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Development of pollen mediated activation tagging system for *Phalaenopsis* and *Doritaenopsis*

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Abstract In the present study, a novel plant transformation system for *Doritaenopsis* and *Phalaenopsis* has been developed. The pollen-mediated activation tagging system was established by artificial pollination. The pollens, co-cultured with *Agrobacterium tumefaciens* strain EHA105 harbouring an activation tagging vector (pTAG-8), were used for pollination. In order to optimize the transformation efficiency, several factors (concentration of *A. tumefaciens*, concentration of acetosyringone during co-cultivation and the duration of co-cultivation) known to influence *Agrobacterium*-mediated DNA transfer were examined. A concentration of 0.5-1 x 10^8 CFU/ml for *A. tumefaciens,* 0.1 mM acetosyringone, and 6 hrs of co-culture period were found to be the optimal condition for high transformation efficiency. Integration of T-DNA into the genome of putative transgenic plants was confirmed by PCR and DNA blot analyses. Single copy of the transgene was observed in all transgenic plants analyzed. Most of the transgenic plants had a morphologically normal phenotype and the overall capsule formation efficiency was similar to control plant. Our results showed a new approach of genetic transformation in orchids and this method can be employed for genetic improvement of the orchids.

Keywords: activation tagging, *Agrobacterium tumefaciens*, *Doritaenopsis* cultivars, *Phalaenopsis amabilis*, transformation

INTRODUCTION

Doritaenopsis and *Phalaenopsis*, an intergenetic hybrid between the orchid genera *Doritis* and *Phalaenopsis*, are grown throughout Asia and Pacific Ocean islands. *Doritaenopsis* often blooms longer and has more colours than the traditional *Phalaenopsis*. Both of them are cultivated for use as cut flowers or as blooming potted plants. Based on their economic value, genetic improvements with respect to the flower colour, flower shape, disease resistance and flowering time are of considerable importance. This has been achieved through sexual crossing techniques; however, the progress in these respects is slow, due to the long duration of growth and reproductive cycle. One of the effective alternatives to this constraint could be *Agrobacterium* mediated genetic transformation. Several studies have been reported on *Agrobacterium*-mediated transformation of *Phalaenopsis* (Belarmino and Mii, 2000; Chai et al. 2002) and other orchid species (Knapp et al. 2000; Yu et al. 2001; Liau et al. 2003). It is essential to optimize the transformation procedure, since plants exhibit wide variation in susceptibility to *Agrobacterium*. Since, tissue culture based methods require optimization of the plant regeneration system and often generate somaclonal variations, therefore, direct gene transfer method is an alternative for transformation. Direct transformation through pollen grains co-cultivated with *A. tumefaciens* has come up as an interesting alternative technique which has been used for transformation in tobacco (Aziz and Machray, 2003) and lily (Kim et al. 2007).

Transformation efficiencies of orchids, like those of other plants or *fungi*, are correlated to the *Agrobacterium* and AS concentrations and many other factors. Meyer et al. (2003) reported that the excess of *A. tumefaciens* cause a decreased transformation efficiency. High *Agrobacterium* concentration (50 x 10^8 cfu/ml) affected the capsule ratio and the recovery of transgenic seedlings, resulting no transformants in this study. On the other hand, several studies have shown that co-culture periods may affect the transformation efficiency. A prolonged co-cultivation period (3 d) has increased transformation efficiency in rice callus (Mohanty et al. 1999), while overnight co-culture has produced maximum transformants in *Phalaenopsis* callus (Belarmino and Mii, 2000). Co-culture of pollen with *A. tumefaciens* and *A. rhizogenes* have been tested in tobacco (Sanford and Skubik, 1986). However, no transformants have achieved in maize, when, pollens were mixed with plasmid DNA before pollination (Booy et al. 1989). No transformants have achieved in tobacco with mature pollens (Negrutiu et al. 1986). In case of *Arabidopsis thaliana*, transgenic plants have been generated by vaccum-infiltration of whole inflorescences with *A. tumefaciens* in MS medium (Bechtold et al. 1993). Moreover, genetic transformations of Petunia hybrid have been generated by pollinating flowers through vacuum-infiltrated with *A. tumefacien* or by applying a drop of *A. tumefaciens* to the stigma immediately or prior to pollination (Tjokrokusumo et al. 2000). Several reports have demonstrated that flavonoid compounds from pollen have activated the *vir* gene in *A. tumefaciens* (Zerback et al. 1989, Hess et al. 1990), while, ethylene has inactivated *vir* gene and ultimately effected transformation efficiency (Nonaka et al. 2008).

