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Original Paper

Agrobacterium-mediated genetic transformation of *Cattleya* with an *Odontoglossum ringspot virus* replicase gene sequence

Lin Zhang[1], Dong Poh Chin[1], Masanobu Fukami[2], Hiroaki Ichikawa[3], Ikuo Nakamura[1], Masahiro Mii[1,*]

[1] Graduate School of Horticulture, Chiba University, Matsudo, Chiba 271-8510, Japan; [2] Chiba Prefectural Agriculture and Forestry Research Center, Chiba 266-0006, Japan; [3] Division of Genome and Biodiversity Research, National Institute of Agrobiological Sciences, Tsukuba, Ibaraki 305-8602, Japan
* E-mail: miim@faculty.chiba-u.jp Tel & Fax: +81-47-308-8852

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Abstract Protocorm-like bodies (PLBs) of *Cattleya* orchid CM2450 cultured either under constant illumination with cool-white-fluorescent lamps or in the dark were cocultivated with *Agrobacterium tumefaciens* strain EHA105 carrying plasmid pSMAHdN627-ORSV harboring genes coding for *Odontoglossum ringspot virus* (ORSV) replicase and hygromycin phosphotransferase. PLBs were maintained in liquid New Dogashima (ND) medium and then added to a bacterial suspension culture (OD600≈0.6) yielding medium dilution ratio of 1 : 10 and incubated for either 30 minutes or 3 h. Hygromycin-resistant secondary PLBs were induced after 4 weeks of culture on 2.5 g l^{-1} gellan gum-solidified ND medium containing 1 mg l^{-1} naphthaleneacetic acid (NAA), 0.1 mg l^{-1} benzyladenine (BA), 10 mg l^{-1} hygromycin, 20 mg l^{-1} meropenem, 10 g l^{-1} sucrose in both light- and dark-cultured PLBs. The number of resistant PLBs generated using dark-cultured PLBs was higher than those cultured under constant illumination. Presence of acetosyringone (AS) in the pre-culture medium was also effective for the transformation. The highest frequency of transformation was obtained when dark-cultured PLBs were pre-cultured with 100 μM AS for 3 days and inoculated with *Agrobacterium* liquid culture for 3 h. Transformation of hygromycin-resistant plantlets regenerated from different sites of inoculated PLBs was confirmed by Southern blot hybridization. Transcription of ORSV replicase gene in transgenic lines was successfully confirmed by Northern blot hybridization.

Key words: *Agrobacterium tumefaciens*, *Cattleya*, *Odontoglossum ringspot virus*, orchid, protocorm-like body.

The Orchidaceae is the most species-rich angiosperm family (Atwood 1986; Benner et al. 1995; Chai et al. 2007) widely distributed around the world and is one of the most attractive ornamental plant groups because of its beautiful flowers and morphological diversity. Among the important cultivated orchid genera, *Cattleya* is one of the most famous and colorful of all the orchid genera. Although traditional breeding efforts have successfully produced diverse array of numerous cultivars of *Cattleya* orchids, there still remain various breeding objectives which are difficult to achieve by conventional methods, including disease and pest resistance, stress tolerance, flower color, scent, size and form modification, and growth habit.

Viral diseases in orchids are a major concern among breeders due to their catastrophic effects on plant yield and quality. At least 25 orchid viruses have been reported around the world. Of these, *Odontoglossum ringspot virus* (ORSV) is one of the most prevalent viruses distributed worldwide and commonly found in more than 20 orchid genera including *Cattleya* (Wong 2002). ORSV induces streak or striped mosaic, diamond-shaped mottle, or ring-spots on leaves, and color breaking on flowers, which have been shown to occur in *Cattleya* severely (McMillan and Vendrame 2005). It also reduces plant vigor and lowers flower quality of the orchids, which adversely affects the orchid industry worldwide (Khentry et al. 2006; Ryu and Park 1995; Wong et al. 1994; Zettler et al. 1990). Therefore, production of cultivars resistant to ORSV has been eagerly desired in *Cattleya* and other important orchids for a long time. However, like as other orchids, production of *Cattleya* cultivars with virus resistant trait has hitherto been difficult to achieve through conventional breeding techniques based on sexual crossing due to the lack of available germplasm for the virus resistance.

