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Genetics, anatomy and expression of antisense ACC oxidase gene in transgenic *Dendrobium draconis* Rchb.f.

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Abstract. This research was aimed to compare chromosome number, anatomy and expression of antisense ACC oxidase gene between nontransgenic and transgenic *Dendrobium draconis* Rchb.f. obtained by Homkrajae (2005). Plant materials were cultivated on modified MS medium supplemented with 5 mg/L hygromycin and 15% coconut water for four months. It was found that the frequency of hygromycin-resistant *D. draconis* was 80 % while 20 % were escape plants. One hundred percent of the hygromycin-resistant plants were viable when further cultured on the same medium for eight months. GUS assay used to verify transient gene expression in transgenic *D. draconis* showed positive results. An anatomical study in leaves, stems and roots of transgenic plants showed positive blue coloration in all tissues while those of nontransgenic plants showed negative results. PCR analysis confirmed the integration of 35S promoter, NOS terminator and antisense ACC oxidase gene in *D. draconis*. A cytological study revealed that there was no change in chromosome number (2n=38). Furthermore, both transgenic and nontransgenic orchids exhibited no significant difference in chlorophyll content.
Key Words: *Dendrobium draconis*, transgenic plant, antisense ACC oxidase gene, chromosome number, cytogenetics.

Rezumat. Acest studiu a dorit să compare numărul de cromosomi, anatomia şi fenotipul genei codificate pe catenă opusă a ACC-oxidazei între o linie normală şi una transgenică de *Dendrobium draconis* Rchb.f. obţinute de Homkrajae în 2005. Materialul vegetal s-a cultivat pe un mediu MS modificat, suplimentat cu 5 mg/L hygromicină şi 15 % lapte de cocos timp de două luni. S-a descoperit ca 80 % din plantele de *D. draconis* s-au adaptat la hygromicină în timp ce restul de 20 % nu s-au adaptat. 100 % din plantele rezistente la hygromicină s-au dovedit viabile în cultură pe acelaşi mediu timp de 8 luni. GUS (β-glucuronidaza) - gena martor folosită pentru a verifica transmiterea secvenţei genetice în plantele de *D.draconis* transgenice a relevat rezultate pozitive. Un studiu anatomic al frunzelor, tulpinilor şi radăcinilor plantelor transgenice a arătat coloraţia albastră pozitivă în toate ţesuturile, în timp ce plantele normale au rămas neschimbate. Analiza PCR a confirmat integrarea promotorului 35S, codonului stop NOS şi a genei codificate pe catenă opusă a ACC-oxidazei în plantele de *D. draconis*. Un studiu citologic a scos în evidenţă faptul că nu există nici o schimbare a numărului de cromosomi (2n=38). Mai mult, ambele linii genetice (atât transgenică cât şi cea normală), nu au manifestat nici o variaţie semnificativă a conţinutului de clorofilă.
Cuvinte cheie: *Dendrobium draconis*, plantă transgenică, genă antisens a ACC-oxidazei, număr de cromosomi, citogenetică.

Introduction. *Dendrobium draconis* Rchb.f. is an orchid and it is cultivated as cut flower; that is considered important to Thailand for it contributes a significant amount of money to the country. At the present time, many researchers, both inside and outside the country, pay more attention to plant improvement by gene transformation as it is inexpensive and easy to manage. This method lies on the basis of gene manipulation prior to introducing into plant cells to produce preferred plants. Homkrajae (2005) has studied gene transformation and has successfully produced transgenic *D. draconis*. Whether transgene introduced into plant cells is stable in laboratory-reared F1, F2 and F3 generations has widely questioned. Therefore, cytogenetics, anatomy, physiology and expression of antisense ACC oxidase gene were important keys to the question. The information derived from this study would provide basic knowledge for orchid

improvement within the country. Antisense ACC oxidase gene plays an important role in delaying flower senescence. This helps improve quality of orchids exported outside the country, reduce capital and enhance capability to compete with other countries. Also, it literally promotes development of the technology for plant production and improvement.

Material and Method

Selection and Micropropagation of Transgenic *D. draconis*. Transgenic orchids were cultivated for 4 months on solid MS medium supplemented with 5 mg/L hygromycin, 1% (w/v) sucrose, 15% (w/v) coconut water and 8 g/l agar at pH 5.6 in order to select transgenic orchids.

Evaluation of *gus* Gene Expression by GUS Assay. Staining for GUS activity was performed according to the method of Jefferson (1987). Leaves, roots and stems of transformed orchids were dissected and transferred into a micro tube containing 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-gluc) and subsequently incubated overnight at 37 °C.

