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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).

Table 1
Primer pairs for semiquantitative PCR amplification

Gene	Primer pairs[a]	Fragment size[b]
OsARD	F, 5′-TCTTCTACACCTTCCAGGCTATCCG; R, 5′-TGATATGCCTTTAACGAGCTTCGACAG	700
OsSAMS	F, 5′-CGCACTTGATACCTTCCTCTTTACC; R, 5′-GGGGTCTCATCAGTTGCATATCC	408
OsACS1	F, 5′-GAATTCGATGGTGAGCCAAGT; R, 5′-AGCGCGTGGGGGTTCTTCT	322
OsACO1	F, 5′-GTCCATGGAAACCGAGACCT; R, 5′-GAGCTCGTCGCGAGTAGTAA	229
ETR2	F, 5′-TAGCGAGAGCGGGAGCGAGATGAT; R, 5′-TGTGATAGGGCGACTGCGACTTGA	375
EIN3	F, 5′-CACAATGTCCAGTGCCCGCATAG; R, 5′-CTCTCCATGATCGTGGCATTGTC	299
ERF3	F, 5′-GGCGTCGGCGGAGGTGTCG; R, 5′-CGGCGGTGCAAGCTTCATCATACG	504
Actin	F, 5′-GGAACTGGTATGGTCAAGGC; R, 5′-AGTCTCATGGATACCCGCAG	775

[a] F, forward primer; R, reverse primer.
[b] Numbers indicate the size (in bp) of amplified fragments.

2.5. Semiquantitative RT-PCR analysis

We used the Trizol reagent (Invitrogen, California, USA) to isolate total RNA from the root tissues. For the first-strand cDNA synthesis, 3 ìg of total RNA from roots of plants was reverse-transcribed in a volume of 20 µl containing 50 ng oligo (dT) 18 primer, 2.5 mM dNTPs and 200 units of M-MLV (*moloney murine leukemia virus*) reverse transcriptase (Promega, Wisconsin, USA) in a reaction buffer. PCR amplifications were performed on first cDNA strand using five specific primer sets (Table 1). Primers of *Actin* (X16280) (Table 1) from rice were used as control. The reaction included an initial 5 min denaturation at 94 °C, followed by 28–34 cycles of PCR (94 °C, 30 s; 58 °C, 30 s and 72 °C, 1 min), and a final extension period at 72 °C for 7 min. PCR products were separated on 1% agarose gels, and stained with ethidium bromide. Each RT-PCR was repeated three times.

3. Results

3.1. Cloning and characterization of OsARD gene

The cDNA of *OsARD* was cloned by RT-PCR with the gene specific primers from a rice root cDNA library constructed in previous work (Yang et al., 2003). *OsARD* (Genbank Accession No. AY955841) contains a coding domain sequence (CDS) of 597 bp (Fig. 1). The CDS predicts a polypeptide of 198 amino acids with a calculated molecular mass of 23.5 kDa, and a putative transit peptide of 19 amino acids predicted by ChloroP 1.1 Server (http://www.cbs.dtu.dk/services/ChloroP) (Fig. 1). The genomic DNA sequence of the *OsARD* gene expands 2171 bp from the initial condon ATG to the terminal codon TAA and comprises seven exons interrupted by six introns (Fig. 1). The exons range from 19 to 127 bp, while the size of introns is in the range of 73–941 bp.

The deduced amino acid sequences of *OsARD* is substantially homologous to some hypothetical proteins containing an ARD domain from different organisms (Fig. 2A), including *Hordeum vulgare* (Genbank Accession No. BAB61039), *Arabidopsis*

