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Development of an efficient leaf protoplast isolation and transient expression system for *Artemisia japonica*

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Abstract

Artemisia japonica Thunb is an important perennial herb containing abundant chemical compounds utilized in conventional medicine for the treatment of malaria, hepatitis, hypertension, and inflammation. The protoplasts-based transient transformation system is a versatile and convenient tool for functional gene analysis in several *Artemisia* species. However, effective protoplast preparation and transformation systems are still lacking for *A. japonica*. We developed an efficient protoplast-based transformation system by optimizing conditions of protoplasts isolation and polyethylene glycol (PEG)-mediated transformation in *A. japonica*. The optimum conditions for the protoplast preparation were: the enzyme solution containing 1.75% (m/v) cellulase R10, 0.5% (m/v) macerozyme R-10, and 0.4 M D-mannitol with proper leaves treatment and pre-plasmolysis treatment. The maximum protoplast yield was 1.93×10^6 protoplasts g^{-1}(FM) and the viability of protoplasts was approximately 87.5% under optimized conditions using an orthogonal experiment. Furthermore, the transient protoplast transformation efficiency was 47.86% in *A. japonica* protoplast under the conditions of 40% (m/v) PEG 4000 for 20 min. The establishment of *A. japonica* protoplasts isolation and transient transformation system can accelerate the gene function studies of *A. japonica* and provide a fast and simple gene expression platform for molecular, biochemical, and functional gene characterization for other *Artemisia* species.

Keywords: *Artemisia japonica*, PEG-mediated transformation efficiency, protoplast isolation, transient gene expression.

Introduction

Protoplast isolation for transient gene transformation is widely used for protein subcellular localization (Yoo *et al.* 2007), promoter screening (Sultana *et al.* 2019), protein-protein interactions (Ehlert *et al.* 2010), signal transduction (Kanofsky *et al.* 2019), plant genome editing analyses, and high-throughput sequencing technology (Jiang *et al.* 2013). Comparison of gene expression in transgenic plants, transient gene expression provides an efficient and convenient system with rapid gene expression for molecular, biochemical, and genetic studies (Kim *et al.* 2009).

Recently, different transformation techniques (PEG-mediated, liposome-mediated, electroporation, and microinjection) have been developed for DNA plasmid transfer. For higher binding of DNA and cells, the PEG-mediated transformation method was regularly used to deliver constructs to protoplasts. Therefore, it is widely used in cellular and molecular studies in different kinds of plants (Wu *et al.* 2017). A large number of protoplast isolation methods have been reported for different kinds of plant species, such as rice (Toriyama and Hinata 1985), maize (Cao *et al.* 2014), *Cannabis sativa* (Beard *et al.* 2021), orchids (Ren *et al.* 2020), castor bean (Bai *et al.* 2020), and strawberry (Gou *et al.* 2020). But the protoplast isolation and transformation systems in other plant species were based on the transient expression system of *Arabidopsis* mesophyll protoplasts that has become a basic reference work (Yoo *et al.* 2007).

Artemisia, one of the largest genera of the *Asteraceae* family, comprises over 500 species and more than 200

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species were found in China (Bora and Sharma 2011). *Artemisia* plants have a wide range of bioactivity due to the existence of a variety of active components or secondary metabolites (terpenoids, phenylpropanoids, and aliphatic compounds), which play a role through diverse modes of action (Sharifi-Rad *et al.* 2017). *Artemisia* plants are a good platform to produce high-valued natural products, such as artemisinin, an antimalaria drug specially produced in *Artemisia annua*. Great efforts have been made on genes function in the biosynthesis and regulation of artemisinin in *A. annua via* an efficient gene transformation system, but less effort on the other *Artemisia* species. Although the genetic transformation was established in some *Artemisia* species, such as *A. annua* (Han *et al.* 2005), *Artemisia absinthium* (Mannan *et al.* 2009), *Artemisia dubia* (Kiani *et al.* 2016), *Artemisia carvifolia* (Dilshad *et al.* 2015), and *Artemisia vulgaris* (Sujatha *et al.* 2013), a transient gene expression technology is required for rapid screening of subcellular protein localization, gene function, and genome editing due to the limit of long period and low effectiveness of stable genetic transformation in *Artemisia* plants.

