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Agrobacterium-Mediated Transformation of Indonesian Orchids for Micropropagation

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1. Introduction

Indonesia is the most biodiverse country in the world after Brazil, and its biodiversity is reflected by the vast species of flora found there. The Orchidaceae are one of the largest and most diverse families in the plant kingdom (Arditti, 1992). In Indonesia, orchids are very popular ornamental crops, both as cutting flowers and as potted plants. Many orchid species belonging to the Orchidaceae family can be found only in Indonesia. Most of these species are classified as tropical orchids, which comprise the greatest part of the orchids' diversity. Orchid flowers have fabulous variations based on size, shape, structure, odor, color, and floriferousness.

Indonesian orchids are very unique and exotic. The black orchid (*Coelogyne pandurata* Lindley), endemic to the provinces of East Kalimantan and Papua, is a very important species. The uniqueness of this orchid is in its flowering characteristics, i.e., a very short blooming period (3-5 days) and difficulties with pollination (Arditti, 1992; Wibowo, 2010). Another important Indonesian orchid is the moth orchid (*Phalaenopsis amabilis (L.)* Blume) which has been used as a parent for creating new hybrids. The pandanus orchid (*Vanda tricolor* Lindley) has spread over Java, Bali, and Sulawesi islands. This orchid is quite popular in those regions, not only because of it odor, but also because of its value as an ornamental plant (Figure 1). The destruction of habitat and difficulties in cultivation, however, are threatening these species (Arditti, 1992). Thus, there is a need to counteract the decrease of the populations of these orchids to conserve them as a genetic resource, either using conservation methods both *in situ* and *ex situ* or micropropagation methods.

Micropropagation methods can be applied using biotechnology. Modern biotechnology is important in agriculture, particularly in the economically important horticulture industry, and methods such as genetic transformation have become increasingly important tools for

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A B C

Fig. 1. A. The Black Orchid (*Coelogyne pandurata* Lindley); B. The Moth Orchid (*Phalaenopsis amabilis)*; C. The Pandanus Orchid (*Vanda tricolor var Suavis* Merapi Form), bars: 1 cm.

improving cultivars and studying gene function in plants. This is particularly true in orchids, which are highly valued ornamental plants that are continually being genetically altered. Of the reports published to date on the genetic transformation of orchids, most have focused on transformation techniques targeting specific genes and areas related to crop improvement. We have used the *BREVIPEDICELLUS (BP)/KNAT1* gene, which is a member of the family of class 1 *KNOTTED*-like homeobox (*KNOX)* genes in *Arabidopsis thaliana,* for transformation studies in Indonesian orchids. One reason to use it is that the *KNAT1* gene is required for the maintenance of the indeterminate state of cells during which the plant produces multiple shoots. Micropropagation of orchids conducted by genetic transformation is potentially very valuable. We hope that the *BP/KNAT1* gene can be used to improve shoot formation for mass propagation of these orchids.

2. Orchids are an important horticultural plant

Horticulture, literally "garden cultivation", is a branch of agriculture concerned with cultivation of crops that also includes agronomy and forestry. Traditionally, horticulture deals with garden crops such as fruits, nuts, vegetables, culinary herbs and spices, beverage crops, and medicinal, as well as ornamental plants.

In Indonesia, orchids are very popular ornamental crops, and many people enjoy keeping orchids in their homes as decorative plants. On the other hand, orchids such as Dendrobium, Cymbidium, Eulophia, and Habenaria are used in traditional medicine as restoratives and to treat various diseases (Puri, 1970). The market for orchids is worldwide, providing opportunities to grow orchids not only as a hobby, but also for commercial purposes. Horticulturally important species are clustered into taxonomic sections including Phalaenanthe, Spatulata (Ceratobium), Latourea, Formosae (Nigrohirsutae), Dendrobium (Eugenanthe), and Callista (Schelpe and Stewart, 1990). The breeding of orchids is common since most species have high crossability with other orchids, including hybridization between different genera.

3. Orchid breeding

Enhancing productivity and qualitative traits are the main objectives in general horticultural breeding programs. Common goals in breeding new varieties of orchids mainly concern

flower size, flower shape, floriferousness, flower color, early flowering, compact growth (dwarfing), resistance to pathogens, and flower longevity. However, breeding programs are successful if the products are marketable regarding extrinsic (color, size, shape) and intrinsic (odor, longevity) traits. Breeding of ornamental plants can be achieved by several methods, either through conventional breeding or biotechnology.

3.1 Classical breeding

Classical or conventional breeding methods using crosses, such as intraspecific and interspecific hybridization of orchid species, are a common way to create new varieties. The French hybrid variety registered in 1934, Dendrobium Pompadour (PPPC), is comprised of three chromosome sets of *D. phalaenopsis* and one chromosome set of *D. discolor*. The genome symbols for each Dendrobium section are P and C for Phalaenanthe and Ceratobium, respectively. Those sections are key contributors to both the cut flower and potted varieties of the new hybrid species. Different ploidies can be found within one species within the same family, and so hybridization between them can generate new species, such as in the family Brassicaceae (Figure 2).

Variability in the percentage of viable progeny is generally determined by the genome or cytogenetic relatedness; more similar genome constitutions have more normal meiotic

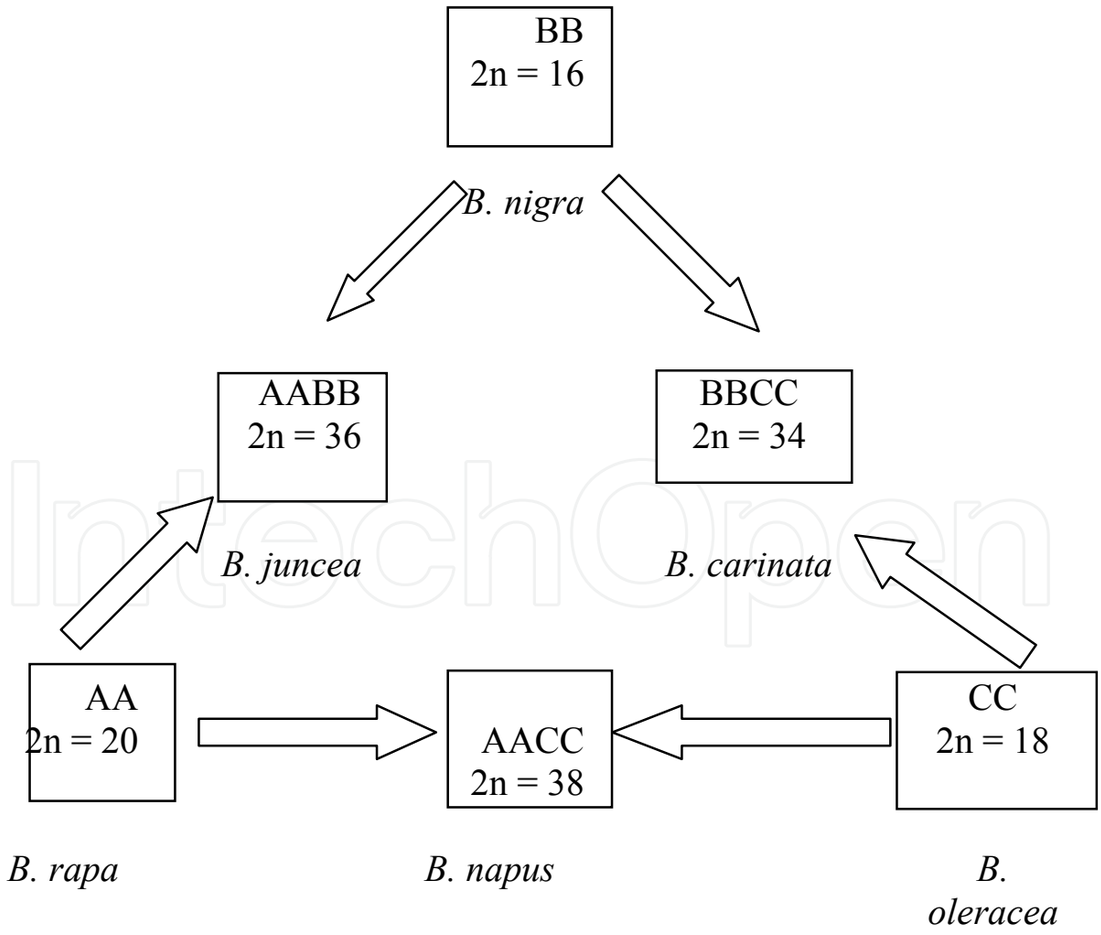


Fig. 2. Genome constitution of species belonging to the genus *Brassica*

pairings (Kanemoto and Wilfret, 1980). The development of new F1 hybrids of orchids allows high flower quality to be achieved, which is an important factor not only for hobbyists but also for commercial purposes.

Creating new varieties can be conducted without hybridization. Polyploidization is the simplest way to induce variability in horticultural plants. Chromosome doubling occurs in existing polyploid plants, but it is more likely to produce a positive effect from a diploid base (Levin, 2002). The induction of chromosome doubling is generally performed *in vitro*. Protocorm-like bodies can be treated with autoclaved 0.1% (w/v) colchicine in orchid seed germination medium for 5, 7, and 10 days at 100 rpm under continuous light before being transferred onto solid medium (Sanguthai et al., 1973).

3.2 Modern breeding through biotechnology

Plant genetic transformation is potentially a powerful tool for orchid breeding as it can break the species barrier and bring in favorable traits from other gene pools that are not easily accessible through traditional breeding techniques. Transformation of orchids has been accomplished mainly using *Agrobacterium*. *Agrobacterium*-mediated genetic engineering is currently the most widely used plant genetic engineering strategy, and was first demonstrated in rice in 1994 (Hiei et al., 1994). The greatest motivation for using *Agrobacterium* appears to be molecular and cytogenetic analyses showing that higher frequencies of transgene loci produced by microprojectile bombardment and other direct DNA delivery methods are more complex compared to transgene loci produced via *Agrobacterium* (Pawlowski and Somers, 1996). Complex transgene loci are associated with problems of transgene expression (Finnegan and McElroy, 1994; Matzke and Matzke, 1995; Pawlowski et al., 1998; Kohli et al., 1999) and inheritance (Christou et al., 1989; Choffnes et al., 2001). In other words, *Agrobacterium*-mediated transformation is a simple and inexpensive method that produces the simplest transgene locus structure. Therefore, this method is readily adopted for plant genetic engineering.

