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Full Length Research Paper

Sonication assisted *Agrobacterium*-mediated transformation of chalcone synthase (*CHS*) gene to Spring *Dendrobium* cultivar 'Sanya'

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In this study, a protocol was developed to obtain stable lines of the Spring *Dendrobium* cultivar 'Sanya' via sonication assisted *Agrobacterium*-mediated transformation (SAAT) of protocorm-like bodies (PLBs). *Agrobacterium tumefaciens* strain LBA4404 was used with the binary vector AG205 containing the chalcone synthase (*CHS*) gene for flower color change and the neomycin phosphotransferase II gene (NPTIIgene) as a selectable marker for kanamycin resistance. PLBs were treated for 10 min using ultrasound (40 KHz) and subsequently immersed in the *Agrobacterium* suspension for 60 min. The addition of 100 µM acetosyringone (AS) during the pre-culture and co-culture achieved the highest efficiency of gene transformation (0.5%). Following co-cultivation, PLBs were cultured on selective medium containing 200 mgL^{-1} kanamycin and 250 mgL^{-1} cefotaxime. Proliferating PLBs were repeatedly selected for kanamycin resistance and stable transformed lines were generated. Incorporation and expression of transgenes were confirmed by PCR analysis, southern blot analysis and reverse transcription-quantitative real-time PCR (RT-PCR). A total of five southern blot positive lines were obtained from co-cultivated PLBs.

Key words: Spring *Dendrobium* cultivar 'Sanya', chalcone synthase (*CHS*) gene, transformation, sonication assisted *Agrobacterium*-mediated transformation (SAAT), protocorm-like bodies (PLBs).

INTRODUCTION

Dendrobium is one of the most famous tropical orchids. Members of the genus are widely used as potted plants, cut flowers and several species exhibit medicinal properties. Spring *Dendrobium* have tropical origins and exhibit spring flowering. These species are used in flower festivals and are in increasingly high demand as the ornamental potted flowers popular in the international flower markets, especially Japan, the United States and Europe (Hu, 2002).

Genetic engineering offers an effective way to develop improved cultivars. In the horticultural and floriculture industry, flower color is one of the most important traits to be addressed in terms of commercial value. The development of transgenic cultivars with increased color value is a more efficient means to increase and enhance color variability in Spring *Dendrobium* than traditional breeding programs. The factors influencing flower color change are numerous. Chalcone synthase is a key enzyme in the formation of several major classes of flavonoids, including flavonols, flavones, isoflavonoids and anthocyanins. Flavonoids are a large class of secondary metabolites in plants, which provide beautiful pigmentation in flowers, fruits, seeds and leaves. They also play key signaling roles between plants and microbes, as antimicrobial agents and feeding deterrents, and in the protection of plants against ultraviolet (UV) light (Forkmann et al., 2001; Winkel-Shirley, 2001; Meer, 1993). The chalcone synthase (*CHS*) gene was first isolated from suspension cells of *Petroselium hortense*

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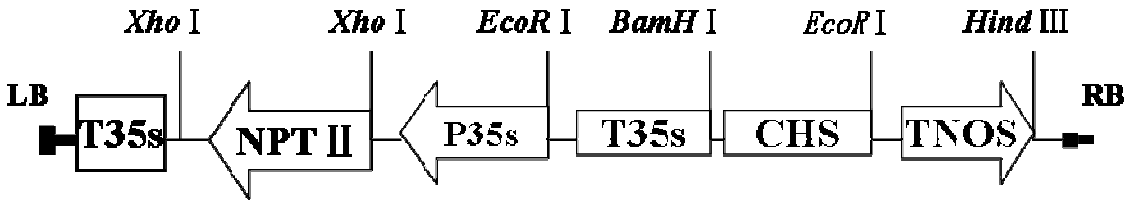

Figure 1. Schematic structure of the T-DNA region of the AG205 plasmid. LB, Left border; RB, right border; T35S, CaMV 35S terminator; *NPT*II, the coding region of the neomycin phosphotransferase gene; P35S CaMV, 35S promoter; CHS, chalcone synthase gene; TNOS, terminator of the nopaline synthase gene.

