J. Trop. Agric. and Fd. Sc. 44(2)(2016): 265 – 276

Assessment of co-transformation technique for Malaysian orchid variety (*Dendrobium* Savin White) with cymbidium mosaic virus coat protein gene

S. Rogayah[1], K.H. Nasir[1], W.C. Yeong[1] and V. Pillai[1]

[1]Biotechnology and Nanotechnology Research Centre, MARDI Headquarters, Persiaran MARDI-UPM, 43400 Serdang, Selangor, Malaysia

Abstract
Enzyme linked immunosorbent assay (ELISA) was conducted to test 300 samples of orchid plants, collected from commercial orchid nurseries around Malaysia. The orchid samples were either apparently healthy or exhibited virus-like symptoms. ELISA results showed that 88% of the samples were infected with cymbidium mosaic virus (CyMV) including the orchid samples that did not exhibit any viral symptoms and looked healthy in appearance. Shoot tips from virus free plants were used to induce protocorm-like bodies (PLBs). The generated PLBs were bombarded with gold particle coated with plasmid pRQ6 and pJIT.CyMVcp containing *hygromycin phosphotransferase* (*hpt*II) selectable marker gene and CyMVcp gene respectively. A total of 500 PLBs were bombarded and after 5 months selection in hygromycin media, 63 putative transgenic lines were recovered. Based on polymerase chain reaction analysis (PCR) on putative transgenic lines, 53 showed positive for the presence of *hpt*II and CyMVcp genes. This study revealed that co-transformation of *hpt*II and CyMVcp genes in two different vectors manage to achieve 10.6% transformation efficiency. The transgenic orchid plants were established in pots and acclimatised in the greenhouse. The transgenic plants were found morphologically similar compared to untransformed plants.

Keywords: *Dendrobium* Savin White, cymbidium mosaic virus coat protein gene, protocorm-like bodies, co-transformation, particle bombardment

Introduction

Orchid is an important floriculture crop in Malaysia and commercially produced for cut flower for export market. One of the leading varieties planted is *Dendrobium* Savin White. The flower is very much in demand for cut flower in both local and international markets. Orchids can be infected by a number of different plant viruses and one of the most important virus infecting cultivated orchids in Malaysia is cymbidium mosaic virus (CyMV). CyMV infection occurs worldwide in all genera, species and hybrids of orchids (Brunt et al. 1996).

The virus can be transmitted between plants during handling or harvesting, through sap and pollen. Orchids infected with CyMV exhibited various symptoms, or can also be symptomless (Wong et al. 1989). Viruses may affect plant growth and reduce flower quality due to striping and flecking (Inouye 2008). The virus infection caused yellow leaves symptom which then forms a mosaic pattern followed by blackening and death of that area of the leaf along the leaf margins (Van Dun et al. 1988). Even though good handling and harvesting practices are able to control spread of the virus disease, development of a virus resistant variety still the most effective way to overcome the problem. Unfortunately, conventional breeding for virus resistance has proved to be long, difficult and costly.

Article history
Received: 27.3.13
Accepted: 9.6.15

Authors' full names: Rogayah Sekeli, Khairun Hisam Nasir, Wee Chien Yeong and Vilasini Pillai
E-mail: lynn@mardi.gov.my

Hence, genetic engineering could serve as an alternative approach which allows insertion of genetic materials from foreign species to develop virus resistant plants. Genetic engineering in orchid has been successfully demonstrated and mainly focused on flower colour and quality production (Kuehnle and Sugii 1992; Chia et al.1994; Nan and Kuehnle 1995). Various reports have shown the success of using biolistic method for gene transfer in *Dendrobium* orchids. Developments of *Dendrobium* resistant to CyMV have been actively carried out by many researchers worldwide. In 2009, Obsuwan et al. transformed a mutant movement protein gene, *mut11* into two different *Dendrobium* hybrids via biolistic method. Their results showed that out of 24 transgenic lines obtained, nine transgenic lines were free from CyMV after 12 months inoculated with CyMV. Another study on transformation of *Dendrobium* variety was carried out by Chang et al. (2005), where the coat protein gene was transformed using biolistic method. When the transgenic plants challenge with CyMV, they exhibited considerably milder symptoms. Effective control of CyMV virus disease allows orchid growers to improve the quality and quantity of orchid production. This study was aimed to develop CyMV resistant *Dendrobium* Savin White using biolistic bombardment method.

