A novel technique for recombinant protein expression in duckweed (*Spirodela polyrhiza*) turions

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Abstract *Spirodela polyrhiza*, from the Lemnaceae family, are small aquatic plants that offer an alternative plant-based system for the expression of recombinant proteins. However, no turion transformation protocol has been established in this species. In this study, we exploited a pB7YWG2 vector harboring the *eYFP* gene that encodes enhanced yellow fluorescent protein (eYFP), which has been extensively used as a reporter and marker to visualize recombinant protein localization in plants. We adopted *Agrobacterium tumefaciens*-mediated turion transformation via vacuum infiltration to deliver the *eYFP* gene to turions, special vegetative forms produced by duckweeds to endure harsh conditions. Transgenic turions regenerated several duckweed fronds that exhibited yellow fluorescent emissions under a fluorescence microscope. Western blotting verified the expression of the eYFP protein. To the best of our knowledge, this is the first report of an efficient protocol for generating transgenic *S. polyrhiza* expressing eYFP via *Agrobacterium tumefaciens*-mediated turion transformation. The ability of turions to withstand harsh conditions increases the portability and versatility of transgenic duckweeds, favoring their use in the further development of therapeutic compounds in plants.

Keywords *Agrobacterium tumefaciens*-mediated turion transformation, Enhanced yellow fluorescent protein, Fluorescence microscopy, *Spirodela polyrhiza*, Turion, Vacuum infiltration

Introduction

Duckweeds, members of the family Lemnaceae, are small aquatic free-floating plants that are widely distributed on the surface of slow-flowing water (Tang et al. 2014). They are the smallest angiosperms and consist of 38 species in 5 genera (Appenroth et al. 2013; Wang 2016), namely, *Lemna*, *Landoltia*, *Spirodela*, *Wolffiella*, and *Wolffia*. Members of the genera *Spirodela*, *Landoltia*, and *Lemna* have only fronds and one to a few roots, whereas the genera *Wolffiella* and *Wolffia* have no roots (Landolt 1986). The leaf-like structures of duckweeds termed fronds are small (Wang et al. 2015), but the mature fronds of *Spirodela polyrhiza* (*S. polyrhiza*), the largest among the duckweed species, average 4- to 10-mm long (Oláh et al. 2008). The fronds of *S. polyrhiza* reproduce asexually through budding of offspring (daughter) fronds from the meristematic pocket to produce true turions (Gordon-Kamm et al. 1990; Lemon et al. 2000; Mejbél and Simon 2018).

The buds form true turions during winters in the dominant phase (Hillman 1961; Krajncić and Slekovec-Golob 1991; Krajncić and Devidé 1979; Wang and Messing 2012; Wang et al. 2014). These turions play a significant role in the survival of vegetative fronds by sinking to the bottom of the water and germinating new fronds under suitable conditions (Landolt 1986; Landolt and Kandler 1987).

Recently, *S. polyrhiza* has been utilized as an efficient plant expression system for the production of protein antigens in the area of biopharmaceutical plant research (Thu et al. 2015) as it is fast-growing, has a short life span (Wang et al. 2014; Lemon et al. 2001), and has no genetic variation (Yang et al. 2021). Therefore, efficient β -glucuronidase (GUS) gene transformation protocols have been developed for *S. polyrhiza* in callus using an *Agrobacterium tumefaciens*-mediated callus transformation method (Yang and Li et al. 2018). However, there have been no techniques published for the *Agrobacterium tumefaciens*-mediated trans-

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formation of turions of *S. polyrhiza*.

The gene coding for green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* has been successfully used as a marker for duckweed transformation (Cantó-Pastor et al. 2015; Vunsh et al. 2007).