Comparison Anatomy of Normal and Transgenic Plants. Leaves, roots and stems of transformed orchids were cross-sectioned and placed on a slide and mounted in distilled water. Area of blue coloration was microscopically observed.

Evaluation of the Integration of Antisense ACC Oxidase Gene by PCR Technique. Total genomic DNA was extracted from the transformants and control by CTAB method (Doyle & Doyle 1987). The primer sequences for PCR were as follows: 35S forward sequence (F)5'- GCTCCTACAAATGCCATCA-3', reverse sequence (R)5'-GATAGTG GGATTGTGCGTCA-3' to yield a 195 bp fragment; NOS (F)5'-GAATCCTGTTGCCGGTCTTG-3', (R)5'-TTATCCTAGTTTGCGCGCTA-3' to yield a 180 bp fragment; ACC (F)5'-CGCGGATCCGCNTGYSARAANTGGGGNTT-3', (R)5'-AAACTGCAGNGGYTCYTINGCYTGRAAYTT-3' to yield a 500 bp fragment. The DNA was denatured at 94 °C for 3 min, followed by 40 cycles of amplification (20 sec at 94 °C; 40 sec at 50 °C; 60 sec at 72ºC). The final incubation at 72 °C was extended to 3 min, and the reaction material was cooled and kept at 4 °C. The PCR products were visualized by running the completed reaction on a 2% agarose gel containing ethidium bromide.

Chromosome Number. Roots of nontransformed and transformed orchids, approximately 1 cm in length were soaked in 0.2% colchicines for 3-5 h at 4 °C. They were then transferred to acetic acid-ethanol (3:1) solution for 30 min at ambient temperature. They were subsequently kept in 70% alcohol at 4 °C for a comparison study on chromosome number in both nontransformed and transformed orchids.

Chlorophyll Content. Leaves of nontransformed and transformed orchids were evaluated for chlorophyll content according to the method of Holden (1967). Leaf weight of 1 g were grinded in a mortar containing 80% acetone volume of 10 ml. Remnant was removed using Whatman No.1 filter paper. The resulted solution was determined for absorption value by spectrophotometer, using acetone as blank, at wave lengths of 645 and 663 nm, respectively. Chlorophyll content was calculated as a following formula.

$$\frac{(20.2A_{645} + 8.02A_{663})V}{1000W}$$

Where,

V is solution volume (mL), W is leaf weight (g), and A_{645} and A_{663} are absorption values at wave lengths of 645 and 663 nm, respectively.

Results and Discussion

Selection and micropropagation of transgenic *D. draconis*. The transgenic *D. draconis* was cultured for 4 months on solid MS medium supplemented with 5 mg/L hygromycin, 1% (w/v) sucrose, 15% (w/v) coconut water and 8 g/l agar at pH 5.6 in order to select transgenic orchids. It was found that the stems of *D. draconis* could grow and develop well on the solid medium (Figure 1a) together with formation of newly ramified shoots on each stem. The percentage of survival is 80. The newly ramified shoots only tolerate hygromycin at the beginning but failed to withstand the condition in the latter period by turning brown in color and finally died (Figure 1b). This suggested that they were escape plants. After screening the transgenic orchids by hygromycin, they were further cultured under the same condition for 8 months. It was found that they totally survived. Belarmino & Mii (2000) found that 50 mg/L hygromycin could be used to identify transgenic and nontransgenic tissues in *Phalaenopsis*. Likewise, 30 mg/L hygromycin was considered appropriate for selection of putative transgenic protocorms in modification of *Dendrobium* (Men et al 2003). Apart from that, Chai et al (2002) have reported that 3 mg/L hygromycin was suitable for distinguishing transgenic *Phalaenopsis* protocorms from nontransgenic ones. However, roots were restrained at a concentration of 3 mg/L.

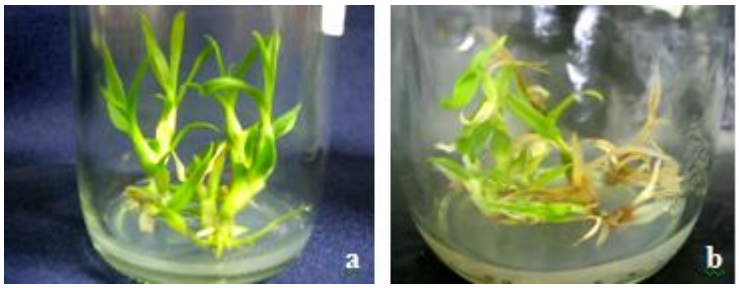

Figure 1. Charateristics of newly ramified shoots of *D. draconis* cultured on solid MS medium supplemented with 5 mg/L hygromycin, 1% sucrose, 15% coconut water and 8 g/L agar with pH of 5.6: (a) *D. draconis* tolerating hygromycin, (b) *D. draconis* failing to withstand hygromycin.