Materials and methods

Plant material

A total of 300 samples of *Dendrobium* Savin White orchid were collected from commercial orchid nurseries in Malaysia. The orchid samples collected that either apparently healthy or exhibited virus-like symptoms were subjected to enyzyme linked immunosorbent assay (ELISA) test. This test was carried out according to the manufacturer's instruction (Agdia, Indiana, USA). Both positive and negative controls were included in this test. Result of the test was based on colour development of the samples after 2 h of incubation at 25 ± 2 ºC and quantified by the absorbent reading obtained using microplate ELISA reader.

Induction of protocorm-like bodies

Shoot tips excised from the non-infected orchids were used as explants for induction of protocorm-like bodies (PLBs). The excised shoot tips were dried for 3 days on working bench before undergone a pre sterilisation step by soaking them in 1% (v/v) decon and rinsed with running tap water for three times. Subsequently, the explants were surface sterilised in the laminar air flow chamber using 80% (v/v) ethanol for 30 s, followed by 10% (v/v) clorox for 10 min and 5% (v/v) clorox for 5 min. The explants were then rinsed three times (5 min each) with sterile distilled water.

After the surface sterilisation step, meristemic region of the shoot tip was isolated and washed with 1% (v/v) clorox solution for 1 min, followed by rinsing with sterile distilled water for three times. Shoot tips were then cultured in Vacin and Went liquid medium (Vacin and Went 1949) supplemented with 20% (v/v) coconut water and 2% (w/v) sucrose. The pH medium was adjusted to 5.2 prior to autoclaving at 121 °C and pressure 15 psi for 15 min. The cultures were agitated on a rotary shaker at 120 rpm, 25 ± 2 °C and 16 h illumination provided by fluorescent tubes. The produced PLBs were subcultured monthly in the same liquid medium for further proliferation. The subculturing process was carried out repeatedly to obtain sufficient amount of PLBs for transformation work. PLBs of 6-month old were used as target tissues for bombardment.

Transforming protocorm-like bodies

Transformation vectors

Cymbidium mosaic virus coat protein (CyMVcp) gene was cloned into pJIT vector in sense orientation resulting pJIT.CyMVcp (*Figure 1*). The inserted gene was driven by the cauliflower mosaic virus 35S promoter but without a selectable marker gene in the vector. Another vector, pRQ6, contains selectable marker gene, *hpt*II which is conferring resistance to antibiotic hygromycin. This selectable marker gene also controlled by the cauliflower mosaic virus 35S promoter. Both vectors were used for co-transformation of *Dendrobium* Savin White orchid using biolistic bombardment.

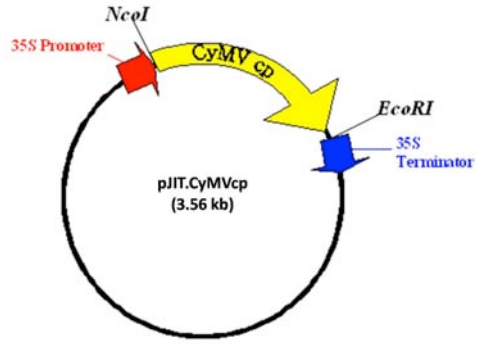

Figure 1. CyMV coat protein gene in pJIT117

Preparation of gold microparticle

Plasmid DNA was coated onto the gold micro particles according to Klein et al. (1987). Gold microparticles weighing 0.012 g (1.0 µm in size) was washed with 200 µl cold ethanol and vigorously vortexed for 2 min, followed by spinning down at 10,000 rpm for 4 s at room temperature. Subsequently, ethanol was discarded and the gold microparticles were resuspended in 200 µl cold sterile distilled water. The suspension was vortexed again for 2 min and spinned down at 10,000 rpm for 6 s. The pellet obtained was then added with 200 µl cold sterile distilled water for further use.

Plasmid DNA (10 µg) was added to 100 µl washed gold microparticles followed by 40 µl of 0.1 M spermidine. Then, 100 µl of 2.5 M $CaCl_2$ was added drop by drop. The mixture was vortexed at 8,000 rpm for 1 min and incubated at room temperature for 10 min. Subsequently, the mixture was vortexed for another 2 min and spinned down at 10,000 rpm for 6 s. The supernatant was then discarded. The plasmid DNA coated with gold microparticles were resuspended with 100 µl cold absolute ethanol. A total of 6 µl of the suspension was dispensed onto macrocarrier and left to dry in laminar air flow before bombardment.

