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Rice Salt-Tolerance Enhancement by Expression of 1-Aminocyclopropane-1-Carboxylic Acid Oxidase Gene from Salt Tolerant Barley

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Abstract

Ethylene, a plant hormone, plays important roles not only in regulation of plant growth and development, but also in regulating plant biological mechanisms in response to various abiotic and biotic stresses. A gene encoding 1-aminocyclopropane-1-carboxylate (ACC) oxidase (ACO), which catalyzes ACC to ethylene, was isolated from salt-tolerant barley. The ACO gene was expressed more constitutively and preferentially in salt-tolerant barley root than in salt-sensitive barley. The T3 generation transgenic (T3) rice with barley ACO gene exhibited decreased frequency of root looping response and increased root elongation, thereby producing gravitropic enhancement of roots. T3 rice in the medium with 200 mM NaCl exhibited the root elongation as well as without NaCl, and showed 65% survival, whereas root elongation of wild type (WT) rice was inhibited by salt treatment and survival percentage was 47%. Subsequent RT-PCR analysis revealed that pathogenesis related-10a (PR-10a) gene in T3 rice was expressed 12 times more than in WT rice, which is known to play a role in salt and drought stress tolerance and which is known to be produced preferentially in salt-tolerant barley, however, gene expression levels of reactive oxygen species-scavenging enzymes in T3 rice, which were required as internal signals for plant survival response, were not different from those of WT rice. These results demonstrate that ethylene enhances salt tolerance with the induction of PR-10a.

Keywords: 1-Aminocyclopropane-1-carboxylic acid oxidase; Barley; Ethylene; Pathogenesis-related protein; Salt stress

Introduction

Salt stress is a severely limiting factor affecting plant growth and agricultural crop productivity. Plants protect themselves from abiotic and biotic stresses by changing expression levels of stress-tolerant genes. Plants develop strategies for responding to environmental conditions through an intricate network of abiotic and biotic stress signal transduction pathways [1-3]. Ethylene is a salt-stress-responsive plant hormone that also regulates plant growth and development [4]. Ethylene is synthesized from methionine by three enzymatic reactions. Methionine is converted to *S*-adenosyl-methionine (SAM) by SAM synthetase. After SAM is converted to 1-aminocyclopropane-1-carboxylate (ACC) by ACC synthase (ACS), ACC is catalyzed to ethylene by ACC oxidase (ACO) [5]. In *Arabidopsis*, the gene expression level of ACS and enzyme activity became higher under salt stress [6,7] and salt-stress-induced transcription of ACS in tobacco and cotton [8,9]. In *Cicer arietinum* root exposed to salt stress, ACO production and activity were increased to enhance the ethylene concentration [10]. The gene expression level of ACO was increased in cotton under salt stress [9]. These results suggest that plants under salt stress produce ACS and ACO for increasing the level of ethylene to adapt to salt stress. By contrast, *acs7* mutant and ethylene signaling defective mutant of *Arabidopsis* were more tolerant of salt stress [11,12]. *Arabidopsis* expressing wheat ACO gene reduced salt tolerance [13]. These results demonstrated that ethylene plays positive or negative roles in plant salt tolerance. Controlling the ethylene level by regulating ethylene synthesizing enzymes and ethylene signalling genes in plants is important to enhance salt tolerance.

Reactive oxygen species (ROS), which are generated by abiotic stress, react with DNA, lipids, and proteins, causing severe cellular damage. Plants have ROS-scavenging systems consisting of antioxidant enzymes to maintain the cellular steady-state concentration of ROS. In contrast, ROS control and regulate biological processes such as growth, cell cycle, cell death, hormone signaling, and abiotic and biotic stresses responses in plants, as does ethylene [14]. Therefore, plants require ethylene and ROS as signals to initiate adaptive survival responses to abiotic and biotic stressors and the relation between ethylene and ROS signaling systems will help to clarify the regulatory network that leads to plant survival responses upon abiotic stress [15].

Previously proteome analysis showed preferential production of pathogenesis related-10a (PR-10a), *O*-methyltransferase (OMT), glutathione *S*-transferase (GST), and dehydroascorbate reductase (DHAR), in salt-tolerant barley [16]. In order to find the relation among these genes for salt tolerance, we defined genes that are expressed preferentially in salt-tolerant barley. One was found to have significant identity with plant ACO gene and was expressed constitutively in salt-tolerant barley. This study found that transgenic rice with barley ACO gene can achieve salt tolerance with decreased root looping response and root gravitropism enhancement. Also, RT-PCR analysis showed the increase of pathogenesis related-10a (PR-10a) gene, which is induced

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under salt stress and known to improve salt and drought tolerance [17], but no difference was found in the gene level of ROS-scavenging enzymes.