The activation tagging system is normally used for generating gain-of function or loss-of function in mutants. By random insertion of a binary vector into the genome, it may enhance gene expression or knock-out the gene. This activation tagging approach has been successfully used in plants (Van Der Fits et al. 2001; Mathews et al. 2003; Hsing et al. 2007) and *fungi* (Chen et al. 2009).

The purpose of the present study was to develop a pollen-mediated activation tagging system (PMATS) for *Doritaenopsis* and *Phalaenopsis,* and to analyze their effect on morphological changes (flower colour, flower shape, flower longevity, disease resistance and flowering time). To date no study has been done on PMATS in orchids. To our knowledge, this is the first report on PMATS in orchids.

MATERIALS AND METHODS

Plant materials

Potted plants of *P. amabilis* (45 cm in height) with white flowers (10-14 cm in diameter), Dpts. cultivars Sinica Sunday (55-62 cm in height) with pink flowers (10-12 cm), and Dpts. cultivars Taisuco Firebird (57-65 cm in height) with dark pink flowers (10-12 cm) (Figure 1) were grown under greenhouse conditions in Chaoyang University of Technology, Taiwan.

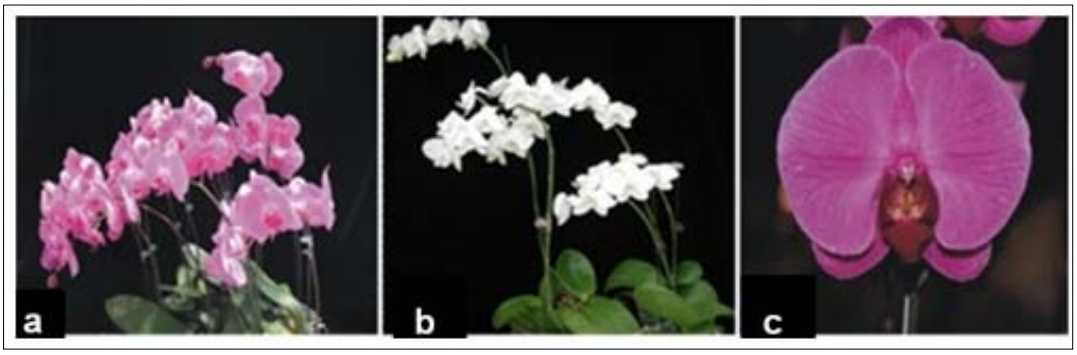

Fig. 1 Potted plants of Dpts. cultivars Sinica Sunday (a), *P. amabilis* (b) and Dpts. cultivars Taisuco Firebird (c).

Agrobacterium strain

A. tumefaciens strain EHA105, carrying a binary vector pTAG8 (Hsing et al. 2007, Chen et al. 2009) was used for the transformation. The T-DNA region of the binary vector contained a selectable marker coding for the hygromycin phosphotransferase (*hptII*) gene under CaMV 35S promoter was kindly provided by Dr. Yu (Academia Sinica). *A. tumefaciens* was cultured on LB medium containing 100 mg/l kanamycin, and 50 mg/l rifampicin at 28ºC for 2 days.