GFP transgene expression was stable over multiple subcultures for plant species, as the GUS system is unsuitable for the rapid screening of primary transformants of living plants (Jefferson et al. 1987). Yellow fluorescent protein (YFP) is a variant of GFP fabricated by changing some amino acid residues, shifting the protein to produce a yellowish emission (Ormö et al. 1996; Tsien 1998). Enhanced yellow fluorescent protein (eYFP) is a modified YFP with an increased quantum yield. It is currently the most widely used version of fluorescent protein (Jusuk et al. 2015). Generally, eYFP gene and genes conferring resistance to antibiotics or herbicides are introduced to plant cells using *A. tumefaciens*-mediated transformation due to the simplicity, reliability, and effectiveness of this technique. Based on herbicides, resistant genes are commonly used to transform selected transgenic plants (Áy et al. 2021). Such as the bialaphos-resistance (*bar*) gene was used to construct transgenic plants resistant to herbicide glufosinate, which is the active ingredient of the commercial herbicide BASTA® (Dröge et al. 1992), and many researchers have heavily used it for genetic modification of various plant crops (Christou et al. 1991; Gordon-Kamm et al. 1990; Janakiraman et al. 2002; Tan et al. 2006; Vasil et al. 1992). The examples of *A. tumefaciens*-mediated transformation protocols have been reported for *S. polyrhiza* callus (Yang and Li et al. 2018) and fronds (Thu et al. 2015) to produce transgenic *S. polyrhiza* which is resistant to antibiotics. However, *A. tumefaciens*-mediated turion transformation in this species has been poorly characterized and transgenic *S. polyrhiza* resistant to the herbicide glufosinate has not been widely reported so far.

In our previously report, we optimized many factors important for the GFP transformation of *S. polyrhiza* turions, such as the preculture period, the turion induction conditions, the culture media, *Agrobacterium* density, the effect of ceftriaxone on *Agrobacterium* growth, acetosyringon concentration, and herbicide glufosinate concentration, to regenerate transgenic duckweeds (Jaiprasert 2008). The efficiency of GFP transformation of *Agrobacteria* was improved by up to 75% using vacuum infiltration, but the protein expression of *S. polyrhiza* turions was not confirmed. In this study, we aimed to create transgenic *S. polyrhiza* expressing recombinant eYFP through *A. tumefaciens*-mediated turion transformation resistant to herbicide glufosinate using 16 h light/8 h dark

and dark conditions during co-cultivation. Protein expression was determined using SDS-PAGE and western blot analysis. These techniques should be portable and capable of tolerating harsh conditions, paving the way for the development of mobile pharmaceutical plant factories in the near future.

Materials and Methods

Plant materials and cultivation of *S. polyrhiza*

S. polyrhiza were collected from natural reservoirs in Burapha University, Chonburi, Thailand. They were sterilized in 0.9% sodium hypochlorite (NaOCl) with 0.05% Tween 20 for 2 minutes and washed five times with sterile deionized water. After washing, 10 fronds of *S. polyrhiza* were cultured on solid Murashige–Skoog (MS) medium (PhytoTech, Lenexa, KS, USA) and incubated at $25^{\circ}\text{C} \pm 2^{\circ}\text{C}$, with a 16 h light/8 h dark photoperiod with $55 \mu\text{mol m}^{-2} \text{s}^{-1}$ from fluorescent light bulbs. After 14 days, *S. polyrhiza* was transferred to Hoagland's E medium and cultivated for 14 days (Jaiprasert 2008).

Turion induction

S. polyrhiza was cultured under starvation conditions to induce turion formation. Turions are morphologically different from fronds but can regenerate to form fronds when the required nutrients are replenished. In this experiment, 15–16 fronds of *S. polyrhiza* were left to starve in sterile deionized water under 16 h light/8 h dark photoperiods with an irradiation of $55 \mu\text{mol m}^{-2} \text{s}^{-1}$ from fluorescent light bulbs at $25^{\circ}\text{C} \pm 2^{\circ}\text{C}$. Turions started to form within 7 days of starvation and sunk to the bottom of the bottles within 45 days (Jaiprasert 2008).