The production of virus-resistant plants by using virus coat protein (CP) based on cross-protection with a mild

Abbreviations: AS, acetosyringone; BA, benzyladenine; Hm, hygromycin; *hpt*, hygromycin phosphotransferase; NAA, naphthaleneacetic acid; ND, New Dogashima; ORSV, *Odontoglossum ringspot virus*; PGR, plant growth regulators; PLB, protocorm-like body
This article can be found at http://www.jspcmb.jp/

strain of a given virus reduces the susceptibility of a plant to a more virulent strain of the same virus (Zaitlin 1976). Since Powell-Abel et al. (1986) reported that plants expressing the CP gene of tobacco mosaic virus (TMV) were resistant to TMV, several studies on the production of virus resistant plants have been reported (Malpica et al. 1998). Apart from the CP-mediated resistance system, Golemboski et al. (1990) transformed *Nicotiana tabacum* cv. Xanthi plants with TMV replicase sequence and demonstrated that transgenic plants containing the TMV 54-kDa gene coding sequence were resistant to infection with TMV. They also showed that the level of resistance in transgenic plants is independent of the number of copies of the inserted sequence, and that transgenic plants containing a single copy of the gene sequence showed the same level of resistance as those with multi-copies to intact virions or viral RNA.

To date, there are no reports on the transformation of *Cattleya* for resistance to viral diseases. In our previous study, we established a transformation procedure in *Cattleya* using PLB as the explant material (Zhang et al. 2010). In the present study, therefore, we report successful result on the production of transgenic plants of *Cattleya* with ORSV replicase gene sequence, which was introduced into PLBs by developing an improved protocol of *Agrobacterium*-mediated transformation system.

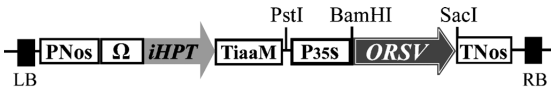
Materials and methods

Plant materials

PLBs of *Cattleya* orchid CM2450 maintained in liquid NDCa medium, the New Dogashima (ND) medium (Tokuhara and Mii 1993) containing 10 g l^{-1} sucrose, 1.0 mg l^{-1} 1-naphthaleneacetic acid (NAA) and 0.1 mg l^{-1} N6-benzyladenine (BA), at pH 5.4 were used in this study. The PLBs were subcultured every month by transferring to fresh medium. For each subculture, PLBs were cut into several pieces of approximately 2 mm, and 1.0 g aliquot was inoculated into each 100 ml flask containing 30 ml liquid medium. Cultures were incubated on a reciprocal shaker by agitation at 60 strokes min^{-1} at 25°C either under constant illumination with cool-white-fluorescent lamps (35 mmol m^{-2} s^{-1}) (National FL30SN, Osaka, Japan) or in the dark. The PLBs thus proliferated were used for transformation.

Cloning of ORSV replicase gene sequence

The total RNA of ORSV strain H-1 (MAFF No. 104037) was extracted from infected *Chenopodium amaranticolor* by using the RNeasy Plant Mini Kit (QIAGEN, Germany) according to the manufacturer's instructions. The DNA fragments of ORSV replicase gene sequence were obtained by RT-PCR (TaKaRa RNA PCR Kit (AMV) Ver.3.0: Takara Bio Inc. Shiga, Japan) using the total RNA with the primer pairs of ORSV803 (5′-ATCTATTCGTACCATGTCCT-3′) and ORSV2429R (5′-CACACATACCCATTCTCTAC-3′) which were designed based on the ORSV sequence database (Accession no. NC_001728, GenBank). The DNA fragments were electrophoresed on 1.5% agarose gel (Wako Pure Chemical Industry Ltd, Osaka, Japan) and purified using a GeneClean kit (Qbiogene). The eluted product was cloned into a TA cloning vector, pT7Blue-2 (Merck, Germany). Sequence of inserted DNA was analyzed with 377 DNA sequencer (PE Applied Biosystems, California, USA).