Optimization of transformation conditions and pollination of flowers with pollen co-cultured with *Agrobacterium*

A. tumefaciens cells were harvested by centrifugation at 2500 x g for 10 min and their OD_{600} was determined in resuspended liquid inoculation medium consisting of MS medium supplemented with 50 g/l sucrose, with or without acetosyringone (AS) at different concentrations (0, 0.1, 1, 10 mM). The cultures were then diluted to different concentrations (0.5×10^8, 1×10^8, 5×10^8, 50×10^8 cfu/ml) and used for the transformation. At full bloom, pollen from the flowers of recipient *Doritaenopsis* or *Phalaenopsis* plant to be pollinated was collected individually in 1.5 ml-centrifuge tubes. To each pollen sample, 100 µl *A. tumefaciens* in different concentrations was added without or with AS. The mixture of pollen grains and *Agrobacterium* was pasted either immediately (0 hr) or after different co-culture periods (3, 6, 12, 24 or 48 hrs), onto the stigmas of the same recipient plant, thus facilitating self-pollination. Flowers without *A. tumefaciens* treated pollen served as control. Four months after the pollination, number of capsules formed under each treatment was recorded. The capsule ratio represents number of capsules divided by the number of flowers pollinated under each treatment.

Screening of orchid seedlings for hygromycin resistance

The optimum concentration of selection pressure for transformants was determined by using non-transformed orchid seeds. Capsules from both control and treatments were surface sterilized by immersion in 70% ethanol for 30-60 sec, followed by soaking in 0.5% sodium hypochlorite for 20-30 min, and then rinsed with sterile water. After disinfected capsules were cut open in a sterile chamber, all seeds were scooped out and inoculated in Petri-dishes containing 1/2X MS basal medium (Murashige and Skoog, 1962) supplemented with different concentrations of hygromycin (0, 0.125, 0.625, 1.25, 2.5, 5 and 10 mg/l). All transformants were selected on 1/2X MS basal medium with optimized concentration of hygromycin (5 mg/l, data not shown) 2 months after culture. The putative seedlings which survived on hygromycin were transferred to growth chamber for 3 weeks and later to green house for further growth.

Identification of activation-tagged *Doritaenopsis* and *Phalaenopsis*

To confirm the transformation, hygromycin-resistant transformants were randomly selected for genomic DNA extraction and PCR analysis. Total genomic DNA from the leaves of these putative transformants was extracted by CTAB method (Doyle and Doyle, 1987). Integration of the foreign gene into the genome was confirmed by PCR using *gus* gene specific primers (GUSF: 5'GAAAGGTTGGGCAGGCCAGC3' & GUSR: 5'TCGCCTGTAAGTGCGCTTGCT3'). The PCR was performed by using 50 ng of genomic DNA, 200 µM of each dNTP, 0.5 µM of each *gus* primer and one unit of Taq polymerase. Reactions were carried out with hot-started at 94ºC for 5 min and subjected to 35 cycles of denaturation at 94ºC for 30 sec, annealing at 60ºC for 30 sec, and elongation at 72ºC for 40 sec. The final extension phase was prolonged for 10 min at 72ºC. The amplified 950 bp fragment from a control plant and different transgenic lines were electrophoresed on 1% agarose gel and visualized under UV after staining with ethidium bromide.

DNA blot analysis

Six putative transformants were selected and their genomic DNA (15 µg) was digested with *Eco*RI and *Hin*dIII, the digested DNA was size-fractionated on 1% agarose gel. The gel was treated with dupurination, denaturation and neutralization buffers and DNA was transferred onto positively charged nylon membranes (Bio-Rad) as described by Sambrook et al. (1989). A PCR-amplified *gus* gene fragment was labelled with digoxigenin-9-dUTP using Dig High Prime Labeling and Detection Starter Kit I (Roche, USA). This labelled probe was used for hybridization at 52ºC and later passed through

series of stringency washes (high stringency at 56ºC) to eliminate unspecific binding. The hybridized probes were immune-detected with anti-digoxigenin-AP, Fab fragments and visualized with the colorimetric substrates NBT/BCIP according to manufacturers instruction.

Statistical analysis

Data were analyzed statistically by using Fisher's protected least significant difference (LSD) test at the 5% probability level.