(Krenzaler, 1979) and serves in the regulation of flower color (Huang et al., 2004). This gene has already been applied to modify flower color in several ornamental plants (Yang et al., 2004; Koes et al., 1989; Zuker et al., 2002) and a medicinal plant *Echinacea purpurea* (Wang and To, 2004). Reports of successful introduction of commercially valuable genes in *Dendrobium* cultivars are limited. Transgenic *Dendrobium* production aimed at developing a transformation system using *Agrobacterium*-mediated and biolistic transformation methods have recently been reported (Men et al., 2003, a b; Yu et al., 2001). However, the protocorm that was used in the earlier mentioned studies as the transgenic receptor material was obtained from seed germination, which is known to exhibit unreliable segregation patterns. Consequently, it is difficult to select for favorable parental traits. It is possible to use this material as a receptor in a transgenic research system; however, the system has no practical value for transferring a functional gene in order to generate a valuable transgenic variety. In this study, protocorm-like bodies (PLBs) induced from 'Sanya' clone explants were used as the transgenic receptor, which produced a more efficient method for the production of transgenic *Dendrobium*.

Trick and Finer (1997) reported that sonication assisted *Agrobacterium*-mediated transformation (SAAT) is an efficient *Agrobacterium*-based transformation technology for soybean, which enhanced the transient expression of a *β*-glucuronidase (GUS) reporter gene in five other species. Pathak and Hamzah (2008) found that the transformation efficiency was more than two times higher with SAAT treatment than simple *Agrobacterium* transformation without sonication in chickpeas. Subsequently, the ultrasound treatment has been successfully employed to enhance transformation efficiency in several plant species, both by direct DNA transfer to protoplasts and tissue cultures, and co-cultivation with vector bacteria (Joersbo and Brunstedt, 1990, 1992; Zhang et al., 1991; Finer and Finer, 2000; Zaragoza et al., 2004; Beranova´ et al., 2008).

The *CHS* gene used in this work was cloned from *Petunia hybrida*. Exogenous transfer of the *CHS* gene into PLBs was conducted to regulate endogenous *CHS* gene expression in transgenic lines to affect synthetic flower color in Spring *Dendrobium* varieties. This investigation served to design and develop an efficient and reliable transformation strategy for Spring *Dendrobium* variety 'Sanya' and transfer the *CHS* gene into "Sanya' PLBs. The transformation efficiency of 'Sanya' by *Agrobacterium* with and without sonication was compared during the transgenic process. Our study tested the efficiency of SAAT to see if it could be in 'Sanya' transformation.

The objectives of this study were to develop a protocol for the transfer of functional genes to Spring *Dendrobium* cultivars, with the specific secondary aim of producing a methodology that would induce color variation in Spring *Dendrobium* 'Sanya' from the overexpression of the *CHS* gene. This would be in order to generate brightly colored Spring *Dendrobium* cultivars with commercial potential. However, the ultimate success of our study will not be determined until the Spring *Dendrobium* flowers, can take up to 2.5 years from young transgenic material.

MATERIALS AND METHODS

Vigorous Spring *Dendrobium* 'Sanya' PLBs were selected as receptor transgenic materials. PLB induction and rapid proliferation followed the methods of Zheng et al. (2009).

Agrobacterium tumefaciens strains and plasmid

A. tumefaciens strain LBA4404 was used for the transformation studies. The binary vector AG205 T-DNA region contains the *CHS* gene from *P. hybrida* responsible for flower color change, and the selectable *NPT*II gene, which encodes the neomycin phosphotransferase to express kanamycin resistance in transgenic lines. *CHS* and *NPT*II genes are under control of the cauliflower mosaic virus (CaMV) 35S promoter. The T-DNA region of the plasmid is shown.

Inoculation and co-cultivation

Single colonies of *A. tumefaciens* strains were selected, and cultured overnight in liquid LB medium containing 50 mg L^{-1} tetracycline and 150 mg L^{-1} streptomycin, agitated (200 rpm) in a shaking incubator at 27°C to an OD_{600} equal to approximately 0.6, and then centrifuged at 4,000 rpm for 8 min. The supernatant was discarded, the pellet was re-suspended in 25 ml of liquid MS medium containing 100 µM acetosyringone (AS), and agitated (50 rpm) again in a shaking incubator at 27°C for 60 min before