Point of view	Classical breeding	Modern breeding (Biotechnology methods)
Source of gene desired	limited	unlimited
Insertion of gene desired	indirect	direct
Application	easy	difficult
Expected result	unfixed	fixed
Time consumed	longer	shorter

Table 1. Comparison between classical and modern (biotechnology) breedings

Transformation using *Agrobacterium* has become common in horticultural plants such as in lettuce (Michelmore et al., 1987), cabbage (Metz et al., 1995; Jin et al., 2000; Lee et al., 2000), kale (Cogan et al., 2001), orchids (Semiarti et al., 2007 and 2010; Chin and Mii, 2011), and petunias (Klee et al., 1987). With genes that can modulate auxin concentrations, transgenic techniques have been used to manipulate endogenous levels of auxin in plants. Insertion of the *iaa*M gene into petunia causes increased apical dominance and reduced stem growth (Klee et al., 1987). Expression of the *iaa*M gene, if under the control of appropriate gene promoters, can enhance the formation of adventitious roots of cuttings of hard-to-root horticultural crops (Li et al., 2004). Zhang and colleagues (2000) reported that endogenously

produced cytokinin can regulate senescence caused by flooding stress, thereby increasing plant tolerance to flooding. A bacteria ethylene-forming enzyme (EFE), cloned into transgenic plants, causes a high rate of ethylene production compared to untransformed plants (Araki et al., 2000). Dwarf morphology was observed in transgenic tobacco that resembled the phenotype of a wild-type plant exposed to excess ethylene. Dwarf Phalaenopsis orchid plants were also produced by over-expression of the gibberellin 2-oxidase gene (Chin and Mii, 2011). Moreover, it is possible to use transgenic technology to manipulate the kinds of hormones in plants to improve performance, such as stress tolerance, in horticultural crops.

Wakita and colleagues (2001) reported that the nutritional value of sweet potato was improved by transgenic changes to the fatty acid composition. Transgenic tomato plants expressing the ACC deaminase gene withstand flooding stress (low oxygen) better than untransformed plants and are less subject to the deleterious effects of root hypoxia on plant growth (Grichko and Glick, 2001). The transgenic plants have greater shoot fresh and dry weight, produce lower amounts of ethylene, and have higher amounts of leaf chlorophyll content.

During 1998-2001, more than 40 orchid genes were discovered. However, only a few of these sequenced genes could be directly applied to orchid production and improvement (Kuehnle, 2007). Some of the published orchid genes are summarized in Table 2.

Gene name	Gene origin	Reference
DCAC	*Dendrobium crumenatum*	Yang et al., 1996
DOH1	*Dendrobium* Madame Thong-In	Yu and Goh, 2000
DOMAD1	*Dendrobium* Madame Thong-In	Yu and Goh, 2000
Ovg14	*Dendrobium* Madame Thong-In	Yu and Goh, 2000
DSCKO1	*Dendrobium*	Yang et al., 2003

Table 2. Some genes discovered in orchids.

Using the orchid genes or other sequenced genes, genetic transformation of orchids can be conducted using three major approaches including particle-bombardment, direct gene transfer to protoplasts, and *Agrobacterium*-mediated transformation. Several studies regarding orchid transformation are summarized in Table 3.

Species	Transformation method	Reference
Phalaenopsis	*Agrobacterium*-mediated	Semiarti et al., 2007
Phalaenopsis	*Agrobacterium*-mediated	Semiarti et al., 2010
Phalaenopsis	*Agrobacterium*-mediated	Belarmino and Mii, 2000
Phalaenopsis	*Agrobacterium*-mediated	Chin and Mii, 2011
Dendrobium	*Agrobacterium*-mediated	Semiarti et al., 2008
Dendrobium	Particle-bombardment	Kuehnle and Sugii, 1992
Vanda tricolor	*Agrobacterium*-mediated	Semiarti et al., 2009
Cymbidium	Particle-bombardment	Yang et al., 2000

Table 3. Several studies on orchid transformation.

3.3 Cultivation and propagation

Another modern biotechnological procedure in orchid breeding is plant tissue culture, which is widely used for orchid cultivation and propagation. The orchid seed has no

endosperm, so the probability of growing naturally in its habitat is very low, necessitating plant tissue culture. Plant tissue culture techniques use rich nutrient culture medium and aseptic conditions with growth in transparent bottles. The orchid seed obtains its nutrients from the media and can grow normally. The phenotype of the plantlet varies from its parent. If we use explants from leaves, shoots, or other vegetative organs, we can produce identical offspring. As we know, orchids need a long time to reproduce, but using plant tissue culture techniques, we can produce offspring in large quantities in a very short time. This is very useful for breeders and orchid conservationists. Plant tissue culture is also a tool that facilitates genetic engineering, so it is being continuously refined and should be studied by orchid researchers.

3.4 Standard techniques of orchid micropropagation

Several aspects that need to be considered in plant tissue culture are culture conditions, media components, and aseptic conditions. Ideal culture conditions for orchid micropropagation depend on the type of medium, pH, illumination, photoperiod, and temperature. Both liquid and solid media can be used, but proliferation is faster and more extensive in liquid media; however, differentiation is always better in solid media. The optimum pH for orchid micropropagation is approximately 5-7. Suitable illumination for orchid tissue is from darkness to 2000 ft-c (ft-candles). Photoperiods used for orchid tissue culture vary from none (constant illumination or darkness) to short and long days (12-18 hours). Orchid tissue culture is usually maintained at a temperature of 22 to 26°C (Arditti and Ernst, 1993). The medium components for orchid micropropagation are mostly the same as those for other plant culture media. It must contain macro elements (C, H, O, N, S, P, K, Ca, Mg, and Fe), microelements, hormones, myo-inositol, vitamins, amino acids, adsorbents, solidifying agents, and complex organic additives (Arditti and Ernst, 1993; George and Sherrington, 1984).

In principle, all plant cells can be cultured because they all have totipotency and autonomic properties. However, in practice, only meristematic cells can be used as explants for plant tissue culture. Micropropagation of orchids was first performed by German orchid expert Hans Thomale in 1957, who used *Orchis maculata* shoot-tips as explants. The parts of the orchid usually used as explants are the shoot-tip, root-tip, leaf, flower bud and segments, stems, cells, and protoplasts (Arditti and Ernst, 1993). The use of phytohormones in the orchid medium is very important, as different combinations of phytohormones can induce different organs to grow. The most common combination in orchid media is auxin and synthetic cytokinin, while giberelin is very seldom used. Combinations of indole-3-acetic acid (IAA) and indole-3-butanoic acid (IBA) can induce root growth in the *Dendrobium* orchid's protocorm-like body (Khatun et al., 2010). The family of cytokinin-like kinetins, including 6-benzyl amino purine (BAP) and dimethyl allyl phosphate (DMAP), are known to induce shoots (Arditti and Ernst, 1993). Thus, the phytohormones used depend on the aim of the orchid culture.

Orchids, like any other plant cultured in medium, need acclimatization before being planted in a pot. Plant tissue culture uses rich media to support orchid growth, which while good for the orchid, has the effect of limiting its ability to produce food for itself. Acclimatization is needed to adapt the orchid to its new environment and to change it from heterotrophic into autotrophic conditions (Arditti and Ernst, 1993). In their natural habitat, orchids cannot always obtain optimum conditions to grow, so we need to adapt orchids to their new environment slowly. This is usually done in a greenhouse.

4. *Agrobacterium*-mediated genetic transformation of Indonesian orchids

4.1 Transformation of *Phalaenopsis amabilis* (L.) Blume

Phalaenopsis amabilis (L.) Blume is one of the national flowers of Indonesia, along with the padma (*Rafflessia arnoldi*) and jasmine (*Jasminum sambac*). This orchid is distributed widely in some islands such as Java, Kalimantan, Sulawesi, Maluku, Papua, and also the Philippines and North Australia. Due to its long-lasting flowering, the well-known *P. amabilis*, with its large white flowers, is one of the most important ancestor species of *Phalaenopsis* hybrids. Hybrids of this orchid are of great economic value as house and garden plants as well as cut flowers. These hybrids are usually clonally propagated. A problem in this respect is that seedlings initially form only a single vegetative shoot (Dressler, 1981). However, additional shoots induced from cut protocorm-like bodies (PLBs) can be efficiently obtained using new *Phalaenopsis* medium, which contains a high concentration of nitrogen (Islam et al., 1998).

To improve the potential of the orchid's micropropagation, we developed a genetic transformant of *P. amabilis* using *Agrobacterium tumefaciens* that harbors T-DNA with a meristem-related gene, *BP/KNAT1*. We started with intact protocorms (developing orchid embryos) of *P. amabilis* that were maintained in a pre-culture NP medium with the addition of 100 g l^{-1} tomato extract (Semiarti et al., 2010). Regenerated plants under the same conditions showed the highest frequency of shooting. A kanamycin resistance gene under the control of the 35S promoter was used as a selective marker. In addition, T-DNA vectors containing the *Arabidopsis* class 1 *KNOX* gene, *BP/KNAT1,* were successfully introduced into protocorms. The protocorms transformed with *BP/KNAT1* produced multiple shoots. Both the presence and expression of the transgene in transformed plants were confirmed by molecular analysis.

To introduce the *KNAT1* gene into *P. amabilis,* the entire coding region of *BP/KNAT1*cDNA was cloned in a pG35S binary vector to generate pG35SKNAT1. To generate pG35S, two DNA fragments, one containing the promoter for 35S RNA from the cauliflower mosaic virus (P35S) and the other the terminator of the nopaline synthase gene, were amplified from pTH-2 (Chiu et al., 1996) by PCR. These two amplified fragments were introduced into the multiple cloning sites of the binary vector pGreen. Disarmed octopine type *A. tumefaciens* strain LBA 4404 was used for transformation. Nucleic acids of the orchids were isolated from the protocorms and leaves of wild-type or putative transgenic plants by the method of Semiarti et al. (2001). cDNA was synthesized from the mRNA using the TimeSaver cDNA synthesis kit (Amersham Biosciences, USA). The cDNA was used as a template for RT-PCR analysis.