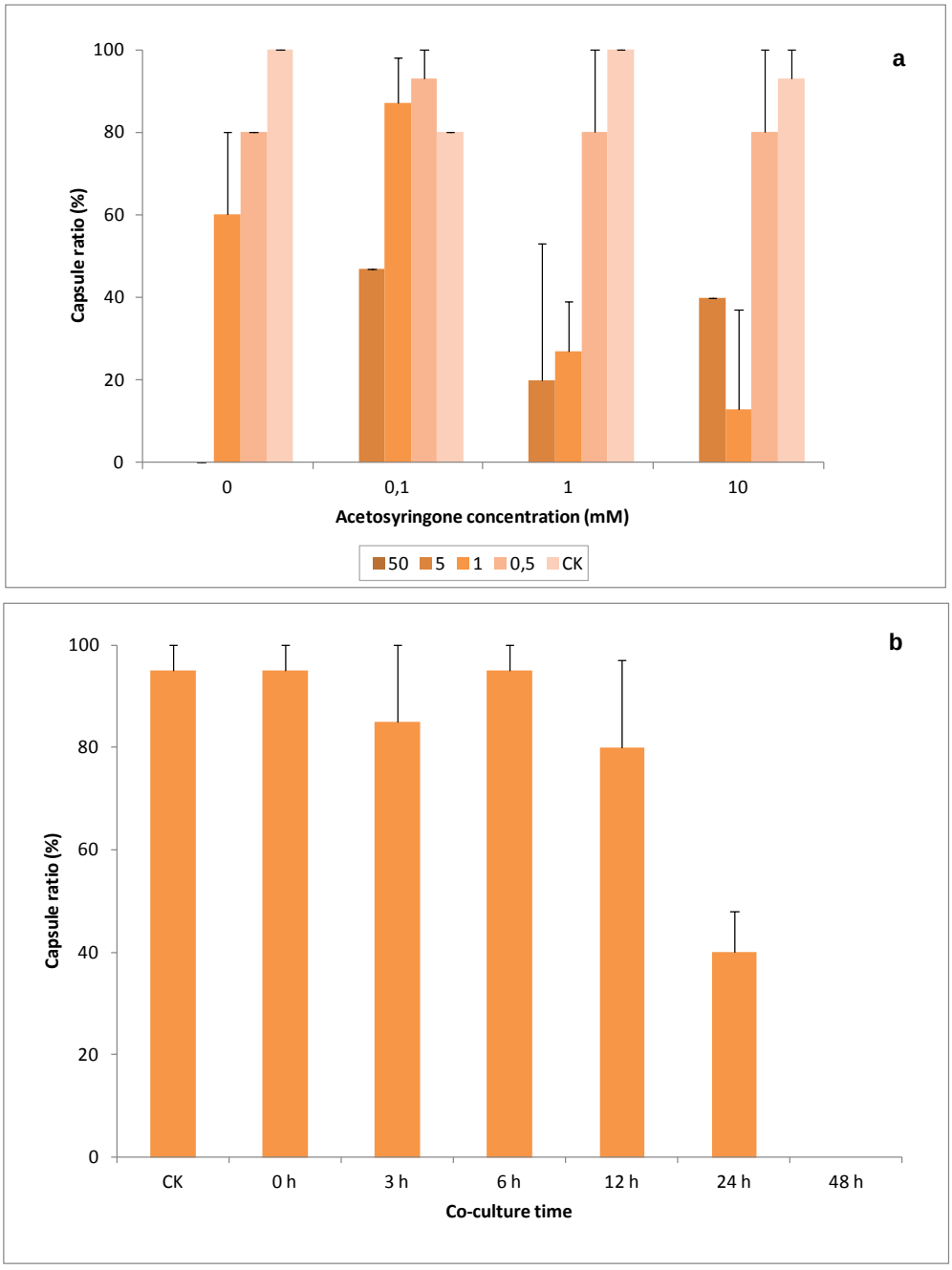

Fig. 2 Effect of acetosyringone concentration (a) and co-culture period of pollen and *Agrobacterium* (b) on capsule ratio.