Materials and Methods

Plant cultivation and stress treatment

Seeds of salt-tolerant barley (*Hordeum vulgare* L.), OUK305, and salt sensitive barley, OUI743, in the barley germplasm collection from various parts of the world preserved in the Barley Germplasm Center, Okayama University [18], were soaked in water at 15°C for 5 h and were subsequently incubated on water-soaked filter paper at 20°C for 3 d in the dark. The seedlings were cultivated in a hydroponic solution consisting of 4 mM KNO_3, 1 mM NaH_2PO_4, 1 mM $MgSO_4$, 1 mM $CaCl_2$, and 1 mg/ml Fe-citrate, pH 5.5 under metal halide light (350 μmol/m^2/s) for 2 d. Plants were transferred to a culture solution containing 100 mM NaCl for salinity stress. After 12 h incubation, the roots were harvested, frozen in liquid nitrogen, and stored at -80°C.

Seeds of rice, *Oryza sativa* L. ssp. *japonica* cv. Nipponbare, were germinated on MS gellan gum consisting of Murashige and Skoog basal medium, 2% sucrose, 0.1% gellan gum, with or without 50 g/ml hygromycin (pH 5.8) filled in a graduated cylinder, of which the side wall and bottom were covered with aluminum foil. Plants were cultivated at 25°C under metal halide light (350 μmol/m^2/s) with a light/dark cycle of 16 h/8 h in a growth chamber.

Subtraction hybridization and generation of subtracted library

Subtraction hybridization [19-21] was performed using a cDNA subtraction kit (the PCR-Selected™; Clontech). Tester and driver cDNAs were prepared from total RNAs isolated from roots of OUK305 and OUI743 exposed respectively to 100 mM NaCl for 12 h. The enriched cDNA fragments were cloned into a pGEM-T vector (Promega Corp.) and were then transformed into *Escherichia coli* XL1-Blue cells. Colonies were hybridized with the driver cDNA; colonies displaying weaker signals were selected as the OUK305 subtracted library, as described in an earlier report [21].

RACE-PCR

The respective primers for 3′-RACE and 5′-RACE PCR, 5′-ACATGGCCAAGCTGCTCAATTTGGAT-3′ and 5′-ATCCAAATTGAGCAGCTTGGCCATGT-3′ were synthesized based on sequences of gene fragments isolated from the subtracted library. RACE-PCR was conducted on the cDNA. The PCR products were cloned into a pGEM-T vector (Promega Corp.). Then the nucleotide sequences on both strands were determined.

Expression in *E. coli* cells and purification of HvACO

The open reading frames of HvACO gene were amplified with total RNA from control roots and the primers,5′-CTCATATGCCGGAGCCCATCCCCGTCA-3′, which creates a Nde I site (shown as underlined), and 5′-GAGGATCCAATTCAGATCTTCAAGGTG-3′, which creates a *Bam*H I. The PCR product of 954 bp length was cloned into the pGEM-T vector. Fragments of the plasmids digested using *Nde* I and *Bam*H I were subcloned into a pET-15 vector in which a polyhistidine tag (Hig-tag) gene is fused upstream from the start codon. The resulting plasmid, pHvACO, was transformed into *E. coli* BL21(DE3) cells. *E. coli* cells harboring pHvACO were grown at 37°C in Luria-Bertani (LB) medium containing 50 μg/ml ampicillin. When the OD_{600} became 0.5, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to the culture at a final concentration of 0.5 mM. After cultivation at 37°C for 8 h, the cells were harvested by centrifugation and were frozen at -80°C for at least 2 h. The frozen cell pellets were suspended in a protein extraction reagent (BugBuster™ HT; Merck and Co. Inc., Darmstadt, Germany) according to the manufacturer's instructions. The resulting recombinant protein, which showed an insoluble form, was dissolved in 20 mM Tris-HCl buffer (pH 8.0) containing 0.5 M NaCl, 5 mM imidazole, and 6 M guanidine HCl (Buffer A). Protein solution was loaded on an Ni-NTA agarose column (Qiagen Inc.) initially equilibrated in Buffer A. The column was washed with Buffer A, followed by 20 mM imidazole in Buffer A, with the absorbed His-tag fused protein eluted with 1 M imidazole in Buffer A. The protein solution was dialyzed against 1 M Tris-HCl (pH 8.0) containing 0.1 M NaCl, 1 mM EDTA, and 1 mM DTT, followed by 50 mM Tris-HCl buffer (pH 8.0) containing 0.1 M NaCl and 1 mM DTT at 4°C overnight. The dialyzed solution was then concentrated using a Vivaspin 4 (Sartorius AG, Göttingen, Germany).

Enzyme and protein assay

The reaction mixture for ACO activity consisted of 100 mM HEPES (pH 7.0), 1 mM ACC, 1 mM sodium ascorbate, 4 mM sodium bicarbonate, 0.1 mM ferrous sulfate, and enzyme solution, in a total volume of 10 ml [22]. The reaction was conducted in a sealed 50 ml volume Erlenmeyer flask at 30°C for 1 h. Ethylene produced and released to the head space was quantitated using a gas detector (MiniWarn; Drägerwerk & Co. AG, Germany). Protein was measured using the method of Bradford with bovine serum albumin as a standard [23].

