FW)$^{-1}$) at the*1*, *2*, *3 level*. *Numbers* in *columns* followed by different *lowercase letters* indicate significant differences. *1*, *2*, *3*: three levels of factors. The larger the *Range* value, the greater the influence of the factors on the test results

a sample under the microscope. The viability of the purified protoplasts was determined using the Fluorescein diacetate (FDA) (Sigma-Aldrich® Co., St. Louis, MO) staining method: FDA solution (5.0 mg of fluorescein diacetate dissolved in 1.0 mL of acetone) was added to the purified protoplasts (1:40, *v/v*), incubated for 10 min at room temperature before counting the green fluorescing cells under a fluorescence microscope (OLYMPUS BX53, Tokyo, Japan) (excitation per emission wavelengths 470–490 and 510–530 nm). The viability of the protoplasts was expressed as (viable protoplasts ÷ total number of protoplasts) × 100%.

Transformation and transient gene expression in 'Ruili Beauty' mesophyll protoplasts The modified PEG-mediated protoplast transfection protocol was based on a method described by Yoo *et al.* (2007). The 'Ruili Beauty' mesophyll protoplasts were resuspended in 1.0 mL of MaMg solution, consisting of 4.0 mM 2-(N-morpholino) ethanesulfonic acid (MES), 0.4 M D-mannitol, and 15 mM $MgCl_2$, at pH 5.6. The protoplasts were incubated on ice for 30 min, then centrifuged at 70*g* for 4 min at room temperature. The supernatant was discarded and 0.5–1.0 mL of MaMg solution was added to adjust the protoplast density to 5×10^5–2×10^6 protoplasts mL^{-1}.

A total of 0.2 mL of protoplast suspension was transferred to a 2.0-mL Eppendorf tube, then 5–25 μg plasmid DNA and an equal volume of 20–50% (*w/v*) PEG-4000 (Guangzhou Jinhuada Chemical Reagent Co., Guangzhou, China)were added in turn and incubated at room temperature in the dark for 10–60 min. To stop the transfection, a two-fold volume of W5 solution (154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl, and 2 mM MES) was added to the tube, centrifuged (70*g* for 2 min) at room temperature, and the supernatant discarded; the centrifugation process was repeated once. The protoplasts were finally resuspended in 0.1 mL of W5 solution and incubated at 23 ± 1°C for 16–24 h in the dark.

The expression of the green fluorescent protein gene (*GFP*) was observed using a laser confocal microscope (LSM780, Zeiss, Jena, Germany) (excitation 488 nm, emission 510–530 nm). The percent transformation efficiency was calculated as (fluorescent protoplasts ÷ total protoplasts) × 100%.

Unless otherwise stated, the tissue culture chemicals including plant growth regulators, sucrose, macronutrient, and micronutrient (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) were bought from Shanghai Jiafeng Horticultural Products Co., Ltd. (Shanghai, China). Other chemicals (Xilong Chemical Reagent Co., Ltd., Shantou, China) were bought from Nanjing Shoude Biotechnology Co., Ltd. (Nanjing, China).

Statistical analysis All variable adjustment experiments were repeated at least thrice; the range analysis of the orthogonal test was based on PASW Statistics 18 software (version 18.0, IBM Information Management, Armonk, NY).

Table 3. Result of the optimal protoplast yield verification test

Treatments	Yields of viable protoplasts × 10^6 (g FW)$^{-1}$			Average
	1	2	3	
Orthogonal T2	4.91	5.00	4.59	4.83 ± 0.22b
Theoretical optimal combination	6.00	5.75	6.08	5.94 ± 0.17a

FW: Fresh weight. Student's *t* test was used for statistical analyses and *numbers* followed by different *lowercase letters* indicate significant differences ($P < 0.05$). Numbers *1*, *2*, *3* represent repetitions of verification test