RESULTS

Optimal conditions for high activation-tagged transformation efficiency in both *Doritaenopsis* and *Phalaenopsis*

In order to obtain the highest number of pollen-mediated activation-tagged transformants of *Doritaenopsis* and *P. amabilis* cultivars, a series of studies on *Agrobacterium* concentration, AS concentration, and co-culture period were carried out. Out of several *Agrobacterium* concentrations tested, 0.5 x 10^8 cfu/ml to 1 x 10^8 cfu/ml was the best for the co-culture. High concentration (50 x 10^8 cfu/ml) of *Agrobacterium* produced no transformants, under different concentration of AS, an inducer of the *vir* gene expression of *A. tumefaciens* (Figure 2a). Higher concentration of AS had negative effect on the capsule ratio while lower concentrations promoted capsule ratio. Thus, capsule ratio was used as one of criteria to select transgenics and with lower concentration of AS (0.1 mM) and *A. tumefaciens* (0.5 x 10^8 cfu/ml to 1 x 10^8 cfu/ml), the capsule ratio increased significantly. Optimizing the co-cultivation time with *A. tumefaciens* to 6 hrs yielded the highest transformation frequency (Figure 2b). These experiments demonstrated that 0.5 x 10^8 cfu/ml to 1 x 10^8 cfu/ml of *A. tumefaciens,* 0.1 mM AS, and 6 hrs of co-culture period are the optimum conditions for the transformation of *P. amabilis* and *Doritaenopsis* cultivars. The seeds obtained from the capsule of putative transformants were germinated on hygromycin (5 mg/l) 2 months after culture (Figure 3b and c), while no germination was observed from the seeds obtained from non transformed capsule (Figure 3a). A total of 30 transformants of *P. amabilis*, 7 transformants of Dpts*.* cultivars Sinica Sunday, and 15 transformants of Dpts*.* cultivars Taisuco Firebird were obtained after selection in 5 mg/l hygromycin.

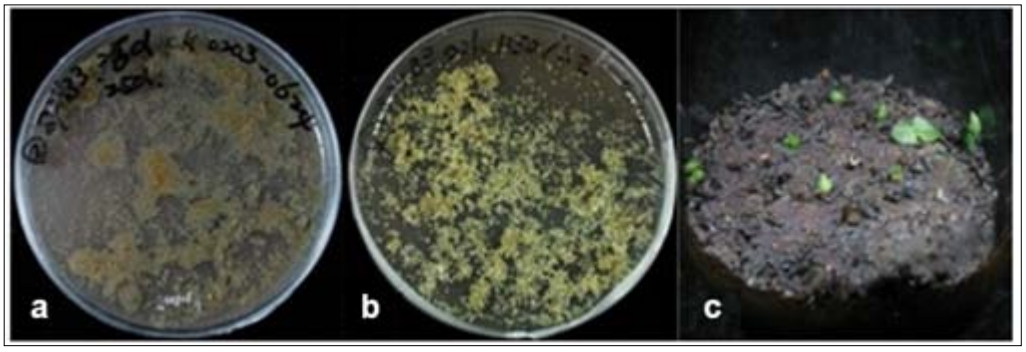

Fig. 3 Germination of transgenic plantlets under selection pressure (b and c). (a) is control.

Identification of T-DNA insertion by PCR

Although the most widely used method for the generation of transgenic plants is the *Agrobacterium*-mediated transformation which precisely delimited DNA sequences with a single integration (Lawrence and Koundal, 2001), the structure of the inserted T-DNA varies widely to include single or multiple copies, at a unique or several loci in the plant genome. Total DNA isolated from the putative transformants was tested for the presence of the transgene. PCR amplification confirmed that the *gus* genes were present in plants grown in hygromycin selection medium. The PCR results of expected 950 bp fragments in four randomly selected (two from *P. amabilis*, one from Dpts*.* cultivars Sinica Sunday and one from Dpts*.* cultivars Taisuco Firebird) putative transgenic orchids are displayed in Figure 2 . In this experiment, no amplification was observed in the untransformed control (Figure 4).