inoculation. 'Sanya' PLBs were pre-cultured for 3 to 4 days on 1/2 MS medium supplemented with 1 mg L^{-1} 6-benzylaminopurine (BA) and 100 µM AS in the dark. The pre-cultured PLBs were then immersed in 20 ml sterile water, and subjected to pulses of ultrasound for 5, 10 and 15 min (40 kHz, 35 W) delivered by the Sonorex HZ-40s apparatus (Boao, Ningbo China). Periods of 0 pulses were used as a control (CK, no ultrasound applied). Following sonication, sterile water was discarded and the PLBs were immersed in the *A. tumefaciens* suspension, and inoculated for 30 and 60 min at 27°C. After treatment, the PLBs were blot dried on sterile filter paper, and co-cultured on 1/2 MS medium with 1 mg L^{-1} BA and 100 µM AS at 25°C in the dark for 2 to 3 days until *A. tumefaciens* growth was observed. After 2 to 3 days of co-cultivation, the cultures were transferred to selective medium [1/2 MS medium containing 1 mg L^{-1} BA, 250 mg L^{-1} cefotaxime (Cef), 200 mg L^{-1} kanamycin (Km), 150 ml L^{-1} coconut water (CW)] and incubated at 25°C under a 14-h (14 h day/10 h night) photoperiod with a light intensity of 20 µmol m^{-2} s^{-1}. Tissues were sub-cultured to a new selective medium every month. Preliminary experiments indicated that 100 µM AS in the inoculation and co-cultivation media increased transient expression efficiency of 'Sanya' PLBs. Kanamycin was included in the medium through the selection and culture phase, however, cefotaxime was omitted after 2 months of selection. Following 4 months of culture on kanamycin medium, surviving and actively growing PLBs were selected and cultured for plant regeneration on 1/2 MS medium (containing 1 mg L^{-1} BA, 0.2 mg L^{-1} NAA, and 150 ml L^{-1} CW) supplemented with 200 mg L^{-1} Km. After 30 days of culture, regenerated plantlets were transferred to 1/2 MS medium (containing 1 mg L^{-1} BA, 0.1 mg L^{-1} NAA, 150 ml L^{-1} CW and 200 mg L^{-1} kanamycin) for root establishment.

PCR analysis

The presence of *CHS* gene in kanamycin resistant and non-transgenic control plants was analyzed via PCR. Genomic DNA was isolated using the WizardR Genomic DNA purification kit (Promega) following the plant DNA isolation procedure. PCR amplification was performed in a Gene System 9600 Thermocycler (Perkin Elmer, USA). A 30 µl PCR reaction mixture was comprised of 100 ng genomic DNA, 1.5 U Taq polymerase (Promega), 1 mM $MgCl_2$ and 0.5 µM mixed *CHS* gene primer set. The following *CHS* primer sequence sets were used: CHS F: 5' –ATGGTGACAG TCGAGGAG-3') and CHS R: 5'-TTAAGTAGCAACACTGTG-3'.

PCR reaction conditions were as follows: 94°C for 120 s; 30 cycles of 94°C for 60 s, 55°C for 120 s, 72°C for 120 s; and one cycle at 72°C for 10 min. The PCR products were separated on a 1.5% (w/v) agarose gel.

Southern blot hybridization

Kanamycin resistant lines and non-transgenic plant genomic DNA (15 µg) were digested with *EcoR*I, separated on 1.2% agarose gels, and blotted onto positively charged nylon membranes (RPN3001 Southern blot kit, Amersham, UK) with 20× SSC according to the manufacturer's recommendations. The *CHS* gene plasmid of AG205 was digested with *EcoR*I, and probe was labeled with ECL biotin (Amersham, UK). Blots were hybridized overnight with the ECL biotin-labeled DNA probes at 42°C in ECL Standard Hybridization buffer. Following hybridization, blots were washed twice for 10 min each in primary washing buffer at 42°C, followed by two 5 min washes with secondary washing buffer at room temperature. Colorimetric detection was performed with NBT and BCIP (Boehringer Mannheim) according to the manufacturer's instructions.