Construction of transgenic rice

The open reading frame of HvACO gene was amplified with total RNA from control shoots and the primers, 5′-AAGGATCCATGCCGGAGCCCATCCCCG-3′, which creates a *Bam*H I site (denoted as underline), and 5′-TTGAGCTCACCAAATATCTTCACTTGA-3′, which creates a *Sac* I. The PCR product of 982 bp length was cloned into the pGEM-T vector. The fragments of the plasmids digested by *Bam*H I and *Sac* I were subcloned into a binary vector p2K-1$^+$ to produce p2K-Ubp-HvACO [24,25]. The resulting plasmid was transformed into *Agrobacterium tumefaciens* and *Agrobacterium*-mediated transformation. Regeneration of rice plants, Nipponbare, was performed according to the method described by Terada et al. [26]. Cultured rice cells were grown in hygromycin selective medium and were transferred to redifferentiative medium. The T3 generation transgenic (T3) rice lines were established. The significance of the difference between T3 rice and wild-type (WT) rice was analyzed by Student's *t*-test.

RT-PCR Analysis

Total RNA isolated from roots was used to synthesize a first-strand cDNA using a cDNA synthesis kit (PrimeScript first strand; Takara Bio Inc., Shiga, Japan). The concentration of first-strand cDNA was adjusted to 0.075 μg/μl. A reaction mixture of 50 μl containing 1 μl of first-strand cDNA, 0.4 mM dNTPs, 1 × PCR buffer, 2.5 U ExTaq HT (Takara Bio Inc.), and 10 pmol of each primer combination (Table 1) was amplified by PCR with the following the thermal cycle profile: 1 cycle of 94°C for 1 min, followed by 25, 25, 35, 25, 40, 30, 30, 25, and 20 cycles of 94°C for 15 s, 60°C for 15 s, and 68°C for 1 min for each of rice SOD (OsSOD), rice APX (OsAPX), rice CAT (OsCAT), rice GST (OsGST), rice PR-10a (OsPR-10a), rice OMT (OsOMT), rice DHAR

Gene	Forward (5'=>3')	Reverse (5'=>3')	Fragment size (bp)	Accession No.
OsSOD	ATGGTGAAGGCTGTTGTTGTGCTTGGT	TCAGCCTTGAAGTCCGATGTTCCCGCA	461	L19435
OsAPX	CTCCTACGCCGACTTCTACCAGCTTGC	CCCAGTTCAGAGAGCTTGAGGTGGGCC	467	D45423, AB053297
OsCAT	TTCCACTGGAAGCCTACCTGTGGTGTC	ACCTCTTCATCCCTGTGCATGAAATTC	502	D29966, D64013, D86611
OsGST	TTATGGGAAGATCAATCCAATTAAGTA	TCAGGATGAAGGTGCATCTGGTTGGTT	513	AF309381, AF402792
OsPR-10a	TCCGGCCTGCGTCTCCGACGAGCACGC	TTAGGCGTATTCGGCAGGGTGAGCGAC	474	AF274850
OsOMT	GTACGGGATGACGGCGTTCGAGTACCA	GCGAGCATGATCATGTCGACGTGGAAC	500	DQ530257
OsDHAR	GCCCGACTGGTTTCTGAAGATCAGCCC	TTACGCATTCACTTTTGGTGCCCATCC	504	AY074786
HvACO	AAGGATCCATGCCGGAGCCCATCCCCG	ATCCAAATTGAGCAGCTTGGCCATGT	460	AK357897
Osα-TB	GGTTTGATGGTGCTCTGAACGTGGATG	CCTCATTCTCGTCGGACTCAGAGCCAA	605	Z11931

Table 1: Primers for RT-PCR.

(OsDHAR), barley ACO (HvACO), and rice α-tubulin (Osα-TB) genes. The reaction mixture (3 μl) was loaded on agarose gel electrophoresis. Amplified fragments were detected using ethidium bromide staining.

Results and Discussion

Identification and function of barley ACO gene

A total of 1000 clones from the subtraction library, which were constructed with cDNAs of OUK305 as a tester cDNA and OUI743 as a driver cDNA, were hybridized with total cDNAs of OUI743 roots treated by NaCl. Negative clones were hybridized with the cDNAs again, yielding 74 clones that showed negative signals. The nucleotide sequences of the insert cDNA fragments were compiled into 9 genes. Northern hybridization revealed that 3 of 9 fragments with 221, 114, and 421 bp length were expressed significantly in OUK305, respectively which showed identities to ACO, OMT, and uncharacterized protein genes. Northern hybridization revealed that the putative ACO gene fragment was expressed constitutively in OUK305. The expression level was 2.5-fold higher than that of OUI743 after NaCl treatment (Figure 1). To obtain the complete nucleotide sequence of the gene, 3′-RACE and 5′-RACE cDNA fragments were amplified using RACE-PCR with specific primers based on the 221-bp fragment sequence and cDNA synthesized from the roots of OUK305 treated by NaCl. The resulting nucleotide sequence, except for the poly(A) sequence, was 1219 bp long, with an open reading frame encoding a polypeptide of 311 amino acid residues with a calculated molecular mass of 35.4 kDa. The nucleotide sequence was identified with the barley mRNA (accession no. AK357897). The deduced amino acid sequence showed 48,39,30 and 27% identities, respectively, with those of ACO from *O. sativa* (accession no. AP003019), *Arabidopsis thaliana* (accession no. AY087779), *Lycopersicon esculentum* (accession no. Y00478), *Nicotiana tabacum* (accession no. X83229), and *Nicotiana attenuata* (accession no. AY426756), by application of the BLAST program [27,28]. Alignment analysis of amino acid sequence using the Clustal W algorithm [29] revealed that HvACO conserved all 12 amino acid residues including H186, D188, and H243 for chelation of the Fe (II) ion, and R253 for binding with ACC carboxyl group among all members of the Fe (II) and ascorbate requiring superfamily of enzymes (Figure 2) [30-33].