Verification of plasmid DNA using PCR

eYFP was amplified using polymerase chain reaction (PCR)-specific primers. The eYFP forward primer was 5'-ACCA TGTGATCGCGCT-3', and the eYFP reverse primer was 5'-TGAACCGCATCGAGC-3'. PCR was performed in a MultiGene OptiMax thermal cycler (Labnet International, Inc., NJ, UK) as follows: the temperature was 94°C during denaturation for 2 min, 40 cycles of 94°C during denaturation for 30 s, 72°C during denaturation for 30 s, annealing temperature of 51°C for 30 s, and final extension time at 72°C for 7 min (Jaiprasert 2008). The PCR product was mixed with 6X DNA loading dye and separated on 1% agarose gels. Finally, the DNA was stained with SYBR™

Safe DNA Gel Stain (Invitrogen, Carlsbad, CA, USA) and visualized on a BLOOK LED transilluminator at 470 nm (GeneDireX, Inc., Taoyuan, Taiwan).

Agrobacterium strain and vector

The binary vector pB7YWG2 (Fig. 1) (Kirami et al. 2002) was used to transform *A. tumefaciens* strain GV3101 and to deliver the *eYFP* gene to *S. polyrhiza* for turion transformation. The expression vector pB7YWG2 consisted of the CaMV 35S promoter to drive the expression of the *eYFP* gene, a spectinomycin resistance (*Sp^R*) gene for *Escherichia coli* selection and a bialaphos-resistance (*bar*) gene for plant selection. A single colony of pB7YWG2 vector after manipulation in *E. coli* DH5 α was confirmed using an *eYFP* gene-specific primer to confirm the presence of the pB7YWG2 plasmid.

Agrobacterium transformation

Agrobacterium stock was grown on Luria-Bertani (LB) agar medium supplemented with 50 μ g/mL of gentamicin and incubated at 30°C for 2 days. The corrected plasmid pB7YWG2 was transferred into *A. tumefaciens* strain GV3101 via heat-shock transformation as described by Sambrook and colleagues (Sambrook et al. 1989), with slight modifications. Briefly, 1.0 μ g of plasmid pB7YWG2 was added to 50 μ L of *Agrobacterium*-competent cells, and the mixture was incubated at 42°C for 2 min and then placed on ice for 30 min. The transformants were spread on LB agar

supplemented with 50 μ g/mL of gentamycin and 200 μ g/mL of spectinomycin and then incubated at 30°C $\pm$ 2°C for 2 days. The white colonies of single transformed *Agrobacterium* carrying plasmid pB7YWG2 were then inoculated into LB liquid medium supplemented with the concentration of antibiotics indicated above. The transformants were incubated at 30°C $\pm$ 2°C overnight with shaking at 200 rpm, sub-cultured at 10% inoculum, and cultivated until the OD₆₀₀ reached 2.0 (Jaiprasert 2008).

Turion transformation

The cell pellet of *Agrobacterium* carrying plasmid pB7YWG2 was harvested via centrifugation at 5,000 rpm and resuspended in MS medium supplemented with 1% sucrose, 100 μ M acetosyringone (Sigma-Aldrich, St. Louis, MO, USA), and 0.2% Tween 80. The cell density was adjusted to 0.5 at an optical density of 600 nm (Jaiprasert 2008). The turions were immersed in *Agrobacterium* suspension and vacuum-infiltrated at -60 mmHg for 10 min (Yang et al. 2018; Yang and Li et al. 2018) before being incubated at room temperature for 30 min. The infiltrated turions were blotted dry on sterile filter paper and co-cultivated on filter paper discs soaked with 10-mL MS liquid medium supplemented with 1% sucrose and 100- μ M acetosyringone (Sigma-Aldrich) (Jaiprasert 2008) and then incubated under either 16 h light/ 8 h dark or dark conditions for 3 days (Chhabra et al. 2011; Thu et al. 2015; Yang et al. 2018). The co-cultivated turions were gently washed four to five times with sterile deionized water containing 750 mg/L