Explant preparation for bombardment

PLBs were prepared following the protocol as described by Vilasini et al. (2000). PLBs of 6-month old were subcultured onto Vacin and Went fresh liquid medium 1 week prior to bombardment. Before bombardment, the PLBs were squashed using sterilised spatula and placed onto osmoticum (OSM) medium for 2 h. The osmoticum medium consisting of half-strength Murashige and Skoog (MS) basal salts medium (Murashige and Skoog 1962) supplemented with 100 mg/l myo-inositol, full-strength MS vitamin, 36 g/l sorbitol, 36 g/l mannitol, 2 mg/l 2, 4-dichlorophenoxyacetic acid (2,4-D) and 0.30% (w/v) gelrite. The pH of the medium was adjusted to 5.8 prior to autoclaving.

Bombardment parameters

Biolistic TM PDS/1000 Helium System (BioRad, USA) was used in this study. The biolistic device parameters used were as follows: rupture disk helium pressure 1100 psi with the microcarrier gap and the target distance combination at 1,4, vacuum pressure 26 inches of mercury (in Hg) and 1.6 µm gold microparticles. A total of two bombardments were carried out for each plate and a total of approximately 500 PLBs were bombarded. Control PLBs were bombarded with non-coated gold

microparticles. A day after bombardment, the bombarded PLBs were transferred into half-strength MS liquid medium for 60 days, with shaking at 120 rpm to induce proliferate PLBs before subjected to antibiotic selection.

Selection and regeneration of transformed tissue

The proliferated PLBs were subjected to antibiotic selection in half-strength MS liquid medium supplemented with 100 mg/l myo-inositol, full-strength MS vitamin, 2% (w/v) sucrose and hygromycin (ranged from 30 to 50 mg/l). The selection process was carried out for a duration of 5 months. Selection process was carried out in selection media consisted 30 mg/l hygromycin for the duration of 1 month followed by increasing the hygromycin concentration to 40 mg/l for another one month and finally increased to 50 mg/l hygromycin for another 3 months. Half-strength MS liquid medium without hygromycin was used as control medium. The survived putative transgenic PLBs were then transferred onto regeneration medium consisted of half-strength MS solid medium supplemented with 50 mg/l mio-inositol, half-strength MS vitamin, 2% (w/v) sucrose, 0.1% (w/w) charcoal and 0.30% (w/v) gelrite for further growth. Sub-culturing onto new fresh media was carried out at one month interval. The rooted putative transgenic plantlets were then planted into plastic pots containing mixture of crushed bricks, charcoal and sphagnum moss at a ratio of 1:1:1. The plants were acclimatisation in a temperature controlled growth chamber (28 ± 2 ºC) for 1 month before planted in transgenic glasshouse.

Polymerase chain reaction analysis of putative transformed plantlets

Polymerase chain reaction (PCR) was carried out using the TPersonal Thermocycler (Biometra GmbH, Goettingen Germany) to analyse the presence of CyMVcp and *hpt*II genes in putative transformed plantlets. Genomic DNA of young leave was extracted using the Qiagen kit (Qiagen, Hilden, Germany) with a starting material of 100 mg for each sample. A total of 50 ng of each extracted genomic DNA sample was used for the PCR analyses. PCR reaction mixture was carried out in a total volume of 20 µl containing template DNA (50 ng), 1X PCR buffer, 10 mM of each dNTPs, 1U of ampliTaq DNA polymerase (Fermentas) and 10 pmol of each forward and reverse primers. Specific primers CyMV1F: 5'-GGTGTGGAATCTGATGCT-3' and CyMV2R: 5'-GTAGGGGGTGCAGGCAGAG-3' were used to detect CyMVcp gene, while H3: 5'-GGGGGGTCGGTTTCCACTA-3' and H4: 5'-ATCGTTATGTTTATCGGGACTTTG-3' were used to amplify *hpt*II gene.

The amplification program started with 5 min at 94 °C, followed by 35 cycles of 30 s denaturation at 94 °C, 30 s primer annealing at 55 °C and 30 s extension at 72 °C for the detection of CyMVcp and *hpt*II genes, respectively. The amplification program ended with 10 min extension at 72 °C. Each PCR amplified products were subjected to 1% (w/v) agarose gel electrophoresis analyses. Amplified products were visualised under UV light and photographed using the Gel Doc XR System equipment (Bio Rad, Corston, United Kingdom).

Results and discussion

Screening for virus free mother plants

ELISA test of total of 300 *Dendrobium* Savin White orchid plants that were randomly collected from several orchid nurseries showed that 88% of the orchid samples were infected with CyMV, included of the samples that did not exhibit any viral symptoms and looked healthy in their appearance (*Plate 1A*). This indicated that

Dendrobium Savin White orchid was highly susceptible to CyMV infection. The viral infection may not affect plant growth and causing plant death immediately. However, it can affect flower quality, causing market value of cut flower to drop and indirectly affecting orchid growers' income.