HvAOC protein, tagged with a His-Tag, was produced in *E. coli* cells. An extra protein with a molecular mass of 37 kDa, which is similar to that calculated from the amino acid sequence, was produced as an inclusion body in *E. coli* cells. It was refolded to a soluble form (Figure 3). The purified HvACO incubated with ACC showed production of ethylene, of which the activity was 37.9 C_2H_4 nmol/h/mg. It had maximum activity at pH 7.0. These results indicate that putative HvACO gene encodes ACO.

Root growth of transgenic rice

Seeds were germinated and grown on MS gellan gum for 9 d. The roots of T3 rice were elongated and straight, whereas those of WT rice showed looping (Figure 4). The depths from the surface to the root tip of T3 rice and WT rice were, respectively, 75 ± 15 mm and 48 ± 11 mm, with respective root lengths of 80 ± 17 mm and 63 ± 14 mm (Figure 5). The depth/length ratios were 0.94 for T3 rice and 0.76 for WT rice, indicating that T3 rice decreases the root looping response frequency. Reportedly, sucrose in the medium promotes root looping, but ethylene overrode this effect [34]. These results suggest that increased ethylene concentration by HvACO enhances the gravitropic response of roots. Ethylene reduces root elongation. However, rice root growth is stimulated at low concentrations (below 1 ppm) of ethylene [35]. Actually, T3 rice expressing HvACO showed more root growth than WT rice did, suggesting that T3 rice produces ethylene, but their total concentration was less than 1 ppm.

Salt tolerance of transgenic rice

T3 rice and WT rice were germinated and grown on MS gellan gum containing NaCl for 9 d. Roots of T3 rice under 100 mM NaCl grew as well as without NaCl, whereas root elongation of WT rice was inhibited by 100 mM NaCl (Figure 6). To evaluate the salt tolerance of T3 rice, plants germinated and grown on MS gellan gum for 9 d were transplanted to hydroponic solution with or without NaCl. Root elongations of T3 rice and WT rice treated with 200 mM NaCl for 2 d were 3.1 ± 2.0 mm and 0.5 ± 0.7 mm; those without NaCl were 2.9 ± 2.7 mm and 3.7 ± 4.7 mm, respectively, indicating that the T3 rice

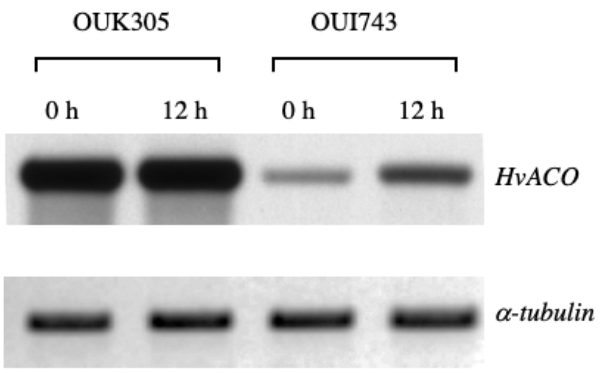

Figure 1: Expression profiles of HvACO gene in the salt-tolerant barley OUK305 and salt sensitive barley OUI743 exposed to NaCl. One microgram of each RNA prepared from roots exposed to NaCl for 0 and 12 h was electrophoresed on 1% agar gel and transferred onto a nylon membrane. The membrane was hybridized with a 221 bp fragment of HvACO. The blot was stripped and hybridized with an α-tubulin gene as a control.

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Hv_ACO      ---------------------------------------------MPEP-IPVIDLGRLS  14
Os_ACO      ----MADESWRTPAIVQELAAAGVEEPPSRYVLGEKDRSDELVAAELPEP-IPVVDLSRLA  56
At_ACO      ----MEKPKFKT---VQEVVAAGEGLPERYLHAPTGDGEVQPLNAAVPEMDIPAIDLNLLL  54
Le_ACO      ---------------------------------------------MENFPIINLEKLN  13
Na_ACO      ---------------------------------------------MENFPIINLEKLN  13

Hv_ACO      ANN---AEEFAKLQSALENWGFFLAVGHGMEPSFLAETMSVSKEFFKLPLEEKQKVSKIAY  72
Os_ACO      G-----ADEAAKLRAALQNWGFFLLTNHGVETSLMDDVLNLAREFFNQPIERKRKFSNLID 112
At_ACO      SSSEDGREELRKLHSALSTWGVVQVMNHGITKAFLDKIYKLTKEFFALPTEEKQKCAREID 115
Le_ACO      GDER--ANTMEMIKDACENWGFFELVNHGIPHEVMDTVEKMTKGHYKKCMEQR--------  64
Na_ACO      GSEK--AATMEMIKDACENWGFFELVNHGIPHEVMDTVEKLTKGHYKKCMEQR--------  64

