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Expression of *Trigonopsis variabilis* D-amino acid oxidase in transgenic rice for cephalosporin production

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ABSTRACT. Transgenic plants have become an effective system to produce recombinant proteins, and there are many examples of transgenic plants that successfully produce functional proteins. In this study, the *japonica* rice cultivar Taiken 9 was transformed through an *Agrobacterium*-mediated method to express D-amino acid oxidase (DAAO) from *Trigonopsis variabilis*. DAAO is a flavoenzyme that catalyzes the oxidation of cephalosporin C to produce the precursor of the cephalosporin antibiotic glutaryl-7-aminocephalosporin acid (Gl-7-ACA). DAAO derived from *T. variabilis* has the highest catalytic activity for cephalosporin C oxidation of DAAO enzymes that have been characterized. *Trigonopsis daao* was expressed in rice under the control of either the rice actin 1 (*Act1*) or maize phosphoenolpyruvate carboxylase (*PEPC*) promoter. Southern blot analysis demonstrated the integration of *Trigonopsis daao* gene into the rice genome. Furthermore, northern blot and western blot analysis demonstrated production of the *daao* transcript and accumulation of its protein in various tissues of transgenic rice plants using either the *Act1* or *PEPC* promoter as compared with the wild type. DAAO activity was detected in both transgenic rice lines with a maximum specific activity of 65.5 ± 7.4 U mg protein^{-1} min^{-1} detected in the leaves of transgenic plants containing the rice *Act1* promoter. The transgenic rice plant with the rice *Act1* promoter exhibited several fold higher DAAO activity than the plant with the maize *PEPC* promoter: 5.3- and 3.7-fold higher in the leaves and sheaths, respectively. No DAAO activity was detected in the grains of transgenic rice containing the *PEPC* promoter. Taken together, these results demonstrate that *Trigonopsis daao* is stably integrated into the transgenic rice genome, transcribed efficiently, and translated into a functional protein.

Keywords: Cephalosporin; D-amino acid oxidase; *Japonica* rice cultivar Taiken 9; Transgenic rice plant; *Trigonopsis variabilis*.

INTRODUCTION

Because rice is the most important food in Asia, improving its additive value will be of economical significance. An effective approach toward this goal is the introduction of economically beneficial gene(s) into rice to produce useful proteins. Many exogenous genes have already been introduced into transgenic rice plants for producing useful foreign proteins. For example, two plant genes from daffodil, phytoene synthase (*psy*) and lycopene β-cyclase (*lcy*), together with phytoene desaturase (*crtl*) from the bacterial provitamin A biosynthesis pathway, have been expressed in rice endosperm to improve the nutritional value of the staple food, Golden Rice (Ye et al., 2000).

Recently, high value recombinant proteins with diagnostic, prophylactic or other potential applications have been expressed in transgenic rice and shown to be biologically active. Molecular farming of transglutaminase (Capell et al., 2004), human α1-antitrypsin (rAAT) (Trexler et al., 2005) and lactoferrin (Conesa et al., 2007; Fujiyama et al., 2004) has been reported. Production of rice containing the vaccine of multiple T-cell epitopes has been proven feasible (Takagi et al., 2005; Takagi et al., 2006). The advantages of using plant systems to produce recombinant eukaryotic proteins are: fast growing, low-cost, easy to scale-up, capable of posttranslational modification, and little risk of bacterial or animal pathogenic contamination (Kusnadi et al., 1997; De Wilde et al., 2000; Daniell et al., 2001).

D-amino acid oxidase (DAAO, EC. 1.4.3.3) is an industrial biocatalyst of 7-aminocephalosporanic acid (7-ACA), an intermediate with high commercial value, from which more than 50 semi-synthetic cephalosporin-type antibiotics are produced (Fernández-Lafuente and Guisán, 1997; Suzuki et al., 2004). The industrial conversion of cephalosporin C into 7-ACA involves two reactions: the first reaction is catalyzed by DAAO and the second is catalyzed by glutaryl-7-ACA acylase (Pilone and Pollegioni, 2002). Although DAAO exists ubiquitously in prokaryotes and eukaryotes, ranging from yeasts to mammal cells (Kawamoto et al., 1977; Pistorius and Voss, 1977; Rosenfeld and Leiter, 1977; Konno and

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Yasumura, 1983; D'Aniello et al., 1993), its bioconversion capacity, in general, is not efficient and the best specific activity of conversion is only 1 μmol cephalosporin C per minute per gram biomass of *T. variabilis* (Pilone and Pollegioni, 2002). cDNA of *T. variabilis* DAAO was also expressed in *Escherichia coli* by using lactose as an inducer, the enzyme activity was up to 20.7 U mg protein^{-1} min^{-1}, and the expression level reached to 15% of total soluble proteins (Hwang et al., 2000). The chimerical *T. variabilis* DAAO accounted for 35% of the total soluble in *E. coli* when fused with a 12-amino acids peptide at N terminus (Dib et al., 2007; Pollegioni et al., 2008). Thus, an economical solution to overcome this inefficiency is to overexpress a large quantity of protein in transgenic organisms.

Eriskon et al. (2004) have introduced *Rhodotorula graculis daao* into *Arabidopsis thaliana* as a selectable marker for transgenic plants. Using transgenic rice to produce a large amount of DAAO protein is the long-term goal of our study. The objective of this report is to introduce the *T. variabilis* gene coding for DAAO into *japonica* rice cultivar Taiken 9, a superior quality rice variety, to produce DAAO with pharmaceutical value. Rice does not contain the counterpart gene. Transformation mediated by *Agrobacterium* was employed to overexpress *daao* under the control of either the rice actin 1 promoter (*Act1*) or the maize phosphoenolpyruvate carboxylase (*PEPC*) promoter. The resulting transgenic rice plants were subjected to Southern and northern analysis to detect *daao*, and DAAO production was evaluated by western blot and enzyme activity.

MATERIALS AND METHODS

Plant material and induction of embrogenic calli

Seeds of *Oryza sativa* L. cv. (Taiken 9) were generously provided by the Rice Germplasm Center of Taichung District Agricultural Research and Extension Station, Council of Agriculture, Executive Yuan. To produce calli, mature rice seeds were sterilized sequentially with 75% ethanol for 30 s and 2.5% sodium hypochlorite for 10 min, then rinsed three times with sterile water. After removing the palea and lemma, seeds without the seed coat were sterilized again with 75% ethanol for 30 s and 2.5% sodium hypochlorite for 30 min, washed three times with sterile water, and finally cultured on N6D medium in the dark with scutella pointing upward (Toki et al., 1997). After 18-21 days, the primary calli (approximately 0.1 cm in diameter) were separated from the scutella and used for transformation.

