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Protocorm wounding enhanced *Agrobacterium*-mediated transformation of *Hygrochilus parishii* (Veitch & Rchb.f.) Pfitz

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Abstract. This study was aimed to encourage the use of protocorm wounding as a substitute of acetosyringone in an attempt to reduce cocultivation time and to increase possibility of successful genetic transformation of *Hygrochilus parishii* (Veitch & Rchb.f.) Pfitz. *Agrobacterium tumefaciens* strain LBA4404 harboring the plasmid *p*BI121 was employed as a transgene carrier. The plasmid *p*BI121 contained *β*-glucuronidase (*gus*) and kanamycin resistance (*npt*II) genes, both of which were driven by the CaMV35S promoter. It was found that wounded protocorms cocultivated with *A. tumefaciens* for 30 min and acetosyringone-treated protocorms cocultivated for 30 min exhibited high gene expressions of 75 and 80%, respectively, while normal protocorms (control) showed gene expression of 60% when cocultivated with *A. tumefaciens* for 45 min. The presence of CaMV35S promoter and NOS terminator detected by PCR technique confirmed a successful T-DNA integration. This suggested that protocorm wounding could be used as a substitute of acetosyringone in enhancement of genetic modification.
Key Words: Transgene, protocorm wounding, acetosyringone, *Agrobacterium tumefaciens*, *Hygrochilus parishii*.

Introduction. The species *Hygrochilus parishii* (Veitch & Rchb.f.) Pfitz. is a wild orchid native to North and Northeast Thailand. It has been utilized as ornamental and cut flowers traded within the country and some foreign countries. Generally, commercial orchid producers employ classical breeding methods through sexual hybridization and selection to produce numerous young plants and to create desirable or novel traits. The classical breeding methods are time-consuming and unable to produce disease- and pest-free orchids (Pimda & Bunnag 2010). For this reason, large-scale micropropagation and genetic engineering technologies are an alternative means required in orchid industry. *Agrobacterium*-mediated transformation is routinely used nowadays to transfer desirable genes into plant genome as it may have good efficiency and may result in low copy number of intact and non-rearranged transgenes. Besides, transgenes are frequently integrated into plant genome when this approach is employed (Stanton 1998). After several factors have been unveiled to influence a successful *Agrobacterium-mediated* transformation, many efficient approaches were developed for agronomically important cereal crops (e.g. rice, wheat, maize, barley and sorghum) (Cheng et al 2004). The use of phenolic compounds (e.g. acetosyringone) (Stanton 1998), a plasmolysis medium (Uzè et al 1997), and use of calcium-reduced medium (Subramaniam et al 2009) or calcium-free medium (Sangwan et al 1992) are among the most common approaches. Temperature has also been reported to have a profound effect on *Agrobacterium*-mediated transformation. Gene expression in calluses of *Phaseolus acutifolius* was detectable when cocultivation was performed at 27°C whereas the gene expression was undetectable when implemented at 29 °C; besides, the gene expression decreased when subjected to the temperature of 22 °C (Dillen et al 1997). Phenolic compounds (e.g. acetosyringone and α-hydroxy acetosyringone) widely used to enhance gene transformation are released from wounded tissues. These compounds activate the signal

molecules for the genes in the *vir* region of Ti plasmid, thus enhancing the frequency of T-DNA integration (Stachel et al., 1985; Wang et al 2007).

In this study, we demonstrated high effectiveness of protocorm wounding tantamount to application of acetosyringone in enhancement of *Agrobacterium*-mediated transformation of *H. parishii*.

Material and Method. Seed-derived protocorms reaching a diameter of 2-3 mm were used as explants in this study. Mature pods of *H. parishii*, collected from the agricultural garden of the Department of Horticulture, Faculty of Agriculture, Khon Kaen University, Thailand, were gently washed with detergent and subsequently rinsed with running tap water for 1 h before surface sterilization. They were then dipped into 70% (v/v) ethanol for 5 min, subsequently soaked in a disinfectant, 25% (v/v) sodium hypochlorite, with a continuous shake for approximately 20 min, and then washed for 3 times with sterile distilled water. Sterilization was ensured by dipping them into 70% ethanol and immediately flaming for 2-3 times. After that, they were longitudinally dissected and seeds were picked off and transferred onto solid new Dogashima (ND) medium. The cultures were kept at 25 ± 2 °C under a long photoperiod (16 h light: 8 h dark) with a photon dose of 40 µmol $m^{-2}s^{-1}$. The experiment was repeated 5 times. A comparison study on the effects of protocorm wounding and acetosyringone on level of *gus* gene expression was implemented following the steps of selecting suitable doses of selective agents and bactericide, determining optimal cocultivation time, evaluating *gus* gene expression by histochemical GUS assay, and confirming transgene integration into plant genome by PCR technique as follows.