DNA blot for transformants

DNA blot analysis were carried out to evaluate further the transfer and insertion of foreign gene lies between left and right border in the genome of the transformed *Phalaenopsis* and Dpts. cultivar. Hybridization of *gus* gene to *Eco*RI and *Hin*dIII digested genomic DNA from the transgenic plantlets is shown in Figure 5 (transformant of Dpts*.* cultivars Sinica Sunday shown inlane 1, transformant of *P. amabilis* in lane 2, and transformants of Dpts*.* cultivars Taisuco Firebird in lane 3-6) respectively. The

results confirmed that all six selected transgenic plantlets, contained bands with sizes larger than the *gus* gene fragment (950 bp) and were indicative of integration of the introduced plasmid part into the host plant genome. The results confirmed the transgenic nature and showed a uniform insertion pattern with only single insertion. No bands were detected in the wild type (Data not shown). The presence of calcium oxalate in the tissues of these species makes it difficult to isolate DNA. Thus, only few transgenic were analyzed through Southern blots.

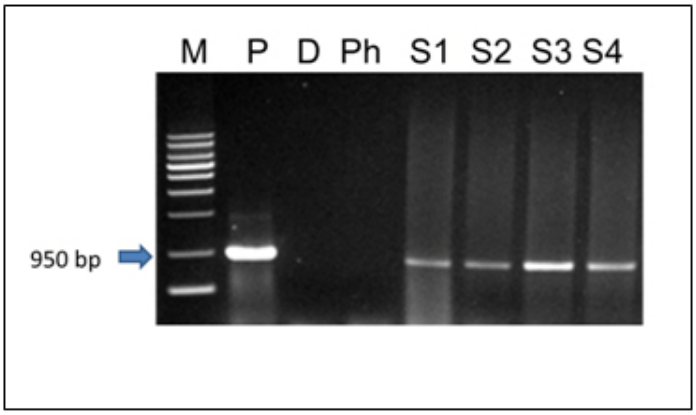

Fig. 4 Identification of randomly selected seedling of putative T1 transformants of *Phalaenopsis and Doritaenopsis* by PCR of *GUS* gene. M: DNA marker; P: pTAG-8 as template; D: WT Dpts. Cultivars; Ph: WT *P. amabilis*; S1-4: putative transformants.

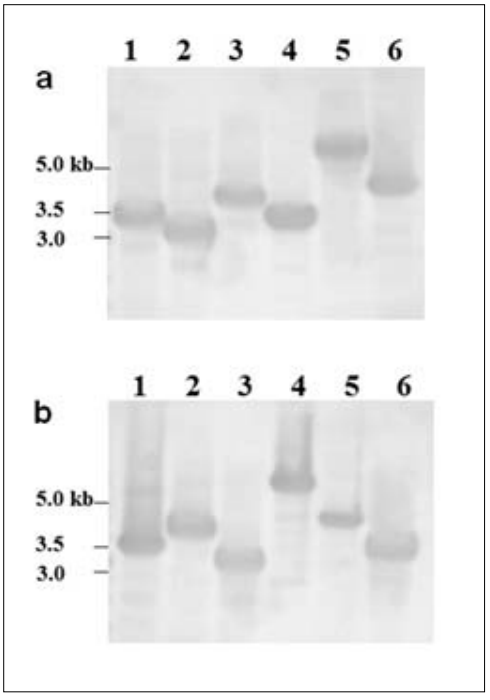

Fig. 5 Confirmation of T1 transformants of *Phalaenopsis and Doritaenopsis* by southern blots. Six randomly selected T1 transformants were digested by *Eco*RI (a) or *Hind*III (b), and hybridized by *GUS* probe. 1: Transformant of Dpts. cultivars Sinica Sunday; 2: Transformant of *P. amabilis*; 3-6: Transformants of Dpts. cultivars Taisuco Firebird.