Fig. 1 The pB7YWG2 mapping we used for turion transformation in *S. polyrhiza*

ceftriaxone to remove excess *Agrobacterium* and dried on sterile filter paper. Ten transformed turions were then transferred to selective MS agar media; MS2 (MS, 1% sucrose, 100- μ M acetosyringone (Sigma-Aldrich), 250-mg/L ceftriaxone, 0.8% agar, pH 5.8) or MS3 (MS, 1% sucrose, 100- μ M acetosyringone (Sigma-Aldrich), 250-mg/L ceftriaxone, 0.01-mM glufosinate, 0.8% agar, pH 5.8). The negative controls of wild-type turions were cultured on MS1 agar medium (MS, 1% sucrose, 0.8% agar, pH 5.8) (Jaiprasert 2008). All turions were cultured under a 16 h light/8 h photoperiod with an irradiation of 55 μ mol m⁻² s⁻¹ from fluorescent light bulbs at 25°C $\pm$ 2°C for 14 days.

eYFP detection under fluorescence microscope

Transgenic *S. polyrhiza* freshly regenerated from transformed turions were mounted on slides filled with deionized water. Yellow fluorescence and chlorophyll autofluorescence were detected using an excitation filter BP450-480 (blue) and a barrier filter BA515 (green and red) under a fluorescence microscope (Olympus BX51, Shinjuku City, Tokyo, Japan). Chlorophyll autofluorescence was detected using an excitation filter BP510-550 (green) and a barrier filter BA590 (red) for control purposes. Images were captured using an Olympus DP22 digital camera.

Protein extraction and western blotting analysis

Regenerated fronds of 100-mg transgenic *S. polyrhiza* were ground in 200- μ L lysis buffer containing 50-mM Tris, 300-mM NaCl, and 2% glycerol at pH 8.0 for 30 min *via* homogenization on ice. The supernatant was collected after centrifugation at 15,000 rpm at 4°C for 20 min and kept at -20°C. This supernatant of the protein extraction was used for western blotting. Proteins were separated on 12% SDS-PAGE gels and transferred to PVDF membranes (Bio-Rad Hercules, CA, USA). After blocking with 5% non-fat dried milk, the blots were incubated with rabbit anti-green fluorescent protein (GFP) antibody in 1:1000 dilution (Cell Signaling Technology, Danvers, MA, USA), followed by incubation with goat anti-rabbit immunoglobulin G coupled to horseradish peroxidase in 1:1000 dilution (Cell Signaling Technology). Immunoreactive signals were detected *via* chemiluminescence (Santa Cruz Biotechnology, Dallas, TX, USA) according to the manufacturer's protocols.

Statistical analysis

The transformation efficiency was calculated as the number of regenerated fronds of transgenic *S. polyrhiza* on selective

media/number of all turions inoculated with *Agrobacterium* $\times$ 100 (%). The transformation efficiency data were evaluated using one-way ANOVA, followed by a Tukey's multiple comparisons test. A *P* value of <0.05 was considered to indicate statistical significance. All visualization and statistical analyses were performed using GraphPad Prism software version 5.0 for Windows (GraphPad Software, San Diego, California, USA).

Results

S. polyrhiza cultivation and turion induction

To cultivate duckweeds axenically in the laboratory, the young fronds of *S. polyrhiza* were submerged in 0.90% NaClO for 2 min (Fig. 2A). NaClO solution was utilized as a sterilizing agent for the surface sterilization of *S. polyrhiza*. Turions are special organs of some species of duckweeds developed for survival under a stressful condition. To induce turion formation, fronds of *S. polyrhiza* were subjected to starvation in sterile deionized water for 45 days. After 7 days, the fronds began to undergo chlorosis, developing a yellow color, with reddish pigments accumulating on the ventral (abaxial) side. After 45 days, turions were observed as dark-green colored organs attached to the mother fronds (Fig. 2B). Subsequently, the turions sunk to the bottom of the vessels (Fig. 2C).