All CyMV tested positive plants were discarded, meanwhile the identified virus free plants were kept in the glasshouse for further used for PLBs induction in this study (*Plate 1B*). Shoot tips excised from these virus free plants were used as explants for PLBs induction. It was reported that CyMV is very stable and able to be transmitted from infected to healthy plants through tools and the pots used (Abouhaider et al. 1999). Therefore, during the sampling of shoot tips for PLBs induction, separate tools such as blade and container were used for excision and collection of shoot tip from each plant. This is an important precaution to avoid any virus infection from occurring although all the used plants in this study were identified as virus free.

Induction of protocorm-like bodies

PLBs are widely used as target tissues in orchid transformation study (Sreeramanan et al. 2008). PLBs proliferate rapidly and easily regenerate into complete plantlets. In orchid industry, most of the commercially important orchids were mass propagated through PLBs (Tokuhara and Mii 2001). PLBs can be induced from various parts of the explants such as shoot tips, callus, leaves and flower stalk buds (Chong et al. 2010). In this study, PLBs were induced by culturing the shoot tip in Vacin and Vent liquid medium. It was reported that the formation of large scale PLBs from *Dendrobium* species was efficiently achieved by using shoot tip as an explant (Malabadi et al. 2005).

Formation of PLBs were observed after 2 to 3 months of shoot tip inoculation. However, more than 30% of shoot tip explants turned brown and some became necrotic finally died. At the initial stage of inoculation, the cultured explants normally produced leaves which were then had been removed. Generally, multiplications of PLBs were observed after 6 months in culture and the PLBs were subsequently transferred to Vacin and Went liquid media for further proliferation (*Plate 1C*). Addition of coconut water in the culture media promoted the proliferation of PLBs. Besides, supplemented of coconut water was also reported to increase the regeneration efficiency of PLBs into healthy intact plantlets (Pavallekoodi et al. 2012). The subculturing process was carried out repeatedly to obtain sufficient amount of PLBs for transformation work. All PLBs produced were re-tested to be sure they were virus-free before used as target tissue for particle bombardment transformation work. The ELISA results showed that all (100%) of the PLBs produced were free from CyMV.

Transformation and regeneration of transformed tissue

PLBs were successfully transformed with CyMVcp and *hpt*II genes using biolistic method (*Plate 1D*). Co-transformation was carried out to transform both CyMVcp and *hptII genes* in two different vectors (pJIT and pRQ6) since there is no selectable marker gene in pJIT vector. Both of these vectors using a cauliflower mosaic virus 35S promoter since a strong constitutive promoter is needed to drive the gene expression to obtain virus resistant plants. *Figure 2* shows the nucleotides and deduces amino acid sequences of isolated CyMVcp gene that was registered in NCBI under accession number AJ42827. pRQ6 construct that harbouring *hpt*II gene was co-transformed because hygromycin had been reported as the best selective agent for *Dendrobium* orchid (Porntip et al. 2007). Pre-treatment of the PLBs with osmoticum

medium is required to plasmolyse the target cells in order to reduce damage during biolistic bombardment (Brettschneider et al. 1997). In this study, the elevation of osmotic pressure was obtained by addition of sorbitol and mannitol, 36 g/l each respectively. Prior to the selection process, the bombarded PLBs were cultured for a period of 60 days into half-strength MS liquid growth medium for recovery of injured cell caused by microparticles penetration and also to promote active proliferation of the PLBs (Yang et al. 1999). Cell injury due to bombardment process can produce large amount of phenolic compound which can affect the growth of transformed PLBs (Yang et al. 1999). Bonga (1981) reported that, the used of liquid culture reduced the toxicity of the phenolic oxidation products.