Transformation was started by preparation of overnight cultures of *A. tumefaciens* that were diluted 1:4 (v/v) using NP (Ishii et al., 1998) liquid medium supplemented with 15% (v/v) coconut water, 2% (w/v) sucrose, 5 μM benzyladenine and 0.01% silvet L-77 (Nippon Unicar Co. Ltd., Tokyo). Prior to modification, orchid protocorms were cultured for 4 days in modified NP solid medium containing 1 mg l^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D) and 100 mg l^{-1} tomato extract, then immersed in the diluted culture of *A. tumefaciens* for 30 min. These protocorms were transferred onto sterile filter paper to dry and then onto modified NP medium containing 1 mg l^{-1} 2,4-D without antibiotics. After 4 days, the explants were transferred onto solid NP medium containing 1 mg l^{-1} 2,4-D and 300 mg l^{-1} carbenicillin, which inhibits the growth of *A. tumefaciens* , and cultured for 4 weeks. Protocorms that grew were rinsed thoroughly with liquid NP medium containing 200 mg l^{-1} kanamycin and 300 mg l^{-1} carbenicillin, then transferred onto medium containing 5 μM N^6-(Δ^2-isopenthenyl)

adenine (2-IP), 0.15 μM synthetic auxin (NAA), 200 mg l^{-1} kanamycin, and 300 mg l^{-1} carbenicillin. The developing protocorms were transferred onto new medium every 3 weeks for further selection of transformants. Developing shoots were screened by PCR using a *BP/KNAT1* specific primer to confirm that they were transformants. When the shoot and roots had grown sufficiently, the plantlets were transferred onto NP medium supplemented with 100 mg l^{-1} kanamycin and 50 mg l^{-1} carbenicillin.

Genomic DNA from the putative 35S::*BP/KNAT1* transformants was analyzed by PCR using primers KNAT1F1 and KNAT1R1, which are specific for the *BP/KNAT1* gene. First, we transformed the construct pG35S, which contains the kanamycin resistance gene. The experiments were performed twice. The protocorms that had been cocultivated for 4 days with *A. tumefaciens* harboring pG35S produced shoots at frequencies of 1.7 and 1.5% on kanamycin-containing medium (Table 4). We obtained 35 shoots out of 2,150 protocorms on kanamycin- and/or carbenicillin-containing medium after cocultivation with *A. tumefaciens* harboring pG35S in these experiments. The thirty-five shoots were independent, since each protocorm produced only one shoot (Figure 3).

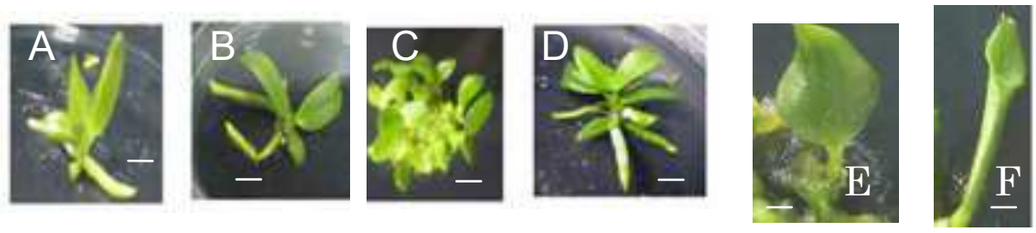


Fig. 3. Phenotype of non-transformant (NT), vector transformant, and 35S::KNAT1 transformant orchid plants. A. NT, B. vector-only transformed plant, C. 35S::KNAT1 multishoot transformant (6 months old), D. 35S::KNAT1 transformant (9 months old), E. abnormal trumpet-like leaf shape, and F. quadrat shape of leaf. Bars: 5 mm in A-D, 1 mm in E and F.

Antisense *DOH1* expression also causes abnormal multiple shoot development in *Dendrobium* orchids, indicating a role for *DOH1*, another member of the class 1 *KNOX* family, in basic plant architecture (Yu et al., 2001).

	Exp	Number of protocorms examined	Number of protocorms producing shoots	Frequency of transforma-tion (%)*	Number of regenerated plants
Non-transformant	1	100	0	0/0	0
PG35S	1	1150	20	1.7	20
PG35S::KNAT1	2	1000	15	1.5	15

Table 4. Frequency of *Agrobacterium*-mediated transformation in *P. amabilis*. * Ratio of the number of protocorms producing shoots to total number of transformed protocorms.

In addition, both *DOH1* sense and antisense transformants exhibit defects in leaf development (Yu et al., 2000; 2001). Since the transformation frequency using pG35SKNAT1 was one-seventh of that using pG35S, the *BP/KNAT1* gene might somehow affect the efficiency of transformation due to multishoot production (Figure 4).

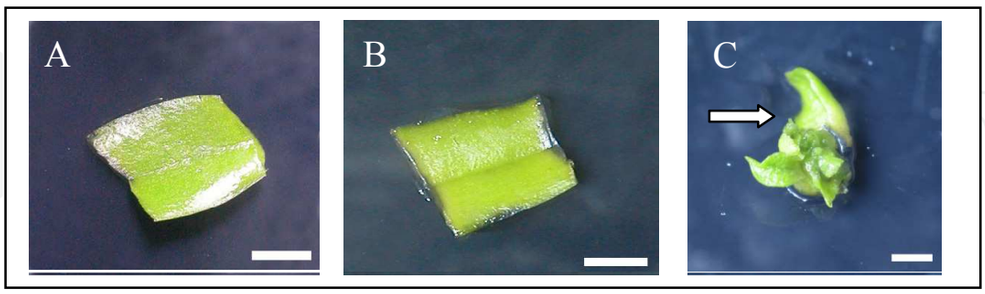


Fig. 4. Multishoot generation from leaf culture of 35S::KNAT1 orchid transformant. A-C. leaf disc of non-transformant (A), transformant with vector only (B), and 35S::KNAT1 transformant (C). Bars: 5 mm. Arrow is pointing to multishoots.

Although the function of members of the class 1 *KNOX* family is not known in *P. amabilis*, further studies using transformed *P. amabilis* plants are also expected to lead to a better understanding of the function of genes that are involved in developmental processes, including shoot and leaf development. In this experiment, we showed that *Phalaenopsis* protocorms transformed with the *KNOX* gene of *Arabidopsis* produced multiple shoots with trumpet-like, medio-laterally unopened blades of leaves that were not observed in rice (Figure 3). First, we thought that some of these phenotypes might be due to over-expression and ectopic expression of the *KNOX* gene. Alternatively, these phenotypes might be produced by suppression of the function of endogenous genes by ectopic expression of the *Arabidopsis KNOX* gene. Finally, however, the phenotype gradually returned to normal after acclimation in a pot under greenhouse conditions. These phenomenon should be investigated in future studies by functional analysis of the class 1 *KNOX* genes of *P. amabilis*. Production of multishoots will have a good impact on micropropagation of orchids through tissue culture.

Maryani (2010, personal communication) introduced the *Wasabi defensin* gene that is responsible for soft rot disease resistance into *P. amabilis* with the *Bar* gene responsible for herbicide resistance. Development of a method for improving *Phalaenopsis* orchids through genetic modification could be extremely valuable for horticulture and, indirectly, also for conservation, as well as contributing to understanding the functions of genes in *Phalaenopsis* orchids.

The core component of genetic modification 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 *A. 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 developed a convenient method for genetic modification of *Phalaenopsis amabilis* orchids using *A. tumefaciens* (Semiarti et al., 2007) in which intact protocorms (young orchid seedlings) were used for transformation.

This method is simple, reproducible, and applicable to other species. However, the transformation efficiency was ≤2%, and further studies are needed to improve this.
For *in vitro* germination of orchid seeds, 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 may promote the germination and growth of protocorms (Arditti and Ernst, 1993). Perl et al. (1996) determined that a combination of polyvinylpolypyrrolidone (PVPP) and dithiothreitol (DTT) as anti-oxidants improved plant viability. Tissue necrosis in *A. tumefaciens* -treated embryogenic calli of grapevine plants was inhibited completely using these anti-oxidants, while the virulence of *A. tumefaciens* remained unaffected. These treatments enabled the recovery of stable transgenic grapevine plants resistant to hygromycin.
In order to improve the frequency of *Agrobacterium*-mediated transformation of *P. amabilis*, we pre-cultured the protocorms in medium containing an extract from fully-ripe tomatoes and/or coconut water and investigated the effect of this pre-culturing treatment on improving the efficiency of regeneration of transformed shoots. Adult plants of *P. 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 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 tomatoes into 1-cm^3 cubes, homogenizing them, and filtering the homogenate through a steel mesh with a 150-μm pore size. The nutrient compositions of the coconut water sample and the tomato extract were analyzed by high performance liquid chromatography.
To determine the growth rates of orchid embryos and protocorms, the sizes, colors, 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 are formed.

4.2 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 acids were isolated from the protocorms and leaves of wild-type or putative transgenic plants by the method of Semiarti et al. (2001) using the QIAGEN DNA

purification kit for isolation of genomic DNA and QIAGEN RNeasy mini kit (QIAGEN GmbH, Germany) for isolation of total RNA. mRNA was isolated from total RNA using Dynabeads Oligo $(dT)_{25}$ (DYNAL, Norway), and cDNA was synthesized from the mRNA using the TimeSaver cDNA synthesis kit (Amersham Biosciences, USA). The cDNA was used as a template for RT-PCR analysis.

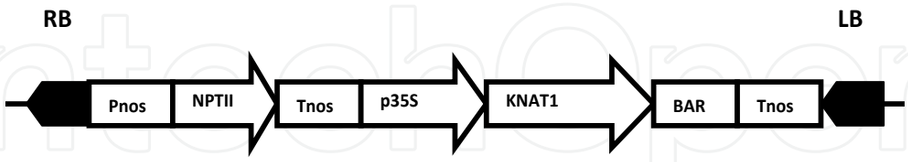


Fig. 5. Schematic structure of 35S::KNAT1 containing T-DNA. LB, left border; RB, right border; 35S: CaMV promoter; KNAT1: BREVIPEDICELLUS (BP)/KNAT1 gene; NPTII: neophosphotransferase II; Tnos: nos terminal.