Artemisia japonica has a broad spectrum of pharmacological activities and has been administered for the treatment of fever, malaria, hepatitis, hypertension, eczema, and inflammation (Giang *et al.* 2014). In early studies, protoplast isolation for *Artemisia* species such as *Artemisia vulgaris*, *Artemisia judaica*, and *Artemisia sieversiana*, has been previously reported (Pan *et al.* 2003, Furuta *et al.* 2004, Sujatha *et al.* 2013). Nevertheless, an efficient system for protoplast isolation and transformation in *A. japonica* is not currently available. Considering the importance of gene function and genome editing, the development of a rapid and efficient protoplast-based transient gene expression system for *A. japonica* was the primary objective to study and produce natural compounds in *A. japonica*.

In this study, an efficient method for protoplast isolation and PEG-mediated transient transformation of *A. japonica* was developed and the GFP protein was successfully expressed.

Materials and methods

Materials and expression vector: *Artemisia japonica* Thumb plants were provided by the Naval Medical University and grown in the greenhouse with a natural irradiance, a 16-h photoperiod, and a temperature set to 25°C. 8 - 10 pairs of leaves grew after 3 - 4 weeks. The upper two to four layers of leaves were collected for protoplast isolation.

The plasmid pHB-GFP was used for transfection. This vector contained a GFP gene driven by CaMV 35S promoter and terminated by a NOS terminator.

Protoplast isolation was conducted according to the previous protocols with several modifications (Yoo *et al.* 2007). The fully expanded green leaves were cut into about 0.5 - 1.0-mm strips with sharp razors (leaves treatment 1; Fig. 1*A*). The lower epidermis of leaves was removed (leaves treatment 2; Fig. 1*B*). The lower epidermis was removed and cut into 0.5 - 1.0-mm strips (leaves treatment 3; Fig. 1*C*). The adhesive tape was used to remove the lower epidermis of leaves (Fig. 1*D-F*).

For pre-plasmolysis treatment, the samples were placed in 0.4 M D-mannitol solution for 60 min. Non-pre-plasmolysis treatment acted as a control. After pre-plasmolysis treatment, the samples were transferred into 10 cm^3 of enzyme solution (0.1% bovine serum albumin, 0.4 M D-mannitol, 20 mM KCl, 10 mM $CaCl_2$, 20 mM MES, pH 5.7) with different concentrations of cellulase R-10 (1.5 - 2%) (*Yakult*, Japan) and macerozyme R-10 (0.5 - 1%) (*Yakult*, Japan). Then, all the samples were vacuumed (0.1 MPa) for 30 min under the dark to improve the penetration of enzyme solution into leaves tissues. After released the air pressure, the enzymatic mixture was incubated for about 4 h on a rotary shaker at 40 rpm and 25°C in the dark for plasmolysis.

After digestion, the digested tissues were added to 10 cm^3 of pre-cooled M-W5 solution (154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl, 2.2 mM MES, pH 5.7), filtered through a 70-μm nylon sieve, centrifuged at 1 000 rpm for 10 min and suspended in 5 cm^3 M-W5 solution. The protoplast pellet was washed and purified once in M-W5 solution by repeated resuspension and centrifugation at 1 000 rpm for 3 min. The resulting protoplasts were resuspended in a 2 cm^3 M-W5 solution, and incubated on ice for 30 min. During the incubation period, protoplasts were counted under the microscope with a hemocytometer. After incubation, protoplasts were centrifuged and resuspended in MMG (0.6 M mannitol, 15 mM $MgCl_2$, 4 mM MES-KOH, pH 5.7) to adjust the protoplasts' density to 10^6 cells cm^{-3}.