Real-time RT-PCR analysis

Total RNA was extracted using Trizol RNA Extraction Kit (Invitrogen, USA), and cDNA synthesis according to the technical guidelines established for the Oligo (dT) method (Beijing Dingguo, China); 2 µl total RNA of each sample was reverse transcribed into cDNA. Fluorescence based real-time quantitative RT-PCR was performed in the ABI PRISM 7700 Sequence Detector (ABI USA) using fluorescent SYBR Green dye I (Invitrogen, USA). β-Actin was employed as the restricted reference gene. The forward and reverse β-actin primer sequences were as follows: Actin F 5' GTTCTCAGTGGTGGTTCTAC 3'; and Actin R 5' GACCCTG ACTCCTCATACTC 3'. Reactions were carried out in a 50 µl volume capillary tube containing 2 µl cDNA, 2.5 µl 10 × PCR Buffer, 0.5 µl 10 mM dNTPs, 0.25 µl 20 pmol/µl primer F, 0.25 µl 20 pmol/µl primer R, 0.5 µl 2 u /µl Taq DNA polymerase, 1 µl 10 × Sybr Green I, and 18 µl ddH_2O. PCR conditions were as follows: 94°C for 120 s; 35 cycles of 94°C for 30 s; 56°C for 30 s; and 72°C for 30 s.

Following real-time RT-PCR, the exogenous *CHS* gene Ct values and the β-actin internal standard gene values were obtained. cDNA expression was calculated for the *CHS* gene in each sample using the following formula: $\Delta Ct = 1000 \times Ct_{CHS} / Ct_{\beta\text{-}actin}$. Expression among the transgenic lines and the control were compared.

Statistical analysis of transformants

Transformation experiments using SAAT and the simple *Agrobacterium-mediated* method were repeated three times using 200 PLB explants in each experiment. Data were analyzed and the results are presented in Table 1. Results represent means ± standard errors. The two treatments (SAAT vs. absence of sonication and inoculation) were statistically tested to determine any significant increase in transformation efficiency. Fisher's (F) protected least significant difference test (LSD) was applied following a two-way analysis of variance (ANOVA). LSD compared treatment group following ANOVA null hypothesis of equal means rejecting the ANOVA F-test.

RESULTS AND DISCUSSION

Transformation, selection and regeneration of transgenic lines

Following six to eight weeks on selection medium with both antibiotics, green-colored, kanamycin-resistant cell clusters (0.5 to 2 mm in diameter) were observed on white PLBs (Figure 2B). The kanamycin-resistant PLBs were selected and sub-cultured on the same selection medium, where the majority of the recovered cell clusters continued to proliferate. Four to six weeks after PLB transfer to the regeneration medium, the plantlets were reestablished. Root formation was initiated when shoots reached 1 to 2 cm in height. The stages involved in the production of transgenic 'Sanya' plantlets from SAAT treated PLB explants is depicted in Figure 2A to D.

A total of 61 kanamycin resistant lines were obtained from 4800 pieces of co-cultivated PLBs in three independent transformations (Table 1). Among the different sonication treatments, SAAT treated for 10 min and then inoculated for 60 min showed better effects than others. The stages involved in production of transgenic

Table 1. The influences of different sonication treatment times and inoculation periods on 'Sanya' transformation efficiency[a,b].

Inoculation time (min)	Time of sonication Treatment (min)	Kan + plants[c] (number)	PCR + plants [d] (number)	Southern blot + plants[e] (number)	Transformation efficiency[f] (%)
60	15	4	1	0	
	15	3	0	0	0.17± 0.29[b]
	15	4	1	1	
	10	9	2	1	
	10	6	3	2	0.5± 0.29[a]
	10	11	1	0	
	5	1	0	0	
	5	3	1	0	0[b]
	5	1	1	0	
	0	0	0	0	
	0	1	0	0	0[b]
	0	0	0	0	
30	15	2	0	0	
	15	1	1	0	0[b]
	15	1	0	0	
	10	4	0	0	
	10	2	1	0	0.17±0.29[b]
	10	6	1	1	
	5	1	1	0	
	5	0	0	0	0b
	5	0	0	0	
	0	0	0	0	
	0	0	0	0	0b
	0	1	0	0	
Account [g]		61	14	5	

Data presents the means ± SE of three independent experiments using 200 PLB explants in each experiment. Two-way analysis of variance (ANOVA) was tested for significant differences in transformation efficiency based on independent transformed plant lines of four different sonication treatment times and three different inoculation times. ANOVA was followed by Fisher's protected least significant test (LSD). Data within the same column followed by the same letter are significantly different at 5% level of significance ($P < 0.05$). [a]100 µM AS was added to both inoculation and co-cultivation media; [b]200 PLB explants were used in each experiment; [c] Kanamycin resistant plant lines. [d]presence of the *NPT*II gene was analyzed by PCR; [e] analysis of the *NPT*II gene by Southern blot; [f] percentage of transformation efficiency was derived by dividing 100 × the number of confirmed transformed plants of independent lines (Southern blot positive) by the number of treated explants; [g]the total number of kanamycin resistant, PCR positive and Southern positive lines were obtained from 4800 pieces of co-cultivated PLBs in three independent transformations.