Determination of antibiotic doses

The protocorms were cultured on solid ND medium supplemented with cefotaxime (0-350 mg/l) or kanamycin (0-20 mg/l). The cultures were kept at 25 ± 2 °C under a long photoperiod (16 h light: 8 h dark) with a photon dose of 40 µmol $m^{-2}s^{-1}$. After 4 weeks of culture, the number of viable protocorm clumps was counted. Each treatment was repeated 5 times.

Preparation of bacteria and determination of cocultivation time

The bacterium *A. tumefaciens* strain LBA4404 (pCAMBIA 1305.1) was cultured overnight at 25 ± 2 °C in liquid Luria Broth medium supplemented with 100 mg/l kanamycin until reaching OD_{600}=1.0. In an attempt to determine the suitable cocultivation time for each tissue type; 3 types of protocorms which were normal protocorms, wounded protocorms gently pierced with a sterile fine needle and normal protocorms soaked in 0.1 M acetosyringone for 30 min were cocultivated with *A. tumefaciens* for 0-60 min. They were then blotted on sterile tissue papers and subsequently transferred onto solid ND medium. The cultures were kept under a 16-hour photoperiod (a light intensity of 40 µmole $m^{-2}s^{-1}$) at 25 ±2 °C for 3 d. Thereafter, they were washed with 250 mg/l cefotaxime for 5 min followed by washing 3 times with sterile distilled water prior to transfer onto solid ND medium supplemented with 250 mg/l cefotaxime and 15 mg/l kanamycin. Then, *gus* gene expression was evaluated in order to determine the most suitable approach for genetic modification in this study regarding low-cost payment, less time-consuming and stable gene integration.

Genetic transformation of the orchid

H. parishii protocorms pierced with a sterile fine needle were cocultivated with *A. tumefaciens* for 45 min. They were then blotted on sterile tissue papers and subsequently transferred onto solid ND medium. The cultures were kept under a 16-hour photoperiod (a light intensity of 40 µmole $m^{-2}s^{-1}$) at 25 ±2 °C for 3 d. After that, they were washed with 250 mg/l cefotaxime for 5 min followed by washing 3 times with sterile distilled water prior to transfer onto solid ND medium supplemented with 250 mg/l cefotaxime and 15 mg/l kanamycin. The cultures were maintained for 4 weeks in order to eliminate *A. tumefaciens* together with selection of putative transformants. Then, the putative transformants were assessed for *gus* gene expression and gene integration.

Histochemical GUS assay

Histochemical staining for GUS activity was performed in accordance with the method of Jefferson (1987). Ten transformed protocorms were transferred into a micro

tube containing 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-gluc) and incubated overnight at 37 °C. Positive histochemical staining of GUS activity would result in blue coloration in the transformed tissues.

Detection of gene integration by Polymerase Chain Reaction (PCR) technique

Total genomic DNA was extracted from the transformants and control by CTAB method (Doyle & Doyle 1987). The primer sequences for PCR were as follows: 35S forward sequence (F)5'- GCTCCTACAAATGCCATCA-3', reverse sequence (R)5'-GATAGTG GGATTGTGCGTCA-3' to yield a 195 bp fragment; NOS (F)5'-GAATCCTGTTGCCGGTCTTG-3', (R)5'-TTATCCTAGTTTGCGCGCTA-3' to yield a 180 bp fragment. The DNA was denatured at 94 °C for 3 min, followed by 40 cycles of amplification (20 sec at 94 °C; 40 sec at 50 °C; 60 sec at 72ºC). The final incubation at 72 °C was extended to 3 min, and the reaction material was cooled and kept at 4 °C. The PCR products were visualized by running the completed reaction on a 2% agarose gel containing ethidium bromide.