Preparation of constructs for plant transformation

Two expression vectors with different promoters (*Act1* and *PEPC*) to drive the expression of *Trigonopsis daao* were used for *Agrobacterium*-mediated transformation. pActdaaoHm2 (Figure 1D), an expression vector derived

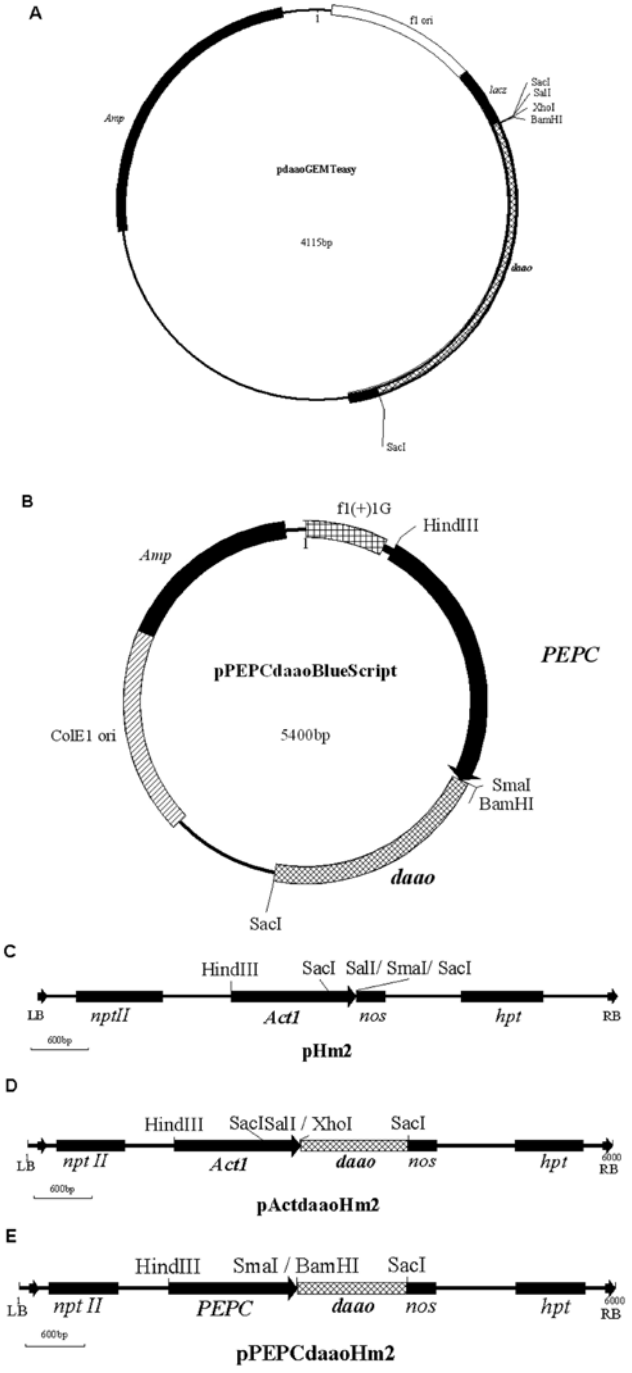

Figure 1. Construction of the *daao* expression vector used for transformation. (A**)** Diagram of the pdaaoGEMTeasy; (B**)** Diagram of pPEPCdaaoBlueScript; (C**)** The T-DNA region of pHm2 carrying the rice *Act1* promoter; (D**)** The *Sal*I/*Sac*I region of pHm2 was replaced by the *Xho*I/*Sac*I fragment of *daao* from pdaaoGEMTeasy to generate pActdaaoHm2. The rice *Act1* promoter was used to the *daao* gene expression; (E) The *Hind*III/*Sac*I region of pHm2 was replaced by the fragment of *PEPC-daao* (the *Hin*dIII/*Sac*I fragment, about 2.4-kb) from pPEPCdaaoBlueScrpit (Figure 1B) to yield pPEPCdaaoHm2. The maize *PEPC* promoter was used to the *daao* gene expression. RB and LB, right and left border repeats, respectively; *hpt*, hygromycin-B-phosphotransferase gene; *npt II*, neomycin phosphotransferase gene; *nos*, nopaline synthase terminator.

from pHm2 (Figure 1C), was a binary vector originating from pBI101 bearing a rice *Act1* promoter and *hpt* as a plant selection marker and an *nptII* as a bacterial selection marker. The *Hind*III/*Sac*I fragment of pHm2 was replaced with *Act1* fragment (the *Hind*III/*Sal*I fragment, about 1.3-kb) from pHm2, combined the 1.1-kb *daao*-carrying *Xho*I/*Sac*I fragment from pdaaoGEMTeasy (Figure 1A) which contained the *Trigonopsis daao* cDNA (Ju et al., 1998). pPEPCdaaoHm2 (Figure 1E) was constructed from pHm2 by inserting the *PEPC-daao* fragment (the *Hin*dIII/*Sac*I fragment, about 2.4-kb) of pPEPCdaaoBlueScript (Figure 1B) into its *Hin*dIII/*Sac*I site. The plasmid pPEPCdaaoBlueScript was a derivative of pBlueScript in which the *Hin*dIII/*Sac*I site was replaced by the 1.1-kb *Bam*HI/*Sac*I *daao* fragment of pdaaoGEMTeasy (Figure 1A) and by the 1.3-kb blunt-ended *Hin*dIII/*Sma*I *PEPC* fragment of pPEPC19 (Matsuoka et al., 1994). *PEPC* is the promoter sequence of the maize C4-type phosphoenolpyruvite carboxylase gene (Matsuoka et al., 1989).

Rice transformation

The procedure for rice transformation was modified from that of Toki (1997). Transformation was initiated by co-culturing the scutellum-derived calli (3-weeks-old) from mature seeds with suspension cultures of *Agrobacterium tumefaciens* carrying either the pActdaaoHm2 or pPEPCdaaoHm2 vector. The *Agrobacterium* suspension cultures were prepared by adding 1 loop of bacteria into AAM-AS medium and growing to an OD_{600} 0.8-1.0. The calli were then immersed for 2 min in the culture supplemented with 10 mg/l acetosyringone, and cultured on N6-AS medium (pH 5.2) for 3 days in the dark at 28°C. After 3 days, the co-cultured calli were washed with N6D liquid medium containing 500 mg/l carbenicillin to eliminate the bacteria, and then transferred onto a solid N6D medium for culture at 28°C for 2 weeks in the dark. Subsequently, the transformed calli were transferred onto MS-NK medium (Kyozuka and Shimamoto, 1991) containing 50 μg/ml hygromycin and 50 μg/ml cefotaxime for regeneration under a light/dark cycle of 8/16 h. The calli were subcultured every 2 weeks until the appearance of green spots on MS medium (Murashige and Skoog, 1962), which were then incubated in sterile culture before transplanting to pots in the greenhouse. At maturity, plants were self-pollinated, and the resulting seeds (T_1 and T_2) were collected for further analysis.