pSMAHdN627-ORSV

Figure 1. Schematic representation of the T-DNA region of pSMAHdN627-ORSV plasmid. PNOS, nopaline synthase promoter; Ω, translation enhancer sequence 5′-untranslated sequence of tobacco mosaic virus; *iHPT*, hygromycin phosphotransferase gene; TiaaM, polyadenylation signal from *Agrobacterium iaaM* gene; P35S, cauliflower mosaic virus 35S transcript promoter; *ORSV*, ORSV replicase gene sequence; TNOS, nopaline synthase terminator.

Construction of the plasmid vector

The 1300 bp fragment encoding ORSV replicase gene sequence was amplified from pT7Blue-2-ORSV with a pair of primers, forward primer: 5′-ATGCGGATCCATGCAATTTTA-TTATGACACTTTGT-3′ and the reverse primer: 5′-CGATGA-GCTCTTAATCGGAAAGATACTTAACAATA-3′. Restriction enzyme sites *Bam*HI and *Sac*I sites were added in the forward and reverse primer respectively. The plasmid pBI221 (accession number AF502128) was digested by *Bam*HI and *Sac*I at 3′ end of the CaMV35S promoter and 5′ end of the nos terminator, respectively. The above-mentioned ORSV replicase gene sequence was ligated to the *Bam*HI and *Sac*I sites of the CaMV35S promoter vector by replacing the *GUS* gene. Then the plasmid was digested by *Pst*I at 5′ end of CaMV35S promoter and *Sac*I at 5′ end of the terminator, the resulting ORSV replicase gene sequence fragment containing a CaMV35S promoter was cloned into the binary vector pSMAHdN627, which was pSMAHdN627-M2GUS (Nakamura et al. unpublished), by removing GUS by digesting with *Sbf*I at 5′ end and *Sac*I at 3′ end of *GUS* gene. This final constructed vector named as pSMAHdN627-ORSV (Figure 1), which also harbored the gene coding for hygromycin phosphotransferase (*hpt*) in the T-DNA region, was introduced into *Agrobacterium tumefaciens* strain EHA105 (Hood et al. 1993) and used to transform *Cattleya*.

Inoculation and co-cultivation with Agrobacterium

A. tumefaciens strain EHA105/pSMAHdN627-ORSV was grown overnight at 28ºC in LB liquid medium containing 50 mg l^{-1} hygromycin (Hygromycin B; Wako Pure Chemical Industries, Osaka, Japan), 25 mg l^{-1} chloramphenicol, 20 mg l^{-1} rifampicin (Wako Pure Chemical Industries, Osaka, Japan), 100 mg l^{-1} spectinomycin (Sigma-Aldrich, St. Louis, MO, USA) and adjusted to pH 7.0. Three days before infection, PLBs were transferred to 30 ml fresh liquid NDCa medium with or without 100 μM acetosyringone (AS) (3′,5′-dimethoxy-4′-hydroxy-acetophenone; Sigma-Aldrich, St. Louis, MO, USA) for pre-culture. For inoculation, 3 ml of *Agrobacterium* suspension culture (OD_{600}≈0.6) was added to 30 ml PLB culture, to which 100 μM of AS was added irrespective of the presence of AS in the pre-culture medium, and incubated for

either 30 min or 3 h, with occasional agitation. The PLBs were then blotted dry, and co-cultivated on 2.5 g l^{-1} gellan gum (Wako Pure Chemical Industries, Osaka, Japan)-solidified NDCa medium supplemented with 100 μM AS in Petri plates for 3–6 d at 20ºC in the dark.