Hv_ACO      GDTLSIEGYGNESVVVENQLLDWNDQCFLIVEPESKRTYTLWPTQPPSFRDILSEYTVKCR 133
Os_ACO      GKNFQVEGYGTDRVVTQDQILDWSDRLFLRVEPKEERNLAFWPDHPESFRDVLNEYASRTK 173
At_ACO      ----SIQGYGNDMILWDDQVLDWIDRLYITTYPEDQRQLNFWPEVPLGFRETLHEYTMKQR 172
Le_ACO      -FKELVASKGLEAVQAEVTDLDWESTFFLRHLPTS--NISQVPDLDEEYREVMRDFAKRLE 122
Na_ACO      -FKELVASKGLEGVEAEVTDMDWESTFFLRHLPVA--NISEVAGLDDQYREVMRDFAKRLE 122

                                                          * *
Hv_ACO      AVANIVLQNMAKLLNLDEEYFTNKFADTSY--TLVGFNYYPPCPKPDHVFGLRPHTDGSAI 192
Os_ACO      RIRDDIVQAMSKLLGLDEDYFFDRLNKAP---ALARFNYYPPCPRPDLVFGVRPHSDGSLF 231
At_ACO      IVIEQFFKAMARSLELEENSFLDMYGESAT--LDTRFNMYPPCPSPDKVIGVKPHADGSAI 231
Le_ACO      KLAEELLDLLCENLGLEKGYLKNAFYGSKGPNFGTKVSNYPPCPKPDLIKGLRAHTDAGGI 183
Na_ACO      NLAEELLDLLCENLGLENGYLKNVFYGSKGPNFGTKVSNYPPCPKPDLIKGLRAHTDAGGI 183

                                                      *           •
Hv_ACO      TVNFIDADVSGLQFEKNGTWYNVPIVPTALVVNIGDVMEILSNGFFKSLMHRVVTNTEKER 253
Os_ACO      TILLVDEDVGGLQIQRDGKWYNVQVTPNTLLINLGDTMEVLCNGIFRSPVHRVVTNAERER 292
At_ACO      TLLLPDKDVGGLQFQKDGKWYKAPIVPDTILINVGDQMEIMSNGIYKSPVHRVVTNREKER 292
Le_ACO      ILLFQDDKVSGLQLLKDEQWIDVPPMRHSIVVNLGDQLEVITNGKYKSVLHRVIAQTDGTR 244
Na_ACO      ILLFQDDKVSGLQLLKDGQWIDVPPMRHSIVVNLGDQLEVITNGKYKSVMHRVIAQKDGTR 244

Hv_ACO      LSLAMFYSLDMEMDIEPVPDLLDDKRPPR----YMKIKNKDYIAKQTYIFATGKQT-IDTL 309
OS_ACO      ISLAMFYSVNDEKDIGPAAGLLDENRPAR----YRKVSVGEFRAGIIGKFSRRERY-IDSL 348
At_ACO      ISVATFCIPGADKEIQPVNELVSEARPRL----YKTVK--KYVELYFKYYQQGRRP-IEAA 346
Le_ACO      MSLASFYNPGSDAVIYPAKTLVEKEAEES-TQVYPKFVFDDYMKLYAGLKFQAKEPRFEAM 304
Na_ACO      MSLASFYNPGSDAVIYPAPALVEKEAAES-KQVYPKYVFDDYMKLYAGLKFQAKEPRFEAM 304

Hv_ACO      KI------------ 311
Os_ACO      KI------------ 350
At_ACO      LI------------ 348
Le_ACO      KAMES----DPIASA 315
Na_ACO      KSIESDVKLDPIATA 319
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Figure 2: Alignment of the deduced amino acid sequences of barley ACO (Hv_ACO) with those of ACO from *O. sativa* (Os_ACO), *A. thaliana*, *L. esculentum* (Le_ACO), and *N. attenuata* (Na_ACO). Gaps, shown as dashes, are introduced into the sequences to maximize the homology. Amino acids conserved across all members of the Fe (II)-requiring and ascorbate-requiring superfamily of enzymes are represented by black boxes. Asterisks and a circle respectively denote the amino acids chelating with the Fe (II) ion and binding with ACC carboxyl group.