Evaluation of *gus* Gene Expression by GUS Assay. The transgenic orchids were tested for *gus* expression by GUS assay in comparison with nontransgenic plants. It was found that the transgenic plants exhibited expression of *gus* gene by blue coloration in all tissues of genetically modified plants while normal plants gave negative results (Figure 2). This indicated that GUS assay could be used to distinguish between transgenic and normal plants. It has been reported that each strain of *Agrobacterium* was specific to plant species. Men et al (2003) have reported that genetic transformation of *Dendrobium* mediated by *A. tumefaciens* strain AGL1 (pCAMBIA1301) gave rise to higher *gus* expression than by *A. tumefaciens* strain EHA 105 (pCAMBIA1301). Likewise, Kamoltham (2002) found that *A. tumefaciens* strain LBA4404 (pBI121) was more suitable for genetic transformation of *Durio zibethinus* Murr. than *A. tumefaciens* strain EHA 105 (pCAMBIA1301) as *A. tumefaciens* strain LBA4404 was easier to eliminate and resulted in higher effectiveness in genetic transformation.

Comparison Anatomy of Normal and Transgenic Plants. The transgenic orchids that were previously screened by hygromycin and GUS assay were used to evaluate in term of anatomy in comparison with normal plants. Leaves, roots and stems were cross-sectioned in order to observe blue coloration in tissues under a microscope. The blue spots were found in parenchyma cells of transgenic plant while normal plants gave rise to negative results (Figure 3).

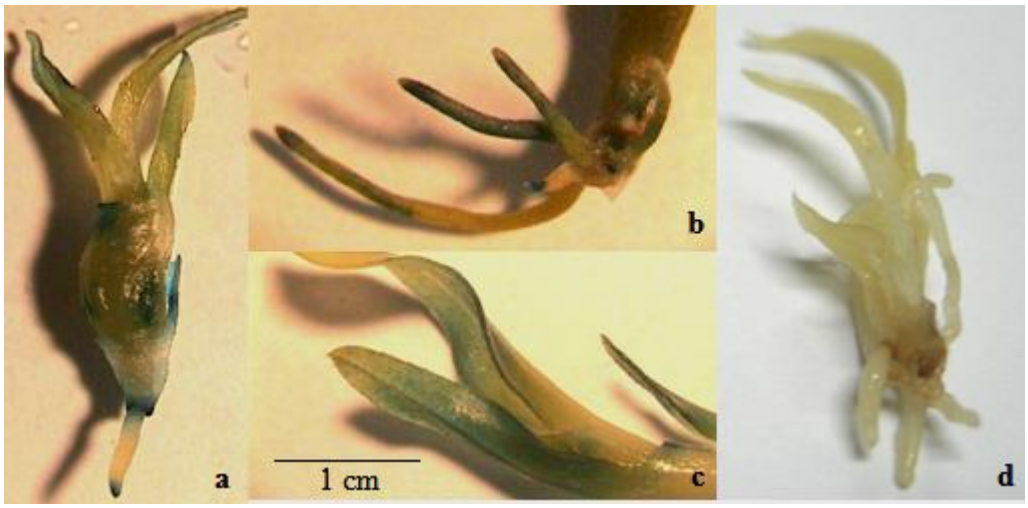

Figure 2. Histochemical staining for gus gene expression: a) stems, b) roots, c) leaves and d) control.