Selection of transgenic PLB and plant regeneration

After 3 d of co-cultivation, the PLBs were washed with liquid NDCa medium containing 10 mg l^{-1} meropenem (Ogawa and Mii 2004, 2007) (Meropen; Dainippon Sumitomo Pharma, Osaka, Japan) to remove excess *Agrobacterium*. PLBs were then transferred onto selection medium (NDCa medium supplemented with 10 mg l^{-1} hygromycin and 20 mg l^{-1} meropenem and solidified with 2.5 g l^{-1} gellan gum). PLBs were subcultured onto a fresh medium with the same composition every 10 d during the first month, and subsequently, every 2 weeks during the next 2 months. Secondary PLBs showing green coloration and reaching approximately 5 mm in diameter produced on the selection medium were cut transversely into several pieces and transferred onto the same medium for proliferation. The newly developed PLBs were then transferred onto 2.5 g l^{-1} gellan gum-solidified phytohormone-free ND medium containing 10 g l^{-1} sucrose, 10 mg l^{-1} hygromycin and 20 mg l^{-1} meropenem for plant regeneration. Regenerated plantlets were selected as putative transformants and transplanted into culture bottles containing the same medium for rooting.

DNA isolation and Southern hybridization

Leaf tissue samples from regenerated plants were subjected to DNA isolation. Total genomic DNA was extracted from leaf tissues (0.5 g fresh weight) using the CTAB method (Murray and Thompson 1980). Ten micrograms of genomic DNA from non-transformed control and hygromycin-resistant plants was digested with *Bam*HI, fractionated on a 0.9% agarose gel and subsequently transferred to a nylon membrane (Immobilon-Ny^+ Transfer Membrane; Millipore Co, Billerica, MA, USA). The ORSV replicase gene (1.1-kb) probe was generated from the plasmid pSMAHdN627-ORSV by labeling with digoxigenin (DIG) using the PCR DIG Probe Synthesis kit (Roche Diagnostics, Mannheim, Germany) and the following set of primers, 5′-TAGATTTTTCTAAATCTGTT-AGTG-3′ and 5′-CACAAGGAGATTCTAAATTCTTCC-3′ were used. Southern blot hybridization and detection were carried out following the manufacturer's instruction (Roche Diagnostics, Mannheim, Germany). For detection of hybridization signals, membrane was exposed to a detection film (Lumi-Film Chemiluminescent Detection Film; Roche Diagnostics, Mannheim, Germany).

RNA extraction and Northern Blot Analysis

Samples (0.2 g fresh weight) from non transformed control and transgenic leaf tissue were quick-frozen in liquid nitrogen and ground to powder by mortar and pestle, and total RNAs were isolated according to the procedure of De Vries et al. (1988). For northern blot analysis, 5 μg of total RNA was separated on 1% agarose containing 2% formaldehyde and transferred on to a nylon membrane (Immobilon-Ny^+ Transfer Membrane; Millipore Co, Billerica, MA, USA). Loading of equal amounts of total RNA for northern blots was determined by visualization of ethidium bromide-stained rRNA bands. Probe for northern blots was generated as Southern blot analysis. Hybridization was performed at 50ºC in the high-SDS hybridization buffer overnight. The blots were washed twice first in 2X SSC, 0.1% SDS for 10 min and in 0.2X SSC, 0.1% SDS at 50ºC for 30 min. They were finally detected with x-ray film (Lumi-Film Chemiluminescent Detection Film; Roche Diagnostics, Mannheim, Germany).

