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BRIEF COMMUNICATION

Selection of co-transformed *Dendrobium* Sonia 17 using hygromycin and green fluorescent protein

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Abstract

Dendrobium Sonia 17 calluses were used for co-transformation study using particle bombardment. The bombarded transformed callus tissues were selected using half-strength MS medium containing 25 mg dm^{-3} hygromycin. Expression of green fluorescent protein (GFP) was observed in the callus and protocorm-like body (PLBs) tissues survived on the selection medium. The presence of green fluorescence protein (s*gfp*), hygromycin-B-phospho-transferase (*hptII*) and β-glucuronidase (*uidA*) genes in the transformed tissues were verified using PCR, Southern blot and dot blot analyses. Based on the results from PCR and expression of *sgfp* and *uidA* genes in the calluses and PLBs survived from hygromycin selection, we reported the co-transformation of s*gfp*, *hptII* and *uidA* genes into *Dendrobium* Sonia 17. GFP- expressing tissues were also observed in the regenerated transformed plantlets.

Additional key words: bombardment, β-glucuronidase, PCR, phosphotransferase, Southern blot.

Growing orchids for the cut flowers is one of the major floriculture industries contributing significantly to Malaysia's economy. In general, *Dendrobium*, *Oncidium*, *Aranda* and *Mokara* are among the orchid genera that are commonly cultivated in Malaysia. In order to stay competitive in the global floriculture industry, it is important to improve the quality of orchid flowers. Reports of several attempts to genetically modify orchids (Belarmino and Mii 2000, Knapp *et al.* 2000, Chai *et al.* 2007, Chin *et al.* 2007) indicated the feasibility of using biotechnology to improve the quality of orchid flowers. It is always the interest of researchers to study the ability of introducing more than one desired traits into transgenics at the same time. A possible approach to introduce multiple desired traits into plants is by co-bombarding the target tissues with different plasmids carrying different genes of interest. Besides, it is also crucial to perform an efficient selection system to select transformants. Green fluorescent protein (GFP) is commonly used as an effective and reliable reporter system to optimize the transformation system (Tee *et al.* 2003, Tee and Maziah 2005). However, if GFP is to be used as the sole selectable marker to select the putative transformants, it could be very laborious and time consuming. The rapid growth of the untransformed tissues might mask the GFP-expressing tissues and cause difficulty in isolating transformed tissues as observed in sugarcane transformation (Elliot *et al.* 1999). Hence, using antibiotic assisted by GFP in the selection process is more efficient in identifying the transformed tissues (Elliott *et al.* 1999) and it is able to reduce the frequency of escapes due to the protection of non-transformed cells

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Abbreviations: GFP - green fluorescent protein; GUS - β-glucuronidase; MS - Murashige and Skoog; PLBs - protocorm-like bodies.
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(Ghorbel *et al*. 1999, Stewart 2001). In most of the orchid transformation studies, antibiotics (Men *et al.* 2003, Chin *et al.* 2007, Suwanaketchanatit *et al.* 2007) and herbicides (Knapp *et al.* 2000, Chai *et al.* 2007) were used as the selectable markers. In this study, *Dendrobium* Sonia 17 calluses were co-bombarded with two plasmids to study the co-transformation event and putative transformants were selected using hygromycin assisted by GFP.

Dendrobium Sonia 17 callus tissues grown as described previously (Tee *et al*. 2003) were used. To transform the callus, 35S- *sgfp*-TYG-nos (p35S) plasmid carries *sgfp* gene driven by the cauliflower mosaic virus (CaMV) 35S promoter (Sheen *et al.* 1995, Chiu *et al.* 1996) and pSMDFR plasmid carries hygromycin-B-phosphotransferase (*hptII*) and *uidA* genes driven by CaMV 35S promoter were used. Both p35S and pSMDFR plasmids were co-bombarded into *Dendrobium* Sonia 17 calluses. Bombardment was carried out using *PDS-1000/He* biolistic particle delivery system (*BioRad*, Hercules, USA). All callus tissues were bombarded using the optimised parameters as described previously (Tee and Maziah 2005). Bombardment was carried out in triplicate and it was repeated. After two weeks post-bombardment, the callus tissues were transferred to the selection medium containing 25 mg dm^{-3} hygromycin and the number of survived callus tissues was recorded. At the same time, the survived tissues were monitored weekly to detect GFP-expressing tissues using a fluorescence microscope (*Leica MZ FLIII*, Wetzlar, Germany) equipped with a *GFP 2* filter. The transformed callus tissues were maintained on the selection medium for 4 to 6 months before proliferating on the hygromycin-free medium for plant regeneration.

