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Overexpression of the *γ-TMT* gene in *Codonopsis lanceolata*

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Abstract

A cDNA-encoding γ-tocopherol methyltransferase (γ-TMT) from *Arabidopsis thaliana* was overexpressed in deoduck (*Codonopsis lanceolata* L.) to improve the tocopherol composition. Deoduck (T_2) containing the *γ-TMT* transgene was produced by *Agrobacterium*-mediated transformation. Transgene expression was confirmed by polymerase chain reaction and RNA gel blot analysis. The transgenic plants produced more leaves than control plants. In addition, the transgenic plants showed higher levels of the *CSOD*, *CTRX*, *CAPX*, *CNADP*$^+$*-IDCH*, and *CSO* transcripts and higher SOD-like activity compared with the control plants.

Additional key words: antioxidant metabolism, CSOD, deoduck, SOD-like activity.

Introduction

Codonopsis lanceolata (*Campanulaceae*) is a perennial medicinal herb distributed in East Asia. Tocopherol-encoding genes, identified in *Arabidopsis*, can alter the tocopherol composition of seed oils, resulting in improved nutritional and food-quality (Grusak and DellaPenna 1999, Van Eenennaam *et al.* 2003). While α-tocopherol is the predominant form in leaves, γ-tocopherol and tocotrienols accumulate in the seeds of many plant species (Demurin *et al.* 1996). The aromatic ring of tocopherol interacts with reactive oxygen species (ROS) and is important in protecting membrane fatty acids from oxidative stress (Fryer 1992, McKersie *et al.* 1990). Furthermore, tocopherol is necessary for scavenging the ROS that result from photosynthesis (Fryer 1992). Increases in *α*-tocopherol content in soybean seed have been demonstrated using the *γ-TMT* gene from *Arabidopsis thaliana* (*At-VTE4*) (Kim *et al.* 2005, Van Eenennaam *et al.* 2003). The overexpression of the *γ-TMT* gene has been also reported in *Perilla frutescens*, lettuce, and *Brassica juncea* (Rimm *et al.* 1993, Yusuf and Sarin 2007). The responses of *γ-TMT*-transgenic deoduck plants to salt and sorbitol stress and methyl viologen treatment were compared with those of the wild type (Brigelius-Flohe and Traber 1999). There have been few reports of the regeneration and morphological characterization of transgenic deoduck plants (Min *et al.* 1992, Shin and Park 2000, Cho *et al.* 1999). The role of metal ions, oxidative stress and antioxidant enzymes is related to salinity stress tolerance in maize (Kholova *et al.* 2009). In the present study, we identified genes whose expression levels change with the overexpression of *Arabidopsis thaliana γ-TMT* gene in transgenic deoduck plants. We investigated whether the *γ-TMT* gene can play a role in antioxidant metabolism by regulating the transcription of several marker genes.

Materials and methods

***Agrobacterium*-mediated transformation of *Codonopsis lanceolata*:** *Agrobacterium tumefaciens* strain LBA4404 harboring the binary vector pYB1130, which contains the neomycin phosphotransferase gene (*npt* II) directed by

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Abbreviations: BA - 6-benzylaminopurine; LB - Luria Bertani; MS - Murashige and Skoog; NAA - α-naphthaleneacetic acid; TAE - Tris-acetate-EDTA.
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the nos promoter as a selectable marker, was used. A single colony of the strain was grown for 24 h at 28 ± 1 °C with shaking at 150 rpm in 20 cm^3 of liquid Luria-Bertani (LB) medium (10 g dm^{-3} tryptone, 10 g dm^{-3} NaCl, 5 dm^{-3} yeast extract, pH 7) containing 100 mg dm^{-3} kanamycin. The cells were collected by centrifugation for 10 min at 7000 *g* at 4 °C and resuspended in liquid inoculation medium (MS salts containing 20 g dm^{-3} sucrose) to a final absorbance (A_{600}) of 1.0 for plant infection. To test the importance of preculturing, *in vitro*-grown *Codonopsis lanceolata* L. leaf explants of 3 to 4-week-old plants were injured slightly across the midrib with a sterile scalpel and incubated for 48 h in preculture medium containing 0.1 mg dm^{-3} α-naphthaleneacetic acid (NAA), 1 mg dm^{-3} 6-benzylaminopurine (BAP), 3 % sucrose, and 0.8 % agar at pH 5.8 prior to inoculation with the bacterial culture. The infected explants were blotted dry using sterile filter paper and transferred to fresh plates of the same medium for co-cultivation for 3 d. Next, the explants were washed in liquid MS medium supplemented with 250 mg dm^{-3} of filter-sterilized cefotaxime, blotted dry with sterile filter paper, and transferred to selection medium consisting of MS salts containing 0.1 mg dm^{-3} NAA, 1 mg dm^{-3} BAP, 3 % sucrose, 0.8 % agar, 100 mg dm^{-3} kanamycin for selection, and 250 mg dm^{-3} cefotaxime to eliminate bacterial growth, at pH 5.8. The cultures incubated in a 16-h photoperiod under irradiance of 45 μmol m^{-2} s^{-1} and temperature of 25 ± 2 °C, were transferred to fresh selection medium containing 50 mg dm^{-3} kanamycin without cefotaxime, and allowed to regenerate for 30 d with subculturing every 2 weeks. The primary regenerated shoots from the leaf explants were then transferred to MS and ½ MS media supplemented with 50 mg dm^{-3} kanamycin for root induction. The rooted plantlets were transferred to plastic pots containing a sterilized mixture of *Vermiculite* and *Perlite* (1:1), incubated at 25 ± 2 °C in a growth chamber for one week in polyethylene bags to maintain high humidity, and then transferred to a greenhouse.