Results and Discussion. The *H. parishii* seeds successfully germinated after one week of sowing on solid ND medium in the absence of plant growth regulator. The seeds gradually extended and developed into protocorms showing swollen spherical green corm-like embryos. The protocorm stage lasted approximately 2 weeks before development into plantlets. Thereafter, they further ramified to form multi-branched structures of plantlets after 4 weeks of germination (data not shown). The results suggested that plant growth regulators could be excluded in the step of protocorm induction and proliferation. This was inconsistent with the study by Pimda & Bunnag (2010) reporting that 3 mg/l BA gave raise to the highest proliferation capacity of *Vanda lilacina* protocorms. However, our results were concordant with the study by Das et al (2007) stating that *Cymbidium devonianum* Paxt. seeds successfully germinated and developed into protocorm in the absence of plant growth regulators.

Antibiotics are crucial in *Agrobacterium*-mediated transformation as they are used as bactericides or selective agents. With respect to the gene components in plasmids, antibiotics used as selective agents may vary. Hygromycin, kanamycin and phosphinothricin (PPT) are among the most common antibiotics which are widely used as selective agents. Vasudevan et al (2007) employed 2 mg/l PPT to select putative transformants in cucumber (*Cucumis sativus* L.) while Pimda & Bunnag (2010) utilized 10 mg/l hygromycin to exclude nontransformants from putative transformants in *Agrobacterium*-mediated transformation of *V. lilacina*. Elimination of *A. tumefaciens* routinely performed using cefotaxime as bactericide. Dose is generally dependent upon plant species and bacterial strains. In this study, cefotaxime and kanamycin were used as bactericide and selective agent, respectively. Both cefotaxime and kanamycin hindered protocorm proliferation. Viability of the protocorms gradually dropped to 82% in the presence of 250 mg/l cefotaxime (Figure 1a). Higher concentration lowered viability of the protocorms. The protocorms cultured on solid ND medium supplemented with cefotaxime failed to proliferate and develop into plantlets. Instead, they exhibited brown coloration, many of which finally died within 3 weeks after culture. The results were concordant with the study previously performed by Pimda & Bunnag (2010) claiming that 250 mg/l cefotaxime was suitable for eliminating *A. tumefaciens* in an attempt to produce fungal-resistant *V. lilacina*. However, Nanditha Krishnan et al (2008) reported that 75 mg/l cefotaxime completely excluded *A. tumefaciens* from transformed calluses of *Centella asiatica* L. Urban. In genetic modification of *C. sativus*, 300 mg/l cefotaxime was required (Vasudevan et al 2002). Besides cefotaxime, 250 mg/l carbenicillin has also been utilized to exclude *A. tumefaciens* from *Oryza sativa* calluses (Saharan et al 2004). Kanamycin greatly suppressed protocorm proliferation and reduced viability of the protocorms. The protocorms were completely inhibited from further development into plantlets in the presence of 15 mg/l kanamycin (Figure 1b). They turned dark brown in color and ultimately died within 2 weeks after culture. The results were inconsistent to the results reported by Nanditha Krishnan et al (2002) using high kanamycin concentration of 100 mg/l as a selective agent in genetic modification of *C. asiatica* calluses. However, our results were consistent with the study conducted by Vasudevan et al (2002) utilizing low kanamycin concentration of 25 mg/l to select *C. sativus* transformants. Likewise,

Jeyaramraja et al (2005) employed 25 mg/l kanamycin as a selective agent in selection of transformed embryonic tissues of tea (*Camelli sinensis* (L.) O. Kuntze). This implied that antibiotics used as bactericides or selective agents are species-dependent.

The bacteria *A. tumefaciens* actively infected the protocorms in a wide range regarding level of gene expression verified by histochemical GUS staining. As cocultivation time was extended, there was an increasing trend in the number of normal protocorms which exhibited *gus* gene expresion. In contrast, the number of wounded protocorms and acetosyringone-treated protocorms which showed *gus* gene expression gradually dropped when cocultivation time was prolonged (Figure 2a). Interestingly, both wounded protocorms and acetosyringone-treated protocorms which did not exhibit *gus* gene expresion were mainly brownish and died afterwards.