4.3 Transformation and transformant regeneration

Overnight cultures of *A. tumefaciens* were diluted 1:4 (v/v) using NP liquid medium supplemented with 15% (v/v) coconut water, 2% (w/v) sucrose, 5 μM benzyladenine, and 0.01% silvet L-77 (Nippon Unicar Co. Ltd., Tokyo). Prior to modification, orchid protocorms were cultured for 4 days in modified NP solid medium containing 1 mg l^{-1} 2,4-D, then immersed in the diluted culture of *A. tumefaciens* for 30 min. These protocorms were transferred onto sterile filter paper to dry and then onto modified NP medium containing 1 mg l^{-1} 2,4-D without antibiotics. After 4 days, the explants were transferred onto solid NP medium containing 1 mg l^{-1} 2,4-D and 300 mg l^{-1} carbenicillin, which inhibits the growth of *A. tumefaciens* , and cultured for 4 weeks. Protocorms that grew were rinsed thoroughly with liquid NP medium containing 200 mg l^{-1} kanamycin and 300 mg l^{-1} carbenicillin, then transferred onto medium containing 5 μM 2-isopentenyl adenine (2-IP), 0.15 μM naphthalene acetic acid (NAA), 200 mg l^{-1} kanamycin, and 300 mg l^{-1} carbenicillin. The developing protocorms were transferred onto new medium every 3 weeks for further selection of transformants. Genomic DNA from putative 35S::GFP transformants was analyzed 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′-CCTGCCCATTCGACCACCAA-3′) and NPTIIR1 (5′-AGCCCCTGATGCTCTTCGTC-3′) for the *NPTII* gene; and GFPF1 (5′-ATGGTGAGCAAGGGCGAGGA-3′) and GFPR1 (5′-GTCCATGCCGTGAGTGATCC-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 distinguishes the red autofluorescence of chlorophyll from the fluorescence of GFP. The images were captured using a Nikon Cool Pix 5000 digital camera system with an adaptor for microscopy (Nikon Corp.).

4.4 DNA analysis by Southern hybridization

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

*Hin*d 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 hybridized with a digoxigenin-labeled 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 hybridized DNA fragments were visualized using the DIG Luminescent Detection Kit (Roche Diagnostics) according to the manufacturer's instructions.

4.4 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 tomato extract based on the number of growing embryos and protocorms found at each stage (Table 5). 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 analyzed growth rates on NP medium with or without coconut water and tomato extract (Figure 6A). 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

Concentration of tomato extract (mg/l)	Total no. embryos examined	Experiment No.	Embryo Stage 0 (%)	Embryo Stage 1 (%)	Embryo Stage 2 (%)	Swollen embryo (protocorm) Stage 3 (%)	Green protocorm Stage 4 (%)	Protocorm with shoot apical meristem Stage 5 (%)
0	806	1	23.9	12.2	3.6	20.8	39.1	0.5
		2	18.7	5.4	7.4	30.5	36.9	1.0
		3	24.4	2.2	4.9	40.1	28.3	0.0
50	1148	1	22.2	3.6	4.3	40.2	29.3	0.4
		2	27.5	6.0	8.8	28.4	28.4	0.9
		3	28.0	6.9	4.0	28.7	31.5	0.9
100	1577	1	24.9	4.7	5.5	13.0	51.1	0.8
		2	31.3	1.9	6.1	9.0	51.2	0.5
		3	36.0	4.1	2.0	15.8	42.1	0.0
150	1417	1	46.0	4.8	3.7	9.5	36.1	0.0
		2	26.2	1.8	5.6	14.6	51.8	0.0
		3	35.5	2.9	4.4	15.0	42.3	0.0
200	1583	1	49.6	3.1	2.8	9.7	34.7	0.0
		2	62.6	7.8	3.6	6.0	20.1	0.0
		3	52.5	5.7	1.1	17.0	23.7	0.0

Table 5. Growth stages of *P. amabilis* cultured on NP medium supplemented with different concentrations of tomato extract for 21 days after sowing. Reproduced from the Journal of Horticultural Science & Biotechnology with permission from the editor.

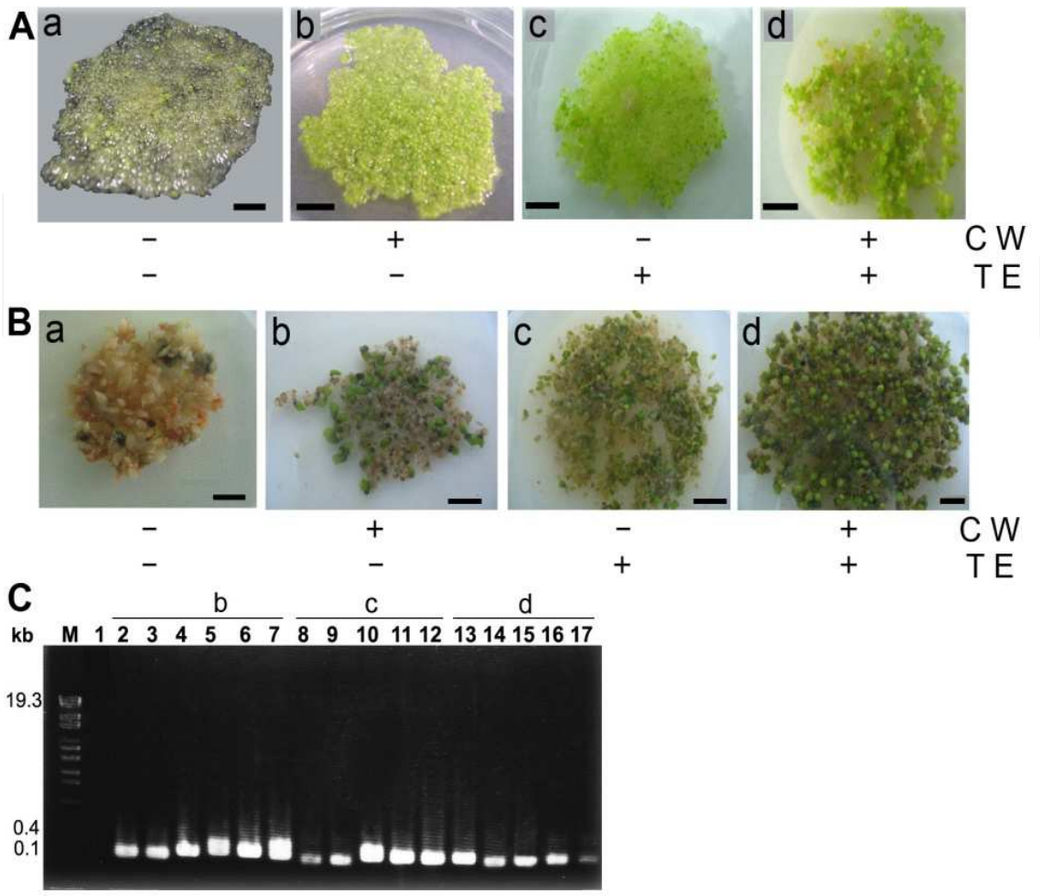


Fig. 6. Main panel A, growth of protocorms of *P. amabilis* on various culture media 3 weeks after sowing. Sub-panel a, NP medium; sub-panel b, NP medium supplemented with 150 ml l^{-1} coconut water (CW); sub-panel c, NP medium supplemented with 100 mg l^{-1} tomato extract (TE); and sub-panel d, NP 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 pBI121 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 pBI121 containing the kanamycin resistance gene (*NPTII*). Main panel C, PCR detection of the kanamycin resistance gene (*NPTII*) in putative transgenic orchid plants harboring pBI121. Fragments from a *Sty*I digest of 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 the 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 (from Semiarti et al., 2010). Reproduced from the Journal of Horticultural Science & Biotechnology with permission from the editor.

Component	Coconut water	Tomato extract
Ash	0.55%	0.31%
Lipid	0.05%	0.47%
Total protein	0.19%	1.78%
(soluble protein)	(0.17%)	(1.46%)
Total sugars	3.22%	3.70%
(reducing sugars)	(3.02%)	(3.39%)
Total carotene	Nd	1.84%
Antioxidants (DPPH)*	Nd	0.024%
Vitamin C	Nd	0.042%
Crude fibre	Nd	1.05%
Phosphate (P_2O_5)	0.013%	0.13%
Inorganic ions		
Mg^{2+}	0.0058%	0.0081%
Mn^{2+}	0.00021%	0.000029%
Na^{+}	0.046%	0.0090%
K^{+}	0.23%	0.16%
pH	5.16	4.34

Table 6. Components of coconut water and tomato extract used in this study. * Total carotene antioxidants measured based on the activity of antioxidants against 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radicals from the sample. Reproduced from the Journal of Horticultural Science & Biotechnology with permission from the editor.

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 6). These components could affect the growth of the embryo.

Oladiran and Iwu (1992) showed that fully-ripe tomato fruit contains 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 is 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 if several compounds have a synergistic effect, on the growth and development of *P. amabilis* seeds.

4.5 Effect of pre-culture of protocorms on NP medium containing tomato extract on the transformation frequency of *P. amabilis* orchids

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 7). 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 7; Figure 6B, panel D). These results were higher than the frequency of transformed regenerated shoots on medium containing coconut water alone (1.2%; Table 7), 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 7). 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 those for 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.

Plasmid	Coconut water	Tomato extract	Total no. protocorms examined	No. protocorms producing shoots (% of total)
None	+	+	1557	0 (0%)
pBI121 (vector)	+	-	1200	14 (1.2%)
pBI121 (vector)	-	+	1200	159 (13.2%)
pBI121 (vector)	+	+	1557	260 (16.6%)
			1500	102 (6.8%)
pBI121-p35S::GFP	+	+	1557	210 (13.5%)
			1500	147 (9.8%)

Table 7. Transformation frequency of *P. amabilis* protocorms following 3 weeks of pre-culture on NP medium supplemented with tomato extract and coconut water. Reproduced from the Journal of Horticultural Science & Biotechnology with permission from the editor.

We used this transformation method on the pandanus orchid (*Vanda tricolor var Suavis* Form Merapi). Protocorms were pre-cultured on NP medium supplemented with coconut water and tomato extract prior to transformation. The transformation frequency of regenerated shoots was 20.3%. This result indicates that the transformation method may be useful for other orchid species.

