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Identification and characterization of a novel water-deficit-suppressed gene *OsARD* encoding an aci-reductone-dioxygenase-like protein in rice

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Abstract

The aci-reductone dioxygenase (ARD) family common to bacteria, plants and animals is involved in the methionine salvage pathway. A water-deficit-suppressed gene, *OsARD* encoding an aci-reductone-dioxygenase-like protein, was identified from rice (*Oryza sativa* L.). Northern blot and reverse transcriptase-polymerase chain reaction (RT-PCR) analysis revealed that the *OsARD* expression is regulated by abiotic stresses and phytohormones. *OsARD* was mainly expressed in roots under flood conditions. It was suppressed by abiotic stresses including water deficit, high salinity and low temperature, and induced by ethylene and gibberellin acid (GA). Our results showed that the genes for *S*-adenosylmethionine (SAM) synthase and 1-aminocyclopropane-1-carboxylic acid (ACC) synthase were upregulated in RNA-interference (RNAi) transgenic rice plants with a significant reduction of *OsARD* expression. Furthermore, the expression of two genes for ethylene signal transduction, *ETR2* and *EIN3*, increased in these RNAi transgenic plants, whereas the expression of *ERF3* was suppressed. These results suggest that *OsARD* may play a role in the metabolism of methionine and ethylene in response to abiotic stresses.

Keywords: Abiotic stress; Aci-reductone dioxygenase (ARD); Methionine salvage pathway; Phytohormone; *Oryza sativa* L

1. Introduction

In response to abiotic stresses, the ethylene and polyamine synthesis of plants are stimulated and result in elevated levels of ethylene and polyamine. These can help plants regulate stress responses, exercise specific tolerance mechanisms and adapt to their environment (Bouchereau et al., 1999; Bleecker and Kende, 2000). *S*-adenosylmethionine (SAM) is an active metabolite involved in many biochemical reactions including the biosynthesis of polyamine and ethylene (Ravanel et al., 1998). Therefore, the metabolism of SAM and its immediate precursor, methionine, has been considered to play important roles in plant stress responses. The enzymatic reactions with SAM in ethylene and polyamine synthesis produce a byproduct, 5′-methylthioadenosine (MTA), that can be recycled to methionine. This methionine salvage pathway is an ubiquitous biochemical pathway that maintains methionine levels, regenerates SAM, and eliminates MTA, thus allowing a high rate of ethylene and polyamine biosynthesis even when the pool of free methionine is small (Schlenk, 1983; Ravanel et al., 1998; Bleecker and Kende, 2000).

The methionine salvage pathway has been described at the biochemical level in plants (Yang and Hoffman, 1984; Miyazaki and Yang, 1987). Recent progress in characterization of the enzymes in this pathway and identification of their corresponding genes is mainly from the work on bacteria (Myers et al., 1993; Wray and Abeles, 1995; Dai et al., 1999, 2001). Two aci-reductone dioxygenases, ARD and ARD′, in the methionine salvage pathway were first identified in *Klebsiella pneumoniae*, that have the same polypeptide sequence but bind

Abbreviations: ABA, abscisic acid; ACC, 1-aminocyclopropane-1-carboxylic acid; ARD, aci-reductone dioxygenase; BA, benzylaminopurine; *EIN3*, ethylene-insensitive 3; *ERF3*, ethylene-responsive element binding factor 3; *ETR2*, ethylene receptor 2; GA, gibberellin acid; MTA, 5′-methylthioadenosine; NAA, naphthaleneacetic acid; oligo, oligodeoxyribonucleotide; *OsACO*, *Oryza sativa* ACC oxidase 1; *OsACS1*, *Oryza sativa* ACC synthase 1; *OsARD*, *Oryza sativa* aci-reductone dioxygenase; *OsSAMS*, *Oryza sativa* SAM synthase; SAM, *S*-adenosyl methionine.

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different metal ions, Ni^{2+} and Fe^{2+}, respectively (Dai et al., 1999, 2001). They react with the same advanced aci-reductone intermediate in the methionine salvage pathway, but yield different products. ARD′ yields the α-keto acid precursor of methionine and forms part of this pathway. ARD yields cytotoxic methylthiopropionate, CO and formate and prevents the recycling of MTA to methionine (Dai et al., 2001). In plants, *IDI1* (Iron Deficiency Induced gene 1) predicting a protein with homology to ARD has been identified from *Hordeum vulgare* (Yamaguchi et al., 2000). However, the studies of the methionine salvage pathway at the molecular level in plants are limited. The members of this pathway and their roles in plant stress responses remain unclear.

Our present study reports a novel water-deficit-suppressed gene in rice, which encodes a protein with homology to aci-reductone dioxygenase (ARD), and is designated *OsARD*. We investigated the expression of the *OsARD* under abiotic stresses and phytohormone treatments, examined the expression of several genes involved in ethylene synthesis and signal transduction in transgenic rice plants, and discussed its roles in methionine and ethylene metabolism and in plant stress responses. Our study of *OsARD* represented the first molecular characterization of a gene in the methionine salvage pathway in rice.