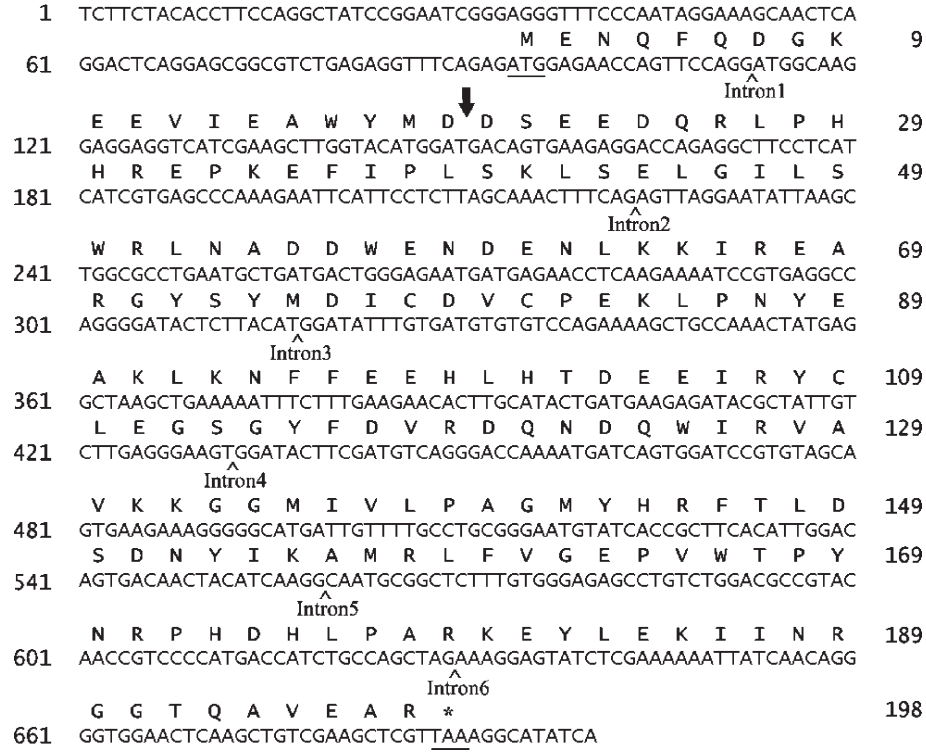

Fig. 1. Nucleotide and deduced amino acid sequences of *OsARD* (Genbank Accession No. AY955841). The transcription start (ATG/M) and stop (TAA/*) codons are underlined. The positions of the six introns presented in the genomic sequence are indicated. Arrow indicates the cleavage sites of putative transit peptides.