Southern blot and northern blot analysis

Genomic DNA was isolated from leaves of T_0 and T_2 transgenic rice plants by the method previously described (Cao et al., 1992). For Southern blot analysis, 30 μg genomic DNA of pActdaaoHm2 or pPEPCdaaoHm2 transgenic plants were digested with restriction enzymes (*Sac*I or *Xba*I for the former, and *Hind*III/*Sac*I, or *Hin*dIII/*Xho* I for the latter), separated by electrophoresis in a 1% agarose gel, and transferred onto a nylon membrane by an alkaline transfer method according to the manufacturer's instructions (NEN® Life Science Products, Boston, MA, USA). The membrane was subsequently hybridized with a ^{32}p-labeled *daao* probe from pdaaoGEMTeasy (a 1.1-kb *Sac*I fragment in Figure 1A), washed with 2% SSC followed by 1% SDS at 65°C for 1 h, and exposed to X-ray film. Southern blot analysis with T_2 transgenic rice was repeated twice, and samples with a clear signal were selected and combined for a composed figure.

For northern blot analysis, total RNA was extracted from mature leaves and mRNA was subsequently isolated using a commercial kit (Stratagene, Los Angeles, CA, USA). mRNA (1 μg) was separated by electrophoresis, transferred on to a nylon membrane, probed with a ^{32}p-labeled *daao* probe from pdaaoGEMTeasy (a 1.1- kb *Sac*I fragment in Figure 1A), washed once with 2% SSC followed by 1% SDS at 65°C three times, and exposed to X-ray film.

RNA isolation and RT-PCR

Total RNA was isolated from 100 mg of leaf tissue using Tri reagent according to the manufacturer's instructions (Sigma, St. Louis, MO, USA). For RT-PCR analysis, 2 μg of total RNA was first reverse transcribed using an oligo (dT) primer and M-MLV reverse transcriptase (Promega, Madison, WI, USA). PCR was subsequently carried out using one-tenth of the first-strand reaction mix and gene-specific primers for *daao*: daao-1 (5′-CCATGGCTAAAATCGTTGTT) and daao-2 (5′-GAGCTCCTAAAGGTTTGGACGAGCAAGG); *Actin*: Rac48 (5′-ATGCTATCCCTCGTCTCGAC) and Rac49 (5′-TAGAAGCATTTCCTGTGCA). PCR conditions for *daao* were 95°C for 120 s followed by 40 cycles of 95° C for 30 s (denaturation), 55°C for 60 s (annealing), 72°C for 60 s (elongation), and finally 72°C for 5 min. The PCR program for *Actin* differed from the protocol in that the annealing condition was 50°C for 60 s. *Actin* expression in rice was as a control.

Preparation of DAAO antibody

Anti-DAAO serum was generated in rabbits using DAAO-His fusion protein produced by *E. coli* strain BL21 bearing a DAAO-His-carrying expression vector (pdaaomET32a$^+$, Figure 7A) according to the manufacturer's instructions (Novagen, Milwaukee, WI, USA). The bacterial culture in LB medium was incubated at 37°C until the OD_{600} reached 0.6, protein expression was induced with 1 mM isopropylthio-β-galactoside (IPTG) and the incubation was continued at 16°C with shaking for 8 h. After centrifugation of bacterial culture at 8000 ×*g* for 10 min at 4°C, the pellet was suspended in 50 mM sodium phosphate buffer (pH 7) containing 300 mM NaCl, and then lysed with sonication. The extract was clarified by centrifugation at 13000 ×*g* for 20 min at 4°C. The fusion protein in the clear supernatants was purified over BD TALON™ metal affinity resins according to the

manufacturer's instructions (BD BioSciences, Palo Alto, CA, USA).

To generate anti-DAAO serum, the DAAO-His fusion protein was treated first with recombinant enterokinase (Novagen), and the DAAO protein was separated by SDS-PAGE. Purified DAAO protein (100 µg) isolated by SDS-PAGE was ground into fine powder, mixed with complete Freund's adjuvant (1:1 [V:V] emulsion; Sigma), and injected subcutaneously into an 8-week-old male New Zealand rabbit. After 10 days, four additional injections (100 µg each; 7-d interval) with incomplete Freund's adjuvant were administered. The serum was collected 2 days after the last injection for titer determination.

Western blot analysis

For protein western blot analysis of transgenic rice plants, total leaf soluble protein was extracted from transgenic plants by the method described (Outchkourov et al., 2003). Briefly, leaves (about 200-300 mg) were homogenized in 500 µl extraction buffer (5 mM EDTA, pH 7.5, 50 mM HEPES, pH 7.5, 10% glycerol, 10% [W/V] polyvinylpolypyrrolidone, and 5 µl protease inhibitor cocktail [Sigma]), and the crude protein extract was centrifuged twice for 10 min each at 13000 ×*g* and 4°C, and separated by SDS-PAGE. Proteins in the gel were transferred onto a polyvinylidene fluoride membrane and probed with anti-DAAO serum. Horseradish-peroxidase-conjugated anti-rabbit antibodies (Jackson, ImmunoResearch, West Grove, PA, USA) were used as the secondary antibodies, and the Immobilon™ Western Chemiluminescent HRP Substrate (Millipore, Watford, UK) was used for signal detection. Alkaline-phosphatase-conjugated anti-rabbit antibodies (Jackson ImmunoResearch, West Grove, PA, USA) were also used as the secondary antibodies, and NBT/BCIP substrate solution (Roche, Basal, Switzerland) was used for signal detection.

DAAO activity assay

An assay of DAAO activity was conducted by following the procedures described previously by Erikson et al. (2004). The assay involved measurement of the change in absorbance of pyruvate at 220 nm (ΔE = 1,090 M^{-1} cm^{-1}) as the enzyme catalyzes the conversion of D-alanine to pyruvate. To extract soluble protein for the enzyme assay, 0.1 g fresh plant sample was pulverized and mixed with 1 ml of 0.1 M potassium buffer (pH 8) before being transferred to a 1.5-ml test tube. The crude extract was cleared by centrifugation at 14000 ×*g* for 10 min and the supernatant was used for the assay. To assay pyruvate, 80 µl of crude extract was mixed with 2.12 ml of 0.1 M potassium buffer (pH 8), and 100 µl of 0.3 M D-alanine and incubated for 2 h at 30°C. The reaction was stopped by boiling for 10 min. The control reaction was the same as above but without D-alanine. One unit (U) of DAAO activity was defined as turnover of 1 nmol substrate per min, and specific activity was expressed as units per minigram protein of crude extract. Enzyme data presented are means ± S.D. from 10 replications of sampling and assay.