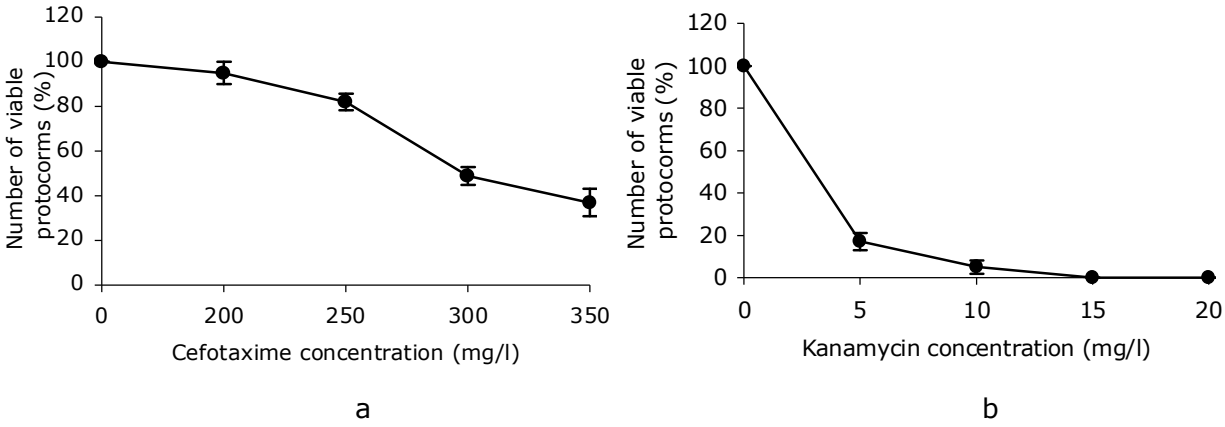


Figure 1. Antibiotic dose on viability of the protocorms after 4 weeks of culture on solid ND medium supplemented with: a) 0-450 mg/l cefotaxime, or b) 0-40 mg/l hygromycin.

It was likely that the level of *gus* gene expression depended upon cocultivation time. The level of *gus* gene expression increased as cocultivation time was extended. In this study, cocultivation time of 30 min was considered suitable for inoculating acetosyringone-treated protocorms with *A. tumefaciens* as it gave rise to high gene expression while maintaining high percentage of protocorm viability. In the same manner, 45 and 60 min cocultivation times were optimal for inoculating wounded and normal tissues with *A. tumefaciens*, respectively (Figure 2b). Genetic transformation mediated by *A. tumefaciens* gave rise to high stability of transgene integration. Minority of the escaped plants was found among all the treatment groups (Table 1). According to the results, protocorm wounding was considered as highly effective approach tantamount to application of acetosyringone during cocultivation, thus suggesting that protocorm wounding could be used as a substitute of acetosyringone in an attempt to produce transgenic plants.

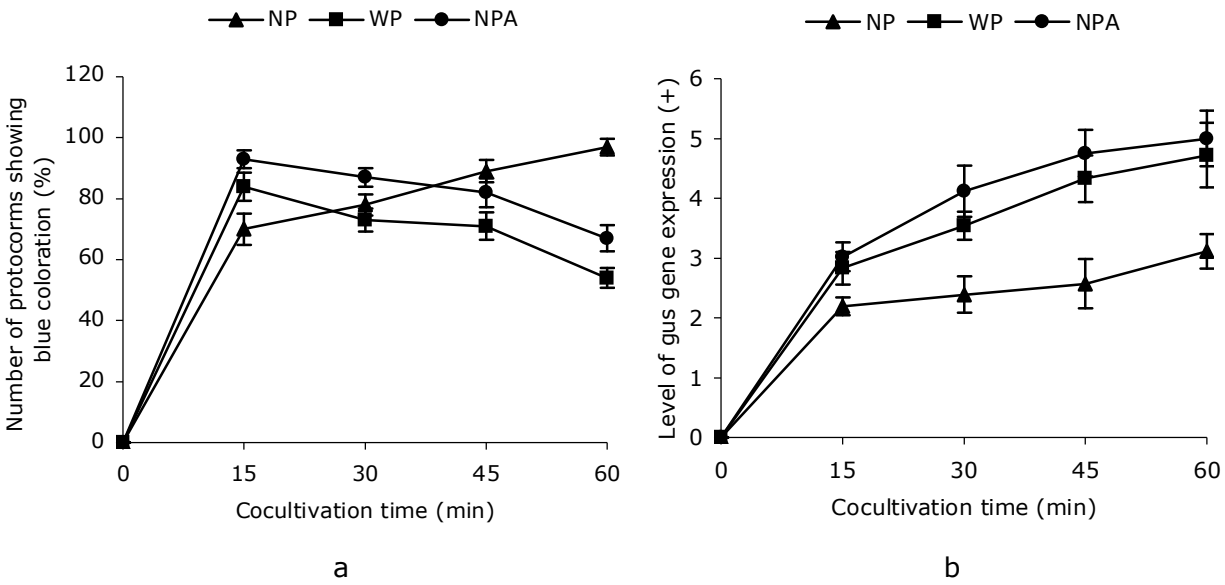
Table 1

Number of the plants undergone genetic modification, and indentified as transgenic and escaped orchid plants after 8 weeks of selection with half-monthly subculture