Protoplast transformation: The polyethylene glycol (PEG) conversion solutions contained 40% (m/v) PEG

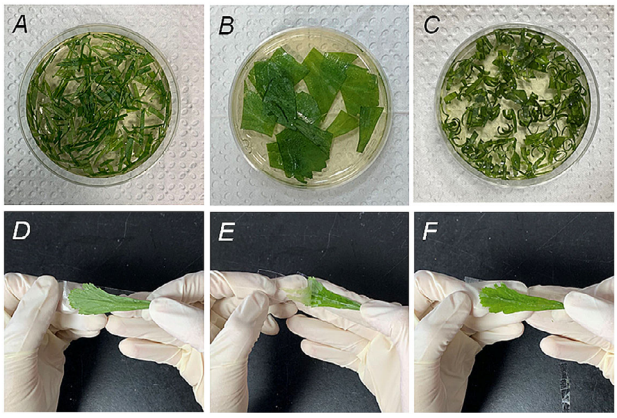

Fig. 1. Treatments of leaves before protoplasts isolation. *A* - *Artemisia japonica* leaves were cut into 0.5 - 1-mm strips. *B* - The lower epidermis of the leaves was removed. *C* - The lower epidermis of leaves was removed and cut into 0.5 - 1-mm strips. *D-F* - The process of removing the lower epidermis from the leaves.

4000 in ddH_2O in addition to 0.2 M D-mannitol and 100 mM $CaCl_2$. Then, for each sample, 10 mm^3 of plasmid DNA solution (10 µg of plasmid) was mixed with 200 mm^3 of protoplasts (approximately 2 × 10^5 cells) in 1.5 cm^3 centrifuge tubes. Subsequently, 220 mm^3 of PEG solution was added, and the tubes were then gently tapped to mix the solutions.

The plasmid, protoplasts, and PEG mixtures were incubated for 5 - 30 min in the dark at room temperature. After termination of transformation by 880 mm^3 of M-W5 solution added, the mixture was centrifuged at 700 rpm for 3 min and the supernatants were gently removed by the pipette. Then, protoplasts were gently resuspended in 1 cm^3 of WI (4 mM MES-KOH, pH 5.7, 0.5 M D-mannitol, 20 mM KCl) and homogenized gently. Finally, the protoplast suspension was transferred into 6-well cell culture plates and incubated in the dark at 25°C for 16 h before microscope observation. The protoplasts expressing GFP protein were observed and calculated with a confocal microscope. The quantity of fluorescent protoplasts/quantity of protoplasts ratio was calculated as transformation efficiency.

Statistical analysis: Statistical analysis was performed using *SPSS* software *version 20.0*. All data represented means ± SEM of three replicates. The significance of differences was determined at $P \leq 0.05$ based on the LSD test.

Results

The protocol described for *Arabidopsis* protoplasts isolation was used for protoplast isolation in *A. japonica* (Yoo *et al.* 2007). The results showed that a low yield of *A. japonica* protoplasts and a large number of cell fragments were generated (Fig. 2*A*). Therefore, leaves treatment, pre-plasmolysis treatment, enzymes concentration, and D-mannitol concentration were optimized to establish an *A. japonica* protoplast isolation method with high yield and viability (Fig. 2*B*,*C*).