Selection of the bombarded PLBs in half-strength MS liquid medium supplemented with hygromycin allowed only the putative transformed PLBs to survive in the selection media (*Plate 1E*). First antibiotic screening process was conducted in liquid medium containing 30 mg/l hygromycin. After 4 weeks of selection, 80% of the bombarded PLBs survived in the selection media but some of the PLBs showed discoloration at the base. The survived PLBs were then continued for second antibiotic screening in medium containing 40 mg/l hygromycin. Survival rate of the bombarded PLBs were further reduced and approximately 50% of the PLBs deteriorated and died. After further 3 months selection on 50 mg/l hygromycin, the untransformed PLBs were totally eliminated and finally a total of 63 putative transgenic lines were recovered (*Plate 1F*). These studies revealed that, medium containing hygromycin at 50 mg/l completely prevent untransformed PLBs to survive, indicating this concentration is effective for selection of transformed PLBs. Gradual exposure selection of transformed cells in increasing concentration of the selective agent has been reported to be beneficial in allowing efficient proliferation and growth of transformed tissues (Ghulam et al. 2007). Such selection process allows the putative transformed tissues to adapt and survive without direct effect of antibiotic.

The survived putative transformed PLBs were then subcultured onto solid hormonal-free MS regeneration media for further growth. In addition, charcoal was added to reduce the potential accumulation of phenolic compound which may affect the plant growth. A total of 63 putative transformed PLBs from *Dendrobium* Savin White were successfully regenerated into plantlets. Multiple shoots were initiated and developed simultaneously. Both transgenic and untransformed plants (control) produced green and healthier leaves. However, the growth and regeneration of putative transformed plants were slower as to untransformed plants. This may due to the effect of too long selection process in antibiotic selection media (5 months). The putative transformed plants required 9 months to regenerate into plantlets while the untransformed plants required only 6 months. Subsequently, root started to produce after the regenerated plantlets reach 3 – 4 cm in height (*Plate 1G*). It was reported that antibiotic and herbicide used for selection of transformed tissue may retard differentiation of adventitious shoot (Ebinuma et al. 1997). Besides, tissue damages caused by bombardment also affected the tissue viability (Perl et al. 1992).

Putative transformed plantlets with good root system, having at least four lateral branches, were individually planted into pots, containing a mixture of crushed bricks, charcoal and sphagnum moss (*Plate 1H*). The pots were then covered with a clear plastic bag punched with 22 holes, and subsequently left in a temperature controlled growth chamber at 28 ± 2 °C until the plants were ready to be transferred into transgenic glasshouse. Low survival rate of rooted

putative transformed plants were obtained during acclimatisation process. The results showed that, only 30% of the transferred plants were survived from a total of 200 putative transgenic plants. Low survival rate of the acclimatised putative transformed plants may due to poor root quality that resulted in poor adaptation during acclimatisation. It was also observed that, plants with shoot and stumpy roots did not survive when transferred into pot. The acclimatisation process need to be further improved to ensure successful transfer of larger number of putative transformed plants for evaluations. In general, growth performance of the untransformed plants was found more vigorous compared to the putative transformed plants. Nevertheless, the putative transformed plants were morphologically similar to untransformed plants in terms of their stem colour, leaf shape and leaf colour.

Analysis of putative transgenic lines

The growth of putative transformed plants in selection media is not a conclusive evidence for successful production of transgenic plants. Therefore, molecular analysis is essential to confirm the integration of the transgene(s) in host plant genome. Hence, PCR analysis was carried out on young leaves of 63 regenerated putative transformed plants to confirm the presence of the transgenes in the genome of these putative transformed orchids. *Plate 2a* and *b* showed the PCR results using *hpt*II and CyMVcp gene specific primers, respectively. A total of 63 independent putative transformed orchid plants were analysed and 53 transgenic lines were tested positive for the presence of both *hptII* and CyMVcp genes of which showed by the amplified of DNA size 821 bp and 306 bp respectively. A total of 10 putative transformed lines that survived the selection process did not contain any of the transgenes. Untransformed controls plants did not showed DNA amplification of genes tested.

In overall, the percentage of the putative transformed lines obtained was 12.6% which was based on the total number of PLBs that survived on hygromycin medium after the selection process per total number of transformed PLBs. PCR analysis showed that transformation efficiency achieved 10.6% based on the total number of PCR positives of the transgenes in the host plant genome per total number of PLBs transformed (*Table 1*). Similar transformation efficiency was reported by Yu et al. (1999) on transformation of *Dendrobium* using microprojectile bombardment. The results in this study indicated that two genes from different vectors can be introduced into plant genome by co-bombardment method. However, their resistance to CyMV is not yet validated.

Conclusion

ELISA assay showed that *Dendrobium* Savin White was highly infected with CyMV. This was explained by the detection of CyMV in non-symptomatic plants. A total of 63 transgenic lines were produced and 53 transgenic lines were tested positive for the presence of *hpt*II and CyMVcp genes using PCR technique. This co-transformation is found to be applicable to introduce two genes from different plasmid into *Dendrobium* orchid. However, functional evaluations of the transgene in transgenic plants need to be carried out to confirm the virus resistant characteristic. The new and improved CyMV resistant *Dendrobium* orchid variety will be valuable to both the commercial growers and exporters.