Results and discussion

Transformation and selection of hygromycin-resistant PLB

3 d after cocultivation with *A. tumefaciens* strain EHA105/pSMAHdN627-ORSV, the PLBs were transferred onto selection medium, on which secondary PLBs were produced within 4 weeks. Three months after the transfer, the number of the hygromycin-resistant PLBs was recorded to compare the transformation efficiency among the different treatments. When PLBs reached approximately 5 mm in diameter, they were cut transversely into several pieces, which subsequently developed into new PLBs and then into plantlets within 3 months after transfer onto PGR-free ND medium. The plantlets were then transferred onto the same fresh medium and maintained for more than 3 months until they produced four to five leaves and several roots. No morphological changes or developmental abnormalities were observed in all the transgenic lines obtained.

The transformation efficiency of *Cattleya* was compared between two kinds of PLBs (light- and dark-cultured). Although hygromycin-resistant PLBs were obtained in both PLB types, the number of resistant PLBs generated using dark-cultured PLBs was higher than those cultured under constant illumination in both 30 min and 3 h of infection treatments and the highest transformation efficiency was obtained when the PLBs were infected for 3 h using dark-cultured PLBs (Figure 2A). These results suggest that dark-cultured PLBs are more susceptible for *Agrobacterium* infection than those cultured under light conditions in *Cattleya*. Similar result has also been reported in *Typha latifolia* (Nandakumar et al. 2005), where the number of GUS spots per gram fresh weight calli was significantly higher in dark-cultured calli than in those cultured under the light condition. Although they suggested the importance of more friable nature of dark-cultured calli for the infection, no such difference was found in PLBs used in the present study except for the discoloration of the PLBs in the dark culture. Therefore, it is necessary to clarify the reason for the effectiveness of dark culture on *Agrobacterium*-mediated transformation of *Cattleya* and other orchids in further studies. The duration of bacterial infection also affected the frequency of hygromycin-resistant PLB formation and infection for 3 h gave higher transformation frequency than that for 30 min in both light and dark

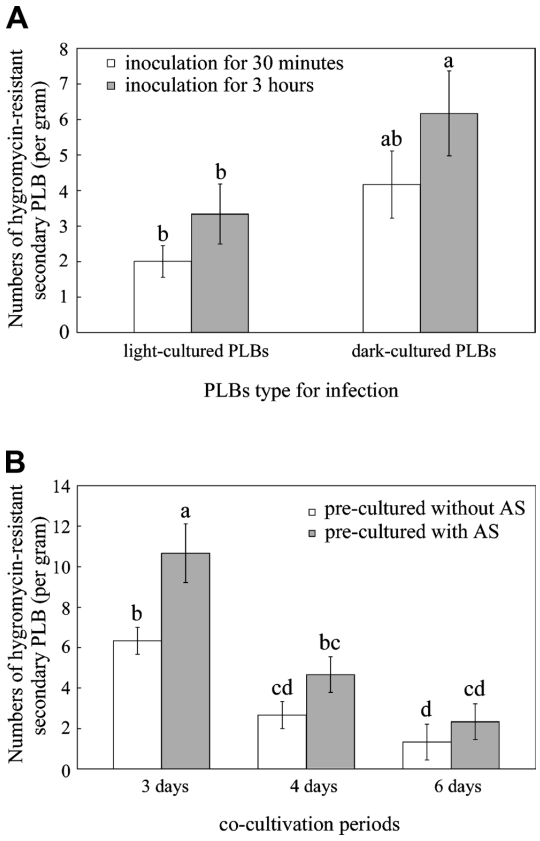


Figure 2. Effects of light condition for culturing PLBs material used for inoculation (A) and co-cultivation periods (B) on the formation of hygromycin-resistant secondary PLBs. (A) Number of hygromycin-resistant secondary PLBs (per gram) after 3 months of culture on selection medium containing 10 mg l^{-1} hygromycin and 20 mg l^{-1} meropenem. Each value represents a mean ±SE of the six independent experiments including different inoculation time using 1 : 10 dilutes of *Agrobacterium* liquid culture (OD_{600}≈0.6) for each infection. Bars with the same letter are not significantly different at 5% level according to the Duncan's new multiple range test. (B) Number of hygromycin-resistant secondary PLBs (per gram) after 3 months of culture on selection medium containing 10 mg l^{-1} hygromycin and 20 mg l^{-1} meropenem. Each value represents a mean ±SE of the three independent experiments using 1 : 10 dilutes of *Agrobacterium* liquid culture (OD_{600}≈0.6) under 3 hours' inoculation for each treatment. Bars with the same letter are not significantly different at 5% level according to the Duncan's new multiple range test.