'Sanya' plantlets from SAAT treated PLB explants is depicted in Figure 2A to D.

PCR assays

PCR and Southern blot analysis were used to confirm the presence of T-DNA in plasmid AG205 and its integration into transgenic lines. *CHS* sequence amplifications from different transgenic lines using *CHS* specific primers produced a 1200 bp fragment. The fragment was not detected in the control PCR amplifications using non-transformed plants and the same primers (Figure 3A). 14 PCR positive lines were identified from the 61 kanamycin resistant lines.

Southern blot assays

Southern hybridization was performed on the PCR positive lines, using a PCR amplified *CHS* probe digested with *Eco*RI, and labeled with ECL biotin. A band of approximately 1.2 kb, which corresponded to the AG205 target fragment digested with *Eco*RI was detected only in the genome of transgenic 'Sanya' lines and not in the control (CK, non-transgenic). Following digestion of genomic DNA with *Eco*RI, the minimum hybridization fragment size was 1.2 kb for the *CHS* gene probe. The results confirmed that *CHS* gene was integrated into the 'Sanya' transgenic lines.

From Southern blot assays, five independent positive 'Sanya' transgenic lines were obtained from 14 PCR

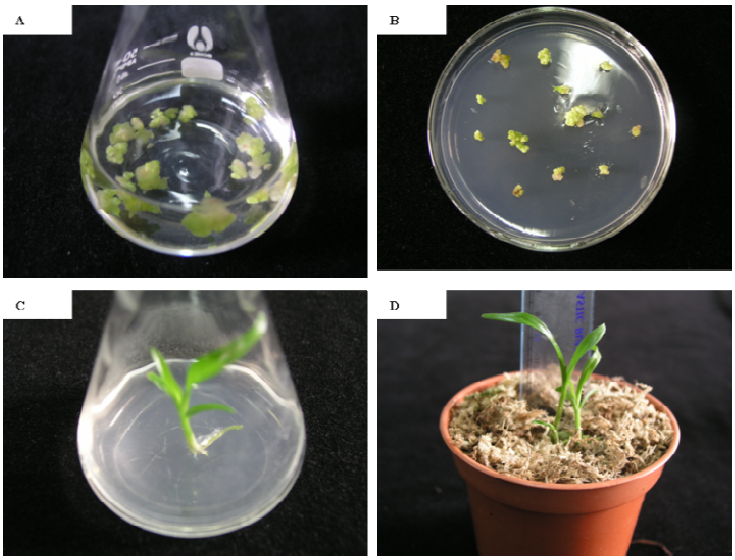

Figure 2. 'Sanya' *CHS* gene transformed transgenic lines. A, PLB explants were inoculated with *A. tumefaciens* strains LBA4404 suspension; B, PLB explants inoculated with *A. tumefaciens* placed on the selective medium (1/2 MS medium containing 1 mg L^{-1} BA, 250 mg L^{-1} Cef, 200 mg L^{-1} Kn, 150 ml L^{-1} CW); C, Kanamycin resistant Spring *Dendrobium* plantlet of A3 cultured on 1/2 MS medium (containing 1 mg L^{-1} BA, 0.1 mg L^{-1} NAA, 150 ml L^{-1} CW) without kanamycin to stimulate root growth; D, transgenic plantlet of kanamycin resistant Spring *Dendrobium* 'Sanya' line A3.