4.6 Molecular analysis of putative transformants

We examined the genomic DNA from *P. amabilis* plantlets regenerated on agar plates containing 200 mg l^{-1} kanamycin for the presence and expression of the kanamycin resistance gene (*NPTII*) using PCR. The predicted 105-bp fragment was amplified from all putative transformants in each treatment (Figure 7C). The plantlets that regenerated after

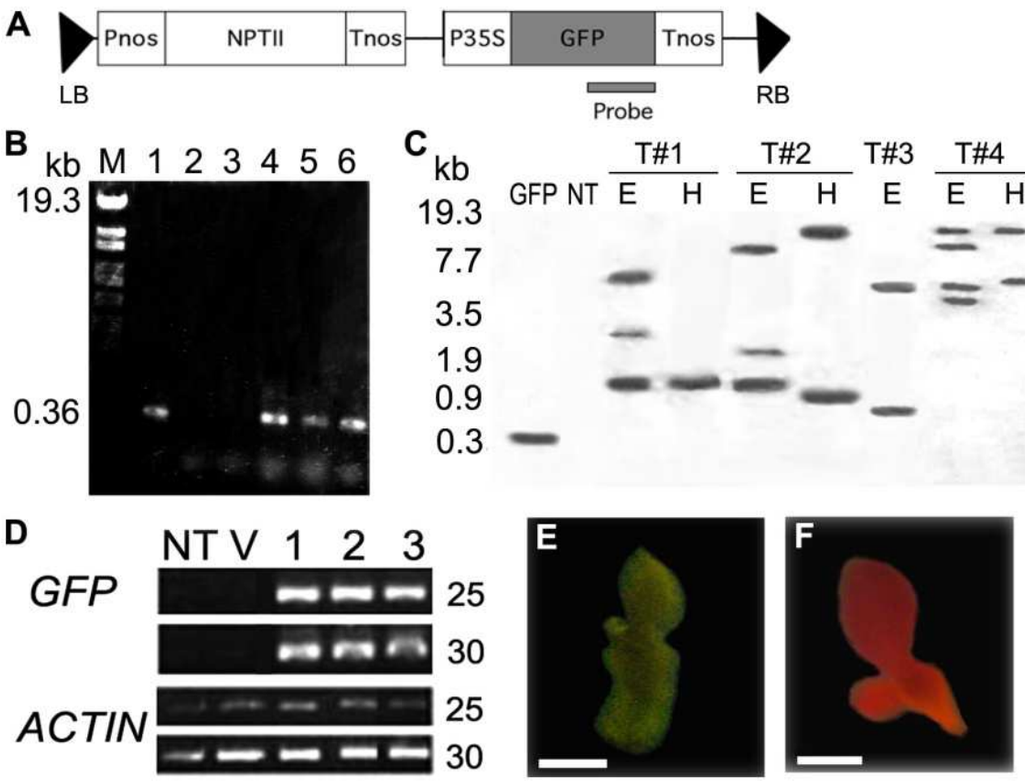


Fig. 7. Analysis of putative *GFP* transformants of *P. amabilis*. A: Schematic representation of the T-DNA region of the binary plasmid pBI121-p35S::GFP. The binary plasmid pBI121-p35S::GFP contained the 720-bp *GFP* gene, which encodes the jellyfish GFP under control of the 35S promoter of cauliflower mosaic virus (CaMV). RB, right border; LB, left border; Pnos, promoter of the nopaline synthase gene; Tnos, polyadenylation site of the nopaline synthase gene; NPTII, neomycin phosphotransferase gene; P35S, 35S promoter of CaMV. A 360-bp fragment from the 3′ end of the *GFP* gene was used as a probe during Southern hybridization. B: PCR analysis of the *GFP* transgene in putative transgenic orchids. Fragments from a *Sty*I digest of λ phage DNA were used as size markers (M), and the specific 360-bp DNA fragment was amplified from the plasmid pBI121-p35S::GFP (lane 1), and fragments were amplified from DNA of an untransformed *P. amabilis* plant (lane 2), a plant transformed with the empty vector pBI121 (lane 3), and three plants independently transformed with pBI121-p35S::GFP (lanes 4-6). No fragment was amplified from DNA from untransformed plants (lane 3) or empty vector-transformed plants (lane 4). C: Southern blot analysis. Lane 1: fragments from a *Sty*I digest of λ phage DNA as the size marker. GFP: GFP probe; NT: genomic DNA from an untransformed plant was digested with *Eco*RI; T#1-4: genomic DNA (5 μg) from transformed plants was digested with *Eco*RI (E) or *Hind*III (H). Fragments were fractionated in a 1% agarose gel, then blotted and hybridized with a digoxigenin (DIG)-labelled *GFP* gene probe. D: expression of the *GFP* gene in putative transgenic *P. amabilis* plants. RT-PCR analysis of transcripts of the *GFP* gene in a wild-type plant (NT), in a plant transformed with the empty vector pBI121 (V), and in pBI121-p35S::GFP-transformed plantlets (1-3). The number of cycles is indicated at the right of each panel. Amplified DNA fragments were separated by electrophoresis in an agarose gel and visualised with ethidium bromide. As a control, the same samples were amplified with primers specific for the *ACTIN* gene transcript. See the Materials

and Methods for details about RT-PCR. E and F: detection of GFP expression in a putative 35S::GFP transformant plantlet (E) and untransformed plantlet (F) under blue light. Bars: 1 cm (from Semiarti et al., 2010). Reproduced from the Journal of Horticultural Science & Biotechnology with permission from the editor.

transformation with the plasmid pBI121-p35S::GFP were examined for the presence of the *GFP* gene by PCR amplification of the 360-bp fragment from the GFP coding region (Figure 7B). 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 hybridizations. Hybridization using an anti-sense probe for the 3' end of the *GFP* gene (Figure 7A) showed two to four copies of the *GFP* gene in each transgenic line (Figure 7C). Since the genomic DNA of each putative transgenic plant showed uniquely-sized bands hybridizing 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-p35S::GFP. We quantified the relative levels of *GFP* gene transcripts (mRNA) using RTPCR with primers specific for *GFP*. PCR products were detected in all three lines of plantlets transformed with pBI121-p35S::GFP, but not in the untransformed plantlet or the plantlet transformed with pBI121 alone (Figure 7D). 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-p35S::GFP showed green fluorescence after excitation with blue light (Figure 7E), whereas untransformed plantlets did not (Figure 7F). 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* by several-fold.

4.7 Transformation of *Vanda tricolor* Lindl. var. Suavis

Vanda tricolor Lindl. var. Suavis is an Indonesian wild orchid that has spread throughout Indonesian territory and is grown in some regions such as Mount Merapi (Central Java and Yogyakarta), Bali, East Java, and West Java. Since the Mount Merapi is a very active volcano with frequent pyrroclastic flows and eruptions, the population of *V. tricolor* in its natural habitat is now extremely rare due to habitat loss. Micropropagation to save the population of this orchid is worthwhile as are *ex situ* conservation efforts. Dwiyani et al. (2011, in press) developed a transformation method based on that previously published by Semiarti et al. (2007) with the use of acetosyringone to improve transformation efficiency of the *V. tricolor* Merapi Form. Acetosyringone was added into the protocorm and *A. tumefaciens* co-cultivation medium.

Vanda tricolor pods (6 months after self pollination) from Mount Merapi were used as seed sources. Orchid pods were washed, sterilized three times by dipping in 70% ethanol, flamed, and then put gently on sterilized petri dishes in a laminar air flow hood. Seeds were then sown in New Phalaenopsis (NP) medium (Islam et al., 1998) enriched with 100 g l^{-1} tomato extract as used by Semiarti et al. (2010). Five weeks after sowing, protocorms were collected to be used for target transformation. A kanamycin test was performed on 8 and 10 week-old protocorms. The disarmed octopine type of *A. tumefaciens* LBA4404 harboring the p35S binary vector containing the *Neophosphotransferase* (*NPTII*) gene as a selectable marker was used for transformation.

Three days before infection, eight week-old germinated protocorms were transferred onto fresh NP medium containing 1 µl l^{-1} of 2,4D. *A. tumefaciens* was cultured overnight in LB liquid medium containing 200 mg l^{-1} kanamycin. Inoculation of *A. tumefaciens* was performed by adding 2 ml *A. tumefaciens* liquid culture into 8 ml NP liquid medium (4 x dilution), 0.01% Tween 20, with or without addition of 25 µl l^{-1} acetosyringone. Precultured protocorms were then immersed in this diluted *A. tumefaciens* liquid culture for 30 minutes, and immediately transferred onto sterilized filter paper for 60 minutes of air drying. These protocorms were then transferred into 0.2% (w/v) gellan gum-solidified NP medium with 1 µl l^{-1} 2,4D added and with or without 25 µl l^{-1} AS for co-cultivation. After 3 days co-cultivation in this medium, protocorms were washed three times with ½ NP liquid medium containing 20 mg l^{-1} meropenem, and then transferred to 0.2% (w/v) gellan gum-solidified NP medium with 5 µM 2-IP and 0.15 µM NAA added (called Shoot Induction Medium or SIM) and 8 mg l^{-1} meropenem to slow the growth of *A. tumefaciens* . Protocorms were cultured in this medium for 3 days.

Three days after maintaining the protocorm in the bacterial-elimination medium, protocorms were washed with ½ NP liquid medium containing 20 mg l^{-1} meropenem three times, then were transferred to selection medium (SIM with 300 mg l^{-1} kanamycin and 8 mg l^{-1} meropenem). Protocorms were mantained in this medium for 5 weeks and subcultured every week or less to eliminate *A. tumefaciens* . Elimination of *A. tumefaciens* was performed by immersing protocorms with ½ NP liquid medium containing 20 mg l^{-1} meropenem for 30 minutes, and then gently washing it using the same medium 2-3 times until it was free of *A. tumefaciens* . After 5 weeks of selection, green protocorms were collected as transformant candidates. Protocorms of *V. tricolor* derived from seeds require 20 weeks after sowing to become plantlets.

The kanamycin test was performed twice on the protocorms of *V. tricolor* Merapi. The first test was performed on eight week-old protocorms and the second on ten week-old protocorms. There were five variations in the concentration of kanamycin applied on each age group of protocorms. Concentrations of 0 mg l^{-1}, 100 mg l^{-1}, 150 mg l^{-1}, 200 mg l^{-1}, and 250 mg l^{-1} were applied on eight week-old protocorms, and concentrations of 0 mg l^{-1}, 200 mg l^{-1}, 300 mg l^{-1}, 400 mg l^{-1}, and 500 mg l^{-1} were used on ten week-old protocorms. The percentage of green (surviving) and brown (dead) protocorms was observed after five weeks of application. As a result, 95% of eight week-old protocorms turned brown at a kanamycin concentration of 250 mg l^{-1}, indicating that the selection medium should have a concentration of kanamycin more than 250 mg l^{-1} (Figure 8). However, for ten week-old protocorms, 20% remained green at a kanamycin concentration of 500 mg l^{-1}. These data indicated that older protocorms were more resistant to kanamycin than the younger ones. Based on these data, we used eight week-old protocorms as the target of transformation and a kanamycin concentration of 300 mg l^{-1} for selection. Besides our results, previous research by Dwiyani (2009, unpublished) also found that more than 50% of *V. tricolor* embryos were resistant to kanamycin. Genetic transformation using the *A. tumefaciens* LBA4404 strain harboring pG35S containing the *NPTII* gene was conducted three times on the eight week-old protocorms. The first transformation used *V. tricolor* from West Java, and the second and the third used *V. tricolor* from Merapi. The difference between the second and the third was the use of pre-culture treatment on the second transformation. Five weeks after growth on selection medium containing 300 mg l^{-1} kanamycin, 100% of control protocorms (without *A. tumefaciens* infection) turned brown in *V. tricolor* Merapi Form (Figure 8).