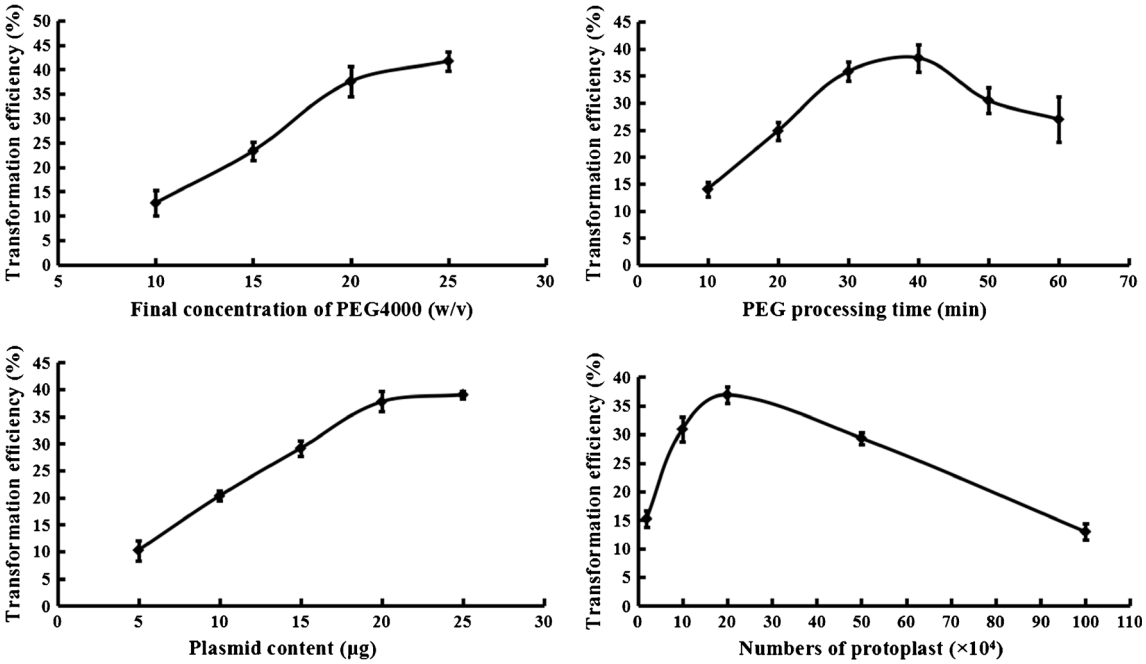

Figure 3. Effects of different factors on protoplast transformation efficiency. Effects of final polyethylene glycol (PEG)-4000 *(w/v)* concentration, PEG processing time (min), amount of plasmid (μg), and numbers of protoplasts (× 10^4) on protoplast transformation efficiency (%). *Bars* represent standard errors (SE).

Results and Discussion

In preliminary experiments, it was found that the growth stage of leaves and growth condition of the aseptic plants were critical for successful protoplast isolation. As indicated in Fig.1*A*, juvenile leaves of healthy 'Ruili Beauty' plants were the best starting material. The successful isolation of protoplasts depended on breaking down the cell wall and releasing the intact protoplasts. The isolated 'Ruili Beauty' protoplasts had a spherical or near-spherical shape with an average diameter of 39 μm (Fig. 1*E*, *F*). Maximum yield of intact protoplasts (viability confirmed by FDA staining) reached 5.94 × 10^6 (g FW)$^{-1}$ (Fig. 1*G*, *H*) after variables were amended.

Effects of single factors on 'Ruili Beauty' mesophyll protoplast isolation To optimize the protoplast isolation protocol, variables, such as enzyme concentration, mannitol concentration, and enzyme digestion duration, were adjusted (Fig. 2). In short, the highest yield of viable protoplasts occurred with 1.5% (*w/v*) Cellulase Onozuka R-10, 0.5% (*w/v*) Macerozyme R-10, 0.5 M D-mannitol, and 6 h of enzymolysis duration.