grown PLBs (Figure 2A). When dark-cultured PLBs were used, the numbers of hygromycin-resistant PLBs per gram of inoculated PLBs were 6.2 when they were infected for 3 h, but reduced to 4.2 when they were infected for 30 min. Since the effectiveness of long-term infection treatment has also been reported in other orchids (Belarmino and Mii 2000; Chin et al. 2007; Mishiba et al. 2005), it might be a general phenomenon for the *Agrobacterium*-mediated transformation in orchid species.

In *Agrobacterium*-mediated transformation of orchids, effectiveness of AS treatment has been reported previously in *Phalaenopsis* (Belarmino and Mii 2000; Mishiba et al. 2005), *Dendrobium* (Men et al. 2003), *Oncidium* (Liau et al. 2003), and *Cymbidium* (Chin et al. 2007). Although AS was added only in infection and/or cocultivation medium in most of the cases, positive effect of AS in the pre-culture medium was reported previously in *Phalaenopsis* (Mishiba et al. 2005). Effectiveness of the presence of AS in the pre-culture medium was also observed in our present study, which showed that the numbers of hygromycin-resistant PLBs obtained after selection were higher when AS was supplemented during pre-culture period, irrespective of the difference in cocultivation period tested (Figure 2B). These results suggest that AS supplemented during pre-culture might accumulate high AS concentration within tissues of the treated PLBs, thereby eliciting higher *vir*-gene-inducing activity in *Agrobacterium* (Mishiba et al. 2005; Nan et al. 1997). In *Agrobacterium*-mediated transformation of *Cattleya*, 3 hour-infection period gave successful results in our previous study (Zhang et al. 2010). Present results confirmed the appropriateness of this infection period since prolonged cocultivation period till 4 to 6 days resulted in the reduction of the number of hygromycin-resistant PLBs due to the difficulty to eliminate *Agrobacterium* and caused browning of PLBs during the early stage of selection period. Consequently, the highest number of hygromycin-resistant secondary PLBs (ca. 11 per 1 g PLBs) was obtained when PLBs were cocultivated for 3 d after infection of 3 day-pre-cultured PLBs for 3 h in the presence of 100 μM AS throughout.

Confirmation of transformation

As shown in Figure 3, Southern blot analysis revealed that one transformed plant had a single copy, while others showed multiple copies of T-DNA integrated into the plant genome. It has been shown that expression of the transgene is dependent upon the copy number and abnormal morphology has been reported in many plant transformation experiments. Although an increased copy number has often been associated with gene silencing (Finnegan and McElory 1994; Matzke et al. 1994), in the present study, northern blot analysis (Figure 4) showed that the expression of the transcript in the transgenic lines was independent of transgene copy number. ORSV replicase gene was expressed in all transgenic lines even in lines 6 and 12 which contained high copy number of the transgene. This result suggests that multiple copies of the ORSV replicase gene sequence did not have inhibitory effect on the expression of the transgene in *Cattleya*. Similar results were also obtained in *Nicotiana tabacum* cv. Xanthi NN plants transformed with TMV replicase sequence by Golemboski et al. (1990).