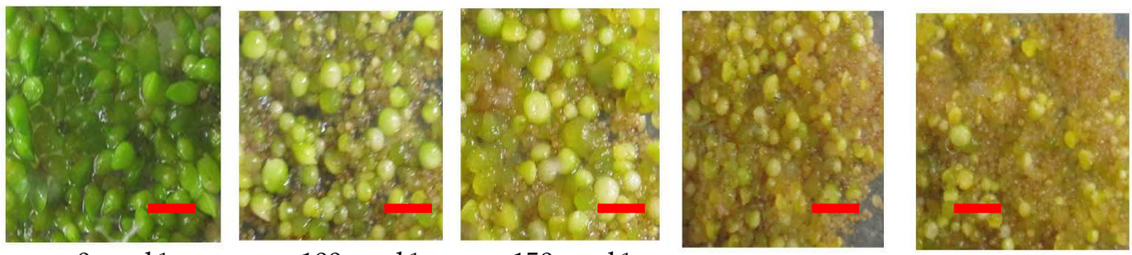


Fig. 8. Kanamycin test on 8 week-old protocorms of *Vanda tricolor* Merapi. From left to right, protocorms after 5 weeks of kanamycin application of 0, 100, 150, 200, and 250 mg l^{-1}. At 250 mg l^{-1}, 5% of protocorms remained green. Bar: 1500 µm. (After Dwiyani et al., 2011, in press).

From the study of Dwiyani et al. described above, two points can be infered: first, the addition of AS into co-cultivation medium is required. The addition of AS into the inoculum increased the percentage of protocorms surviving in the selection medium above that with the addition of AS into co-cultivation only. AS supplementation in this step of transformation (inoculation) might stimulate higher concentrations of AS within the tissues of the treated protocorm, thereby eliciting higher *vir* gene-inducing activity in *A. tumefaciens* (Nan et al., 1997). The presence of AS during transformation is known to induce *vir* gene activity and stimulate T-DNA transfer into plant cells (Zupan and Zambryski, 1995; Gelvin, 2003). This result was in line with Jacq et al. (1993); Robischon et al. (1995); and Mishiba et al. (2005), who found that pre-culture of transformation targets prior to infection resulted in higher transformation efficiency. Cell-cycle progression might be induced during pre-culture (Mishiba et al., 2005), and T-DNA transfer is likely to occur in cells with this condition (Villemont et al., 1997).

The resistancee of *V. tricolor* protocorms to kanamycin varies depending on the developmental stage of the protocorms and the orchid form. Older protocorms are more resistant than younger ones. The West Java form is more resistant than the Merapi form. The addition of AS (especially at the inoculation step) and pre-culture treatment of protocorms prior to infection with *A. tumefaciens* is necessary for *Agrobacterium*-mediated-transformation of *V. tricolor* protocorms.

4.8 Transformation of the black orchid (*Coelogyne pandurata* Lindley)

The black orchid (*Coelogyne pandurata* Lindley), which is endemic to the province of East Kalimantan, Indonesia, is very important. The black orchid is an epiphytic sympodial orchid characterized by a large greenish flower with a black labellum (Figure 1A). This orchid exhibits some pseudobulbs that grow parallel with two leaves each. Five to seven flowers are arranged in a raceme and are fragrant. The diameter of each flower is 7-12 cm. Sepals and petals are green and the labellum (lip) is black. Seeds are microscopic in size, and are located inside the fruit. The uniqueness of this orchid is in the very short blooming period (3-5 days) and its difficulties with pollination (Arditti, 1992; Wibowo, 2010). According to Wirakusumah (2009, personal communication), *in vitro* seed germination of the black orchid needs special conditions such as incubation in the dark prior to germination for 3-4 months. For successful cultivation, *in vitro* seed germination is the key step (Arditti and Ernst, 1993). In order to obtain optimal conditions for *in vitro* seed germination of this orchid, some experiments using various culture media have been carried out (Semiarti et al., 2010), and

we have used 35S::KNAT1 containing T-DNA constructs to get multishoot production similar to our work with *P. amabilis*.

4.9 Developmental phases of the black orchid (*Coelogyne pandurata* Lindley) embryo

The development of black orchid embryos during seed germination can be classified into six phases based on growth and morphology: phase 1) yellowish embryo, phase 2) green embryo, phase 3) bipolar embryo, phase 4) first leaf formed embryo, phase 5) second leaf formed embryo, and phase 6) third leaf formed embryo. The time-course of embryo development shows that the embryo starts to change from yellowish (phase 1) into green (phase 2) at one to two weeks after sowing. At three to four weeks, the green embryo forms a bipolar structure (phase 3), with one side darker than the other. The darker pole of the embryo changes into leaf primordia (phase 4) at the fifth week, a protocorm with two leaves at seven weeks (phase 5), and a protocorm with three leaves at eleven to twelve weeks (phase 6) (Table 8).

At twelve weeks after sowing, based on the growth rate of embryos, the data revealed that ½ NP medium is the best to support and accelerate growth that will result in seed germination. Approximately 86% of protocorms grew up to phase 5 (Table 8). The results show that for embryo development during seed germination in black orchids, a half-strength concentration of complete element-containing medium is the best. It might be that the content of macro- and micro-elements in the half strength medium provides a suitable consentration to promote the development of the embryos, so it is not necessary to use full-strength basic medium. As described by Arditti and Ernst (1993), tissue culture is an empirical science. It is difficult to predict the type of explant, media, and conditions that are suitable for a specific genus or species or clone. It is not possible to explain why certain media and culture conditions lead to success while others fail. In the black orchid, the half-strength NP medium may be the best for seed germination, so that the embryo will respond better to genetic transformation than that if we used full-strength NP medium.

Variation of Medium	No of seeds	Percentage of growing embryos at each phase						Death of protocorms
		Phase 1	Phase 2	Phase 3	Phase 4	Phase 5	Phase 6	
½ NP	193	0.00%	0.00%	0.00%	13.47% (26)	86.53% (167)	0.00%	0.00%
NP	112	0.00%	0.00%	0.00%	18.75% (21)	57.14% (64)	4.46% (5)	19.64% (22)
NP+CW	105	0.00%	0.00%	0.00%	1.90% (2)	72.38% (76)	8.57% (9)	17.14% (18)

Table 8. Growth of black orchid embryos on NP medium.

4.10 Insertion of the *KNAT1* gene in the black orchid

For *in vitro* cultivation of orchids, various media are used for seed germination and shoot induction, such as Knudson C (KC), Murashige-Skoog (MS), and Vacin and Went (VW), with the addition of some organic complexes such as coconut water (Arditti and Ernst, 1993; Widiastoety and Syafril, 1994; Demasabu et al., 1998; Untari and Puspaningtyas, 2006). Islam et al. (1998) used *New phalaenopsis* (NP) medium for callus induction of *Phalaenopsis*. Semiarti et al. (2007) also used the NP medium for growing *Phalaenopsis* orchids before and after

genetic transformation treatment using *A. tumefaciens*. Our previous experiments (Semiarti et al., 2007) indicated that the insertion of the *KNAT1* gene into *Phalaenopsis* protocorms induced multishoot production (about 31 shoots from one protocorm), and further results showed that the level of multiplication increased to more than 90 shoots from one embryo. This is a very valuable and promising technique for micropropagation of black orchids. Multishoot occurrence in *KNAT1* transgenic plants has also been reported by Chuck et al. (1996) in transgenic *Arabidopsis* and Nishimura et al. (2000) in *Nicotiana*. In the hybrid orchid *Dendrobium* "Madame Thong In", Yu et al. (2001) obtained multishoots from calli derived from cut-off protocorms transformed with the *DOH1* gene (a *KNAT1* homologous gene in *Dendrobium*). Each shoot could independently grow into a plantlet. Genetic transformation of the *KNAT1* gene under the control of *Cauliflower Mosaic Virus* (CaMV) in a pGreen vector using *A. tumefaciens* strain LBA 4404 into intact protocorms is a useful means of micropropagation.

Genetic transformation of plasmid 35S::KNAT1 and pGreen vector into orchids was carried out according to the method of Semiarti et al. (2007), except that the liquid medium used to rinse the protocorm was half-strength NP medium with 300 mg l^{-1} cefotaxim. SIM (Shoot Induction Medium; 0.15 µM NAA + 5 µM 2-IP) supplemented with 100 mg l^{-1} kanamycin for selecting independent transformants. Into each step 75 mg l^{-1} acetosyringone was added to improve the efficiency of T-DNA insertion as described by Semiarti et al. (2010). The frequency of transformation was measured as the ratio of the number of surviving protocorms per total number of transformed protocorms (Table 9).

No.	Treatment	Kan, 300 mg l^{-1} (+/-)	No. of protocorms	Percentage of Kan-resistant plants	
				Surviving	Death
1.	Non-transformant (NT)	-	100	98% 98/100	2% 2/100
2.	NT	+	237	37.6% 89/237	62.4% 148/237
3.	pGreen	+	707	66.0% 467/707	34.0% 240/707
4.	p35S::KNAT1	+	701	61.6% 475/701	38.4% 296/701

Table 9. Frequency of transformation by pGreen and pKNAT1 in regeneration medium, two months after selection on kanamycin-containing medium.