DNA extraction and PCR analysis: All of the putative transgenic plants were grown under identical conditions. The transgenic plants were analyzed by PCR and Northern blotting for the expression of the transgenes. Total DNA samples were used as template to amplify the *γ-TMT* gene. For PCR amplification of a 700-bp fragment of the *npt II* gene, two primers were designed: N-1 (5'-GAGGCTATTCGGCTATGACT-3') as the forward primer and N-2 (5'-AATCTCGTGATGGCAGGTTG-3') as the reverse primer. The amplification conditions were 30 cycles of 1 min at 94 °C, 1 min at 60 °C, and 1 min at 72 °C, with a final extension at 72 °C for 7 min. To amplify a 1.07-kb fragment of the *γ-TMT* gene, the following primers were designed: *γ*-TMT forward (5'- GAATTCATGAAAGCAACTCTAGC-3') and *γ*-TMT reverse (5'-TAATCGATTAGACTTAGAGTGGCTTC-3'). The PCR products were resolved by 1 % agarose gel electrophoresis in 0.5× Tris-acetate-EDTA (TAE) buffer along with a 1-kb DNA ladder as a marker (*Invitrogen*, USA). For Northern blot analysis of transgenic deoduck plants, 5 μg of RNA from each line were loaded along with ethidium bromide. Following blotting onto nylon membranes, the membranes were hybridized with a full length *γ-TMT* cDNA labeled with [^{32}P]-dCTP using a random-primed labeling kit (*Promega*, Madison, USA).

Examination of altered gene expression by reverse transcriptase-PCR: Total RNA was isolated from the transgenic deoduck plants (Yi *et al.* 2004). Plant materials (1 g) were frozen in liquid nitrogen and homogenized in 10 cm^3 extraction buffer [4 M guanidine isothiocyanate, 25 mM sodium citrate at pH 7.0, 0.55 % (m/v) N-lauryl sarcosine, 0.1 M 2-mercaptoethanol]. A mixture of 2 M sodium acetate (pH 4.0), water-saturated phenol, and chloroform : isoamyl alcohol (24:1) was mixed with the homogenate. After centrifugation, the pellet was suspended in 2 M LiCl and incubated at 4 °C for 18 h. Total RNA samples were treated with 1 U DNase for 10 min at 37 °C. First-strand cDNA was synthesized from the DNase-treated total RNA (1 μg) using *AccuPower PCR Premix* (*Bioneer*, Korea) containing oligo(dT) primers and Moloney murine leukemia virus RT (M-MLV RT, *Invitrogen*). The primers used for RT-PCR were as follows in Table 1. PCR reactions were carried out as follows: an initial