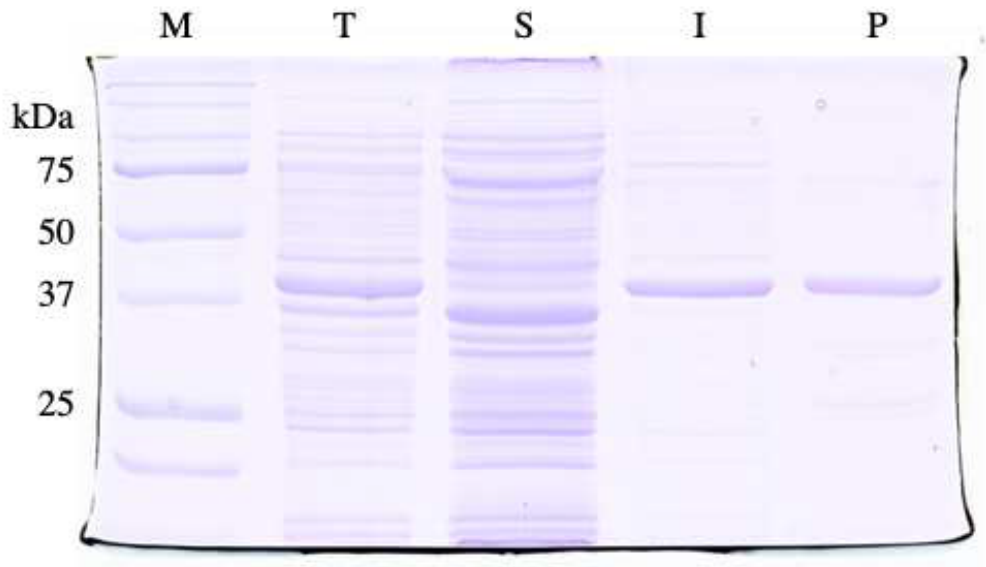

Figure 3: Analysis of expression of barley ACO in *E. coli* cells using SDS-polyacrylamide gel. *E. coli* cells harboring pHvACO were harvested after IPTG induction at 37°C for 8 h. Total cell lysate (T), soluble protein (S), insoluble protein (i), and refolded HvACO purifying with Ni-NTA column (P) were subjected to 12% SDS-PAGE with molecular mass marker (M) followed by Coomassie Brilliant Blue R-250 staining.

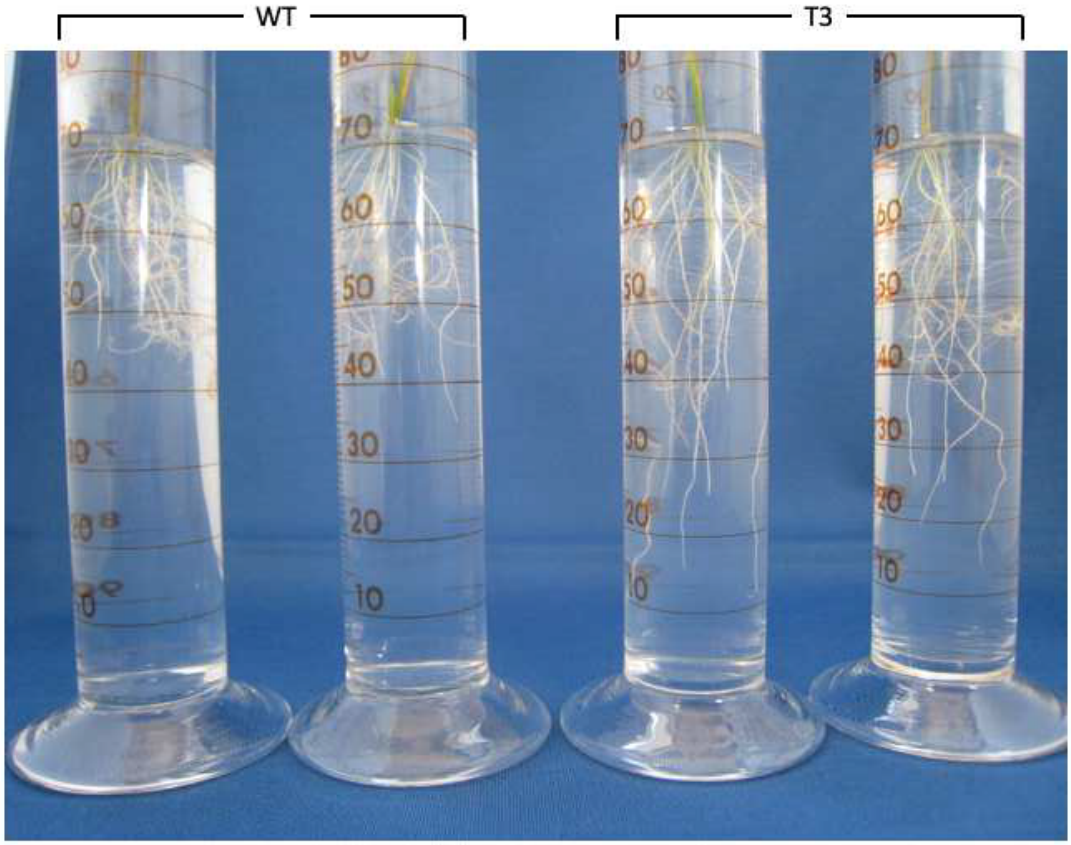

Figure 4: Root growth of T3 rice and WT rice in MS gellan gum. Seeds of WT and T3 rice were germinated and grown on a graduated cylinder filled with MS gellan gum, of which the side wall and bottom were covered with aluminum foil, for 9 d at 25°C with a light/dark cycle of 16 h/8 h.