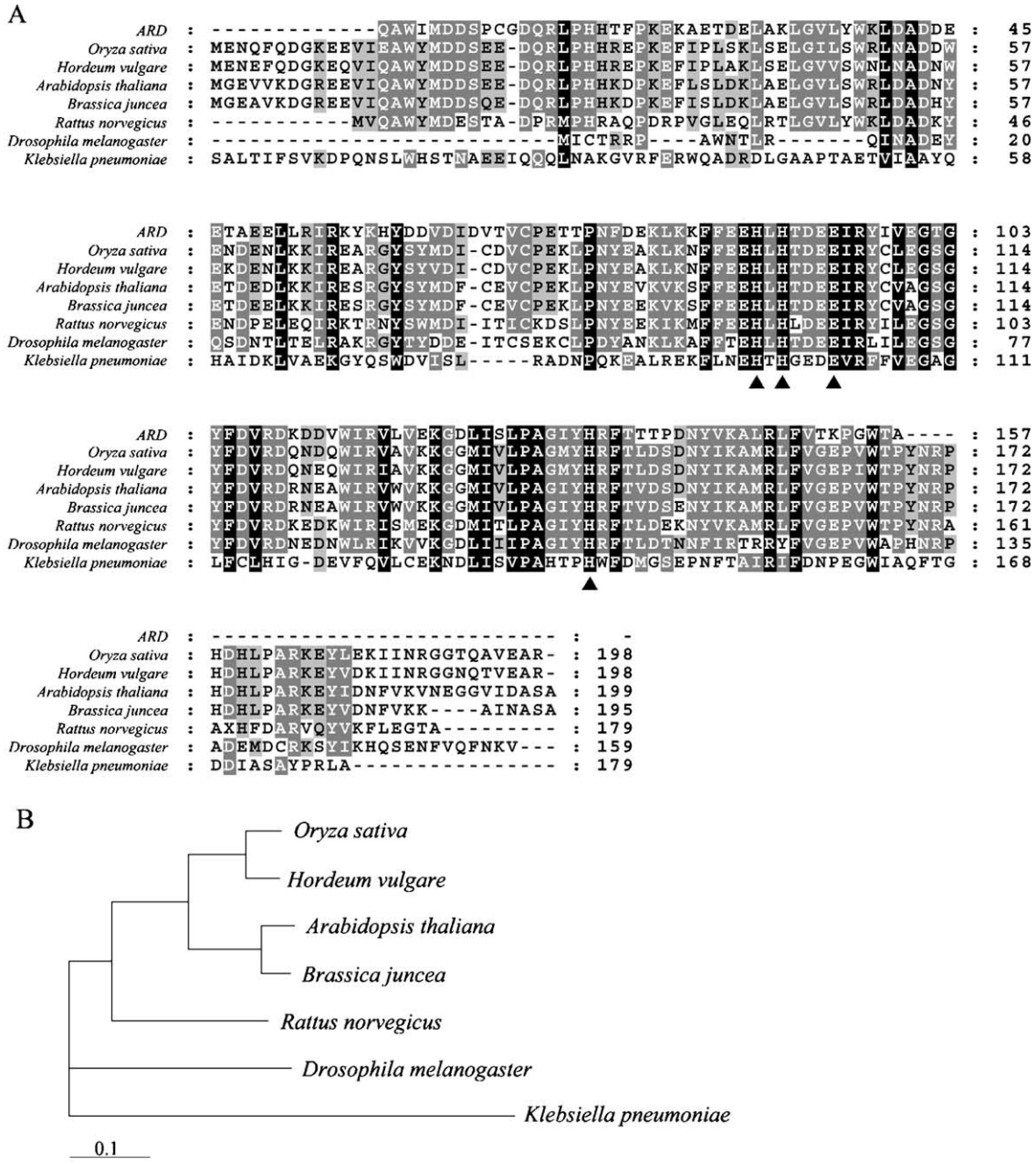

Fig. 2. Sequence comparison and phylogenetic analysis of ARD/ARD′ proteins from seven organisms including *Oryza sativa*, *Hordeum vulgare* (BAB61039), *Arabidopsis thaliana* (AAM63805), *Brassica juncea* (AAR03591), *Rattus norvegicus* (AL024210), *Drosophila melanogaster* (CG32068-PA) and *Klebsiella pneumoniae* (AAD11793). (A) The amino acid sequence alignments of ARD/ARD′ proteins and the ARD domain. Residues conserved in all eight sequences are shown on black background. Residues conserved in at least five out of seven sequences are shaded in gray. The three histidines and one glutamic acid, possibly involved in the binding of metal ions, are underlined with triangles. (B) Phylogenetic tree of the sequences shown in (A) indicates the relationship of the proteins of the ARD/ARD′ family. The alignment and the proposed rectangular cladogram was generated using Clustal X implementation of Clustal W, and analyzed by Genedoc and Treeview.

thaliana (Genbank Accession No. AAM63805), *Brassica juncea* (Genbank Accession No. AAR03591), *Rattus norvegicus* (Genbank Accession No. AL024210), *Drosophila melanogaster* (Genbank Accession No. CG32068-PA) and *Klebsiella pneumoniae* (Genbank Accession No. AAD11793). Three histidine residues and one glutamic acid residues, marked with triangles, are conserved among all the members of the ARD family (Fig. 2A). These amino acids are potential sites for metal ion ligation (Pochapsky et al., 2002). Phylogenetic analysis of ARD homologous proteins from these seven organisms indicated that *OsARD* was closer to the barley *ARD* gene, and the evolutionary relationship between plant and animal was closer than that between plant and hexapod or bacteria (Fig. 2B).

Southern blot analysis was performed to determine the copy number of the *OsARD* gene in the rice genome. The presence of a distinct single band was detected using the enzymes of *Apa*I, *Dra*1, *Eco*RV and *Xba*I, suggesting that *OsARD* should be a single copy gene in the rice genome (Figs. 3 and 4).