The expression of the *KNAT1* gene in black orchid transformants can be judged from their ability to form multishoots similar to another natural orchid, *Phalaenopsis amabilis* (Semiarti et al., 2007). Yu et al. (2001) obtained multishoots of a hybrid orchid from callus protocorms that were transformed by the *DOH1* gene, in which each shoot could independently grow into a plantlet. PCR analysis has confirmed the existance of the *KNAT1* gene in the genomes of surviving plants in antibiotic-containing medium for black orchid transformants. For other transformant candidates, confirmation of the transgene is still in progress. Images in Fig. 8B and C show that non-transformant plants produce only one shoot from the embryo, but a transformant produces seven shoots from one protocorm. The addition of cytokinins as phytohormones such as kinetin or 2-penthenyl adenine (2-IP), when combined with NAA (synthetic auxin) in medium, might induce and increase the number of multishoots from

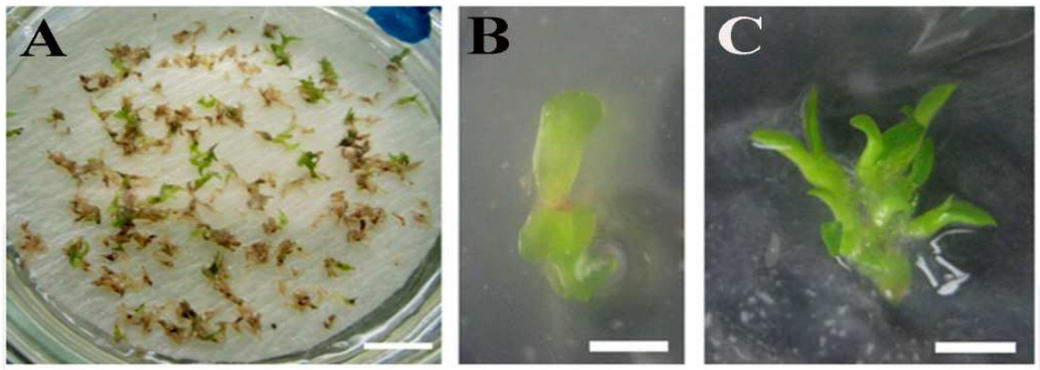


Fig. 8. Black orchid transformants. A. Two-month old candidates of transformants grown on kanamycin-containing medium in a selection plate. B. a shoot grows from a non-transformant protocorm. C. multishoots grow from one 35S::KNAT1 transformant protocorm. Bars: 1 cm in A, 0.5 cm in B-C.

transformants. Further results of transformation with *P. amabilis* indicate that maintaining transformants on NP medium with 3 µM 2-IP + 0.15 µM NAA induces high levels of multishoot production, up to 91 shoots per embryo. It is worth trying this method in black orchids. The high propensity for shoot production will strongly support both conservation efforts and agribusiness of this orchid.

5. Conclusions and future prospects

Agrobacterium-mediated transformation of the *KNAT1* gene into *P. amabilis* orchids resulted in multishoot production of transformants, which is helpful for micropropagation of Indonesian orchids such as *P. amabilis*, *Vanda tricolor*, and *Coelogyne pandurata.* Three-week-old intact protocorms are convenient for use in *Agrobacterium*-mediated transformation. This method is a highly effective technique to enhance the efficiency of micropropagation of Indonesian orchids, which is useful due to the rareness of their populations in their natural habitat. Utilizing advanced orchid biotechnology may facilitate the improvement of Indonesian orchid production for commercial use or for orchid conservation. These tools will also be helpful for creating new traits in orchids.

6. Acknowledgments

We thank all students who work in the laboratory of Plant Tissue Culture, Faculty of Biology Universitas Gadjah Mada. This research was supported in part by the Indonesian DGHE Research Competition grant HB XVII 2009-2010 and the Japanese Academic Frontier Research Grant to CM from 2005-2010. We thank the Bunga Rintee Orchid Nursery for the gift of black orchid fruit and to Mr. Wirakusumah for the gift of 4-month-old protocorms and for valuable discussion on black orchid culture techniques.

7. References

Anzai, H., Ishii, Y., Shichinohe, M., Katsumata, K., Nojiri, C., Morikawa, H. & M. Tanaka. (1996). Transformation of Phalaenopsis by particle bombardment. Plant Tissue Cult. Lett. 13: 265-271.

Araki, S., M. Matsuoka, M. Tanaka, & T. Ogawa. (2000). Ethylene formation and phenotypic analysis of transgenic tobacco plants expressing a bacterial ethylene-forming enzyme. Plant and Cell Physiology 41(3):327-334

Arditti J. (1992). Fundamentals of Orchid biology. John Wiley and Sons, Inc. New York. P. 691

Arditti, J, & R. Ernst. (1993). *Micropropagation of orchids*. Jhon Willey & Son Inc. New York,pp.6-8, 25-65.

Belarmino M.M, & M. Mii. (2000). *Agrobacterium*-mediated genetic transformation of a *Phalaenopsis* orchid. Plant Cell Reports 19:435-442

Chan YL, Lin KH, Sanjaya, Liao LJ, Chen WH, Chan MT (2005) Gene stacking in *Phalaenopsis* orchid enhances dual tolerance to pathogen attack. *Transgenic Res* 14: 279–288

Chansean, M. & S. Ichihashi. (2007). Conservation of wild orchids in Cambodia by simple aseptic culture method. Proceedings of Nagoya International Orchid Conference 2007, Nagoya, Japan. 13-20.

Chin, D.P., & M. Mii. (2011). Dwarf Phalaenopsis Plant Produced by Overexpression of Gibberellin2-Oxidase gene. Proceeding of NIOC, Nagoya Japan, 11 Maret 2011.

Chiu W-l, Niwa Y, Zeng W, Hirano T, Kobayashi H, Sheen J (1996) Engineered GFP as a viral reporter in plants. *Curr Biol* 6:325-330

Chia TF, Chan YS, & Chua NH. (1994). The firefly luciferase gene as a non-invasive reporter for *Dendrobium* transformation. *Plant J* 6: 441-446

Choffnes, D.S., R. Philip, & L.O. Vodkin. (2001). A transgene locus in soybean exhibits a high level of recombination. In Vitro Cellular & Developmental Biology-Plant 37:756-762

Christou, P., W.F. Swain, N.S. Yang, & D.E. McCabe. (1989). Inheritance and expression of foreign genes in transgenic soybean plants. Proceedings of the National Academy of Science of the United State of America 86:7500-7504.

Chuck G, Lincoln C, & Hake S (1996) *KNAT1* induces lobed leaves with ectopic meristems when overexpressed in *Arabidopsis*. *Plant Cell* 8:1277-1289

Cogan, N., Harvey, E., Robinson, H., Lynn, J., Pink, d., Newbury, H.J., & Puddephat, I. (2001). The effects of anther culture and plant genetic background on *Agrobacterium rhizogenes*-mediated transformation of commercial cultivars and derived double-haploid *Brassica oleracea*. Plant Cell Reports 20:755-762.

Cunningham, F. X. Jr., Pogson, B., Sun, Z.,Mcdonald, K. A., Dellapenna, D. & E. Gantt. (1996). Functional analysis of the B and E lycopene cyclase enzymes of Arabidopsis reveals a mechanism for control of cyclic carotenoid formation. The Plant Cell, 8, 1613-1626.

Demasabu, Sofia, B. Dodo, dan D. Kojoh (1998), Penggunaan limbah Air Kelapa dan Bahan Substitusi Agar pada kultur jaringan pisang, krisan, dan anggrek. *Prosiding Seminar Nasional Sains dan Teknologi II, tanggal 17-18 November 2008. Universitas Lampung.*

Dressler RL (1981) The Orchids: Natural History and Classification. Harvard University Press, USA, pp 332.

Dwiyani, R. A. Purwantoro, A. Indrianto, & E. Semiarti. (2011). Improvement of Genetic Transformation Technique in *Vanda tricolor* Orchid Using Acetosyringone.

Finnegan, J. & D. McElroy. (1994). Transgene inactivation: Plants fight back!. Bio/Technology 12:883-888

George, E.F, & P.D. Sherrington. (1984). *Plant Propagation by Tissue Culture*. Exergetics Ltd. Hants, pp, 184-200.

Grichko, V.P., & B.R. Glick. (2001). Flooding tolerance of transgenic tomato plants expressing the bacterial enzyme ACC deaminase controlled by the 35S,rolD or PRB-1b promoter. Plant Physiology and Biochemistry 39:19-25

Hiei, Y., S. Ohta, T. Komari, & T. Kumashiro. (1994). Efficient transformation of rice (*Oryza sativa* L.) mediated by *Agrobacterium* & sequence analysis of the boundaries of the T-DNA. The Plant Journal 6:271-282

Hoekema A, Hirsch PR, Hooykaas PJJ, Schilperoort RA (1983) A binary plant vectorstrategy based on separation of vir- and T-region of the *Agrobacterium tumefasciens* Ti-plasmid. *Nature* 303:179-180

Ishii Y, Tanaka M, Takamura T, Goi M (1998) Callus induction and somatic embryogenesis of *Phalaenopsis*. *Plant Cell Rep* 17:446-450

Islam MO, Ichihasi S, & Matsui S. (1998). Control of growth and development of protokorm like body derived from callus by carbon sources in *Phalaenopsis*. *Plant Biotechnol* 15:183-187.

Islam, M. O., Rahman, A. R. M. M., Matsui, S. & A. K. M. A. Prodhan. (2003). Effects of complex organic extracts on callus growth and PLB regeneration through embryogenesis in the Doritaenopsis orchid. Japan Agricultural Research Quarterly, 37, 229-235.

Jacq B, Lesobre O, Sangwan RS, & Sangwan-Norreel BS. (1993). Factors influencing T-DNA transfer in *Agrobacterium*-mediated transformation of sugarbeet. *Plant Cell Rep* 12: 621-624

Jin, R.G., Lui, Y.B., Tabashnik, B.E., & Borthakur, D. (2000). Development of transgenic cabbage (*Brassica oleracea* var. *capitata*) for insect resistance by *Agrobacterium tumefaciens*-mediated transformation. In Vitro Cell. Develop. Biol. Plant 36:231-237

Kanemoto H. & G.J. Wilfret. (1980). Inter- and multi-sectional *Dendrobium* hybrid. In: M.R. Sukshom Kashemsanta (ed.), Proc. 9th World Orchid Conf. 9th World Orchid Conference Committee, Bangkok, pp 255-261

Khatun. M.M., Khatun, H., Khanam, D., & Al-Amin, Md. (2010). *In Vitro* Root Formation And Plantlet Development In Dendrobium Orchid. *Bangladesh J. Agril. Res. 35(2): 257-265*

Klee, H.J., R.B. Horsch, M.A. Hinchee, M.B. Hein, & N.L. Hoffmann. (1987). The effects of overproduction of two *Agrobacterium tumefaciens* T-DNA auxin biosynthetic gene products in transgenic petunia plants. Gene and Development 1:86-96

Kohli, A., D. Gahakwa, P. Vain, D.A. Laurie, & P. Christou. (1999). Transgene expression in rice engineered through particle bombardment: Molecular factors controlling stable expression and transgene silencing. Planta 208:88-97

Kuehnle A.R., & Sugii N. (1992). Transformation of *Dendrobium* orchid using particle bombardment of protocorms. Plant cell Reports 11:484-488

Kuehnle. A.R. (2007). Orchids. Dendrobium. In: Anderson (ed.). Flower Breeding and Genetics, 539-560

Lee, Y.H., Lee, S.B., Suh, S.C., Byun, M.O., & Kim, H.I. (2000). Herbicide resistant cabbage (*Brassica oleracea* ssp. *capitata*) plants by *Agrobacterium*-mediated transformation. J.Plant Biotechnol. 2:35-41

Levin, D.A. (2002). The Role of Chromosomal Change in Plant Evolution. Oxford Series in Ecology and Evolution. Oxford University Press. 230p.