Type of explants	*Total protocorms*	*Transgenic orchids*[Z]	*Escaped orchids*[Z]
A	100	85±2.5[a]	15±2.5[a]
B	100	87±3.2[a]	13±3.2[a]
C	100	92±3.7[b]	8±3.7[b]

[Z] – Each value is the mean of five replicates, means with the same alphabet (a or b) are not significantly different at p=0.05 using Duncan's multiple range test, A – normal protocorms cocultivaed for 60 min, B - wounded protocorms cocultivated for 45 min, C – normal protocorms previously soaked in 0.1 M acetosyringone and cocultivated for 45 min.

Genetic transformation of *H. parishii* mediated by *A. tumefaciens* was repeated in an attempt to ensure the effectiveness of protocorm wounding in enhancement of bacterial intrusion, leading to successful delivery and integration of transgenes into plant genome. The results exhibited high gene expression as a previous investigation. Also, only minor number of the escaped plants was found.



NP – normal protocorms, WP – wounded protocorms, NPA – normal protocorms soaked in 0.1 M acetosyringone for 30 min before cocultivation with *A. tumefaciens*.

Figure 2. Histochemical GUS assay: a) Number of protocorms exhibiting blue coloration , b) Level of *gus* gene expression regarding an area of blue coloration.

Histochemical GUS staining is a useful tool for primary observation on a success in T-DNA delivery and integration into plant genome. However, in an attempt to prove integration of transgenes, PCR method is a compulsory requirement. In this study, PCR method proved the integration of transgenes by displaying the bands of 195 and 180 bp fragments in the putative transformants, thus indicating that transgene was genuinely integrated into plant genome. The two bands detected were specific for the CaMV35S promoter and the NOS terminator, respectively (Figure 3). PCR amplification of the bands of 195 and 180 bp fragments was also utilized to confirm transgene integration in *V. lilacina* (Pimda & Bunnag 2010), rice (Maneewan et al 2005) and *Dendrobium secundum* (Atichart et al 2007). PCR method has been widely used for detection of T-DNA integration into plant genome in many plant species, Yu et al (2001) employed PCR technique to detect the integration of *DOH1* antisense gene into the genomic DNA of putative transformants of *D.* Madame Thong-In genetically modified using the plasmid pBI121-*DOH1* driven by the CaMV35S promoter and NOS terminator. The PCR technique displayed a 700 bp fragment spanning the 3' end of the CaMV 35S promoter and the 5' end of the *DOH1* antisense gene in transgenic *D*. Madame Thong-In. In the same way, Abang Masli et al (2009) used the PCR technique to examine the integration of *bar* gene, coding for phosphinothricin acetyltransferase, into the genome of transformed embryogenic calluses of *Elaeis guineensis* Jacq. and this technique provided reliable results.

With the understanding that cells at the wound site initiate the synthesis of phenolic compounds (e.g. acetosyringone and α-acetosyringone), which are thought to be produced as antibacterial agents, are recognized by invading *A. tumefaciens* and serve as signals for T-DNA transfer, the phenolic compounds, especially acetosyringone, are widely used for the activation of virulence functions within *Agrobacterium* in an attempt to enhance genetic transformation efficiency in many plant species (Stachel et al 1985).

Sunilkumar & Rathore (2001) reported that acetosyringone significantly increased stable transformation efficiency in cotton (*Gossypium hirsutum* L.). When acetosyringone was omitted, the level of transient gene expression obviously dropped and stably transformed rice or onion could not be regenerated (Rashid et al 1996; Zheng et al 2001).