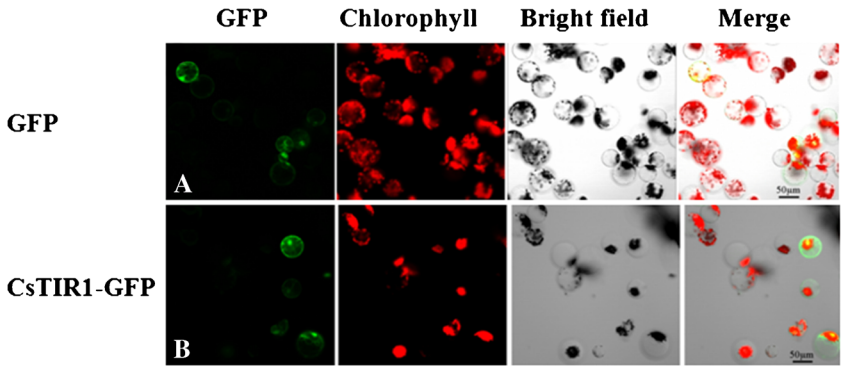

Figure 4. Transient gene expression in protoplasts derived from *Phalaenopsis* hybrid cultivar 'Ruili Beauty' mesophyll cells. (*A*) The expression of the green fluorescent protein (*GFP*) gene in 'Ruili Beauty' mesophyll protoplasts observed under fluorescence microscopy (excitation 488 nm, emission 500–530 nm). *Chlorophyll* fluoresces in *red* (emission 650–750 nm) when excited at 488 nm. *Bright-field* micrograph of the same view. *Merge* indicates computer-generated composite of fluorescent and bright-field images. (*B*) Transient expression of the *CsTIR1-GFP* gene in 'Ruili Beauty' mesophyll protoplasts, observed under both fluorescence and bright-field microscopy, as described above. *Bars*: 50 μm.

Refinement of *Phalaenopsis* mesophyll protoplast isolation variables using orthogonal test An L9 (3^4) orthogonal test was designed as shown in Table 1, to further optimize the abovementioned variables in protoplast isolation. A range analysis of the orthogonal test was also conducted to predicate the best combination of these variables (Table 2). From the range analysis, cellulase R-10 (R: 1.3354) and enzymolysis time (R: 1.1176) had crucial influence on the yield of viable protoplasts. By analyzing the *k* value *via* the Duncan test (Table 2), the optimal level of each factor was kA1, kB3, and kD2, respectively. As for C factors, kC1 and kC2 were significantly higher than kC3, while no significant difference existed between C1 and C2. Because C1 had a lower D-mannitol dosage compared to C2, A1B3C1D2 was deemed the best choice of isolation variables, while curtailing expenditures.

The best theoretical combination was further compared to the optimal combination of T2 (identified in Table 1) to verify the orthogonal test prediction. As shown in Table 3, the yield of 5.94×10^6 (g FW)$^{-1}$of viable mesophyll protoplasts in the theoretical optimal combination had minimal cell debris and was significantly higher than that of T2 [4.83×10^6 (g FW)$^{-1}$]. Therefore, the best system for the isolation of mesophyll-derived protoplasts was established as 1.0% (*w/v*) Cellulase Onozuka R-10, 0.7% (*w/v*) Macerozyme R-10, and 0.4 M D-mannitol, with an enzymolysis time of 6 h. Previously, the best yield of *Phalaenopsis* mesophyll protoplast was 3.8×10^6 (g FW)$^{-1}$, as reported by Shrestha *et al.* (2007). Thus, using the optimized protocol presented here, the yield of viable protoplast was about 57.9% higher than previously reported.

Establishment of a transient gene expression system in *Phalaenopsis* protoplasts To optimize the transient gene expression system in 'Ruili Beauty' mesophyll protoplasts, variables including concentration of PEG, density of protoplasts, amount of plasmid, and incubation time, were adjusted as shown in Fig. 3. In brief, the best transformation efficiency (~ 41.7%) was achieved by using 20% (*w/v*) PEG-4000 (Supplementary Table 1), 30 min of incubation duration (Supplementary Table 2), 20 μg plasmid DNA (Supplementary Table 3), and 2×10^5 mL^{-1} of protoplasts used for transformation (Supplementary Table 4). Using this protocol, a GFP-fusion cucumber TIR1 protein was successfully detected and localized in both the nucleus and cell membrane (Fig. 4), consistent with previously reported findings by Xu *et al.* (2017). This system could easily be applied to the subcellular localization of other proteins.

Conclusions

The variables for protoplast isolation and PEG-mediated gene transformation were optimized for 'Ruili Beauty' mesophyll cells. The improved protoplast isolation protocol reached an efficiency as high as 5.94×10^6 (g FW)$^{-1}$, representing a 57.9% increase over previously reported yields by Shrestha *et al.* (2007). Furthermore, the optimized PEG-mediated transfection protocol reached an efficiency of 41.7%, sufficient for subcellular localization studies or other investigations where a transient protoplast gene expression system would be desirable.

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