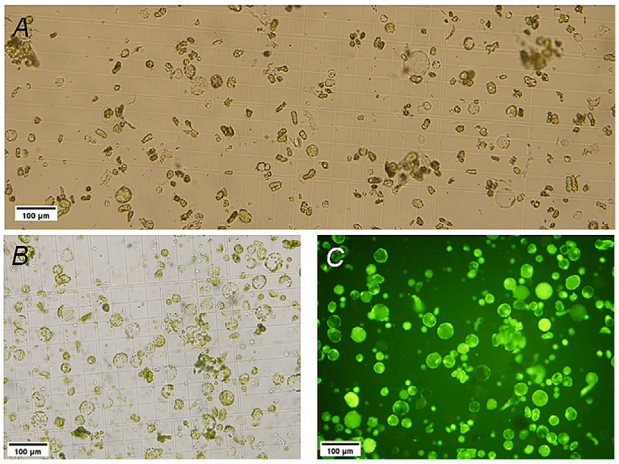

Fig. 2. The viability of protoplasts isolated from *A. japonica*. *A* - Protoplasts isolated from *A. japonica* leaves using the protocol described by Yoo *et al.* (2007) under bright light. *B* - Protoplasts isolated from *A. japonica* leaves using optimal protocol under bright light. *C* - FDA-dyed protoplasts under UV radiation. *Scale bars* = 100 µm.

The effect of leaf treatments during the early stage of enzyme culture was investigated. It was assumed that the lower epidermis of the leaves removed and cut into 0.5 - 1.0-mm strips (leaves treatment 3) would increase the yield of protoplasts by improving the surface area of enzyme solution action. The results showed that leaves treatment 3 had the highest yield [6.60 × 10^5 protoplasts g^{-1}(FM)] among the three leaves treatments (Fig. 3*A*). Although leaves treatment 2 contributed to the highest viability (77.78%), there is no significant difference in protoplast viability among the three leaves treatments.

Pre-plasmolysis treatment for *Solieria filiformis* and *Albizia julibrissin* leaves could improve protoplast yields and enhance the viability of isolated protoplasts (Pinchetti *et al.* 1993, Rahmani *et al.* 2016). In our study, the leaves with pre-plasmolysis for 60 min resulted in a higher yield [1.01 × 10^6 protoplasts g^{-1}(FM)] and higher viability of

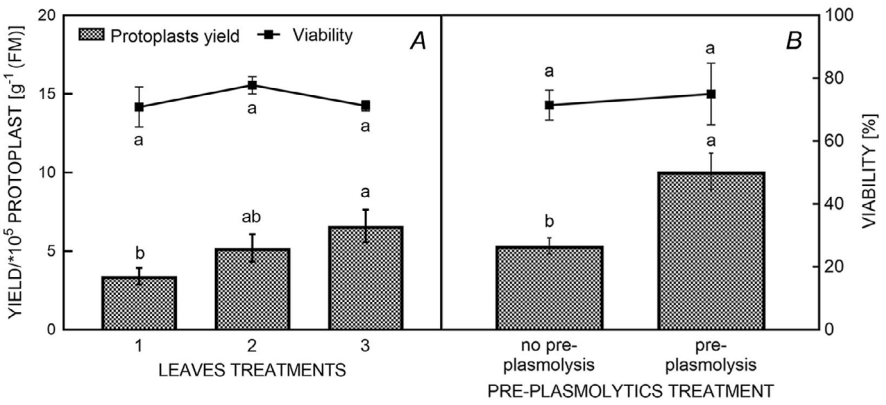

Fig. 3. Effects of leaves treatments (*A*) and pre-plasmolysis treatments (*B*) on protoplast isolation from *A. japonica* leaves. Means ± SEs, $n = 3$, *different lowercase letters* indicated statistically significant differences at $P = 0.05$, according to the LSD test.

protoplasts (74.98%) (Fig. 3*B*) compared to the leaves without pre-plasmolysis.