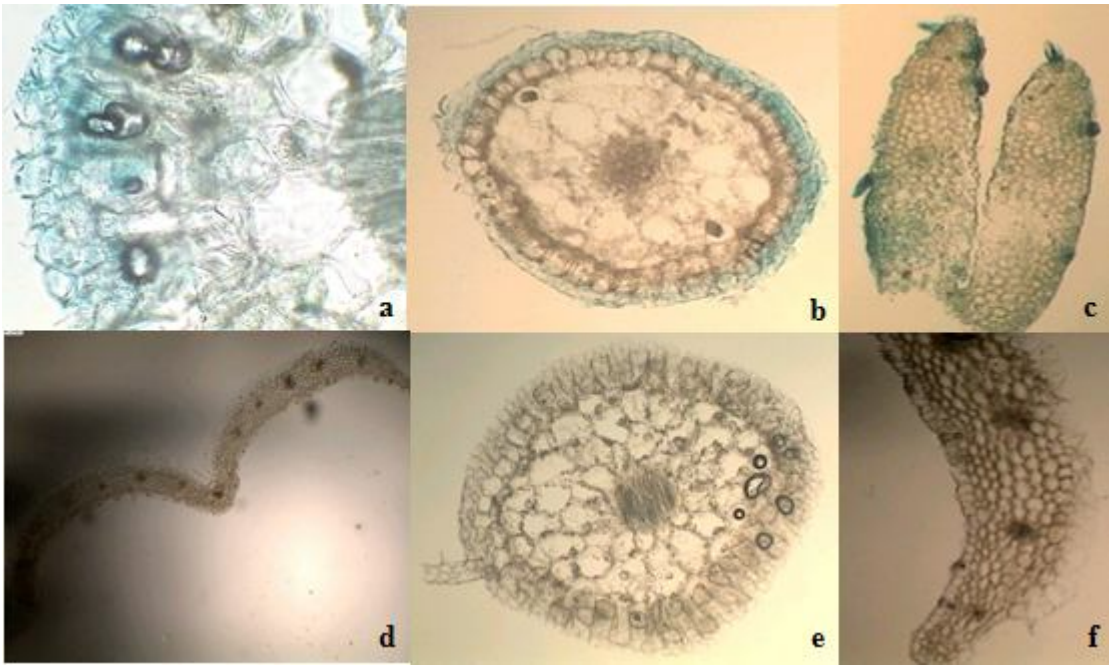

Figure 3. Cross section of normal and transgenic plants: (a, b) roots of transgenic plants, (c) leaves of transgenic plants, (d, e) leaves of normal plants, (f) roots of normal plants.

Evaluation of the Integration of Antisense ACC Oxidase Gene by PCR Technique. DNA extracted from normal and transgenic plants were multiplied by PCR technique utilizing 3 primers: 35S, NOS and ACC. There appeared to be 2 DNA bands (500 and 195 bp) (Figure 4a) when the primer 35S was used in DNA multiplication whereas there was one band of 180 bp DNA when the primer NOS was used (Figure 4b). The primer ACC resulted in 843 bp DNA (Figure 4c) which had the same size as the DNA band obtained from the plasmid pCAMBIA1305.1, a DNA template possessing antisense ACC oxidase gene. This indicated that there was the integration of antisense ACC oxidase gene mediated by *A. tumefaciens* strain LBA4404 (pCAMBIA 1305.1) into plant genome.

Chromosome Number. Root tip cells of *D. draconis* (both nontransgenic and transgenic) were assessed for chromosome number. It was found that there was no change in chromosome number (2n = 38) (Figure 5). The study was consistent to Azhankanandam et al (2000). They work on genetic transformation of rice varieties Pusa Basmati 1, Taipei 309 and Tiwana mediated by *A. tumefaciens*. They successfully induced calluses to develop into plantlets with the same charateristics as normal plants. Moreover, a study on cytogenetics showed that there was no change in chromosome number (2n = 24).

Chlorophyll Content. Leaves of nontransgenic and transgenic *D. draconis* were evaluated for chlorophyll content. It was found that there was no significant difference in chlorophyll content between nontransgenic and transgenic orchids (Figure 6).