Replicase-mediated resistance was first reported for TMV (Donson et al. 1993; Golemboski et al. 1990), and then for other viruses such as cucumber mosaic virus (Anderson et al. 1992; Carr et al.1994; Wintermantel et al. 1997, 2000), potato virus X (Braun and Hemenway

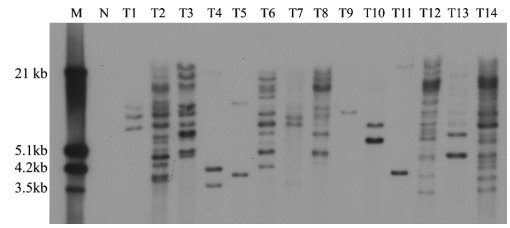


Figure 3. Southern blot analysis of transgenic plants. Genomic DNA was digested with *Bam*HI and hybridized with a digoxigenin (DIG)-labeled ORSV replicase gene sequence probe. *Lane M* marker; *Lane N* non-transformed plant; *Lanes T1–T14* transgenic plants obtained by inoculation with *A. tumefaciens* strains EHA105/pSMAHdN627-ORSV.

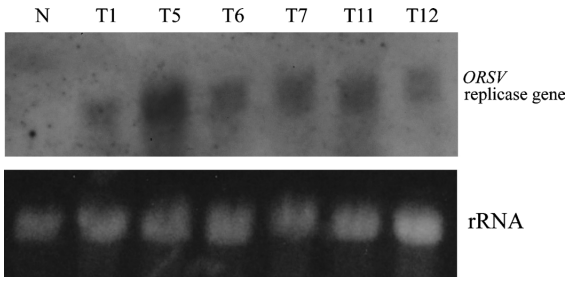


Figure 4. Northern blot analysis of ORSV replicase gene expression in transgenic *Cattleya*. Total RNAs from a non transgenic (lane N) and transgenic plants (lanes T1, T5, T6, T7, T11, T12) were extracted according to the procedure of De Vries et al. (1988). Total RNAs were separated on 1% agarose containing 2% formaldehyde and transferred on to a nylon membrane and hybridized with a digoxigenin (DIG)-labeled ORSV replicase gene sequence probe.

1992; Longstaff et al. 1993), potato virus Y (Audy et al. 1994), cowpea mosaic virus (Sijen et al. 1995), pea early browning virus (MacFarlane and Davies 1992), pepper mild mottle virus (Tenllado et al.1995), cymbidium ringspot virus (Lupo et al. 1994; Rubino and Russo 1995) and African cassava mosaic virus (Hong and Stanley 1996). Compare to coat protein-mediated resistance, transgenic plants with replicase sequence have some obvious advantages such as the level of resistance in transformed plants, which was independent on the number of copies of the inserted sequence. The resistance observed is in many cases stronger than coat protein-mediated resistance, as it is maintained even when higher titers of viral inoculums are applied.

Agrobacterium-mediated method has been successfully applied for the transformation of several orchids such as *Dendrobium* (Men et al. 2003; Nan et al. 1998; Yu et al. 2001), *Phalaenopsis* (Belarmino and Mii 2000; Chai et al. 2002; Mishiba et al. 2005; Sjahril and Mii 2006), *Cymbidium* (Chen et al. 2002; Chin et al. 2007) and *Oncidium* (Liau et al. 2003). In our previous study (Zhang et al. 2010), we used PLBs derived from shoot-tip culture of a commercial cultivar as explants and developed a protocol for *Cattleya* orchid transformation by employing the *Agrobacterium*-mediated transformation system. In this study, we have succeeded to increase the transformation efficiency by employing different culture conditions of PLBs and different periods of AS supplementation. Because of the relatively high transformation efficiency obtained in the present study, this protocol might be applicable for the transformation of various cultivars of *Cattleya* and its intergeneric hybrids.

Although inoculation of many other plant tissues with *Agrobacterium* has most often been carried out in liquid medium for a few minutes, in our previous experiment (Zhang et al. 2010), although the hygromycin-resistant PLBs could be obtained within a relatively short period (30 min of infection) in *Cattleya*, three hours inoculation period was suitable for obtaining hygromycin-resistant PLB. Similar results were observed in the present study. These results indicate again that a relatively long period might be needed for adhesion of *Agrobacterium* on the surface of target tissues in orchids.

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