For molecular analyses, genomic DNA was extracted using the modified CTAB method adopted from Doyle and Doyle (1990). PCR was carried out to amplify *sgfp*, *hptII* and *uidA* genes. For *uidA* and *sgfp* genes, an initial denaturation step at 94 °C for 5 min followed by 30 amplification cycles, including 94 °C (30 s), 60 °C (1 min) and 72 °C (2 min), and an extension cycle at 72 °C (7 min) were performed. The amplification of *hptII* gene was carried out by denaturing the DNA at 94 °C for 3 min before the 30 amplification cycles which included 94 °C (1 min), 60 °C (1 min) and 72 °C (2 min) and an extension cycle at 72 °C (7 min). Southern blot and dot blot analyses were carried to verify the integration of trangenes in the genome. The PCR amplified product from plasmid DNA (p35S or pSMDFR) was DIG-labeled and used as the probe for DNA hybridisation. The DNA labeling and detection were carried out following the supplier's instructions (*Roche*, Mannheim, Germany).

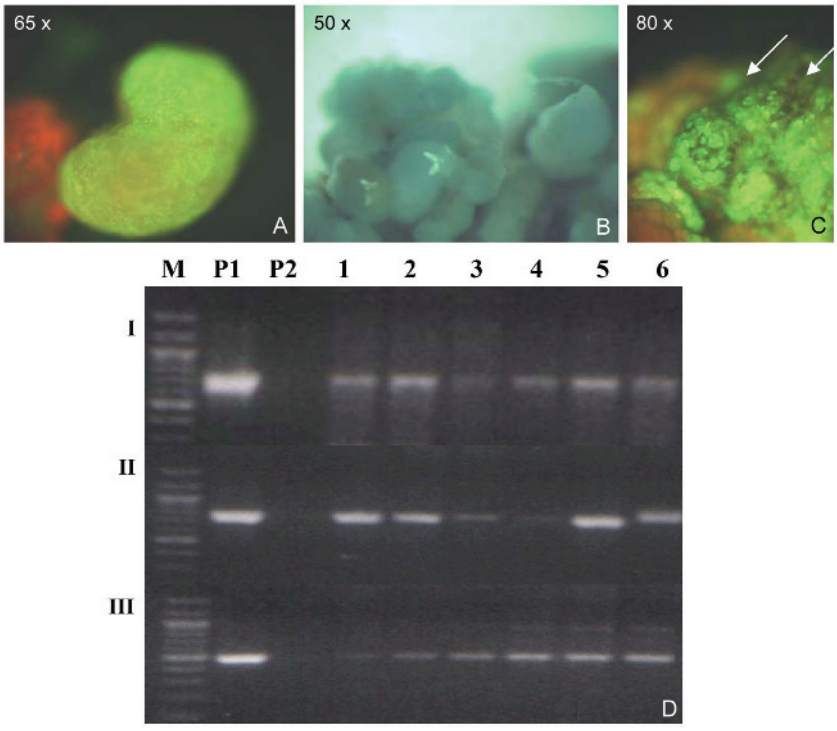

Fig. 1. GFP- expressing tissues observed in the *Dendrobium* Sonia 17 callus and presence of transgenes in the transformed tissues were amplified by PCR. *A* - isolated GFP- expressing tissues; *B* - tissues expressing the *uidA* gene; *C* - browning (*arrows*) appeared on the GFP-expressing tissues; *D* - detection of *sgfp*, *uidA* and *hptII* genes using PCR: I - *sgfp* gene (750 bp), II - *uidA* gene (780 bp) and III - *hptII* gene (500 bp), where *lane* M is 100 bp DNA ladder, P1 is positive control, P2 is untransformed sample and *lanes* 1 to 6 are transformed samples.