RESULTS

Production and phenotypic characteristics of transgenic rice plants

Healthy calli (2-2.5 mm in diameter) were generated from mature embryos of the rice cultivar Taiken 9 after three weeks of induction (Figure 2A). After infection with *A. tumefaciens* harboring the pActdaaoHm2 (Figure 1D) or pPEPCdaaoHm2 (Figure 1E) construct carrying the *Trigonopsis daao*, the calli were incubated on a cefotaxime- and hygromycin-containing selection medium for two weeks in continuous darkness. To induce the green spots from the calli, (Figure 2B and C), the calli were then transferred to a regeneration medium containing hygromycin under a light/dark cycle of 8/16 h. Calli containing green spots were further incubated on MS medium containing hygromycin to induce shoot and root formation (Figure 2D). Four plants were regenerated from numerous calli; each was derived from a single independent callus. Two of these carried the pActdaaoHm2 construct (Act:daao plants) whereas the other two plants (PEPC:daao plants) carried the pPEPCdaaoHm2 construct. The two Act:daao plants were designated A1 and A2 and the two PEPC:daao plants P1 and P2.

The four plants (T_0 plants) were subsequently transplanted to pots and grown in a greenhouse. Relative to the wild type, these plants looked feeble, grew slowly and had low fertility (except A2) and the seeds produced had a lower germination rate. The two PEPC:daao plants possessed distinctive characteristics: P1 had a short stand and P2 had light green leaves. Grains produced from the four T_0 plants were germinated and grown in the greenhouse to obtain T_1 plants. All T_1 and wild type plants developed poorly due to unexpected greenhouse conditions and yielded few or no panicles except for A2 and P1, which bore a sufficient number of grains. Thus, A2 and P1 were selected for further physiological characterization. Seedlings of the two plants were first established in the greenhouse and then transplanted to the field (T_2 plants). At maturity, the PEPC:daao plants (P1) were considerably shorter (~50 cm) than the wild type (~100 cm) and produced an average of 200 grains/plant as compared to ~1000 grains/plant for the wild type (Figure 2F). In contrast, the Act:daao plants (A2) possessed plant height and fertility similar to wild type (Figure 2E).

Molecular characterization

To determine the number of *daao* insertion site in the rice genome, genomic DNA from the four T_0 plants and the wild type were digested with *Sac*I, *Xba*I, *Hind*III combined with *Sac*I, or *Hind*III combined with *Xho*I. DNA fragments were separated and then probed with ^{32}P-labeled *daao* cDNA. As shown in Figure 3A, no signal was evident for

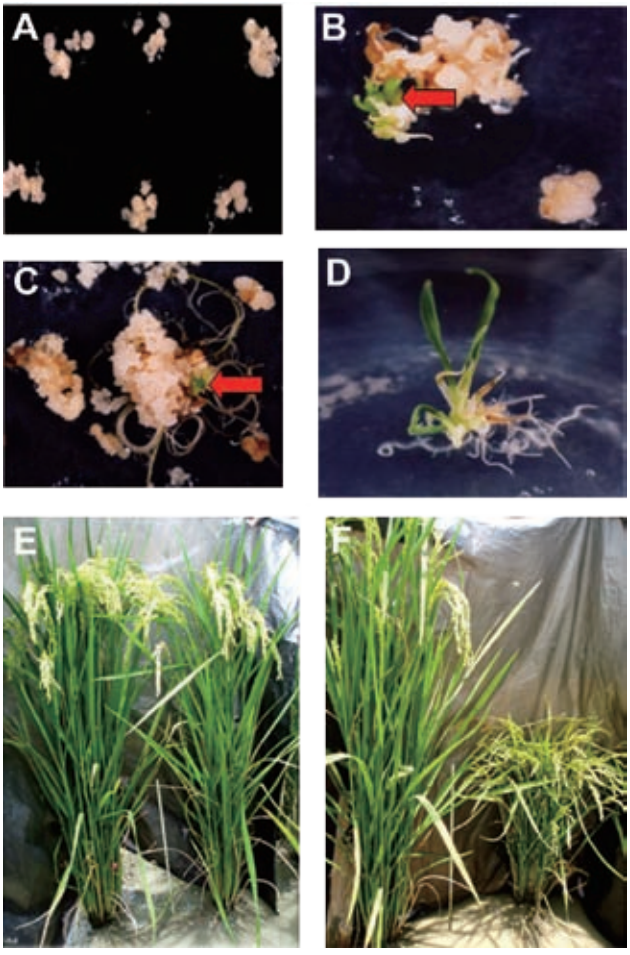

Figure 2. Calli and plants resulting from *Agrobacterium*-mediated transformation. (A) Embryogenic calli derived from mature kernels for three weeks; (B) and (C) Hygromycin-resistant calli on a regeneration medium plus hygromycin. Green spots on some calli are indicated by arrows; (D) Shoots growing from the green spots were transferred to MS medium plus hygromycin; (E) Transgenic rice (T_2) with pActdaaoHm2 compared with wild type grown in the field. (F) Transgenic rice (T_2) with pPEPCdaaoHm2 compared with wild type grown in the field. WT, wild type.

the wild type, and only a single major band appeared in the *Sac*I digest of A1 and A2 plants. However, the *Xba*I digest of the same DNA from A2 gave rise to two bands, 6.5-kb and 8.5-kb, but a single band of 9-kb for A1. Because there was no internal *Xba*I restriction site in the T-DNA of pActdaaoHm2, the size of the *Xba*I band associated with A1or A2 could not be predicted. The band sizes were different in the Act:daao plants, indicating that the two Act:daao plants had different T-DNA integration locations in the rice genomes. Similar results were observed in P1 and P2 (Figure 3B). For P2, only a single 2.4-kb band was observed in the *Hind*III/*Sac*I digest, but three bands (6.0-kb, 8.1-kb, and 24-kb) were presented in the *Hin*dIII/*Xho*I digest. The equivalent P1 digests displayed only a single band with a fragments size of 2.4-kb (*Hind*III/*Sac*I) and 9.2-kb (*Hin*dIII/*Xho*I), respectively. This result was expected, as *Hin*dIII and *Sac*I were used for cloning the *PEPC-daao* fragment into the Ti plasmid, but *Xho*I was not. The band sizes were different in P1 and P2 plants when digested with *Hin*dIII and *Xho*I, which also indicated different T-DNA integration locations in the rice genomes of the PEPC:daao plants. Taken together, these Southern blot results indicated a single *daao* integration site for A1 and P1 plants, but two or three for integration sites of A2 and P2 plants, respectively.

