High-frequency genetic transformation of *Phalaenopsis amabilis* orchid using tomato extract-enriched medium for the pre-culture of protocorms

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**SUMMARY**

The development of an efficient methodology for the genetic transformation of orchids is needed in order to support the genetic engineering of orchids. It is therefore important to identify those factors affecting the transformation process. Previously, we reported a convenient method for the transformation of *Phalaenopsis amabilis* using *Agrobacterium tumefaciens*, in which intact protocorms were used. We also found that embryos cultured on a medium containing tomato extract grew more rapidly than those cultured on a medium with coconut water. When we used protocorms grown on a medium containing tomato extract, we obtained regenerated shoots that had been transformed with a kanamycin resistance gene at relatively high frequencies (7 – 17%). These results suggest that the rate of growth of pre-cultured protocorms may be important for the successful regeneration of transformed shoots. We also obtained regenerated shoots that had been transformed with the green fluorescent protein (GFP) gene at a high frequency (10 – 14%). Both the presence and expression of these transgenes were confirmed in transformed plants by molecular analyses and by the detection of green fluorescence following excitation with blue light.

It is important and of practical value for the orchid industry to generate novel traits such as improved floral characters to satisfy consumer demand and appreciation for aesthetics and novelty. A common trend in current orchid biotechnology is the application of molecular techniques for orchid improvement, which allows the introduction of desirable traits by introducing specific genes. The core component of the molecular breeding of orchids is the need to create efficient and reproducible gene transformation systems. A reproducible methodology for the genetic transformation of orchids, and better recognition of the factors affecting the transformation process, are needed in order to support this objective. Previous studies have reported orchid transformation either directly through the delivery of marker genes such as those encoding *Escherichia coli* β-glucuronidase (*GUS*) and *Aequorea victoria* green fluorescent protein (*GFP*) into plant cells by particle bombardment (Anzai et al. 1996), or indirectly through the use of *Agrobacterium tumefaciens* (Belarmino and Mii, 2000; Chia et al., 1994; Mishiba et al., 2005; Chan et al., 2005; Sjahril et al., 2006; Sjahril and Mii, 2006). Recently, we have developed a convenient method for genetic modification of *Phalaenopsis amabilis* orchid using *A. tumefaciens* (Semiarti et al., 2007) in which intact protocorms (young orchid seedlings) were used for transformation. This method is also simple, reproducible, and applicable in other species. However, the transformation efficiency was ≤ 2%, and further studies were needed to improve this.

For *in vitro* germination of orchid seed, organic substances such as coconut water and tomato extract are commonly used as media supplements. The presence of anti-oxidants such as vitamin C, sugars, and other compounds in tomato extracts could promote the germination and growth of protocorms (Arditti and Ernst, 1993). Perl et al. (1996) determined that a combination of polyvinylpyrrolidone (PVPP) and dithiothreitol (DTT) as anti-oxidants improved plant viability. Tissue necrosis in *Agrobacterium*-treated embryogenic calli of grapevine plants was inhibited*
completely by using these anti-oxidants, while the
virulence of Agrobacterium remained unaffected. These
treatments enabled the recovery of stable transgenic
grapevine plants resistant to hygromycin.

In the present study, in order to improve the frequency of
Agrobacterium-mediated transformation of P. amabilis, we pre-cultured the protocorms on medium
containing an extract from fully-ripe tomato fruit and
investigated the effect of this pre-culturing treatment on
improving the efficiency of regeneration of transformed
shoots.

MATERIALS AND METHODS
Plant material, growth conditions, and culture medium

Adult plants of Phalaenopsis amabilis (L.) Blume
from Java were obtained from Royal Orchids (Prigen,
East Java, Indonesia). Seeds were derived from cross-
pollinated plants that had been sown on a modified, new
Phalaenopsis (NP) medium (Islam et al., 1998) and
maintained under continuous white light. Adult plants
were maintained in a glasshouse at room temperature.
Seeds were sown on modified NP medium with various
concentrations of coconut water (50 – 150 ml l⁻¹) and/or
tomato extract (50 – 200 mg l⁻¹) and grown for 3 weeks to
produce protocorms, which were used for transformation.
Coconut (Cocos nucifera from Java) and the
tomato (Lycopersicon esculentum) cultivar ‘Arthaloka’ from West Java were obtained from local
markets. Tomato fruit extract was prepared by cutting
fruits from ‘Arthaloka’ from West Java were obtained from local
markets. Tomato fruit extract was prepared by cutting
fruits into 1 cm³ cubes, homogenising them, and
filtering the homogenate through a steel mesh with a
150-µm pore size. The nutrient compositions of the
cocnut water sample and the tomato extract were
analysed by high performance liquid chromatography at
the Food and Product Technology Laboratory of the
Faculty of Agricultural Technology of Gadjah Mada
University.