2. Materials and methods

2.1. Plant material and culture

An upland tropical *japonica* rice variety Azucena was used in this study. The plants were grown in a growth chamber under a diurnal photoperiod of 12 h light (~200 μmol m^{-2} s^{-1}) at 22/28 °C (night/day) and 80% relative humidity. For water deficit treatment, a sand growth medium culture experiment was performed as described (Yang et al., 2003). Germinated seeds were directly sown into the sands under flooding conditions for 7 days. Then seedlings were harvested after drainage treatment at 0, 6, 12, 24, 48 and 72 h.

For other stresses, 14-day-old seedlings were grown in rice culture solution (Yoshida et al., 1976) under cold stress at 4 °C for 24 h, or salt stress with 150 mM NaCl for 24 h. Hormonal treatments were performed by adding 10 μM exogenous ethephon (the ethylene-producing compound), 1 μM gibberellin acid (GA_3), 1 μM abscisic acid (ABA), 1 μM auxin (naphthaleneacetic acid NAA) and 1 μM cytokinin (6-benzylaminopurine, 6-BA) to the culture solution for 14-day-old seedlings for 24 h. The root samples were harvested after each treatment.

2.2. Gene cloning and characterization

A differential expression analysis was performed in order to identify genes that respond to water deficit in root tips of upland rice plants (Yang et al., 2003). One expressed sequence tag (EST) (Genbank Accession No. CN487723) represented a gene with homology to aci-reductone dioxygenase (ARD) was obtained and designated *OsARD*. A rice BAC clone OJ1607A12 of *japonica* cultivar (*Oryza sativa* L.) having high sequence similarity with the EST was obtained by the BLAST searching of the rice genome database (http://rgp.dna.affrc.go.jp/). RiceGAAS (http://ricegaas.dna.affrc.go.jp/) annotation programme was used to predict the open reading frame of *OsARD* in the BAC. The gene specific primers 5′-TCTTCTACACCTTCCAGGCTATCCG-3′ and 5′-TGATATGCCTTTAACGAGCTTCGACAG-3′ were designed from the prediction. The cDNA of *OsARD* was cloned using reverse transcriptase-polymerase chain reaction (RT-PCR) with these primers from a rice root cDNA library constructed in previous work (Yang et al., 2003). PCR conditions were 94 °C for 5 min, followed by 30 cycles, 94 °C for 30 s, 58 °C for 30 s, 72 °C for 1 min plus 30 s, and a final extension period at 72 °C for 7 min. The PCR product was cloned in pUCm-T vector and sequenced. Homology searches were performed with the GenBank/EMBL database using BLAST program. An alignment and a phylogenetic tree analysis were performed using the Clustal X implementation of Clustal W and analyzed by Genedoc and Treeview program.

2.3. Construction of overexpression and RNA-interference vectors and the development of transgenic plants

For the overexpression construct, the *OsARD* cDNA clone with the complete CDS was digested with *Pst*I, and subcloned into the corresponding site of the modified pCAMBIA-1301 vector between the cauliflower mosaic virus (CaMV) 35S promoter (inserted by *Eco*RI/*Bam*HI sides) and a Nos Poly-A (inserted by *Pst*I/*Hind*III sides).

A DNA fragment of 196 bp of 5′-terminal regions of *OsARD* cDNA was obtained by digesting *OsARD* cDNA with restriction enzyme *Eco*RI to make the RNA-interference (RNAi) construct. The forward fragment, the second intron of *NIR1* in Maize, and reverse fragment were individually cloned into the modified pCAMBIA-1301 vector. *Agrobacterium tumefaciens* stain EHA105 harboring these constructs was used to transform the rice cultivar Azucena as described (Chen et al., 2003).

2.4. DNA and RNA gel blot analysis

Genomic DNA was isolated from young leaves using the cetyl trimethyl ammonium bromide method and total RNA was extracted from the roots, leaves and stems of plants using the Trizol reagent according to the procedure recommended by the manufacturer (Invitrogen, California, USA). Genomic DNA (10 μg) were digested with restriction enzymes *Apa*I, *Dra*1, *Eco*RV and *Xba*I, and separated on 0.8% agarose gel. Total RNA (20 μg) were separated on 1.0% agarose gel denatured with formaldehyde. After electrophoresis, the digested DNA and total RNA were blotted onto a Hybond-N^+ nylon membrane (Amersham Pharmacia Biotech, New Jersey, USA). ^{32}P-dCTP-labeled cDNA was used as a probe. The blots were hybridized and washed at 65 °C under stringent conditions. After washing, the blots were analyzed using a Typhoon-8600 (Amersham Pharmacia Biotech, New Jersey, USA).