Selecting the proper combinations of different concentrations of cellulase R-10, macerozyme R-10, and D-mannitol in the enzyme solution (tested firstly in *A. japonica*) was found critical for the yield and viability of isolated *A. japonica* protoplast. The maximum protoplast yield and the highest viability were obtained with 1.75% cellulase R-10, reaching 1.04×10^6 protoplasts g^{-1}(FM) and viability 82.67% (Fig. 4*A*). However, there was no significant difference in protoplast viability between cellulase R-10 concentrations from 1.0 to 2.0%. When the concentration of macerozyme R-10 was at 0.75%, both the protoplast yield and percentage of viable protoplasts reached the peak of 8.93×10^5 protoplasts g^{-1}(FM) and 77.67%, respectively (Fig. 4*B*). Protoplast production and viability significantly decreased with the higher concentration of macerozyme R-10 ranging from 0.75 - 1.5%. To investigate the optimal concentrations of D-mannitol during enzymatic digestion, different concentrations of D-mannitol (0.3, 0.4, 0.5, 0.6, and 0.7 M) were added to the enzyme solution. The result showed that the 0.4 M D-mannitol exhibited a higher yield [1.27×10^6 protoplasts g^{-1}(FM)], significantly different from 0.6 - 0.7 M D-mannitol. The 0.4 M D-mannitol gave the highest viability (78.33%) but was only significantly different from 0.7 M D-mannitol (Fig. 4*C*).

Next, to optimize the enzyme mixture for *A. japonica* protoplast isolation, an orthogonal design with 9 treatments was used to find the optimal cellulase-R10, macerozyme R-10, and D-mannitol concentration as shown in Table 1. The maximum protoplast yield [1.93×10^6 protoplasts g^{-1}(FM)] and largest protoplasts viability (87.5%) among 9 treatments were 1.75% cellulase R-10, 0.5% macerozyme R-10, and 0.4 M D-mannitol in the enzyme solution the most suitable for protoplast isolation. Thus these concentrations were selected as satisfactory.

To improve the transient gene expression system of *A. japonica* protoplasts, PEG 4000 concentration and incubation time were optimized (Fig. 5). The results indicated that transformation efficiency first increased and then sharply declined along with the continuing increase of PEG 4000 concentration. The transformation efficiency of 44.09% reached the peak level by using 40% (m/v) PEG 4000 (Fig. 5*A*). The incubation time also affected the transformation efficiency. We found that transfection time at 20 min gave rise to maximum transformation efficiency (approximately 47.86%). However, it was observed that the incubation time of 30 min resulted in a non-significant decline in transformation efficiency (Fig. 5*B*). In brief, 40% PEG 4000 and 20 min of incubation were the optimized conditions for a high transformation efficiency of *A. japonica* protoplasts. Using this developed transformation system, the GFP showed clear signals in both the protoplast membrane and nucleus of *A. japonica* transformants (Fig. 6).

Discussion

Protoplast transient gene expression systems have become an efficient technique used for protein subcellular localization, promoter screening, protein-protein interactions, signal transduction, and promoter activity in many plants (Tan *et al.* 1987, Fischer and Hain 1995, Yoo *et al.* 2007, Ehlert *et al.* 2010, Pitzschke and Persak 2012, Kanofsky *et al.* 2019, Sultana *et al.* 2019). However, the protoplast transient gene expression systems have not been developed for *A. japonica*, since a general protoplast isolation protocol resulted in a poor yield and viability in *A. japonica*. Rare active protoplasts were observed within a large number of cell fragments of *A. japonica* (Fig. 2*A*). Therefore, an efficient protoplast isolation method was developed for transient gene expression in *A. japonica*.

Several factors affect protoplast preparation, including plant tissue choice, types and concentrations of enzymes, the osmolality of enzymolysis solution, and enzymolysis time (Davey *et al.* 2005, Yao *et al.* 2016). As the source of plant materials, young leaves are usually ideal materials for protoplast isolation. By preliminary experiments, the leaves of *A. japonica* from 4-week-old seedlings were selected as the experimental materials. In many studies, young plant leaves were cut into 0.5 - 1-mm filaments, to obtain a larger leaf surface for enzymatic reaction together