Li, Y., Duan, H., Wu, Y.h., McAvoy, R.J., Pei, Y., Zhao, D., Wurst, J., Li, Q., & Luo, K. (2004). Transgenics of Plant hormones and Their Potential Application in Horticultural Crops. In: Liang and Skinner (ed.). Genetically modified Crops, pp 101-117

Matzke, M.A. & A.J.M. Matzke. (1995). How and why do plants inactive homologous(trans)genes? Plant Physiology 107:679-685

Metz, T.D., Dixit, R., & Earle E.D. (1995). *Agrobacterium tumefaciens*-mediated transformation of broccoli (*Brassica oleracea* var *italic*) and cabbage (*Brassica oleracea* var *capitata*). Plant Cell Reports 15:287-292

Michelmore, R., Marsh E., Seely S., & Landry B. (1987). Transformation of lettuce (*Lactuca sativa*) mediated by *Agrobacterium tumefaciens*. Plant Cell Reports 6:439-442

Mishiba K, Chin DP, & Mii M. 2005. *Agrobacterium*-mediated transformation of *Phalaenopsis* by targeting protocorms at an early stage after germination. *Plant Cell Rep* 24: 297-303

Nishimura A, Tamaoki M, Sakamoto T, Matsuoka M (2000) Over- expression of tobacco *knotted1*-type class1 homeobox genes alters various leaf morphology. *Plant Cell Physiol* 41: 583–590

Oladiran, A. O. & Iwu, L. N. (1992). Changes in ascorbic acid and carbohydrate contents in tomato fruits infected with pathogens. Plant Foods for Human Nutrition, 42, 373-382.

Pawlowski, W.P. & D.A. Somers. (1996). Transgenic inheritance in plant genetically engineering using microprojectile bombardment. Molecular Biotechnology 6:17-30

Pawlowski, W.P., K.A. Torbert, H.W. Rines, & D.A. Somers. (1998). Irregular patterns of transgene silencing in allohexaploid oat. Plant Molecular Biology 38:597-607

Robichon MP, Renou JP, & Jalouzot R. (1995). Genetic transformation of *Pelargonium x hortorum*. *Plant Cell Rep* 15:63-67

Perl A, Lotan O, Abu-Abied M, & D. Holland. (1996). Establishment of an Agrobacterium-mediated transformation system for grape (Vitis vinifera L.): the role of antioxidants during grape-Agrobacterium interactions. Nat Biotechnol 1996 Nov;14(11):1521.

Puri, H.S. (1970). American Orchid. Society Bulletin 39:723

Raghavan, V. (1997) Molecular Embryology of Flowering Plants 1st Edition Cambridge Univ. Press, Cambridge, UK. 525-532.

Sanguthai O., Sanguthai S., & H. Kamemoto. (1973). Chromosome doubling of a Dendrobium hybrid with colchicines in meristem culture. Na Okika o Hawaii 2:12-16

Schelpe S, & J. Stewart. (1990). *Dendrobium*, an introduction to the species in cultivation. Orchid Sundries Ltd, Dorset, Great Britain.

Semiarti E, Ueno Y, Tsukaya H, Iwakawa H, Machida C, Machida Y (2001) The *ASYMMETRIC LEAVES2* gene of *Arabidopsis thaliana* regulates formation of a symmetric lamina, establishment of venation and repression of meristem-related homeobox genes in leaves. *Development* 128:1771-1783.

Semiarti, E, Indrianto, A., Purwantoro, A, Suseno, N., Isminingsih, S., Yoshioka, Y., Iwakawa, H., Machida, Y. & Machida C. (2007). *Agrobacterium*-mediated transformation of the wild orchid species *Phalaenopsis amabilis.* Plant Biotechnology 24(3).

Semiarti, E., A. Indrianto, A. Purwantoro, I.N.A. Martiwi, Y.M.L. Feroniasanti, F. Nadifah, I.S. Mercuriana, R.1, H. Iwakawa, Y.Yoshioka, Y. Machida, & C. Machida. (2010). High-frequency genetic transformation of *Phalaenopsis amabilis* orchid using tomato extract-enriched medium for the pre-culture of protocorms. Journal of Horticultural Science and Biotechnology 85(3):205-210

Sjahril, R. & Mii, M. (2006). High-efficiency Agrobacterium-mediated transformation of Phalaenopsis using meropenem, a novel antibiotic to eliminate Agrobacterium. J. Hort. Sci. Biotech. 81: 458-464.

Sjahril, R., Chin, D.P., Khan, R.S., Yamamura, S., Nakamura, I. Amemiya, & Y. Mii, M. (2006). Transgenic *Phalaenopsis* plants with resistance to *Erwinia carotovora* produced by introducing wasabi defensin gene using *Agrobacterium* method. Plant Biotechnology 23: 191-194

Untari, R. & D.W. Puspitaningtyas (2006), Pengaruh Bahan Organik dan NAA terhadap Pertumbuhan Anggrek Hitam (Coelogyne pandurata Lindl.) dalam kultur in vitro. *Biodiversitas, 7(3), 344-348*.

Villemont E, Dubois F, Sangwan RS, Vasseur G, Bourgeois Y, & Sangwan-Norreel BS. (1997). Role of the host cell cycle in the *Agrobacterium*-mediated genetic transformation of *Petunia*: evidence of an S-phase control mechanism for T-DNA transfer. *Planta* 201: 160-172

Wakita, Y., M. Otani, T. Hamada, M. Mori, K. Iba, & T. Shimada. (2001). A tobacco microsomal ω-3 fatty acid desaturase geneincreases the linolenic acid content in transgenic sweet potato (*Ipomoea batatas*). Plant Cell Reports 20:244-249

Wibowo, R.U.W. (2010), Polinasi pada Tanaman Anggrek Hitam (*Coelogyne pandurata* Lindl.), Fakultas Biologi UGM.

Widiastoety, D. dan Syafril. (1994), Pengaruh Air Kelapa terhadap pembentukan protocorm like bodies (plb) dari anggrek Vanda dalam medium cair, *Jurnal Holtikultura, 4 (2), 71-73*4), Pengaruh Air Kelapa terhadap pembentukan protocorm like bodies (plb) dari anggrek Vanda dalam medium cair, *Jurnal Holtikultura, 4 (2), 71-73*

Yang J, Lee H.J., Shin D.H., Oh S.K., Seon J.H., Paek K.Y., & Han K.H. (2000). Genetic transformation of *Cymbidium* orchid by particle bombardment. Plant Cell Reports 18:978-984

Yang X.H., Pua E.C., & Goh. C.J. (1996). Isolation of a cDNA clone encoding 1-aminocyclopropane-1-carboxylase synthase from *Dendrobium crumenatum* PGR96-088 (Accession No. U64031). Plant Physiol. 112:1383

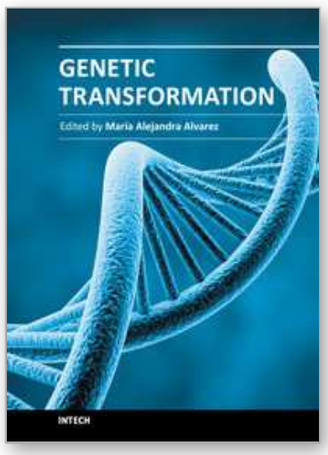
Yang, S.H, Yu H, Goh CJ (2003) Functional characterisation of a cytokinin oxidase gene DSCKO1 in *Dendrobium* orchid. Plant Mol. Biol. 51: 237-248.

Yu H., & C.J. Goh. (2000). Differential gene expression during floral transition in an orchid hybrid *Dendrobium* Madame Thong-In. Plant Cell reports 9:926-931

Yu, H., Yang S.H. Goh CJ (2000) *DOH1,* a class 1 *knox* gene, is required for maintenance of the basic plant architecture and floral transition in orchid. *Plant Cell* 12:2143-2160

Yu H, Yang SH, Goh CJ (2001) *Agrobacterium*-mediated transformation of a *Dendrobium* orchid with the class1 *knox* gene *DOH1*. *Plant Cell Rep* 20:301-305

Zhang, J., T. Van Toai, L, Huynh, & J. Preiszner. (2000). Development of Flooding-tolerant *Arabidopsis thaliana* by autoregulated cytokinin production. Molecular Breeding 6:135-144



Genetic Transformation

Edited by Prof. MarÃa Alvarez

ISBN 978-953-307-364-4
Hard cover, 328 pages
Publisher InTech
Published online 06, September, 2011
Published in print edition September, 2011

Genetic transformation of plants has revolutionized both basic and applied plant research. Plant molecular biology and physiology benefit from this power fool, as well as biotechnology. This book is a review of some of the most significant achievements that plant transformation has brought to the fields of Agrobacterium biology, crop improvement and, flower, fruit and tree amelioration. Also, it examines their impact on molecular farming, phytoremediation and RNAi tools.

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Endang Semiarti, Ari Indrianto, Aziz Purwantoro, Yasunori Machida and Chiyoko Machida (2011). Agrobacterium-Mediated Transformation of Indonesian Orchids for Micropropagation, Genetic Transformation, Prof. MarÃa Alvarez (Ed.), ISBN: 978-953-307-364-4, InTech, Available from: http://www.intechopen.com/books/genetic-transformation/agrobacterium-mediated-transformation-of-indonesian-orchids-for-micropropagation

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