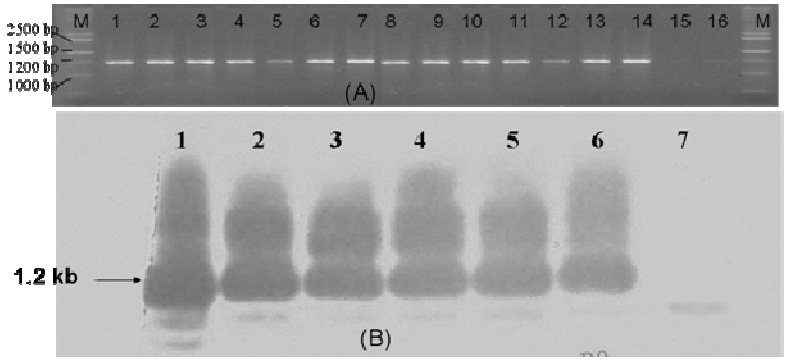

Figure 3. Molecular analysis of the transgenes in transgenic Spring *Dendrobium* plants. (A.) PCR analysis of transgenic 'Sanya' lines using *CHS* specific primers; M, λDNA markers *Hind*III + *ECOR*I; 1 to 14, PCR positive 'Sanya' lines; 15, non- transgenic CK; 16, AG205 positive plasmid. (B) Southern hybridization blot of 'Sanya' transgenic lines obtained by the SAAT method to detect the genomic integration of the *CHS* gene in transgenic plants. Genomic DNA from the non-transgenic control plant (CK) and different lines of 'Sanya' transgenic plants were digested with *EcoR*I and size fractionated via electrophoresis. DNA blots were hybridized with an ECL biotin probe containing *chs* cDNA. 1, Positive plasmid; 2 to 6, 'Sanya' transgenic lines A1 (2), A2 (3), A3 (4), A6 (5) and A10 (6); 7, non-transgenic CK.

positive lines. The maximum transformation efficiency of 0.5% was achieved under this condition. Identified transgenic lines were coded as A1, A2, A3, A6 and A10 (Figure 3B).

Real-time RT-PCR assays

Transgenic line A1, A2, A3, A6 and A10 and the control (CK) were analyzed by fluorescence based real-time

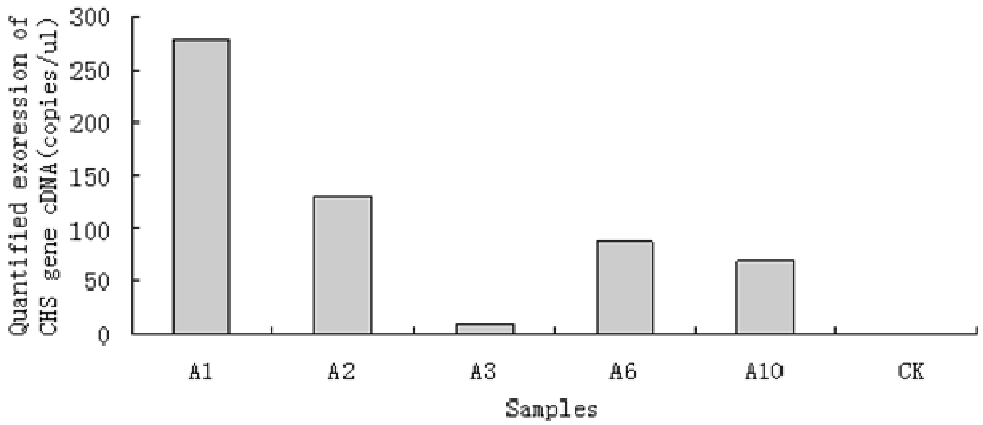

Figure 4. Quantified expression of the *CHS* gene cDNA of 'Sanya' transgenic lines and non-transgenic control samples using the comparative Ct method in real-time RT-PCR, cDNA (copies µl $^{-1}$): $\Delta Ct = 1000 \times Ct_{chs} / Ct_{\beta\text{-}actin}$.

quantitative RT-PCR analysis. *CHS* gene Ct values and the β-actin internal standard gene values were obtained (Figure 4). Results indicated that the *CHS* gene cDNA expression in different transgenic lines and the CK differed. Among these five transgenic lines, the highest and lowest cDNA expression levels of *CHS* were found in transgenic line A1 and A3, respectively (278.14 vs. 9.28 copies/µl). The non-transgenic control exhibited negligible expression levels. These results revealed that among the five different 'Sanya' transgenic lines and the non-transgenic plant, RNA expression levels of the *CHS* gene exhibited marked variation.

The results of real-time quantitative RT-PCR analysis are not conclusive in ascertaining if the difference in treatment concentrations among transgenic lines will have a relationship with changes in flower color. Additional research is required to verify this hypothesis. As stated previously, the ultimate success of our study will not be determined until Spring Dendrobium flower, takes up to two and half years from young transgenic material.