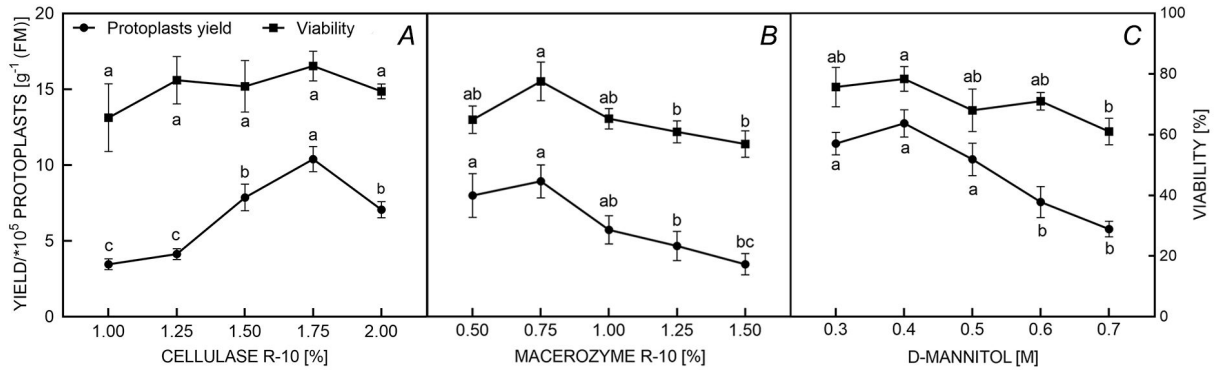

Fig. 4. Effects of cellulase R-10 concentration (*A*), macerozyme R-10 concentration (*B*), and D-mannitol concentration (*C*) on protoplast isolation from *A. japonica* leaves. Means ± SEs, $n = 3$, *different lowercase letters* indicate statistically significant differences at $P = 0.05$, according to the LSD test.

Table 1. Orthogonal test of three factors affecting the yield and viability of protoplasts isolated from *Artemisia japonic*a. k1, k2, k3, K1, K2, and K3 represent the mean values of evaluation index for each level of one factor. For example, when the factor is cellulase-R10 and the level is 1.5%, then k1 = (0.82 + 0.45 + 0.33)/3 = 0.53. Ry and Rv represent the range of protoplast yield (k1, k2, and k3) and protoplast viability (K1, K2, and K3), respectively. Rank represents order of importance.

Treatment number		Cellulase-R10 [%]	Macerozyme R-10 [%]	D-mannitol [M]	Protoplast yield [× 10^6 protoplasts g^{-1}(FM)]	Protoplast viability [%]
1		1.5	0.5	0.3	0.82	74.26
2		1.5	0.75	0.4	0.45	48.28
3		1.5	1	0.5	0.33	36.36
4		1.75	0.5	0.4	1.93	87.50
5		1.75	0.75	0.5	1.58	80.11
6		1.75	1	0.3	0.89	60.20
7		2	0.5	0.5	1.76	75.01
8		2	0.75	0.3	1.20	79.27
9		2	1	0.4	1.64	85.82
Protoplast yield	k1	0.53	1.50	0.97		
[× 10^6 protoplasts·	k2	1.47	1.08	1.34		
g^{-1}(FM)]	k3	1.53	0.95	1.22		
	Ry	1.00	0.55	0.37		
	Rank	cellulase-R10 > macerozyme R-10 > D-mannitol				
Protoplast viability	K1	52.97	78.92	71.24		
[%]	K2	75.94	69.22	73.87		
	K3	80.03	60.79	63.83		
	Rv	27.07	18.13	10.04		
	Rank	cellulase-R10 > macerozyme R-10 > D-mannitol				

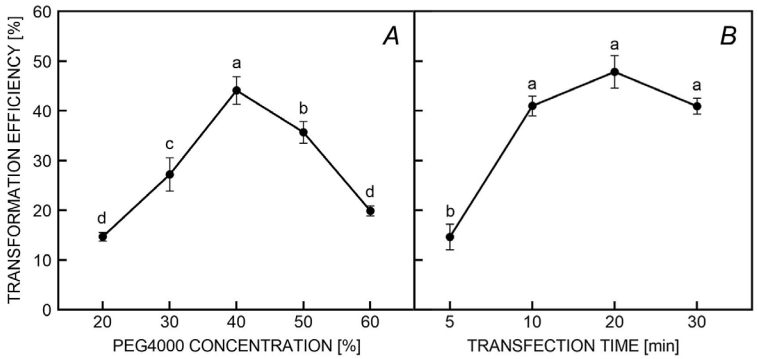

Fig. 5. Effects of PEG concentration (*A*) and transfection time (*B*) on *A. japonica* protoplast transformation efficiency. Means ± SEs, *n* = 3, *different lowercase letters* indicated statistically significant differences at *P* = 0.05, according to the LSD test.