Developmental stages of P. amabilis

To determine the growth rates of orchid embryos and
protocorms, the sizes, colours, and shapes of the embryos
or protocorms were evaluated as described by Dressler
(1981). At Stage 0, each intact seed (270 – 400 µm-long)
with its embryo (100 – 200 µm-long) is coated by a layer
of net-like cells, the testa. At Stage 1, the testa spreads
apart and the embryo swells into an ovoid-shaped mass
of cells. At Stage 2, the seed coat cracks and the mass of
cells grows outside the coat (0.5 – 1.0 mm-long). At Stage
3, the mass of cells elongates gradually into a cone-
shaped body (1.0 – 1.4 mm-long). At Stage 4 (the
protocorm), root hairs emerge from the basal portion of
the cone-shaped body, which turns green. At Stage 5, the
photosynthetic protocorm forms a leafy shoot at its apex
and forms new root hairs. After Stage 5, seed
germination is complete, two leaves gradually emerge
and roots form.

Plasmid vector and bacterial strain

Using the binary plasmid vector pBI121 (Clontech
Laboratories Inc., Otsu, Japan), containing a kanamycin
resistance gene and the 35S CaMV promoter with the
3' nos terminator, a PCR-amplified fragment containing
the entire coding region of the GFP gene was used to
generate a plasmid that we designated pBI121-
p35S::GFP. This construct was introduced into the
disarmed, octopine-type A. tumefaciens strain LBA4404
(Hoekema et al., 1983).

Nucleic acid isolation and purification

Nucleic acids (genomic DNA, total RNA, mRNA, and
cDNA) were prepared according to Semiarti et al.
(2007).

Transformation and transformant regeneration

Transformants were obtained and regenerated using the
methods described by Semiarti et al. (2007). Genomic
DNA from putative 35S::GFP transformants was
analysed by PCR using the following selective forward
(F) and reverse (R) primers to detect both the
kanamycin resistance gene (neomycin phosphotransferase
II; NPTII) and the GFP gene: NPTIIF1 (5’-
CTTGCCCCATTGCACCCCAA-3’) and NPTIIR1 (5’-
AGCCCCCTGTGCTCTTCGTC-3’) for the NPTII
gene; and GFPF1 (5’-ATGGTGAGCAAGGGCGAG
GA-3’) and GFPFR1 (5’-GTCCATGCCGTAGTG
ATCC-3’) for the GFP gene. PCR was performed with 30
cycles of 94°C for 1 min, 60°C for 30 s, and 72°C for 90 s.
As an internal control, genomic DNA was amplified
using primers for the ACTIN gene, as described by
Semiarti et al. (2007). To detect GFP gene expression in
the transformants, seedlings or plant tissues were
excited with blue light (495 nm) using a Nikon Diaphot
300 microscope (Nikon Corp., Tokyo, Japan) equipped
with a B2 filter, which distinguished the red
autofluorescence of chlorophyll from the fluorescence
of GFP. The images were captured using a Nikon Cool
Pix 5000 digital camera system, with adaptor for
microscopy (Nikon Corp.).

DNA analysis by Southern hybridisation

Genomic DNA from 9-month-old leaves of five
independent transgenic lines of P. amabilis that expressed
GFP fluorescence was digested using the restriction
enzymes Eco RI and Hind III. These plants also yielded
the predicted 360-bp PCR product using a primer pair
designed for the GFP coding region. The digested
genomic DNA fragments were transferred to a nylon
membrane (Amersham Hybond-N'; GE Healthcare,
Cambridge, UK) and hybridised with a digoxigenin-
labelled probe for the GFP gene derived from the plasmid
pBI121-GFP (12.6 kbp) using the DIG DNA
Labeling Kit (Roche Diagnostics, Tokyo, Japan). The
hybridised DNA fragments were visualised using the
DIG Luminescent Detection Kit (Roche Diagnostics)
according to the manufacturer's instructions.

RESULTS AND DISCUSSION
Effect of tomato extract on the formation of shoots from
protocorms of P. amabilis

We tested coconut water and tomato extract as
potential supplements to accelerate the growth of
Phalaenopsis embryos, especially at the early
developmental Stages, using embryos grown on NP
medium with or without either supplement.