Further Southern analysis was performed with T_2 plants. Genomic DNA was digested with *Sac*I and *Hin*dIII/*Sac*I in A2 and P1 plants, respectively. As shown in Figure 4, 16 A2 and 15 P1 plants were analyzed, and all exhibited a single *daao* signal, indicating that the T_1 plants that gave rise to these T_2 (A2 and P1) plants were likely carrying the *daao/daao* genotype (likelihoods of -99% and 97.5-%, respectively). In other words, homozygous lines for both Act:daao and PEPC:daao plants were established for future study.

Expression of *daao* transcript in transgenic rice

Northern blot hybridization with mRNA extracted from leaves of both Act:daao and PEPC:daao plants (T_0) was performed to determine the transcriptional expression of *daao*. As shown in Figure 5, a 1.1-kb signal was detected in all Act:daao and PEPC:daao plants. Taking loading variation into account, mRNA signals for the P1 and P2 plants were more intense than those of the A1 and A2 plants, indicating that the *PEPC* promoter outperformed the *Act1* promoter. Further analysis by RT-PCR also detected stable *daao* expression in the T_2 generation of Act:daao (A2) and PEPC:daao (P1) plants (Figure 6).

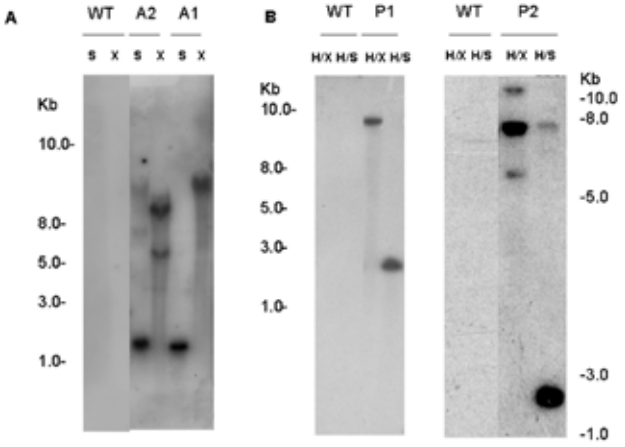

Figure 3. Autoradiogram of Southern hybridization of genomic DNA isolated from wild type and transgenic rice plants (T_0) transformed either with pActdaaoHm2 (A) or pPEPCdaaoHm2 (B). The genomic DNA of pActdaaoHm2 and pPEPCdaaoHm2 transgenic plants was digested with restriction enzymes and probed with the *daao* fragment of pdaaoGEMTeasy. WT, wild type; A1, and A2, pActdaaoHm2 transgenic plants; P1, and P2, pPEPCdaaoHm2 transgenic plants; S, *Sac*I, X, *Xba*I for pActdaaoHm2; H, *Hind*III and S, *Sac*I or X, *Xho*I for pPEPCdaaoHm2.

Expression of DAAO protein and enzyme activity in the transgenic plants

To detect the accumulation of DAAO protein in transgenic rice plants, an antiserum against purified DAAO was produced in rabbits. After induction recombinant protein (DAAO-His fusion protein) was synthesized using an overexpression system in *E. coli* strain BL21 carrying a DAO-His-containing pET32a$^+$ expression vector (Figure 7A). The recombinant DAAO-His protein was purified from a bacterial lysate using a metal affinity column (Figure 7B) before injection into a rabbit. As shown in Figure 7C, the rabbit anti-DAAO serum displayed affinity for the recombinant DAAO-His protein produced by *E. coli*.

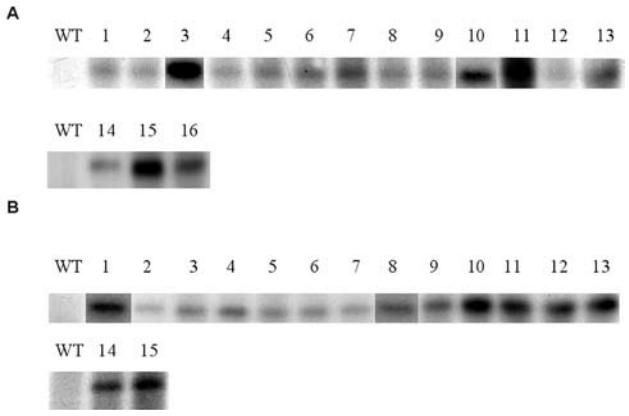

Figure 4. Autoradiogram of Southern hybridization of genomic DNA isolated from wild type and transgenic rice plants (T_2) transformed with either pActdaaoHm2 (A**)** or pPEPCdaaoHm2 (B**)**. Genomic DNA was digested with either *Sac*I (pActdaaoHm2) or *Hind*III/*Sac*I (pPEPCdaaoHm2) and probed with the *daao* fragment of pdaaoGemteasy. WT, wild type; lanes 1-16, different transgenic plants.

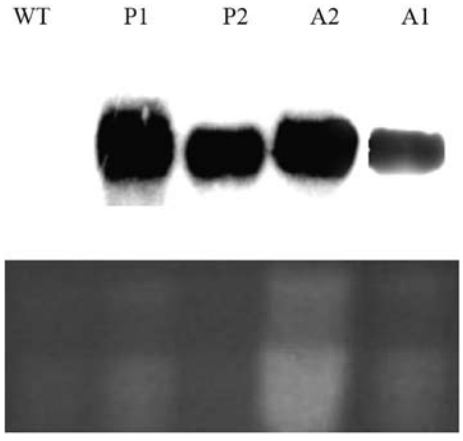

Figure 5. Northern blot analysis of *daao* expression in wild type and transgenic rice plants. Total RNA was extracted from wild type and several transgenic lines (lower panel), blotted and hybridized with the *daao* probe (upper panel). WT, wild type; P1 and P2, pPEPCdaaoHm2 transgenic plants; A1 and A2, pActdaaoHm2 transgenic plants.