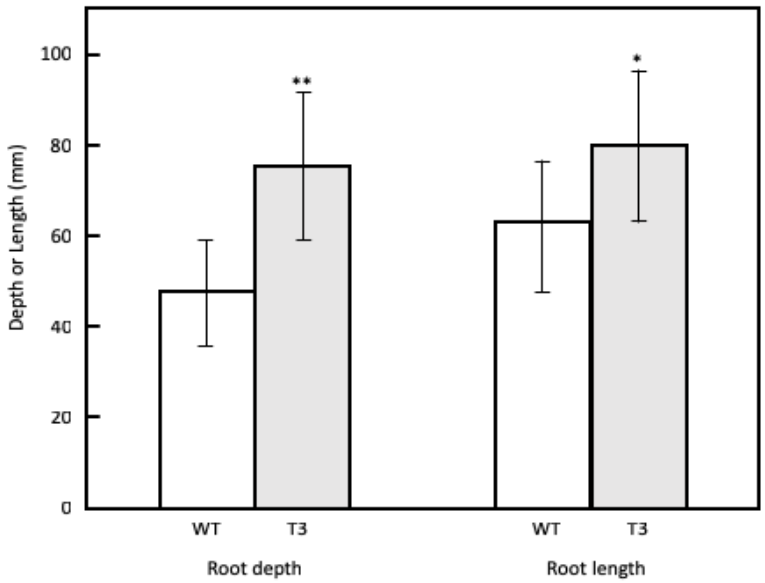

Figure 5: Root depth and length of T3 rice and WT rice in MS gellan gum. Seeds of WT rice (n=9) and T3 rice (n=8) were germinated and grown in a graduated cylinder filled with MS medium, of which the side wall and bottom were covered with aluminium foil, for 9 d at 25°C with a light/dark cycle of 16 h/8 h. $^{*}p$<0.05, $^{**}p$<0.01.

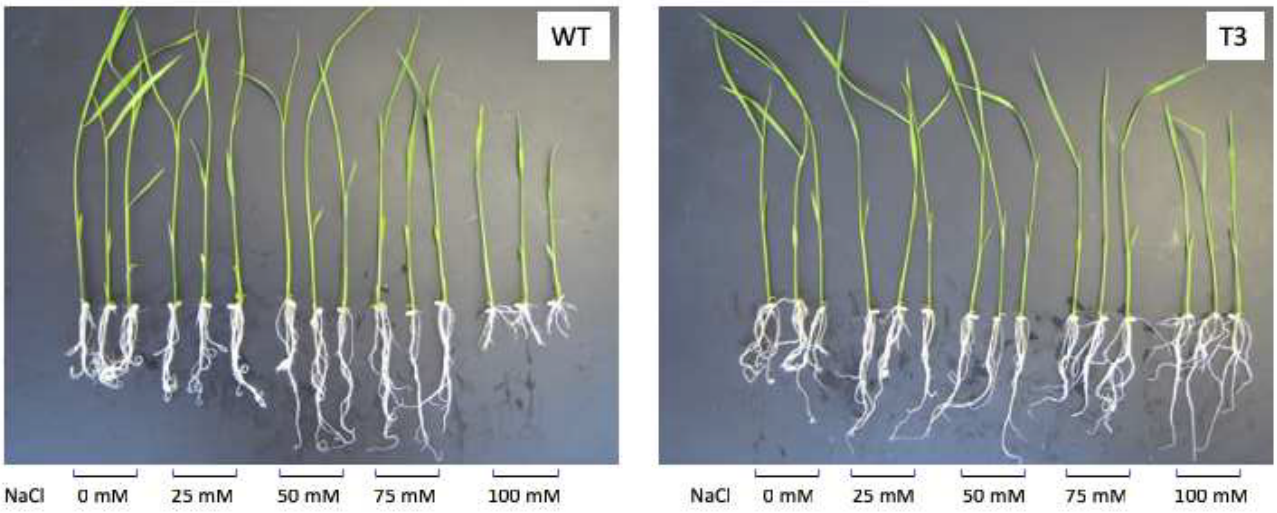

Figure 6: Growth of T3 rice and WT rice on MS gellan gum containing NaCl. The seeds of T3 rice and WT rice were germinated and grown on MS gellan gum containing 0, 25, 50, 75, and 100 mM NaCl for 9 d.

root elongation was unaffected by salt stress (Figure 7). After 2 d of treatment with NaCl, the plants were transferred to the hydroponic solution without NaCl. After prolonged cultivation, the survival percentages of T3 rice and WT rice treated with NaCl for 2 d were, respectively, 65 and 47%. These results indicate that T3 rice exhibits enhanced salt tolerance.