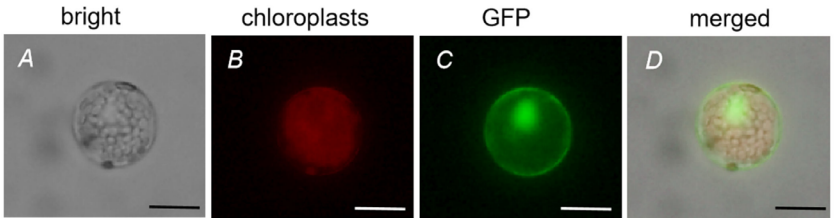

Fig. 6. Application of the optimal protoplast isolation on transient expression of GFP in protoplasts of *A. japonica*. *A* - bright field image, *B* - chloroplast autofluorescence image, *C* - GFP fluorescence image, *D* - merged image. *Scale bars* = 10 μm.

with more enzymes and a higher yield of protoplasts (Nanjareddy *et al.* 2016, Li *et al.* 2018, Liu *et al.* 2019, Ren *et al.* 2020). But the isolation of protoplast from *A. japonica* was challenging due to the wax cuticle layer on the leaf surface. Fortunately, the wax layer on young *A. japonica* leaves was soft and can be removed using adhesive tape. In this study, leaves treatment 3 was to remove the lower epidermis and cut into 0.5 - 1.0-mm strips, showing the highest yield of protoplasts [6.60×10^5 protoplasts g^{-1}(FM)] among three leaves treatments (Fig. 3*A*). Concerning the viability there was no significant difference among the three leaf treatments.

An early study showed that direct plasmolysis of cells in the enzyme solution led to decreased yields of protoplasts due to bursts of cells under osmotic imbalance. That can be avoided by pre-plasmolyzing the cells before enzyme digestion (Friedlander 2007). In this study, 60 min pre-plasmolysis with 0.4 M D-mannitol contributed to a higher yield of protoplasts [1.01×10^6 protoplasts g^{-1}(FM)], which was nearly twice as large as the protoplast yield of non-pre-plasmolysis treatment (Fig. 3*B*), but there was no significant difference in protoplast viability. Therefore, these results suggest that the leaves treatment 3 and pre-plasmolysis treatment were suitable treatments for further optimization experiments.

A suitable combination of enzymes is one of the most important factors for the process of protoplast isolation. This is a key step in mesophyll cell isolation and protoplast release (Sun *et al.* 2018). Macerozymes have multiple functions as pectinase, hemicellulase, and cellulase. Therefore, the combination of cellulases and macerozymes is the most frequently used to reduce cost (Yoo *et al.* 2007, Wang *et al.* 2015). Different plant species have different optimal enzyme concentrations. For instance, 1.0% cellulase R-10 and 0.1% macerozyme R-10 were optimal for hypocotyls of cauliflower protoplast yield and viability (Sheng *et al.* 2011), while 2.5% cellulase R-10 and 0.5% macerozyme R-10 were optimal for *Saccharum spontaneum* protoplast isolation (Wang *et al.* 2021). The optimal enzyme concentration for the isolation of oil palm protoplast was 3.0% cellulase R-10 and 1% macerozyme R-10 (Fizree *et al.* 2021). In this study, the highest *A. japonica* protoplast yield of 1.93×10^6 protoplasts g^{-1}(FM) and the highest protoplast viability of 87.5% were obtained by an orthogonal design (Table 1). From the results of the range analysis, the critical factors contributing to protoplast yield and viability were in the order of cellulase R-10, macerozyme R-10, and D-mannitol. Enzyme concentration remains the most dominant factor affecting the yield and viability of protoplast (Ma *et al.* 2020). Considering the yield and viability of protoplast, 1.75% cellulase R-10 and 0.5% macerozyme R-10 were the best combinations of enzymes, and their combination gave the highest yield of viable protoplasts.