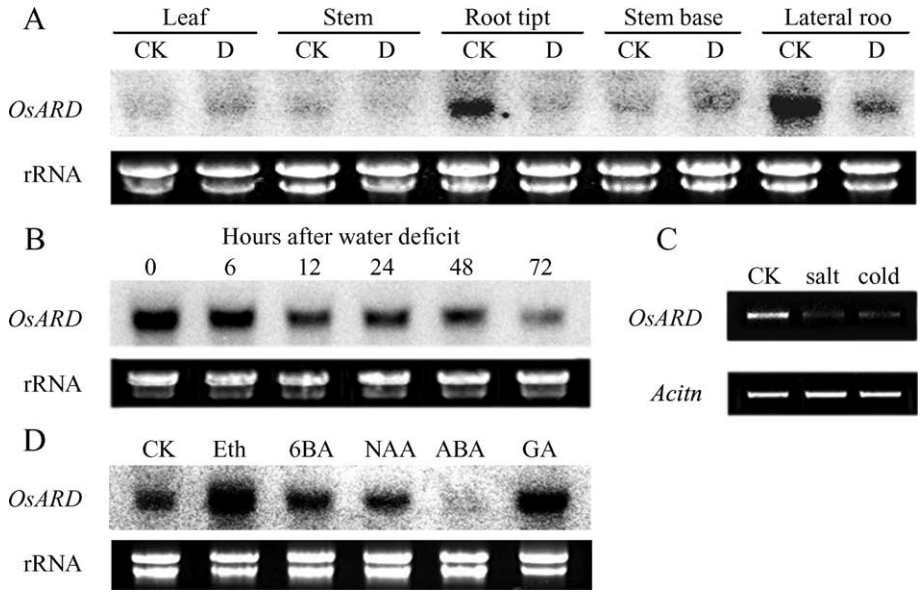

Fig. 3. *OsARD* expression patterns during abiotic stresses and hormonal treatments. (A) Northern blot analysis of *OsARD* during water deficit in different tissues of 7-day-old rice seedling harvested at 0 h (CK) and 72 h (D) after treatment. Total RNA (20 μg) isolated from different tissues was hybridized with α^{32}P labeled cDNA of *OsARD*. An ethidium bromide-stained gel is shown at the bottom. (B) Northern blot analysis of time course of *OsARD* expression during water deficit. Seven-day-old rice seedling roots were harvested at 0, 6, 12, 24, 48 and 72 h after water deficit treatment. Total RNA (20 μg) isolated from seedling roots was hybridized with α^{32}P labeled cDNA of *OsARD*. An ethidium bromide-stained gel is shown at the bottom. (C) RT-PCR analysis of *OsARD* expression under cold and salt stresses. Fourteen-day-old seedlings were grown in culture solution under cold stress at 4 °C for 24 h, or salt stress with 150 mM NaCl for 24 h. Seedling roots were harvested after treatments. The lower panel shows loading control of *Actin* transcripts in each sample. (D) Northern blot analysis of *OsARD* in response to phytohormonal treatments in rice seedling roots. Exogenous 10 μM ethephon (Eth), 1 μM 6-BA, 1 μM NAA, 1 μM ABA, and 1 μM GA were added into the culture solution for 14-day-old seedlings grown for 24 h. Seedling roots were harvested after treatments. Total RNA (20 μg) isolated from seedling roots was hybridized with α^{32}P labeled cDNA of *OsARD*. An ethidium bromide-stained gel is shown at the bottom.

3.2. Expression pattern of OsARD under different stresses and hormonal treatments

Northern blot analysis, using the cDNA of *OsARD* as a probe, was performed to investigate the responses of the *OsARD* gene to water deficit in different rice tissues. *OsARD* was highly expressed under flood conditions in the root tips and lateral roots of 7-day-old seedlings. Water deficit for 3 days remarkably suppressed the gene expression. In contrast, the water deficit induced a low level of the gene expression in the leaf and stem base (Fig. 3A). Northern blot analysis also revealed the time dependent process of *OsARD* accumulation in rice roots during water deficit. *OsARD* mRNA quickly decreased with time, and reached a low level at 72 h of water deficit (Fig. 3B). Semiquantitative RT-PCR analysis was used to examine the effects of salt and cold stresses on *OsARD* expression in rice roots. Salt and cold stresses also suppressed the gene expression as in the case of water deficit (Fig. 3C).