Gene expression of ROS-scavenging enzymes and preferentially produced proteins of salt-tolerant barley in transgenic rice

Plants require ethylene and ROS as a signal to start adaptive survival responses to abiotic and biotic stresses [15]. However, proteome analysis showed preferential production of PR-10a, OMT, GST, and DHAR in salt-tolerant barley [21]. To ascertain what genes of intermediators, affect salt tolerance, gene expression of ROS-scavenging enzyme and preferentially produced protein of salt-tolerant barley were evaluated using RT-PCR in T3 rice (Figure 8). In roots, the respective signal intensities and α-tubulin genes in WT and T3 rice (WT, T3) (13,900, 15,900) were found, and also SOD (17,900, 17,800), APX (36,800, 27,400), CAT (28,500, 14,800), GST (33,300, 30,600), PR-10a (1,740, 20,900), OMT (0, 0), DHAR (55,600, 33,800), and ACO (0, 32,600). These results demonstrated that expression levels of SOD, APX CAT, GST, OMT, and DHAR were not different between T3 rice

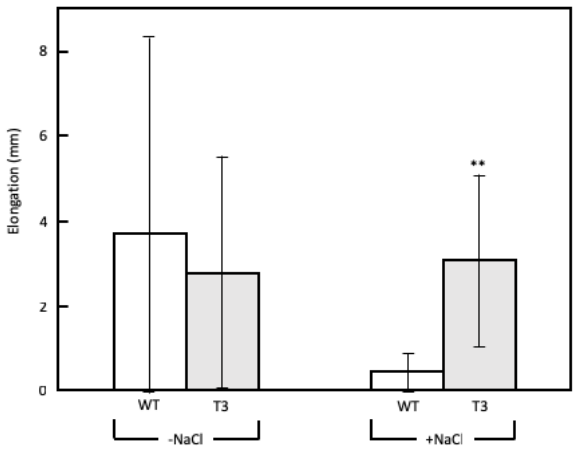

Figure 7: Root elongation of T3 rice and WT rice in MS gellan gum after 2 d of NaCl treatment. Plants of WT rice (n=17) and T3 rice (n=19) germinated and grown in a graduated cylinder filled with MS gellan gum, of which the side wall and bottom were covered with aluminum foil, for 9 d at 25°C with a light/dark cycle of 16 h/8 h, were transplanted to hydroponic solutions with or without 200 mM NaCl for 2 d. * p<0.05.

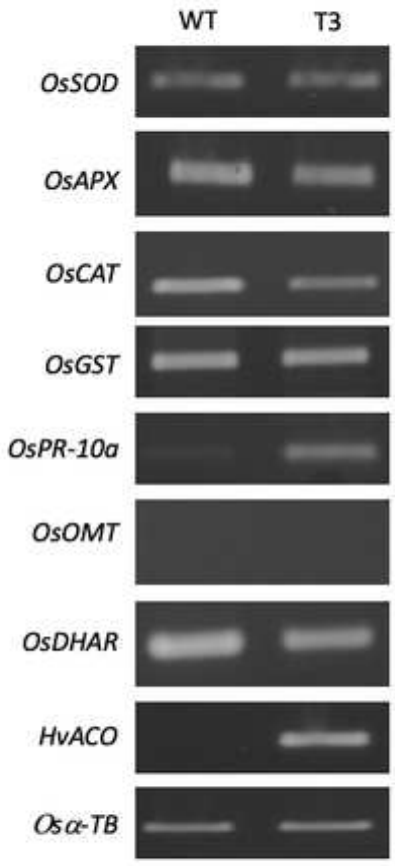

Figure 8: Gene expression of HvACO, ROS-scavenging enzyme, and preferentially produced proteins of salt-tolerant barley in T3 rice and WT rice. Total RNAs isolated from roots of T3 rice and WT rice grown for 9 d were subjected to RT-PCR.

and WT rice. However, the PR-10a gene level in T3 rice was 12 times greater than in WT rice. Actually, PR-10a was studied primarily in the context of attacks by pathogens that are also known to play roles in salt and drought stress tolerance. Proteome analysis demonstrated that roots of Pisum sativum, a salinity-tolerant peanut callus cell line, had significantly elevated concentrations of PR-10 protein [36] as did salt-tolerant barley [21]. Overexpression of potato PR-10a gene in potato suspension cells and PR-10a gene from Jatropha curcas in tobacco exhibited significantly increased salt and osmotic tolerance [37,38]. Most PR proteins are induced through the plant hormones in addition to abiotic stress such as salt and drought and biotic stressors such as pathogens [39]. Although PR-10 protein was enhanced by plant hormones, few reports have described the response of PR-10 to ethylene. The molecular function of PR-10 protein has been suggested as the RNase activity, ligand binding activity, and post-translational modification (phosphorylation). However, its molecular function improving salt tolerance and role in signal transduction under salt stress remains unclear. Taken together, our results show that ethylene enhanced salt tolerance with the induction of PR-10a gene will help in elucidating the potential role and relation among ethylene, PR-10a, and salt tolerance.

Conclusions

ACO gene expressed preferentially in salt-tolerant barley was identified. The protein produced in *E. coli* cells with HvACO gene showed ethylene production from ACC. Transgenic rice expressing HvACO gene showed the decrease of root looping response, the enhancement of root elongation, normal root growth in 200 mM NaCl, and 65% survival, whereas root elongation of WT rice was inhibited with 47% survival. RT-PCR revealed that PR-10a gene level in T3 rice was 12 times greater than in WT rice, but gene levels of ROS-scavenging enzymes were not different between T3 rice and WT rice. These results suggest that ethylene can improve salt tolerance with PR-10a and the potential roles and relations among ethylene, PR-10a, and salt tolerance.

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