Plate 1. Process of producing transgenic Dendrobium Savin White orchid. A. ELISA assay showing the infected and non infected sample. B. Virus free mother plants in the glasshouse. C. Production of PLBs (6 months-old). D. The bombarded PLBs with CyMVcp and hygromycin genes. E. Selection of transformed tissues. F. Regenerated of putative transformed PLBs. G. Rooted putative trangenic plants and H. Acclimitisation process of putative transgenic plants

```
atg gga gag ccc act cca act cca gct gcc act tac tcc gct gcc gac ccc act tct gca 60
 M   G   E   P   T   P   T   P   A   A   T   Y   S   A   A   D   P   T   S   A  20
ccc aag ttg gcc gac ctg gct gcc att aag tac tca cct gtc acc tcc tcc att gcc acc 120
 P   K   L   A   D   L   A   A   I   K   Y   S   P   V   T   S   S   I   A   T  40
ccc gaa gaa atc aag gcc ata acc caa ttg tgg gtt aac aac ctt ggc ctc ccc gct gac 180
 P   E   E   I   K   A   I   T   Q   L   W   V   N   N   L   G   L   P   A   D  60
acc gta ggt acc gcg gcc att gac ctg gcc cgc gcc tac gct gac gtc ggg gcg tcc aag 240
 T   V   G   T   A   A   I   D   L   A   R   A   Y   A   D   V   G   A   S   K  80
agt gct acc ctg ctc ggt ttc tgc cct acg aaa cct gat gtc cgt cgc gcc gct ctt gcc 300
 S   A   T   L   L   G   F   C   P   T   K   P   D   V   R   R   A   A   L   A 100
ggc aga tct ttg tgg cca acg tca ccc ccc gcc agt ttt tgc gct tac tac gca aaa gtg 360
 G   R   S   L   W   T   S   P   P   A   S   F   C   A   Y   Y   A   K   V   P 120
gtg tgg aat ctg atg ctg gcc act aac gat ccg ccc gcc aac tgg gcc aag gct ggt ttc 420
 V   W   N   L   M   L   A   T   N   D   P   P   A   N   W   A   K   A   G   F 140
cag gag gat acc cgg ttt gcc gcc ttt gac ttc ttc gat gcc gtc gat tcc act gct gcg 480
 Q   E   D   T   R   F   A   A   F   D   F   F   D   A   V   D   S   T   A   A 160
ctg gag cct gct gaa tgg cag cgc cgc ccg act gac cgt gaa cgt gct gcg cac tcg atc 540
 L   E   P   A   E   W   Q   R   R   P   T   D   R   E   R   A   A   H   S   I 180
ggg aag tac ggc gcc ctt gcc cgt cag cgt atc caa aac ggc ggc ctc atc acc aac att 600
 G   K   Y   G   A   L   A   R   Q   R   I   Q   N   G   G   L   I   T   N   I 200
gcc gag gtc aac cag ggg cca tct tgg tcc acc aac act ctc aat gct ctg cct gca ccc 660
 A   E   V   N   Q   G   P   S   W   S   T   N   T   L   N   A   L   P   A   P 220
```

Figure 2. The nucleotides and deduce amino acid sequences of isolated CyMVcp gene with accession number AJ42827

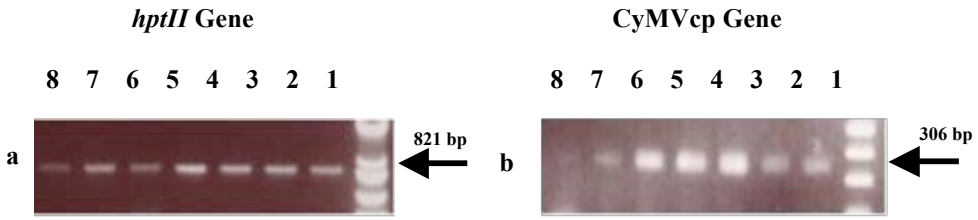

Plate 2. PCR analysis of transgenics Dendrobium Savin White orchid with hptII (821bp) and sense CyMVcp (306bp) genes. Lane 1: GeneRulerTM 1 kb DNA Ladder (Fermentas), Lane 2 to 7 are different line of the transformed orchid, Lane 8: positive control on the plasmid used for transformation. Arrow indicates the transgene

Table 1. Transformation efficiency of *Dendrobium* Savin White by co-transformation method

Experiment	Total no. of PLBs transformed	No. of putative transgenic lines obtained	No. of plants consists gene of interest	No. of regenerated transgenic plants
1	100	13	11	11
2	100	10	9	9
3	100	12	12	12
4	100	13	11	11
5	100	15	10	10
Total	500	12.6 % (of total PLBs transformed	10.6 % (of total PLBs transformed	100 % (of total positive transgenic lines obtained

Acknowledgements

This research was supported by IRPA grant No: 01-03-03-0117-EA001, Ministry of Science, Technology and Innovation, Malaysia.