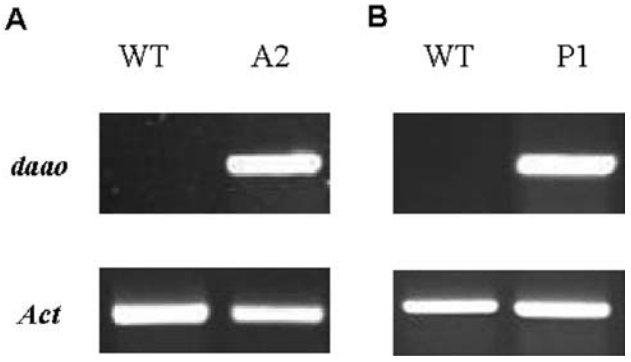

Figure 6. RT-PCR analysis of *daao* expression of transgenic plants (T_2). Total RNA was extracted from wild type and transgenic rice plants. (A**)** *daao* transcription of transgenic pActdaaoHm2 plants; (B**)** *daao* transcription of transgenic pPEPCdaaoHm2 plants. WT, wild type; A2, transgenic pActdaaoHm2 plants; P1, transgenic pPEPCdaaoHm2 plants. *Actin* (*Act*) levels were analyzed in all samples.

Table 1. DAAO activity in different tissues of wild type and T_2 transgenic rice plants.

Genotype	Leaf	Sheath	Grain
Act:daao plant (A2)	65.5 ± 7.4[a]	34.3 ± 6.8	22.4 ± 7.9
PEPC:daao plant (P1)	12.3 ± 2.5	9.3 ± 1.7	0
Wild type	0	0	0

[a]The unit of DAAO activity is expressed as U mg protein^{-1} min^{-1}.

Total soluble proteins extracted from leaves, sheaths and grains from the homozygous T_2 transgenic (*daao/daao*) and wild type plants were subjected to western immunoblot analysis. As shown in Figure 8, no DAAO signal was observed in the wild type. In contrast, a 39-kDa polypeptide corresponding to DAAO was detected in the leaf and sheath of both transgenic (T_2) plants (A2, P1). We consistently observed that the accumulation of DAAO protein in all tissues was more pronounced in Act:daao plants than in PEPC:daao plants (A2 and P1, Figure 8).

To test if the DAAO protein detected by western blot was functional, DAAO activity was measured in leaves, sheaths and grains of A2 and P1 (T_2) plants. As shown in Table 1, the three tissues of the wild type plant showed no DAAO activity, but those of A2 and P1 exhibited various levels of activity. The DAAO activity in Act:daao plants exceeded that in PEPC:DAAO plants by 5.3-fold in leaves and by 3.7-fold in sheaths. Although no activity was measured in the grains of PEPC:daao plants, substantial activity (22.4 U mg protein^{-1} min^{-1}) was detected in the same tissue of Act:daao plants. In general, the specific activity of DAAO in both transgenic rice plants was highest in leaves, followed by sheaths and grains.

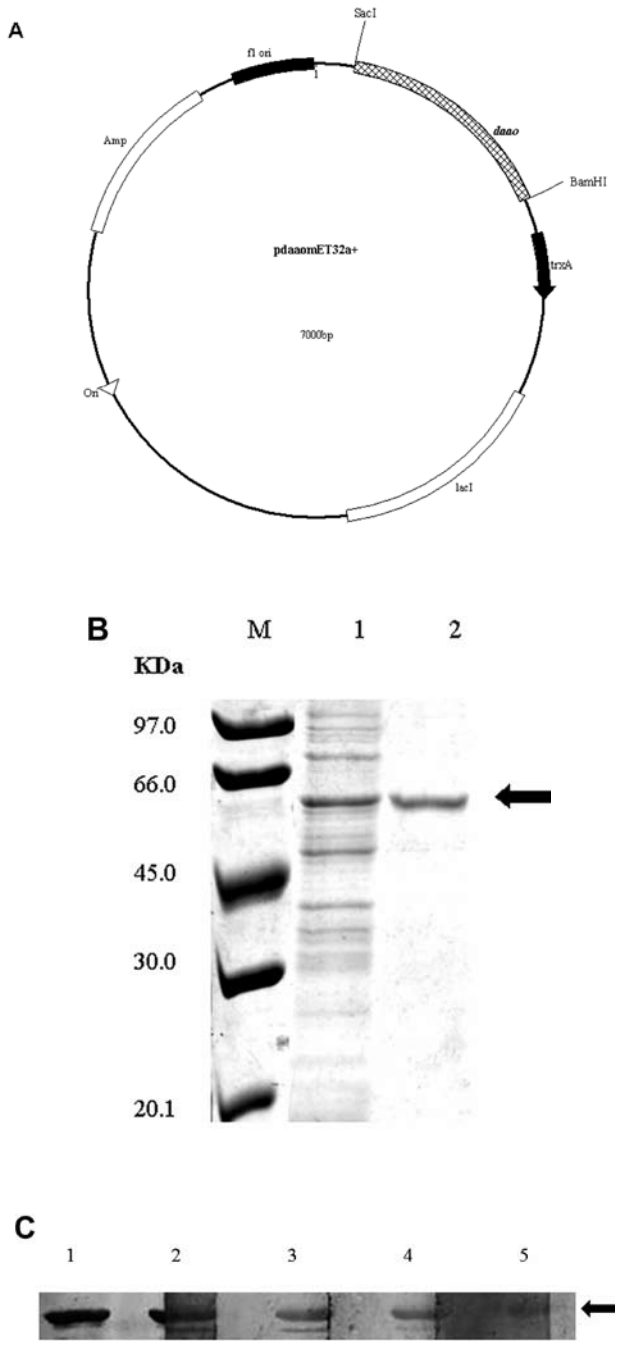

Figure 7. Recombinant DAAO-His protein purification and detection with anti-His antibody and anti-DAAO serum. (A**)** Diagram of pdaaomET32a^{+}; (B**)** Recombinant DAAO protein was over-expressed in *E. coli,* purified with a Talon metal affinity column, separated by SDS-PAGE and stained with Coomassie blue. M, protein marker; lane 1, soluble protein of bacterium culture after 1 mM IPTG induced; lane 2, recombinant DAAO-His protein eluted with 1 M imidazole from Talon metal affinity column. Arrow indicates the over-expressed protein (55-kDa); (C) Western blot analysis of recombinant DAAO. Lane 1, anti-His antibody. Lanes 2-5, various titers of anti-DAAO serum, the titers are 1:1000, 1:2500, 1:5000, 1:10000, respectively. Arrow indicates the recombinant DAAO protein (55-kDa).