Based on the growth Stage classification described
above, we determined the optimal concentration of
TABLE I
Growth stages of *P. amabilis* seeds cultured on NP medium supplemented with different concentrations of tomato extract at 21 days after sowing

<table>
<thead>
<tr>
<th>Concentration of tomato extract (mg l⁻¹)</th>
<th>Total no. embryos examined</th>
<th>Experiment No.</th>
<th>Embryo Stage 0 (%)</th>
<th>Embryo Stage 1 (%)</th>
<th>Embryo Stage 2 (%)</th>
<th>Swollen embryo protocorm Stage 3 (%)</th>
<th>Green protocorm Stage 4 (%)</th>
<th>Protocorm with shoot apical meristem Stage 5 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>806</td>
<td>1</td>
<td>23.9</td>
<td>12.2</td>
<td>3.6</td>
<td>20.8</td>
<td>39.1</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>18.7</td>
<td>5.4</td>
<td>7.4</td>
<td>30.5</td>
<td>36.9</td>
<td>1.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>24.4</td>
<td>2.2</td>
<td>4.9</td>
<td>40.1</td>
<td>28.3</td>
<td>0.0</td>
</tr>
<tr>
<td>50</td>
<td>1,148</td>
<td>1</td>
<td>22.2</td>
<td>3.6</td>
<td>4.3</td>
<td>40.2</td>
<td>29.3</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>27.5</td>
<td>6.0</td>
<td>8.8</td>
<td>28.4</td>
<td>28.4</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>28.0</td>
<td>6.9</td>
<td>4.0</td>
<td>28.7</td>
<td>31.5</td>
<td>0.9</td>
</tr>
<tr>
<td>100</td>
<td>1,577</td>
<td>1</td>
<td>24.9</td>
<td>4.7</td>
<td>5.5</td>
<td>13.0</td>
<td>51.1</td>
<td>0.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>31.3</td>
<td>1.9</td>
<td>6.1</td>
<td>9.0</td>
<td>51.2</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>36.0</td>
<td>4.1</td>
<td>2.0</td>
<td>15.8</td>
<td>42.1</td>
<td>0.0</td>
</tr>
<tr>
<td>150</td>
<td>1,417</td>
<td>1</td>
<td>46.0</td>
<td>4.8</td>
<td>3.7</td>
<td>9.5</td>
<td>36.1</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>26.2</td>
<td>1.8</td>
<td>5.6</td>
<td>14.6</td>
<td>51.8</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>35.5</td>
<td>2.9</td>
<td>4.4</td>
<td>15.0</td>
<td>42.3</td>
<td>0.0</td>
</tr>
<tr>
<td>200</td>
<td>1,583</td>
<td>1</td>
<td>49.6</td>
<td>3.1</td>
<td>2.8</td>
<td>9.7</td>
<td>34.7</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2</td>
<td>62.6</td>
<td>7.8</td>
<td>3.6</td>
<td>6.0</td>
<td>20.1</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>52.5</td>
<td>5.7</td>
<td>1.1</td>
<td>17.0</td>
<td>23.7</td>
<td>0.0</td>
</tr>
</tbody>
</table>

FIG. 1
Main Panel A, growth of protocorms of *P. amabilis* on various culture media 3 weeks after sowing. Sub-Panel a, NP medium; sub-Panel b, NP medium supplemented with 150 ml l⁻¹ coconut water (CW); sub-Panel c, NP medium supplemented with 100 mg l⁻¹ tomato extract (TE); and sub-Panel d, NP medium supplemented with 150 ml l⁻¹ CW and 100 mg l⁻¹ TE. Main Panel B, development of shoots from protocorms of *P. amabilis* that had been cultured on NP medium supplemented with coconut water (CW) and/or tomato extract (TE) for 3 weeks. Protocorms were selected from NP medium containing 200 mg l⁻¹ kanamycin after *Agrobacterium*-mediated transformation with pBH21 after 5 weeks. Sub-Panel a, unregenerated protocorms on medium containing 200 mg l⁻¹ kanamycin (Km); sub-Panels b–d, kanamycin-resistant seedlings produced from protocorms that had been transformed with pBH21 containing the kanamycin resistance gene (*NPTII*). Main Panel C, PCR detection of the kanamycin resistance gene (*NPTII*) in putative transgenic orchid plants harbouring pBH21. Fragments from a Sty I digest of λ phage DNA were used as size markers (M). No amplified DNA fragments from the kanamycin resistance gene were seen in DNA from untransformed *P. amabilis* orchid plants (lane 1). The specific 105-bp PCR fragment of the *NPTII* gene was amplified from DNA of putative transgenic orchid seedlings from sub-Panels b (lanes 2–7), c (lanes 8–12), and d (lanes 13–17) in Main Panel A. Bars = 5 mm.
tomo extract based on the number of growing embryos and protocorms found at each Stage (Table I). The number of seeds developing to Stage 4 was increased at higher concentrations of tomato extract in the NP medium, achieving an optimal number at 100 – 150 mg l\(^{-1}\) tomato extract. Therefore, 100 mg l\(^{-1}\) tomato extract was used in the following experiments.