Fig. 2 *S. polyrhiza* culture and turion induction. (A) *S. polyrhiza* was cultured axenically for 14 days on MS medium. (B) Turions started to appear 7 days after induction via starvation and (C) completely sunk to the bottom of the vessel 45 days after induction. They were grown in 25 $\pm$ 2°C with a 16 h light/8 h dark photoperiod under 55 μ mol m⁻² s⁻¹ fluorescent light bulbs

PCR amplification of plasmid pB7WGY harboring *eYFP*

The integration of target genes into the plasmid vectors was confirmed *via* PCR screening. The PCR products obtained from the amplification of plasmid pB7WGY with gene-specific primers (eYFP-F and eYFP-R) showed positive bands of 298 base pairs (Fig. 3, lanes 4, 5 and 6). The plasmid pB7WGY vector harboring *eYFP* could then be used for recombinant protein expression, eYFP, through *A. tumefaciens*-mediated turion transformation.

Transgenic turion and frond regeneration of *S. polyrhiza*

The frond regeneration of transgenic *S. polyrhiza* carrying the *eYFP* gene was tested on three media: MS1 for wild-type *S. polyrhiza* cultivation; MS2 with 250 mg/L ceftriaxone for *Agrobacterium* growth inhibition; and MS3 with 250 mg/L ceftriaxone and 0.01 mM glufosinate for transgenic *S. polyrhiza* selection. The proportions of transformants were 90% and 50%, respectively, on the two types of media, and were significantly different between the selective media. Different co-culture conditions 16 h light/8 h dark conditions, or dark co-cultivation of turions with *Agrobacterium* did not produce significant differences within the same selective medium (Table 1). The MS2 selective medium without glufosinate produced better frond regeneration under the 16 h light/8 h dark conditions than MS3.

Fig. 3 Agarose gel electrophoresis of plasmid vectors and PCR products. Lanes 1 and 2, pB7WGY plasmids; lanes 3 and 6, 1.5 kb ladder; lanes 4–6, PCR amplification of *eYFP* gene-specific primers (eYFP-F and eYFP-R) showing the size of 298 base pairs, respectively

eYFP detection

The eYFP expression in transgenic duckweeds *S. polyrhiza* was detected after the regeneration of turions to fronds on selective media (MS2 and MS3), as demonstrated *via* the fluorescence of eYFP under the fluorescence microscope, compared with those from wild-type *S. polyrhiza* (Fig. 4A) and transgenic fronds of *S. polyrhiza* (positive control) (Fig. 4B). The autofluorescence of chlorophyll and fluorescence of eYFP were distinguished by the fluorescent colors, as the autofluorescence of chlorophyll was red when excited by green light (Fig. 4A–F). In addition, the fluorescence of eYFP was yellow when excited with green light, mixed with autofluorescence of chlorophyll (Fig. 4B–F). The results of frond regeneration on the selective media, MS2 and MS3, were clearly distinguishable from those on wild type (Fig. 4A–F). Transgenic *S. polyrhiza* were successfully transformed by employing the agroinfiltration method using turions of *S. polyrhiza* and observing yellow fluorescence. Co-cultivation with *Agrobacterium* was successful in transforming the eYFP protein in turions under 16 h light/8 h dark conditions.