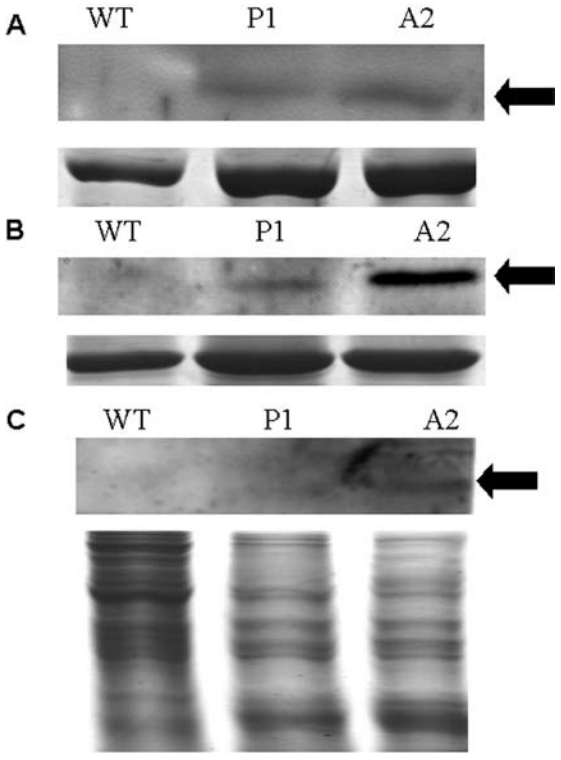

Figure 8. Western blot of DAAO in leaves, sheaths and grains. Total protein was extracted from wild type and transgenic plants (T_2). Upper panels, DAAO detected by rabbit anti-DAAO serum in leaves (A**)** in sheaths **(**B**)** and grains (C**)**. WT, wild type; P1, pPEPCdaaoHm2 transgenic plants; A2, pActdaaoHm2 transgenic plants. Lower panel, the ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) proteins stained with Coomassie blue in leaves (A) and sheaths (B); total proteins stained with Coomassie blue in grains (C); Arrow indicates the position of DAAO (39-kDa).

DISCUSSION

In this study, the *daao* gene of *T. variabilis* was successfully introduced into the *japonica* rice cultivar Taiken 9 via *Agrobacterium*-mediated transformation. This gene was not only transcribed but also translated into functional protein in transgenic rice plants. Substantial activities of DAAO were observed in the leaf, sheath, and grain of transgenic plants containing the rice *Act1* promoter and in the leaf and sheath of transgenic plant containing the maize *PEPC* promoter. As expected, no DAAO activity was detected in the wild type plants. From the limited number of transgenic plants obtained, it appears the rice *Act1* promoter can drive a higher level of *Trigonopsis daao* gene expression than the maize *PEPC* promoter. However, a larger number of transgenic plants for each line are needed for analysis before a clear conclusion regarding the strength of the two promoters can be drawn.

The PEPC:daao plants (P1) were shorter than the wild type; in contrast, the Act:daao plants (A2) possessed plant height and fertility similar to wild type (Figure 2E and F).

The phenotypic variation of the PEPC:daao plants may be due to the T-DNA insertion site, and not attributed to *daao* gene expression, because the phenotype of the Act:daao plants, which also expressed *daao*, was the same as wild type plants.

The *japonica* rice cultivar Taiken 9 was used for the gene transformation because of the high quality of its rice. Our goal is to further increase the value of this superior rice by insertion of *daao*. Over 1000 calli were derived from Taiken 9 by transformation with pActdaaoHm2 or pPEPCdaaoHm2, but only two successful transgenic rice plants containing each plasmid were generated from these calli. The regeneration rate of Taiken 9 transgenic rice plants was very low, so we generated only four transformants of the T_0 generation. Those plasmids with *daao* in transgenic plants maybe existed low expression or didn't express which result to transgenic rice plants can't grow well on the hygromycin selection regeneration medium. The low regeneration rate for *daao* transgenic rice plants wasn't due to the DAAO toxic in plants, because the *daao* transgenic *A. thaliana* grew well (Eriskon et al., 2004), and the next generation (T_2) of DAAO transgenic rice plants also developed well.

As an important pharmaceutical product, DAAO efficiently catalyzes the bioconversion of cephalosporin C to Gl-7-ACA, the first intermediate in the two-step route from cephalosporin C to 7-ACA (Pilone and Pollegioni, 2002). Therefore, improvement in DAAO's stability, enzyme activity and yield is vital for the pharmaceutical industry. Ju et al. (2000) has substituted the critical methionine residues of DAAO from *T. variabilis* with leucine to enhance its resistance to hydrogen peroxide. Hörner et al. (1996) added D- and DL-amino acid derivatives to minimal medium to improve DAAO yield in *T. variabilis*. DAAO is normally produced by fermentation by *T. variabilis* (Hörner et al., 1996; Gabler et al., 2000) or *E. coli* (Alonso et al., 1999). Although fermentative production of DAAO can reach a significant level for pharmaceutical industrial scale, the specific activity was 2 fold lower than that obtained in shaking flasks of *T. variabilis* (Pilone and Pollegioni, 2002). In this study, the *Trigonopsis daao* was introduced into rice with the objective of exploiting the plant system as a bioreactor for large-scale production of DAAO. Using a plant system for production of recombinant proteins is advantageous, as they can be generated in large scale much more economically than by fermentation or by using *E. coli* as a bioreactor.

The level of *daao* expression in the transgenic plants was probably not on the number of integrated *daao* copies in the chromosome(s) in our study (Figure 5). The P1 plants contained a single integrated *daao* copy, but its *daao* RNA accumulation was higher than the P2 plants that carried two *daao* copies. Our result is in agreement with that of Law et al. (2004), who found that the copy number of a humanized monoclonal antibody (*mAb*) gene in the transgenic maize genome is not proportional to the amount of protein produced. The same situation was also noted for the expression of a mosaic green fluorescent protein (*gfp*) in tobacco, in which multiple insertions of *gfp* rendered variable protein production (Bastar et al., 2004). Other has observed that the expression of a transgene gene is often proportional to the number and genomic position of integrated genes (Meyer and Saedler, 1996; Ku et al., 1999). It is conceivable that the variable level of transgene expression in the P1 and P2 plants may be attributable to divergence in the chromosome position of the *daao* integration sites, which were not determined in this study. Alternatively, it may be the result of silencing of some of the multiple *daao* insertions in the plant, as has been observed for other transgenes (Cheng et al., 1998; Matzke et al., 1995).

Two promoters (*Act1* and *PEPC*) were used in this experiment to drive the expression of *Trigonopsis daao* in rice, and both promoters have been previously demonstrated to be effective in many transgenic plants. The *Act1* promoter of rice is an effective regulator of foreign gene expression in transgenic rice (McElory et al., 1990; Zhang et al., 1991; Su and Wu, 2004). The maize *PEPC* promoter from C4-type phosphoenolpyruvite carboxylase has been introduced into rice, a C_3 plant, where it was successfully translated in a light-dependent manner into the expected product in mesophyll cells of leaf blades and sheaths (Häusler et al., 2002; Matsuoka et al., 1994). Gel-retardation assay have consistently shown that nuclear proteins with DNA-binding specificity similar to maize nuclear proteins are present in rice (Matsuoka et al., 1994; Taniguchi et al., 2000), which would permit the maize *PEPC* promoter to function properly in rice.