We also analysed growth rates on NP medium with or without coconut water and tomato extract (Figure 1A). The fastest rate of embryo development was observed on NP medium supplemented with both coconut water and tomato extract. Protocorms cultured on NP medium containing tomato extract alone appeared to change from yellow to green more rapidly than those cultured on NP medium containing coconut water alone. Tomato extract thus appeared to affect the growth rate at all Stages of embryo development, including the formation of the shoot apical meristem prior to the emergence of the leaf primordia. The tomato extract contained carotene, vitamin C, and other anti-oxidants which were not detected in coconut water (Table II). These components could affect growth of the embryo.

Oladiran and Iwu (1992) showed that fully-ripe tomato fruit contained basic nutrients and essential vitamins, as well as trace elements. Among these, carotenoids with cyclic end-groups were essential components of all photosynthetic membranes and played several roles, including protection against photo-oxidation (Cunningham et al., 1996). These are potential candidates for the growth-promoting compounds in the tomato extract, as it was rich in carotenoids. We therefore tested a single carotenoid, lycopene, for its possible effects on growth promotion, but found no significant effect at concentrations typically found in tomato extracts (≤ 0.1% (w/w)), while high concentrations of lycopene inhibited seed growth (data not shown). Further studies on other components found in tomato extract are needed to determine whether any single compound has an effect, or several compounds have a synergistic effect, on the growth and development of \emph{P. amabilis} seed.

<table>
<thead>
<tr>
<th>Component</th>
<th>Coconut water (w/w)</th>
<th>Tomato extract (w/w)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ash</td>
<td>0.55%</td>
<td>0.31%</td>
</tr>
<tr>
<td>Lipid</td>
<td>0.05%</td>
<td>0.47%</td>
</tr>
<tr>
<td>Total protein</td>
<td>0.19%</td>
<td>1.76%</td>
</tr>
<tr>
<td>(Soluble protein)</td>
<td>(0.17%)</td>
<td>(1.46%)</td>
</tr>
<tr>
<td>Total carbohydrates</td>
<td>3.22%</td>
<td>3.70%</td>
</tr>
<tr>
<td>(Reducing sugars)</td>
<td>(3.02%)</td>
<td>(3.39%)</td>
</tr>
<tr>
<td>Total carotene</td>
<td>Nd</td>
<td>1.84%</td>
</tr>
<tr>
<td>Anti-oxidants (DPPH)*</td>
<td>Nd</td>
<td>0.024%</td>
</tr>
<tr>
<td>Vitamin C</td>
<td>Nd</td>
<td>0.042%</td>
</tr>
<tr>
<td>Crude fibre</td>
<td>Nd</td>
<td>1.05%</td>
</tr>
<tr>
<td>Phosphate (P(_2)O(_5))</td>
<td>0.013%</td>
<td>0.13%</td>
</tr>
<tr>
<td>Inorganic ions</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mg(^{2+})</td>
<td>0.0085%</td>
<td>0.0081%</td>
</tr>
<tr>
<td>Na(^{+})</td>
<td>0.00021%</td>
<td>0.000092%</td>
</tr>
<tr>
<td>K(^{+})</td>
<td>0.046%</td>
<td>0.000090%</td>
</tr>
<tr>
<td>pH</td>
<td>5.16</td>
<td></td>
</tr>
</tbody>
</table>

*\(\text{DPPH}\), 1,1-diphenyl-2-picrylhydrazyl, an ingredient that prevents oxidation.

The samples used were coconut water from \emph{Cocos nucifera} from Java and an extract from the tomato cultivar \emph{Arthaloka} (\emph{Lycopersicum esculentum}) from West Java. All values are percentages (w/w). All samples contained 91 – 95% (w/w) \(\text{H}_2\text{O}\). Values are based on the average of duplicate samples. Nd, not detectable.

### Molecular analysis of putative transformants

First, we examined the genomic DNA from \emph{P. amabilis} plantlets regenerated on agar plates containing 200 mg l\(^{-1}\) kanamycin for the presence and expression of the kanamycin resistance gene \emph{(NPTII)} using PCR. The predicted 105-bp fragment was amplified from all putative transformants in each treatment (Figure 1C).