To stabilize and maintain the osmotic potential of protoplasts, D-mannitol is commonly used as an osmotic stabilizer for protoplast isolation (Chen *et al.* 2015). We determined the most suitable D-mannitol concentration (0.4 M) for *A. japonica* protoplasts in this study (Fig. 4*C*), which is consistent with *Arabidopsis* protoplast isolation (Yoo *et al.* 2007).

Although transient gene expression systems with *Arabidopsis* protoplasts have been developed to study gene function, heterologous expression systems may result in difficulties (Zhang *et al.* 2011, Wu *et al.* 2017). Besides, *Artemisia* species contain a more complex secondary metabolism than *Arabidopsis*, which has the potential for enhancing artemisinin accumulation by genetic transformation (D'Auria and Gershenzon 2005, Tu 2016). Artemisinin is extracted from the stems and leaves of *Artemisia annua* (Christen and Veuthey 2001). The discovery of artemisinin has greatly changed the development of antimalarial drugs for many years. Early studies have reported that only *A. annua* contains a meaningful quantity of artemisinin (Tu 2016). Further research showed that transgenic *Artemisia carvifolia* increased 2.3- to 7-fold artemisinin content (Dilshad *et al.* 2015). Building a protoplast-based transient transformation system in *A. japonica* can contribute to a potential platform for artemisinin production. Therefore, we have established a PEG-mediated transformation system for the possible introduction of exogenous DNA into *A. japonica* protoplasts and assessing transformation efficiency by using GFP fluorescence. Transformation efficiency can be optimized by altering PEG concentrations and transformation time (Negrutiu *et al.* 1987, Armstrong *et al.* 1990, Yao *et al.* 2016). We found that 40% PEG 4000 was optimal for the transformation of protoplasts isolated from the *A. japonica* leaves (Fig. 5*A*). Higher PEG 4000 concentrations inhibited transformation and caused a significant decrease in protoplast viability. PEG incubation time also affects transformation efficiency. Different plant species have their optimal PEG incubation time. It was reported that a 15-min PEG 4000 treatment was optimal for a sweet cherry, Chinese kale, and strawberry protoplast transformation (Yao *et al.* 2016, Sun *et al.* 2018, Gou *et al.* 2020). In addition, the best transformation time was 30 min for castor bean protoplast transformation (Bai *et al.* 2020). Our results showed that 20-min PEG 4000 treatment gave the maximum transformation efficiency of protoplasts isolated from *A. japonica* leaves, although there was no significant difference in transformation efficiency when transformation time was 10 to 30 min (Fig. 5*B*). The clear GFP signals showed in both protoplasts membrane and nucleus of *A. japonica* indicated that the system successfully expresses functional genes. The results demonstrated that the protoplasts transient transformation system of *A. japonica* could be a fast and efficient platform to study subcellular protein localization, gene expression, and functional assays.

Conclusions

In conclusion, the efficient protoplast isolation in *A. japonica* was developed by optimizing leaves treatment, pre-plasmolysis treatment, the combination of enzyme solution, and additional procedures. The high-quality protoplasts for the transient transformation

system were obtained. This system is a suitable candidate for studying molecular mechanisms of artemisinin biosynthesis, including gene expression assays, the subcellular localization of proteins, and protein-protein interactions. The highest efficiency of protoplast transient transformation in *A. japonica* was 47.86%. The transient protoplast transformation system in *A. japonica* could be a useful and fast platform for transgenic, protein, and molecular studies of *Artemisia* species.

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