The levels of DAAO expression differed between the two transgenic plants with the two different promoters. The activity of DAAO in the Act:daao plant surpassed that in the PEPC:daao plant (Table 1). The DAAO activities in these transgenic plants were consistent with the DAAO content as determined by western blot analysis (Figure 8). More DAAO accumulated in the leaf and sheath of Act:daao plant than in PEPC:daao plant. However, DAAO was not detect in the grain of PEPC:daao plant (Figure 8C). This is expected because the maize C_4-specific *PEPC* gene promoter is light-dependent. The low DAAO activities found in the PEPC:daao plant (Table 1), however, the northern blot (Figure 5) and the RT-PCR (Figure 6) did not show any sign of decreased transcription level in PEPC:daao plant. The mention above condition may be attribute to the low mass of DAAO proteins production in transgenic PEPC:daao plants could be result of instability or miss-folding of the yeast proteins in the transgenic rice cells to cause low enzyme activities. Another probable reason could be a bias in codon usuage between yeast and rice plants, which would cause a low efficiency of translation of the mRNA coding for the foreign protein (Mason et al., 1980). Integration position effect of *daao* in the rice genome was also maybe influenced transcription of the transgene and thus accumulation and activity of

the DAAO protein. Similar results were reported by De Neve et al. (1999) regarding the gene that encodes the F_{ab} antibody polypeptide; different integration positions in the genome of transgenic *A. thaliana* led to instability of antibody production, and contributed to low antibody accumulation in transgenic plants.

The DAAO activity presented in Table 1 were compared with Eriskon's (2004) study, although the goal of the Eriskon study was use to DAAO as a selectable marker for transgenic plants. They introduced the *R. graculis daao* gene into *A. thaliana* and discerned high DAAO activity, which was calculated on a fresh-weight (g) basis. In our study, the specific activity of DAAO was expressed in term of a protein weight (mg) in the crude extract. Therefore, it is difficult to compare the expression levels achieved in transgenic *A. thaliana* (Eriskon et al., 2004) with those in the transgenic rice in the present study.

Comparing the DAAO specific activities of transgenic rice plant were deeply lower than of overexpressing in *E. coli*. According to Hwang's (2000) study, in lactose-induced *E. coli* BL21, the enzyme activities from expressed His6-tagged DAAO were reached to 20.7 U mg protein^{-1} min^{-1}. Dib et al. (2007) used IPTG as an inducer to elevate *Trigonopsis* DAAO amount in *E. coli*, and the enzyme activities were for up to 58 U mg protein^{-1} min^{-1}. One unit (U) of DAAO activity was defined as the amount of the enzyme for producing 1 μmol substrate per min in *E. coli*, however, in our study, one unit (U) of DAAO activity was defined as turnover of 1 nmol substrate per min. The best DAAO activities in transgenic rice of Act:daao plants were only 65.5 U mg protein^{-1} min^{-1}, which were shown lower 316-886 folds than that in *E. coli*.

The ultimate objective of this study is to be able to extract a large quantity of DAAO from transgenic rice for pharmaceutical uses. Our present study demonstrated that it is feasible to produce DAAO in transgenic rice plants. Enhancement of promoter strength to achieve even higher expression to maximize enzyme is our next effort. For example, the sugar response sequence (SRS) of α-amylase gene can be integrated in the *Act1* promoter for increasing the promoter expression (Chen et al., 2002). Acquisition of more transgenic rice plants for screening a high expression line is underway. It is important for industrial application to maximize enzyme yield in transgenic plants to achieve large-scale production. For the application of DAAO from transgenic rice plants to industrial scale, extraction of the DAAO protein from the transformants crude extract through DAAO antibody-affinity column is a desirable method (Hashimoto and Komastu, 2007). Alternatively, using Berg's method (1976) to purify DAAO from transgenic plants may be another possibility, which by precipitating protein with acetone and ammonium sulfate, and applying gel and ion-exchange chromatography to purify protein further.

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在轉殖水稻中表現三角酵母菌 D-型胺基酸氧化酶以供應頭孢菌素生產

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轉殖植物已成為製造重組蛋白質的有利系統，而且已有許多轉殖植物生產具功能性蛋白質的成功例子。在此篇研究中，利用農桿菌基因轉殖技術表現三角酵母菌 (*Trigonopsis variabilis*) 的 D- 型胺基酸氧化酶 (DAAO) 於粳稻栽培種 — 台粳 9 號 (Taiken 9) 中。DAAO 為一種黃素酵素 (flavoenzyme)，可催化使頭孢菌素 C (cephalosporin C) 經氧化作用成為頭孢菌素類抗生素之前驅物 glutaryl-7-aminocephalosporin acid (GL-7-ACA)。其中三角酵母菌的 DAAO 對於頭孢菌素 C 則有最好的氧化催化能力。在水稻中，分別以來自水稻本身的啟動子 —actin 1 (*Act1*) 和玉米的啟動子 —phosphoenolpyruvate carboxylase (*PEPC*) 調控三角酵母菌的 *daao* 基因表現。並以南方墨點分析證實三角酵母菌的 *daao* 基因皆有插入水稻染色體，且進一步以北方墨點和西方墨點分析，不論使用 *Act1* 或 *PEPC* 啟動子調控 *daao* 基因，證實相較於未轉殖株，在轉殖株的不同組織中皆可偵測其基因正常表現，並有其蛋白之累積。偵測 DAAO 活性分別於兩種調控啟動子的水稻轉殖株，發現以 *Act1* 為啟動子的轉殖植物的葉子有最大比活性 (65.5 ± 7.4 U mg protein^{-1} min^{-1})。利用水稻 *Act1* 啟動子的轉殖水稻具有的 DAAO 活性高於使用玉米 *PEPC* 啟動子的好幾倍，且在葉子和莖桿部位分別提高了 5.3-3.7 倍。具 *PEPC* 啟動子的轉殖水稻的穀粒則偵測不到 DAAO 活性。由上述結果皆可證實，三角酵母菌的 *daao* 基因可以穩定存在於轉殖水稻染色體中，且此基因可行正常的轉譯以及轉錄成具功能性的蛋白質。

關鍵詞 ： 三角酵母菌 ；D- 型胺基酸氧化酶 ; 頭孢菌素 ; 轉殖水稻植株 ; 粳稻栽培種 — 台粳 9 號。