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Coconut water</th>
<th>Tomato extract</th>
<th>Total no. protocorms examined</th>
<th>No. protocorms producing shoots (% of total)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>+</td>
<td>-</td>
<td>1.557</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>pBI121 (vector)</td>
<td>+</td>
<td>-</td>
<td>1.200</td>
<td>14 (1.2%)</td>
</tr>
<tr>
<td>pBI121 (vector)</td>
<td>-</td>
<td>+</td>
<td>1.200</td>
<td>159 (13.2%)</td>
</tr>
<tr>
<td>pBI121 (vector)</td>
<td>+</td>
<td>+</td>
<td>1.557*</td>
<td>260 (16.6%)</td>
</tr>
<tr>
<td>pBI121-p35S::GFP</td>
<td>+</td>
<td>+</td>
<td>1.557*</td>
<td>102 (6.8%)</td>
</tr>
<tr>
<td>pBI121-p35S::GFP</td>
<td>+</td>
<td>+</td>
<td>1.500*</td>
<td>210 (13.5%)</td>
</tr>
</tbody>
</table>

*Data are from two independent transformation experiments in each case.

**Effect of pre-culture of protocorms on NP medium containing tomato extract on the transformation frequency of \emph{P. amabilis}**

Protocorms were pre-cultured on NP medium supplemented with coconut water and/or tomato extract, prior to transformation, to determine the effects of pre-culture supplementation on the frequency of transformation (Table III). The transformation efficiency was determined based on the percentage of protocorms that produced shoots on the selective medium out of the total number of protocorms examined. The transformation frequency of regenerated shoots was increased from 1.2% on NP medium with coconut water alone to 13.2% on NP medium containing 100 mg l\(^{-1}\) tomato extract alone, and to between 6.8 – 16.6% on NP medium containing both coconut water and tomato extract (Table III; Figure 1B, Panel D). These results were higher than the frequency of transformed regenerated shoots on medium containing coconut water alone (1.2%; Table III), confirming the observations made by Semiarti et al. (2007).

In the case of transformation with pBI121-p35S::GFP, transformed regenerated shoots were produced at frequencies of 9.8 – 13.5% following pre-culture on NP medium supplemented with both coconut water and tomato extract (Table III). Overall, the transformation frequencies of protocorms pre-cultured on NP medium supplemented with tomato extract alone, or with both coconut water and tomato extract were higher than that of protocorms pre-cultured on NP medium supplemented with coconut water alone, suggesting that the growth rate of protocorms was related to the pre-culture conditions which are therefore important for the regeneration of transformed shoots.

Several studies have examined the use of rich sources of nutrients, vitamins, and phytohormones, including coconut water, carrot, maize, or potato extracts, as possible supplements for stimulating the germination of various orchid species (Arditti and Ernst, 1993; Raghavan, 1997; Islam et al., 2003; Mishiba et al., 2005; Chansean and Ichihashi, 2007). More studies on other sources of nutrients may be required to establish an optimum method for transformation.
The plantlets that regenerated after transformation with the plasmid pBI121-p3SS::GFP were examined for the presence of the GFP gene by PCR amplification of the 360-bp fragment from the GFP coding region (Figure 2B). Of the 210 plantlets examined, 191 were positive for the GFP gene fragment. To confirm the presence of the GFP gene, and to assess the gene copy number in plants that also showed kanamycin resistance, we performed Southern hybridisations. Hybridisation using an anti-sense probe for the 3' end of the GFP gene (Figure 2A) showed two-to-four copies of the GFP gene in each transgenic line (Figure 2C). Since the genomic DNA of each putative transgenic plant showed uniquely-sized bands hybridising to the GFP anti-sense fragment, this T-DNA fragment was confirmed to be inserted into the genome at different independent sites, and in multiple copies in each putative transgenic plant line.

For further analysis, we purified total poly(A)⁺ RNA from individual leaves of an untransformed wild-type plant, a plantlet transformed with pBI121, and three lines transformed with pBI121-p3SS::GFP. We quantified the relative levels of GFP gene transcripts (mRNA) using RT-PCR with the primers specific for GFP. PCR products were detected in all three lines of plantlets transformed with pBI121-p3SS::GFP, but not in the untransformed plantlet or the plantlet transformed with pBI121 alone (Figure 2D). Thus, transcripts of the GFP gene had accumulated in the leaves of the transformants, confirming expression of the GFP transgene in these plants. Plantlets transformed with pBI121-p3SS::GFP showed green fluorescence after excitation with blue light (Figure 2E),
whereas untransformed plantlets did not (Figure 2F).

Taken together, the molecular analyses of the transformants strongly suggests that supplementation using tomato extract during pre-culture in NP medium improved the transformation efficiency of *P. amabilis* several-fold.

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