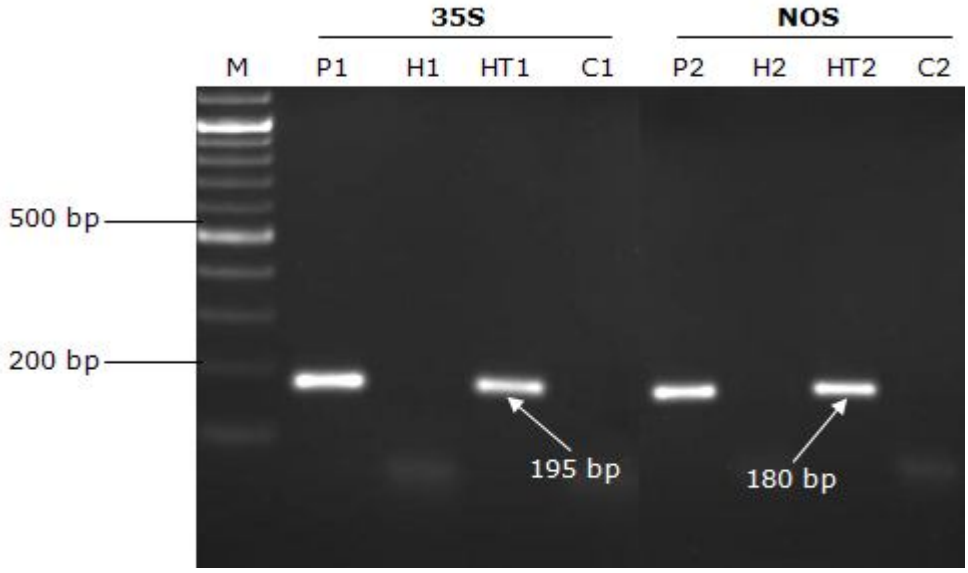


Figure 3. Detection of transgene integration by PCR technique using specific primers to detect the CaMV35S promoter (lane P1-C1) and the NOS terminator (lane P2-C2): M) 100 bp DNA ladder, P) pCAMBIA1305.1, H) Nontransformed protocorms, HT) Transformed protocorms, C) Control.

Many approaches have been introduced to be applied along with addition of acetosyringone during cocultivation. Kumar et al (2006) assessed *Agrobacterium rhizogenes*-mediated transformation efficiency under the influence of sonication and acetosyringone treatment in *Nicotiana tabacum* L. It was found that manual wounding resulted in 21% transformation frequency whereas sonication gave rise to 2.2 fold increase in transformation frequency. Interestingly, sonication with acetosyringone treatment yielded 4.1 fold increase in transformation frequency. This indicated that thousands of microwounds caused by sonication were deeper than those produced by manual wounding allowing *A. rhizogenes* to travel deeper into the tissues. Sonication with acetosyringone treatments was far more effective as acetosyringone induced *A. rhizogenes* to invade wounded cells more often, thus elevating transformation frequency. This approach was useful for genetic modification in higher plants recalcitrant to *Agrobacterium*-mediated transformation. Sometimes acetosyringone treatment could be omitted provided that cavitations yielded sufficient amount of endogenous acetosyringone. In our study, we found that manual wounding alone was sufficient to trigger wounded cells to release a large amount of acetosyringone, thus enhancing transformation frequency in *H. parishii*.

Here, we suggested that acetosyringone treatment could be omitted in genetic modification of *H. parishii* using wounded tissues as explants.

Conclusions. Many factors play a key role in seed germination and development into protocorms and plantlets. Species, pod age, medium type and inclusion or exclusion of plant growth regulators have a profound effect on a failure or success of seeds to develop. Here, the *H. Parishii* seeds successfully germinated and developed into protocorms and plantlets in the absence of plant growth regulators, suggesting that plant growth regulators can be excluded in the steps of explant preparation. The results also suggested that protocorm wounding could be used as a substitute of exogenous acetosyringone in enhancement of *Agrobacteium*-mediated transformation. The optimal

condition was as follows. The wounded protocorms were cocultivated with *A. tumefaciens* for 45 min, dried on sterile tissue papers and then transferred to solid ND medium for 3 d. Thereafter, they were washed thoroughly with sterile distilled water supplemented with 250 mg/l cefotaxime and subsequently transferred to the selective medium supplemented with 250 mg/l cefotaxime and 15 mg/l kanamycin for 4 weeks. Transient gene expression verified by histochemical GUS assay and gene integration detected by PCR technique were recommended to implement every month in order to exclude the escaped plants from transgenic plants.

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