In our study, four transformed lines were obtained from 240 bombarded callus tissues with approximately 1.7 % transformation efficiency. These bombarded callus tissues were pre-cultured for two weeks before subjecting to the hygromycin selection medium. In similar study, Yu *et al.* (1999) achieved 5 - 10 % transformation efficiency when transforming *Dendrobium*. They reported that the pre-culturing period before transferring the protocorms to selection medium might affect the transformation efficiency as only longer pre-culturing period (2 - 3 months) yielded transformed protocorms in their studies. However, early selection (2 to 10 d post- bombardment) was found to be more efficient for selecting the transformed PLBs of two *Dendrobium* species using 30 mg dm^{-3} hygromycin as no transformed line was obtained when selection was carried out after 30 d post-bombardment (Men *et al.* 2003). GFP reporter system was also used to aid in selection process in this study. Many studies have been carried out to study the efficiency of using GFP and a selective agent to select transformed tissues, for examples selective agents such as kanamycin (Darnowski and Vodkin 2002), *Basta* (Richards *et al.* 2001) and hygromycin (Ponappa *et al.* 1999) were used together with GFP to screen transgenic plants. In this study, GFP-expressing tissues were observed from the callus tissues survived in the selection medium for more than four months. They were isolated and multiplied on the antibiotic-free medium for two months before transferring back to the selection medium again to further eliminate the non-transformed tissues. Eventually, GFP expression was observed on the whole transformed tissues (Fig. 1*A*) survived on the selection medium containing hygromycin indicating the successful co-transformation. In order to further verify this, the GFP-expressing tissues were used for β-glucuronidase (GUS) assay. The blue stains observed on the tissues evident the presence and expression of *uidA* gene in the putative transformed tissues (Fig. 1*B*). This indicated the co-transformation of transgenes into the bombarded callus tissues. High co-delivery frequency of two plasmids in which the un-linked transgenes can be introduced into the same tissues has been reported in plant transformation study (Romano *et al.* 2001). A similar study reported that a marker gene and the gene of interest separately carried by two plasmids were co-introduced into wheat (Rooke *et al.* 2003).

All the selected putative transformants were proliferated in the selection medium. However, the regeneration process was very slow, more than six months. Nan and Kuehnle (1995) reported that 3 to 9.5 months was required to recover and regenerate the *Dendrobium* transgenic from protocorms. It is not clear if