Fig. 4 eYFP fluorescence under the microscope (score bar = 500 μ m) of frond regeneration on three types of MS media (MS1, MS2, and MS3) after cultivation for 14 days. (A) Wild type on MS medium. (B) Transgenic *S. polyrhiza* as a positive control. (C–D) Transgenic *S. polyrhiza* on MS2 and MS3 co-cultivated with *A. tumefaciens* under 16 h light/8 h dark conditions. (E–F) Transgenic *S. polyrhiza* on MS2 and MS3 co-cultivated with *A. tumefaciens* under dark conditions, respectively

Table 1 The effect of selective media on frond regeneration and the transformation efficiency of *S. polyrhiza* turions. Co-cultivation represents

Selective media	Co-cultivation	Total number of turions	Number of transformed cells	Transformation efficiency (%)
MS2	16 h light/8 h dark	10	10	90.00±8.16 ^{A, #, ##}
		10	8	
		10	9	
	Dark	10	8	83.33±4.71 ^{*, **}
		10	8	
		10	9	
MS3	16 h light/8 h dark	10	6	50.00±8.16 ^A
		10	5	
		10	4	
	Dark	10	5	30.00±16.33
		10	3	
		10	1	

Each cultivation group consisted of 10 turions ($n = 10$). Percentage transformation efficiency is expressed as mean $\pm$ standard deviation (SD) of three replicates. Uppercase letters (A) indicate no significant difference between the 16 h light/8 h dark and dark co-cultivation conditions on the same selective medium. The symbol # indicates significant differences on the selective MS2 medium cultivated under 16 h light/8 h dark (# $p < 0.05$; ## $p < 0.01$) compared with selective MS3 medium cultivated under 16 h light/8 h dark and dark co-cultivation conditions, respectively. The symbol * indicates significant differences on the selective MS2 medium cultivated under dark (* $p < 0.05$; ** $p < 0.01$) compared with selective MS3 medium cultivated under 16 h light/8 h dark and dark co-cultivation conditions, respectively.

SDS-PAGE and western blot analysis

The expression of the eYFP protein in transgenic *S. polyrhiza* was confirmed *via* western blot analysis using a monoclonal anti-rabbit GFP primary antibody. The SDS-PAGE profiles were generated for the total soluble proteins extracted from whole *S. polyrhiza* plants, including wild type and transformants. All protein samples were stained with Coomassie Brilliant Blue, and examples of western blot are presented in Fig. 5A and B. Non-specific bands were detected in the Coomassie Brilliant Blue-stained gel (Fig. 5A), and western blotting with a monoclonal anti-rabbit GFP primary antibody revealed an additional band at a molecular weight of about 28 kDa when compared with the positive control transgenic fronds (Fig. 5B). The effects of the co-cultivation of turions with *Agrobacterium* under 16 h light/8 h dark conditions and only dark conditions were compared. Co-cultivation under 16 h light/8 h dark conditions produced good eYFP protein expression on the selective MS2 medium and a pale band on selective MS3 medium during co-cultivation with *Agrobacterium* (Fig. 5B, lane 4 and 5), as well as good eYFP protein expression on the selective MS2 medium under dark conditions (Fig. 5B, lane 6) when compared with the positive control of transgenic duckweed. In contrast, no eYFP protein expression was observed on selective MS3 medium under dark conditions (Fig. 5B, lane 7), and no eYFP protein was detected in the wild type. This result indicated that the eYFP protein was successfully expressed

and produced in the transgenic turions of *S. polyrhiza* *via* *A. tumefaciens*-mediated turion transformation under co-cultivation under 16 h light/8 h dark conditions to a greater extent than under dark conditions.

Fig. 5 SDS-PAGE and western blot analysis of the eYFP protein expression in turions. (A) SDS-PAGE. (B) Western blot: lane 1, prestaining protein marker; lane 2, wild type; lane 3, transgenic duckweed as a positive control; lanes 4-5, transgenic duckweed on MS2 and MS3 co-cultivated with *A. tumefaciens* under 16 h light/8 h dark conditions; and lanes 6-7, transgenic duckweed on MS2 and MS3 co-cultivated with *A. tumefaciens* under dark conditions, respectively

Discussion

We developed eYFP expressed in transgenic turions of *S. polyrhiza* to facilitate gene transformation. Some methods of transformation of *Spirodela* plants have been developed and refined to increase the transformation efficacy and stability of the expression of target genes (Vunsh et al. 2007). *A. tumefaciens*-mediated transformation is a highly effective method for gene transformation and protein production in duckweed plants (Firsov et al. 2015; Ko et al. 2011; Vunsh et al. 2007).