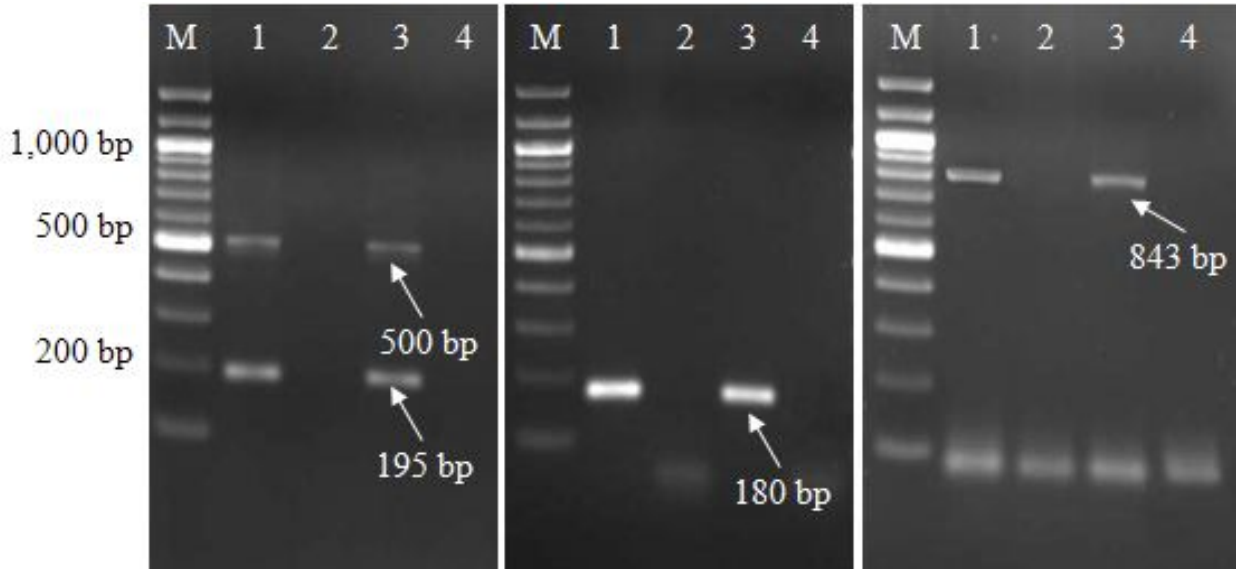

Figure 4. Evaluation of the integration of antisense ACC oxidase gene by PCR technique using: (a) 35S and anti-35S, (b) NOS and anti-NOS and (c) ACC and ACC-reversed as primers [lane M--100 bp DNA ladder, lane 1--the plasmid pCAMBIA1305.1, lane 2--DNA of normal plants, lane 3--DNA of transgenic plants and lane 4--control].

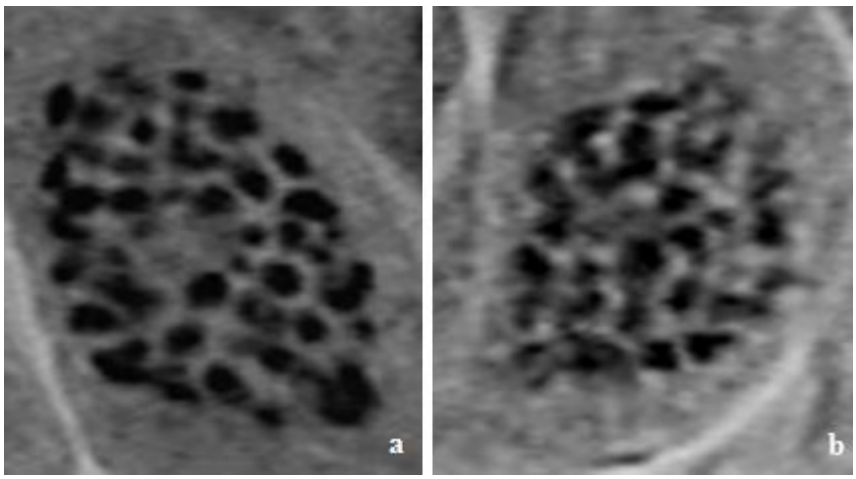

Figure 5. Chromosome number: (a) control, (b) transgenic *D. draconis*.

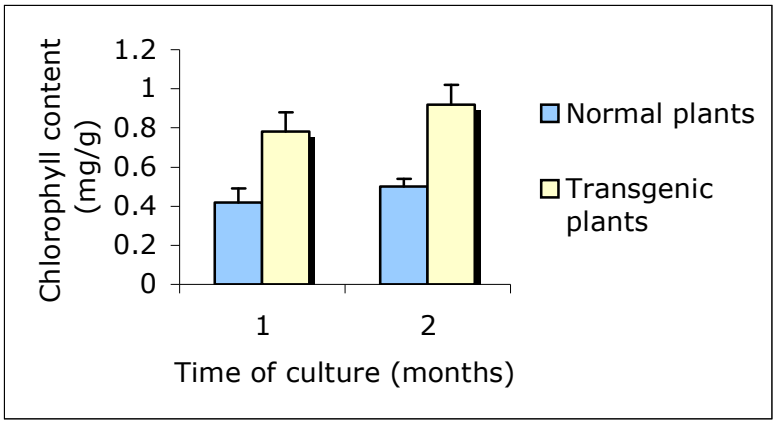

Figure 6. Chlorophyll content in orchid leaves.

Conclusions. It could be concluded that transformation of *D. draconis* with antisense ACC oxidase gene via *A. tumefaciens* strain LBA4404 (pCAMBIA 1305.1) had no results on chromosome number, anatomy and chlorophyll content. It also suggested that transgene was stable in laboratory-reared F1, F2 and F3 generations.

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