Table 1. The primers used for reverse transcriptase-PCR

Gene	Forward primer (5'→3')	Reverse primer (5'→3')
γ-TMT	GAAGCTATTCGGCGGCTATGACTG	ATCGGGAGCGGCGGCGATACCCTA
CActin	CGAGAAGAGCTACGAGCTACCCGA	CTCGGTGCTAGGGCAGTGATCTCTTTG
CHSP18	ACGTCTTCGACCCATTCTCC	CGGTCTTCTCTTCCTGCTCC
CCoAOMT	TCTCCCTGATGATGGCAAGA	CCGATCAGACCTCCAACCTT
CCCR	CTTCATCAATTGGTGCGGTC	AGCGGAGCCAGTGAGGTACT
CSOD	TTACCCAAGAGGGAGATGGC	TAACATTCCCGAGGTCACCA
CTRX	GCCATTAATCAAAGCTCCCC	CATCAGCACAAATGTTGGCA
CAPX	TGCCACTAAAGGCACAGACC	TCATCCGCTGCGTACTTCTC
CNADP$^+$-ICDH	TCGAAATGGCGTTTGAGAAG	CAGTGCCAATACAGGCTGCT
CSO	TTTCAATGCTGAGCCACCTC	TAGCAGAAACATCCCACCCA
CMet2	TGTGGAGGAAACTGTGGGTG	CTTGGATTTGGGTGCAACAC

5-min denaturation at 94 °C was followed by 21 cycles of 30 s at 94 °C, 30 s at 58 °C, and 30 s at 72 °C, with a final extension of 7 min at 72 °C. Samples of the reaction products were separated on 1 % agarose gels and visualized after staining with ethidium bromide. All experiments were performed in triplicate.

Superoxide dismutase (SOD)-like activity: The reaction mixtures were prepared by mixing 0.2 cm^3 of sample, 3 cm^3 Tris buffer (50 mM Tris-HCl, 10 mM EDTA, pH 8.5), and 0.2 cm^3 7.2 mM pyrogallol, and the mixtures were incubated at 25 °C for 10 min. After stopping the reaction by the addition of 1 cm^3 of 1 M HCl, the absorbance of each at 420 nm was measured using a UV/VIS spectrophotometer (*U-2000*, Hitachi, Tokyo, Japan). SOD-like activity is expressed as follows: SOD-like activity [%] = [1 - (absorbance of test solution/ absorbance of control solution)] × 100 (Jo *et al.* 2005).

Results and discussion

The vector pYB1130 contains a single T-DNA harboring the *npt II* and *γ-TMT* genes driven by the 35S promoter (Fig. 1). The mixture method, which uses *Agrobacterium* strain LBA4404 to deliver multiple T-DNAs to plant cells, was used to introduce the pYB1130 binary vector into deoduck plants.

Regeneration of shoots from calli was induced using regeneration medium, and the shoots were transferred to rooting medium (Fig. 2*A*). T_2 transgenic lines were propagated and compared with control T_2 plants. The transgenic T_2 generation showed obvious growth differences as compared to the control plants (Fig. 2*B*). *Agrobacterium*-mediated transformation of deoduck was previously performed with the *bar* gene, encoding phosphinothricin acetyltransferase (PAT), to develop a bialaphos-resistant transgenic *C. lanceolata* line (Cho *et al.* 1999).

PCR was performed to confirm whether the *npt II* and *γ-TMT* genes were integrated into the genomes of the T_2 transgenic plants, using primer pairs specific for these genes and designed to produce 0.7- and 1.07-kb amplification fragments. No DNA band was amplified from control or non-transformed plants (Fig. 3*A*). The PCR analysis guided the selection of T_2 transgenic deoduck lines yielding the expected band. Northern blot analysis was conducted using a 1.07-kb fragment of *γ-TMT* as a probe, and expression of this gene was confirmed in the transgenic lines (Fig. 3*D*). The transformation frequency of deoduck was extremely low (data not shown). Evaluation of the transgenic plants under field conditions will be necessary to determine the effect of the transgene on the agronomic traits of the crop, to verify that the plants pose no environmental risks (Kiffin *et al.* 2006). Altered phenotypes caused by *γ-TMT* gene expression have been reported for several important species, including *Perilla frutescens* and *Brassica juncea* (Min *et al.* 1992).

ROS can act as ubiquitous signal molecules, and are a central component in the stress responses of higher plants. Antioxidants and ROS comprise an important interacting system that has different functions in higher plants (Borland *et al.* 2006, Del Rio *et al.* 2006) In the present study, to elucidate the mechanisms triggered by biotic and abiotic stresses in *γ-TMT*-overexpressing plants, the expression patterns of several genes were monitored in control and *γ-TMT*-overexpressing plants. Semi-quantitative RT-PCR analysis was performed with RNA prepared from *γ-TMT*-overexpressing deoduck leaves (Fig. 4). α-Tocopherol scavenges lipid peroxy radicals produced by the action of other antioxidants in the green parts of plants, yielding a tocopheroxyl radical (Hare *et al.* 1998, Igamberdiev and Hill 2004, Kiffin *et al.* 2006). ROS generated during photosynthesis and metabolism can induce lipid peroxidation in plant cells and α-tocopherol content increases in response to a variety of abiotic stresses in photosynthetic plant tissues (Noctor 2006). α-Tocopherol probably prevents oxidative damage to lipid components during seed storage and seed germination (Venkata *et al.* 2007).