In this study, we report the expression of the eYFP protein in *S. polyrhiza* using *A. tumefaciens*-mediated turion transformation. The vector contains the *eYFP* gene, which encodes the eYFP protein, and has been extensively used as a reporter marker to visualize the gene expression in plants under the *CaMV35S* promoter with the *bar* gene as a selection marker to confer glufosinate resistance. Rapid transgenic-positive visual screening in the frond regeneration of turions in *S. polyrhiza* using this method provides a convenient method for visual screening under fluorescence microscope, and the protein expression was determined *via* western blotting. Different levels of red and yellow autofluorescence were observed in frond regeneration. The fronds regenerating from turions after 14 days demonstrated red autofluorescence due to the presence of chlorophyll, together with the part of the fronds emitting yellow autofluorescence from the transformants. The regenerated wild-type fronds were devoid of fluorescence. Western blotting confirmed that the target gene was actually expressed in the duckweed. The results confirmed the efficacy of the co-cultivation system used in this study. The eYFP protein exhibited good expression on the selective MS2 medium and produced a pale band on the MS3 medium under 16 h light/8 h dark conditions but was not detected in wild-type plants. The performance of the co-cultivation of turions with *Agrobacterium* was compared under two different conditions: under a 16 h light/8 h dark cycle and only in the dark. Light strongly promotes gene transfer from *A. tumefaciens* to plant cells (Zambre et al. 2003). However, *Agrobacterium* co-cultivation with duckweed plants resulted in a successful gene transformation under both conditions (Boehm et al. 2001; Vunsh et al. 2007; Yamamoto et al. 2001; Yang and Li et al. 2018; Yang et al. 2018).

In this study we confirmed that co-cultivation under 16 h light/8 h dark conditions produced good eYFP expression, similar to that of positive control transgenic fronds, was detected using western blotting, and the efficiency of transformation was 90%. The *bar* gene, which confers glufosinate resistance, is a selectable marker gene for generating

transgenic dicotyledonous and monocotyledonous plants (Budhagatapalli et al. 2016; Penna et al. 2002; Zhang et al. 2018). Preliminary optimization studies of eGFP expression in turions were reported in our group, using a selective MS medium consisting of 0.01 mM glufosinate, which efficiently screened out transformants with frond regeneration. The transformation frequency was detected as the frequency of *bar* and *eGFP*, and was up to 75% (Jaiprasert 2018). However, the eYFP protein was not highly expressed on the selective medium that consisted of 0.01-mM glufosinate. These results indicated that the concentration of glufosinate should be lower than 0.01 mM to regenerate fronds on selective medium. Otherwise, the presence of herbicide at high levels in the plant tissue culture would terminate wild-type cells and inhibit the growth of the transformants, leading to delays in plant regeneration (Boehm et al. 2001). The accumulation of herbicides in plant tissues exerted toxic and necrotic effects on plant cells by decreasing their growth and yield (Sikorski et al. 2019). High herbicide resistance might lead to the rapid natural development of plant populations (Rainbolt et al. 2004). Further studies are needed to investigate the reduction of herbicide concentration and stability of gene transformation in turions. Codon-optimized gene constructs could also be utilized to express proteins in the turions of *S. polyrhiza* for high protein expression levels.

Conclusions

We established a simple, novel transformation protocol for *Spirodela polyrhiza* via *Agrobacterium tumefaciens*-mediated turion transformation using the vacuum infiltration method. This system will provide a foundation for the genetic transformation of turions under co-cultivation with *Agrobacterium tumefaciens* in the 16 h light /8 h dark conditions. These findings will pave the way for the use of duckweeds as tools in biotechnology and for therapeutic applications.

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