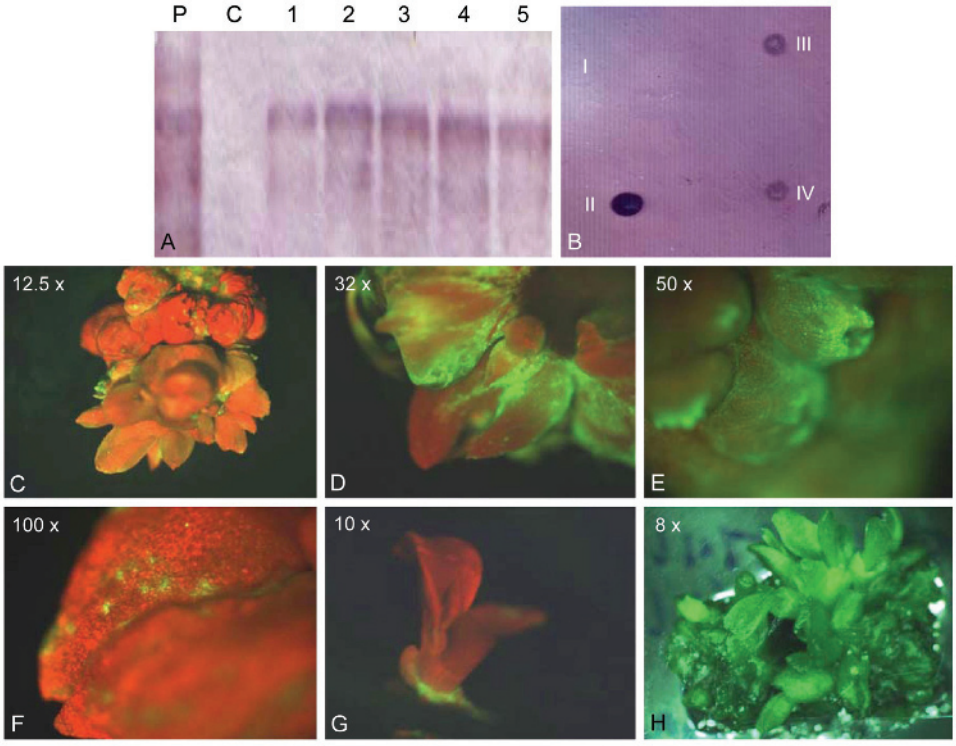

Fig. 2. DNA hybridisation analyses and GFP-expressing tissues of *Dendrobium* Sonia 17. *A* - Southern blot analysis using PCR amplified *sgfp* products: *lanes* 1 to 5 are the transformed samples, *lane* P is positive control and *lane* C is untransformed sample; *B* - dot blot analysis shows hybridisation of genomic DNA with *sgfp* probe: I - untransformed sample, II - plasmid p35S and III to IV - transformed samples; *C* to *E* - actively dividing GFP-expressing regenerated meristem tissues; *F* - weak GFP- expressing cells are observed on the leaf; *G* - a transformed GFP-expressing plantlet; *H* - regenerated transformed plantlets.

the transgenes inserted had influenced the regeneration of the transformed tissues as mild GFP toxicity was reported in some plant species (Haseloff and Amos 1995, Haseloff *et al.* 1997). In our study, the strongly GFP-expressing tissues turned brown (Fig. 1*C*) which might be due to the mild GFP toxicity. Nevertheless, the fluorescent shoots were successfully obtained in the hygromycin-free medium and were transferred to the selection medium containing higher concentration of hygromycin (30 mg dm^{-3}) again to further eliminate the potential escapes. Yu *et al.* (2000) and Suwanaketchanatit *et al.* (2007) applied the same approach in which the selection pressure was increased gradually to select the transformed *Dendrobium* hybrids.

The presence of the *uidA*, *sgfp* and *hptII* genes in the transformed tissues was verified using PCR. PCR was commonly used to detect the presence of the transgenes (Chai *et al.* 2007, Purkayastha *et al.* 2010). Approximately 41 putative transformed tissues survived on the selection medium were randomly chosen and screened using PCR. From the results obtained, specific DNA fragments of *uidA* (789 bp), *sgfp* (750 bp) and *hptII* (500 bp) were successfully amplified (Fig. 1*D*). The PCR results showed 66 % of the samples were GFP positive, 98 % hygromycin positive and 100 % GUS positive from the 41 samples investigated. The PCR results indicated high frequency of co-transformation event in which *sgfp* gene carried by p35S and *uidA* and *hptII* genes carried by pSMDFR were successfully introduced into the transformed tissues. Incorporation of fragmented genes from a plasmid vector during or after the bombardment process was reported in transforming orchids using bombardment (Nan and Kuehnle 1995, Yang *et al.* 1999, Men *et al.* 2003). Plasmid DNA might be sheared during bombardment and resulted in separate integration and deletion of the transgenes (Men *et al.* 2003). Various co-transformation frequencies were obtained from different studies, 48 % for grapevine (Vidal *et al.* 2003), 66 % for wheat (Rasco-Gaunt *et al.* 2001) and 96 % for sugarcane (Elliott *et al.* 1999). Dot blot and Southern blot analyses carried out using genomic DNA and PCR products (Fig 2*A*,*B*) showed the integration of the *sgfp* gene in the transformed plantlets. In general, GFP-expressing tissues were observed in the actively dividing tissues such as the meristem regions (Fig. 2*C*,*D*,*E*) and only faint fluorescent cells were observed in developed leaves (Fig. 2*F*,*G*). It is commonly agreed that the developed chlorophyll in fully developed transgenic plantlets can obscure the GFP observation as also observed by Ghorbel *et al.* (1999). In conclusion, co-transformation was achieved for *Dendrobium* Sonia 17 by co-bombarding two plasmids into callus tissues and the transformed lines were selected using hygromycin and GFP.

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