In summary, we successfully transformed the *CHS* gene into Spring Dendrobium 'Sanya' PLBs using the SAAT procedure. The highest gene transformation efficiency (0.5%) was achieved using an ultrasound (40 KHz) treatment for 10 min followed by 60 min inoculation with an *Agrobacterium* suspension (Table 1). Five total PCR and Southern blot positive lines were obtained from all co-cultivated PLBs. Real-time RT-PCR in different 'Sanya' transgenic lines facilitated the calculation of the *CHS* gene cDNA expression. The results revealed that among the different 'Sanya' transgenic lines, RNA expression levels of the *CHS* gene exhibited marked variation (Figure 4).

Men et al. (2003a) reported higher transformation efficiency than that observed in this study. A possible explanation is the different transgenic receptor material used in each protocol. Men et al. (2003a, b) chose protocorm obtained by seed germination, but in this study, PLBs induced from 'Sanya' clone explants were chosen as the transgenic receptor. In addition, PLBs may be more useful in functional gene transfer. Furthermore, *Dendrobium* species are highly diverse and it is likely that different evaluation methods are required to achieve transformation efficiency among different taxa. This is consistent with the reports by Pathak and Hamzah (2008) and Polowick et al. (2004) in transgenic chickpeas. Therefore, the most important outcome of this study was the development of a practical new transformation system for Spring *Dendrobium*. This plant was consequently transformed with functional genes, from which new cultivars with commercial potential were generated.

We identified several factors affecting SAAT procedure efficiency in transgenic 'Sanya'. The results suggest that sonication-assisted timing is a major factor significantly affecting transformation efficiency. An extension in the sonication-assisted time from 0 to 15 min increased the transformation efficiency from 0 to 0.5%. A 60 min inoculation and 10 min ultrasound assisted treatment exhibited a significantly increased transformation effi-ciency when compared with SAAT for 5 and 0 min (CK), and slightly higher when compared with SAAT for 15 min (Table 1). Pathak and Hamzah (2008) reported that this technique involved subjecting plant tissues to brief periods of ultrasound in the presence of *Agrobacterium*. However, our preliminary experiments demonstrated that between PLBs treated by ultrasound concurrently with *Agrobacterium tumefaciens* inoculation or ultrasound treatment and a subsequent immersion in *A. tumefaciens* suspension for inoculation, the latter was more effective. We suggest that ultrasound treatment forms micro-wounds on the surface of PLBs and facilitates *Agrobacterium* infection. The ultrasound treatment has been successfully employed to enhance transformation efficiency in several other plant species (Joersbo and Brunstedt, 1990, 1992; Zhang et al., 1991; Finer and Finer, 2000; Zaragoza et al., 2004; Beranova´ et al.,

2008). The impact of different ultrasonic power on transgenic 'Sanya' requires further study.

Inoculation time also affected transformation efficiency. According to the result of Fisher's (F) protected least significant difference test (LSD) by Table 1, average transformation efficiency of 60 min inoculation was 0.38%, and showed significantly higher efficiency than that of 30 min inoculation ($P < 0.05$).

Fluorescence based real-time quantitative RT-PCR analysis is a time saving approach that does not require a large number of tissue samples and has the benefit of non-radioactive detection, it is not only a highly sensitive and specific nucleic acid molecule qualitative detection method, but can also serve as a powerful tool to precisely quantify nucleic acid molecules (Gibson et al., 1996). Fluorescence based real-time quantitative PCR technology, particularly because of its high accuracy and stability, can provide early identification of genetically modified organisms. This provides more efficient and convenient breeding programs of new genetically modified varieties. The effectiveness of this methodology has already been confirmed in transgenic cotton (Liu et al., 2007) and jujube (He et al., 2004). In this study, the cDNA concentration in different lines was measured with the main purpose of reflecting the level of *CHS* gene expression at the RNA level in different 'Sanya' transgenic lines. The cDNA concentration of transgenic line A1 (copies μl^{-1}) was much higher when compared with that of transgenic line A3, and higher than the non-transgenic control (Figure 4). However, whether or not the concentration difference among lines is related to color change must be verified by further study.

The potted species of Spring Dendrobium have a long life history. It will take at least two and half years from the time of planting to flowering. Consequently, whether the flower color change in 'Sanya' transgenic lines has practical value is a subject for future determination.

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