The role of γ-TMT in the synthesis of α-tocopherol was demonstrated by the overexpression in *Arabidopsis* (Shintani and DellaPenna 1998). We examined the *C. lanceolata* genes deposited in GenBank, which have not been studied in detail previously. We then carried out RT-PCR analysis using primers designed for a subset of these genes. Lignin content and composition have been shown to be altered by the underexpression of lignin biosynthetic genes, including *CCoAOMT* (encoding caffeoyl-CoA-O-methyltransferase) and *CCR* (encoding cinnamoyl-CoA reductase) (Rogers and Campbell 2004). CCR is thought to be a limiting enzyme that regulates lignin content in tobacco and *Arabidopsis* (Piquemal *et al.* 1998, Goujon *et al.* 2003). However, no altered expression of the *CCCoAOMT* or *CCR* genes was

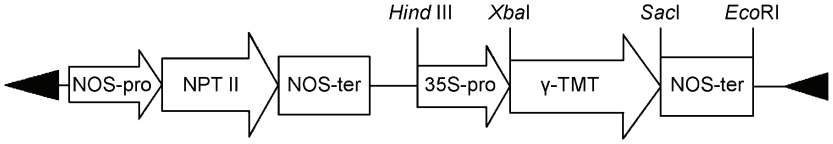

Fig. 1. Construction of the plant expression vector. Diagram of the *Arabidopsis thaliana γ-TMT* gene inserted into the plant gene expression vector pYB1130 (a modified version of pBI121).

Fig. 2. Regeneration and T_2 generation of transgenic *Codonopsis lanceolata*. *A* - Regeneration of putative transgenic shoots from embryogenic callus of leaf explants on selection medium. *B* - Growth of control and T_2 transgenic *Codonopsis lanceolata* in the greenhouse (C - control, 3 - transgenic plant).

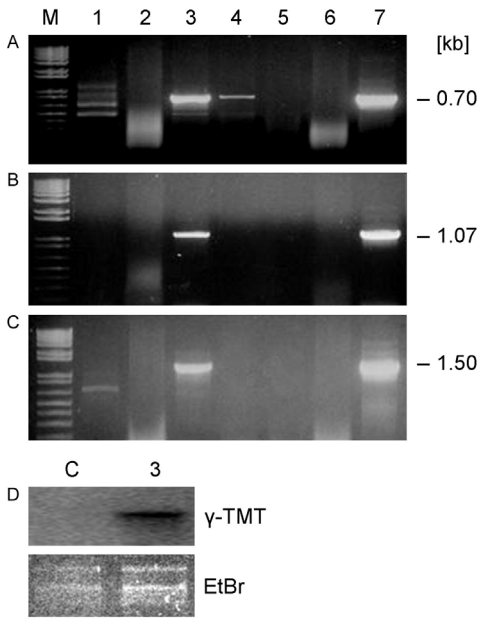

Fig. 3. PCR and RNA expression of *γ-TMT* in transgenic deoduck plants. *A*, *B*, *C* - PCR analysis was performed on transgenic T_2 deoduck plants using NPTII primers, *γ-TMT*-specific primers, and the nos reverse primer, respectively (M - 1-kb DNA ladder, 1 to 5 - independent transformed deoduck lines, 6 - control plant, 7 - plasmid DNA positive control. *D* - *γ-TMT* RNA expression in transgenic T_2 deoduck plants. Total RNA was extracted from fully expanded leaves of 8-week-old deoduck plants (C - control, 3 - transgenic plant).