References

Abouhaider, M.G. and Gellatly, D. (1999). Encylopedia of virology, 2nd edition (Granoff, V. and Webster, R.G., eds.), p. 1364 – 1368, San Diego: Academic Press

Bonga, J.M. (1981). Organogenesis *in vitro* of tissues from mature conifers. *In Vitro* 17: 511 – 518

Brettschneider, R., Becker, D. and Lorz, H. (1997). Efficient transformation of scutellar tissue of immature maize embryo. *Theor. Appl. Genet*. 94: 737 – 748

Brunt, A.A., Crabtree, K., Dallwitz, M.J., Gibbs, A.J., Watson, L. and Zurcher, E.J. (1996). Plant Viruses Online: Descriptions and Lists from the VIDE Database. URL: http://biology.anu.edu.au/Groups/MES/vide/.

Chang, C., Chen, Y.C., Hsu, Y.H., Wu, J.T., Hu, C.C., Chang, W.C. and Lin, N.S. (2005). Transgenic resistance to Cymbidium mosaic virus in *Dendrobium* expressing the viral capsdi protein gene. *Transgenic Research* 14: 41 – 46

Chia, T.F., Chan, Y.S. and Chua, N.H. (1994). The firely luciferase gene as a non-invasive reporter for *Dendrobium* transformation. *Plant Journal* 6: 441 – 446

Chong, S.T., Ching, Q.W., Xui, L.L. and Mahmood, M. (2010). Preliminary study of protocorm like-bodies (PLBs) induction using leaf explants of *Vanda* and *Dendrobium* orchids. *Asia Pacific Journal of Molecular Biology and Biotechnology* 18(1): 189 – 191

Ebinuma, H., Sugita, K., Matsunaga, E. and Yamakado, M. (1997). Selection of marker-free transgenic plants using the isopentenyl transferase gene. *Proceedings of the National Academy of Sciences of the United States of America* 94: 2117 – 2121

Ghulam, K.A.P., Na'imatulapidah, A.M., Alizah, Z. and Omar, A.R. (2007). Determination of minimal inhibitory concentration of selection agents for selecting transformed immature embryos of oil palm. *Asian Pacific Journal of Biotechnology* 15(3): 133 – 146

Inouye, N. (2008). Viruses of orchids, symptoms, diagnosis, spread, and control, Zuid: Blue bird publishers

Klein, T.M., Wolf, E.D., Wu, R. and Sanford, J.C. (1987). High-velocity microprojectiles for delivering nucleic acids into living cells. *Nature* 327: 70 – 73

Kuehnle, A.R. and Sugii, N. (1992). Transformation of *Dendrobium* orchid using particle bombardment of protocorm. *Plant Cell Report* 11: 484 – 488

Malabadi, R.B., Mulgund, G.S. and Kallappa, N. (2005). Micropropagation of *Dendrobium* nobile from shoot tip sections. *Journal Plant Physiology* 162: 473 – 478

Murashige, T. and Skoog, F. (1962). A revised medium for rapid growth and bioassay with tobacco tissue culture. *Plant Physiology* 15: 473 – 497

Nan, G.L. and Kuehnle, A.R. (1995). Factor effecting gene delivery by particle bombardment of *Dendrobium* orchid. *In Vitro Cellular & Developmental Biology-Plant* 31: 131 – 136

Obsuwan, K., Hieber, D.A., Mudalige-Jayawickrama, R.G. and Kuehnle, A.R.A. (2009). Dysfunctional CymMV movement protein confers resistance to CymMV in *Dendrobium* Orchid. *Acta Horticulturae* 836: 149 – 154

Pavallekoodi, G., Ranjetta, P., Maziah, M., Mohd. R. S. and Sreeramanan, S. (2012). Effects of complex organic additives on improving the growth of PLBs of Vanda Kasem's Delight. *Australian Journal of Crop Science* 6(8): 1245 – 1248