Phenotypic characterization of dominant mutant in *Doritaenopsis* and *Phalaenopsis*

The selected T1 transformants of *Phalaenopsis* and Dpts. cultivars were transferred to green house for acclimation to *ex vivo* conditions and their phenotypic characteristics were analyzed in two years of their growth. Most of transformants are morphologically similar to wild types. A small number of T1 transformants showed distinct phenotypic variations. Stunted growth was observed in the transformants of *P. amabilis* and of Dpts. cultivars (22 cm, one half of the wild type; Figure 6a, 6b and 6f) among the selected 30 transgenic *P. amabilis* (3%), 7 transformants of Dpts. cultivars Sinica Sunday (14%) and 15 transformants of Dpts. cultivars Taisuco Firebird (6%). Small size flowers were observed in two of the 15 transformants of Dpts. cultivars Taisuco Firebird (Figure 6c and 6e) and delayed flowering was observed in another selected transgenic Dpts. cultivars Taisuco Firebird (Figure 6d).

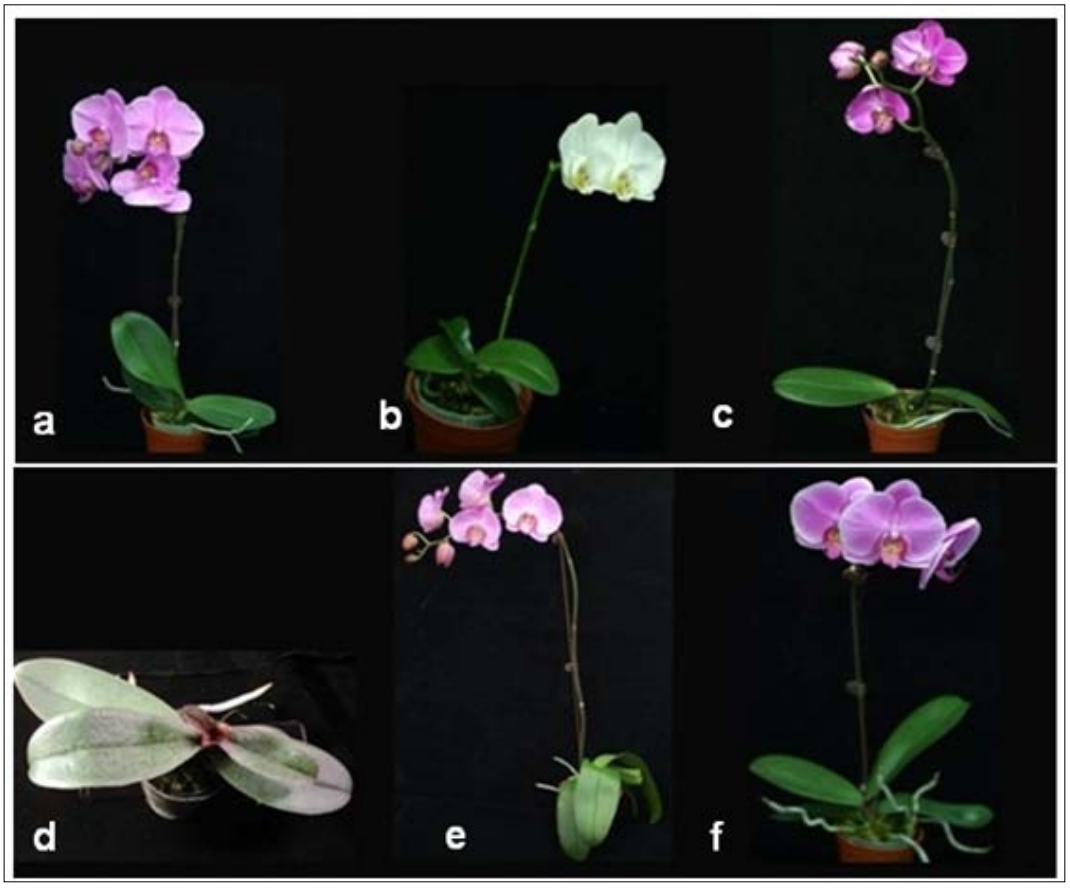

Fig. 6 Phenotypic characterization of dominant mutants in *Phalaenopsis and Doritaenopsis.* (a) transformant of Dpts. cultivars Sinica Sunday; (b) transformant of *P. amabilis*; (c-f) transformants of Dpts. cultivars Taisuco Firebird.