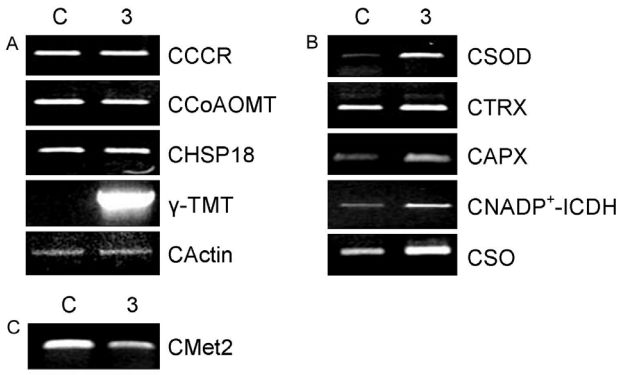

Fig. 4. RT-PCR analysis of genes encoding enzymes involved in plant defense, lignin biosynthesis, and ROS signaling metabolism in control and *γ-TMT*-overexpressing deoduck T_2 progeny. Total RNA was extracted from leaf tissue of control (C) and transgenic (3) plants. The expression of the pathogen- and lignin biosynthesis-related genes *CCCR* (AB243011), *CCoAOMT* (AB243012), and *CHSP18* (BAE48789) (*A*) and the antioxidant-related genes *CSOD* (AY833718), *CTRX* (AB223035), *CAPX* (AB243015), *CNADP*$^+$*-ICDH* (AB243085), and *CSO* (AB243086) (*B*) were compared by RT-PCR. *C* - The expression level of the *CMet2* (AAV97748) gene was downregulated in transgenic deoduck plants. *CActin* was used as a positive control.

observed in transcript level (Fig. 4*A*).

In the *γ-TMT*-overexpressing deoduck plants, but not the control plants, the expression of several genes encoding ROS-scavenging enzymes was found to be

altered. The expression levels of these genes (*CSOD*, *CTRX*, *CAPX*, *CNADP$^+$-IDCH*, and *CSO*) were examined by RT-PCR using primers specific for each (Fig. 4*B*). All of these genes were upregulated in the *γ-TMT*-over-expressing deoduck plants. The SOD-like activities were 61 % in the *γ-TMT*-overexpressing deoduck transgenic lines and only 50 % in control plants. This result was correlated to *SOD* gene expression, as shown by RT-PCR. The gene encoding cytosolic APX is regulated by various environmental stresses (Shigeoka *et al.* 2002). Both the differential regulation of *APX* expression by diverse environmental stresses and its developmental regulation suggest its role in stress-signaling pathways of rice (Agrawala *et al.* 2003). TRX proteins confer heavy-metal tolerance on various organisms. Laloi *et al.* (2004) reported that expression of the gene encoding thioredoxin h5 is observed upon wounding, abscission, senescence, pathogen attack, and oxidative-stress in *Arabidopsis* (Laloi *et al.* 2004). The functions of the thioredoxin h-encoding gene *CTRX* in *C. lanceolata* are largely unknown (In *et al.* 2005a). We observed the induction of *CTRX* in the transgenic plants, but the mechanism of *TRX* regulation in *C. lanceolata γ-TMT*-overexpressing plants (Fig. 4*B*) requires further study. NADP$^+$-mtICDH (mitochondrial isocitrate dehydrogenase) can be involved in tricarboxylic acid cycle flux and the reductive modulation of alternative oxidase (AOX) (Gordon *et al.* 2004). Plant mitochondria contain NAD+-dependent (NAD-IDH) and NADP+-dependent (mtICDH) (Møller 2001). The physiological role of the NADPH-generating enzyme NADP$^+$-mtICDH in the reductive activation of AOX, and its relationship with the *γ-TMT* gene, needs to be studied further. In plants, resistance to SO_2 varies with the plant species and external factors such as the soil, season, and temperature (Lang *et al.* 2007). Sulfite-oxidizing (SO) activities in various organisms have been known for many years. SO enzymes are well characterized on the molecular and biochemical levels, and their co-regulation with sulfate assimilation in plants is well established (Hänsch *et al.* 2007).

In *et al.* (2005b) were the first who characterized the *CMet2* gene and to demonstrated the possibility of high heavy metal and oxidative-stress tolerance in *C. lanceolata* (In *et al.* 2005b). We suggest that *γ-TMT* is involved in the downregulation of CMet2 in transgenic plants (Fig. 4*C*). However, the role of CMet2 in the *γ-TMT*-overexpressing transgenic line is still not understood.

A γ-tocopherol-overexpressing transgenic line has been shown to have increased osmoprotection *in vivo* (Yusuf and Sarin 2007). However, it is unknown whether *γ-TMT*-overexpressing transgenic deoduck shows enhanced tolerance to diverse stresses. In the present study, we found that γ-TMT may play a role in the heavy metal and oxidative stress response pathways in transgenic deoduck plants. Further studies are needed to understand the physiological functions of γ-TMT in the *γ-TMT*-overexpressing transgenic deoduck line.

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