Perl, A.H., Kless, A., Blumenthal, G., Galili and Galun, E. (1992). Improvement of plant regeneration and GUS expression in scutellar wheat calli by optimization of culture conditions and DNA microprojectile delivery procedures. *Molecular and General Genetics* 235: 279 – 284

Porntip, A., Sumontip, B. and Piyada, T. (2007). *Agrobacterium*-mediated transformation of *Dendrobium* sundum (Bl.) Lindl with Antisense ACC Oxidase. *Asian Journal of Plant Science* 6(7): 1065 – 1071

Sreeramanan, S., Vinod, B., Sashi, S. and Xavier, R. (2008). Optimization of the transient-gusA gene transfer of *Phalaenopsis* violacea orchid via *Agrobacterium tumefaciens*: An assessment of factors influencing the efficiency of gene transfer mechanisms. *Advances in Natural and Applied Sciences* 2: 77 – 88

Tokuhara, K. and Mii, M. (2001). Induction of embryogenic callus and cell suspension culture from shoot tips excised from flower stalk buds of *Phalaenopsis (Orchideceae). In Vitro Cellular and Developmental Biology-plant* 37: 457 – 461

Vacin, E. and Went, F. (1949). Some pH changes in nutrients solutions. *Botanical Gazette* 110: 605 – 613

Van Dun, C.M.P., Overdium, B., Van Vloten-Doting, L. and Bol, J.F. (1988). Transgenic tobacco expressing tobacco streak virus or mutated alfalfa mosaic virus coat protein does not confer cross protection against alfalfa mosaic virus infection. *Virology* 167: 649 – 642

Vilasini, P., Abd Hamid, A.H., Siti Shaleha, T., Mohd Shaib, J. and Umi, A.B. (2000). An efficient transformation system for Malaysian orchids. *Proceedings of the 12th national biotechnology seminar, 12 – 15 Nov. 2000,* Perak, p. 421 – 422

Wong, S.M., Soo, F., Ching, C.G. and Lim, G. (1989). Cymbidium mosaic virus and Odontoglossum ringspot virus in commercial orchids in Singapore. (Abstr.) *Phytopathology* 79: 1149 – 1150

Yang, J., Lee, H.J., Shin, D.H., Oh, S.K., Seon, J.H., Paek, K.Y. and Han, K.H. (1999). Genetic transformation of Cymbidium orchid by particle bombardment. *Plant Cell Report* 18: 978 – 984

Yu, Z.H., Chen, M.Y., Nie, L., Lu, H.F., Ming, X.T., Zheng, H.H., Qu, L.J. and Chen, Z.L. (1999). Recovery of transgenic orchid plants with hygromycin selection by particle bombardment to protocorms. *Plant Cell Tissue and Organ Culture* 58: 87 – 92

Abstrak

Enzyme-linked immunosorbent assay (ELISA) telah dijalankan untuk menguji 300 sampel pokok orkid yang diambil daripada beberapa nurseri orkid komersial di Malaysia. Sampel orkid adalah yang sihat atau yang mempamerkan simptom virus. Keputusan ELISA menunjukkan 88% sampel orkid *Dendrobium* telah dijangkiti dengan virus cymbidium mosaic (CyMV). Ini termasuklah sampel orkid yang tidak menunjukkan sebarang simptom dan kelihatan sihat. Bahagian pucuk daripada tumbuhan yang bebas virus digunakan untuk menghasilkan jasad seperti protokom (PLBs). PLBs yang terhasil ditembak dengan plasmid pRQ6 yang mengandungi gen penanda *hygromycin phosphotransferase* (*hpt*II) dan plasmid pJIT.CyMVcp yang mengandungi gen CyMVcp. Sejumlah 500 PLBs telah ditembak dan selepas 5 bulan proses pemilihan dalam medium yang mengandungi hygromycin, 63 barisan putatif transgenik berjaya diperoleh. Berdasarkan analisis tindak balas berantai polimer (PCR) ke atas titisan pokok yang putatif transgenik, 53 titisan menunjukkan positif kehadiran gen *hpt*II dan CyMVcp. Kajian ini menunjukkan bahawa kaedah ko-transformasi antara gen penanda *hpt*II dan gen CyMVcp dalam vektor yang berlainan berjaya menghasilkan kecekapan transformasi sebanyak 10.6%. Pokok transgenik dipindahkan ke dalam pot dan diaklimitasi di dalam rumah hijau. Pokok transgenik mempunyai morfologi yang serupa dengan pokok yang tidak ditransformasi.