DISCUSSION

Activation tagging has been used as a powerful tool to discover gene functions as well as to identify promoter regions. Many transgenic plants (Tani et al. 2004) or *fungi* (Chen et al. 2009) have been generated by this method. In the present study, a successful activation-tagged system was established in *Doritaenopsis* and *Phalaenopsis* via pollen-mediated *Agrobacterium* transformation. *P. amabilis*, Dpts. cultivars Sinica Sunday, and Dpts. cultivars Taisuco Firebird were used as the starting material for establishing PMATS because of their remarkable economic values.

The pollen grains were co-cultured with *Agrobacterium* for different time periods and maximum transformation efficiency were obtained 6 hrs after co-cultivation, the possible reason for maximum transformants after 6 hrs could be the counterfeit effect of flavonoids over ethylene which enhanced v*ir* gene activation and ultimately total transformation efficiency. When co-culture period extended further the ethylene accumulation might have surpassed the flavonoid thus, reduced transformation efficiency. Flavonoids itself act as a chemical inducer and due to the presence of these flavonoid compounds in pollen required low levels of AS (0.1 mM) sufficient for good transformation efficiency in *P. amabilis*, and Dpts. cultivars. In the present study, a pollen-mediated transformation system with 0.1 mM AS, 0.5 x 10^8 cfu/ml to 1 x 10^8 cfu/ml of *A. tumefaciens* and co-culture for 6 hrs was established in *P. amabilis*, and Dpts. cultivars.

Integration of the T-DNA (from pTAG8) into the putative transgenic plant genomes was further confirmed by PCR and Southern blot analyses. Plants grew in selective media were randomly selected for molecular analysis. PCR analyses were conducted using the primer set for the *gus* gene and showed the expected size bands of 950 bp, in all randomly selected plants (Figure 4). The amplified bands were not detected with *gus* primer set in untransformed plants. Southern blot analysis was carried out using the *gus* gene as probe. Genomic DNA from six putative transgenic plants was digested with *Eco*RI and *Hin*dIII, which only cut *gus* gene once and allowed to hybridize with the *gus* probe. Single copy insertion was observed in all six T1 transformants: one from Dpts. cultivars Sinica Sunday (Figure 5a, lane1; Figure 5b, lane 1), one from *P. amabilis* (Figure 5a, lane 2; Figure 5b, lane 2) and four from Dpts. cultivars Taisuco Firebird (Figure 5a, lane 3-6; Figure 5b, lane 3-6). Single or low-copy-number integration of transgenes through *Agrobacterium* have been observed in several plant species (Fu et al. 2000; Ming et al. 2000; Frame et al. 2002). Although a single-copy insertion may facilitates the recovery of DNA sequences of the affected gene in most fungal genomes (Michielse et al. 2005), it is very difficult to isolate the flanking sequence of T-DNA in orchids by using TAIL-PCR or inverse PCR.

Without knowing the sequence of the affected gene, the PMATS developed in the present study is very useful in improving flower phenotypes or flowering time. Among 52 transformants (30 transformants of *P. amabilis,* 7 transformants of Dpts. cultivars Sinica Sunday and 15 transformants of Dpts. cultivars Taisuco Firebird) obtained, a small number of T1 transformants showed distinct phenotypic variations: stunted growth, small size flowers and delayed flowering. The occurrence of these changes might be caused by the integration of transgene or due to somaclonal variations. These variations frequently occur in *in vitro* culture of orchids (Chen et al. 2005). These variations are more frequent when culture goes through prolonged sub-culturing and longer duration of induction (Thorpe, 1994).

An efficient, rapid and simple PMATS was developed in *P. amabilis*, Dpts. cultivars Sinica Sunday, and Dpts. cultivars Taisuco Firebird. This is the first report on PMATS in orchids and it can be used for genetic improvement in both *Phalaenopsis and Doritaenopsis*.

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