The effects of hormonal treatments on *OsARD* gene expression in rice roots were also examined by Northern blot analysis. Ethephon and GA treatments induced *OsARD* expression, while ABA treatment suppressed the expression (Fig. 3D). NAA and 6-BA treatments showed no visible effects (Fig. 3D).

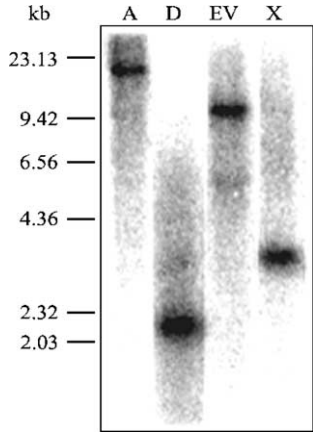

Fig. 4. Southern blot analysis of rice genomic DNA. DNA was digested using the indicated restriction enzymes: *A*, *Apa*I; *D*, *Dra*I; *EV*, *Eco*RV and *X*, *Xba*I. The blot was hybridized with α^{32}P labeled cDNA of *OsARD*. The positions of *Hind*III λ-DNA size markers are indicated on left.

3.3. OsARD is involved in ethylene biosynthesis

To assess the possible function of *OsARD*, the RNAi and overexpression constructs of *OsARD* were used to transform the rice variety Azucena. Three independent RNAi transgenic lines (designated RNAi1, RNAi2 and RNAi3), with significant reduction of the *OsARD* expression, and three independent overexpression transgenic lines (designated 35S1, 35S2 and 35S3), with distinct enhancement of the *OsARD*, expression were obtained (Fig. 5). Since *OsARD* was mainly expressed in roots under flood conditions and suppressed by water deficit (Fig. 3A), we investigated the expression of three genes

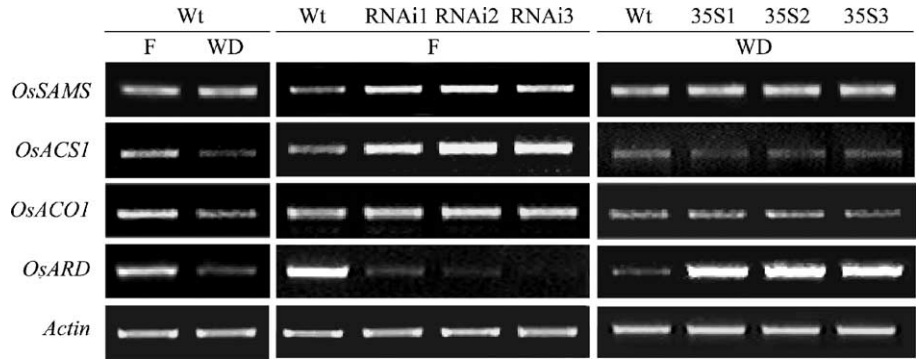

Fig. 5. RT-PCR analysis of three genes involved in SAM and ethylene synthesis in the wild-type and transgenic plants. Seven-day-old seedling roots of the wild-type (Wt), three independent RNAi transgenic lines (RNAi1, RNAi2 and RNAi3) and three independent overexpression transgenic lines (35S1, 35S2 and 35S3) were harvested at 0 h (Flooding, F) and 72 h (Water Deficit, WD) after water deficit treatment. Expression of three genes, *OsSAMS*, *OsACS1* and *OsACO1*, are shown. The lower panel shows loading control of *Actin* transcripts in each sample.

involved in ethylene biosynthesis in RNAi transgenic plants under flood conditions and in overexpression transgenic plants under water deficit. Compared with the wild-type plants, the transcript levels of both *OsSAMS* (*Oryza sativa* SAM synthase, Genbank Accession No. Z26867) and *OsACS1* (*Oryza sativa* ACC synthase 1, Genbank Accession No. M96673) were increased in the RNAi transgenic plants, while the expression of *OsACO1* (*Oryza sativa* ACC oxidase 1, Genbank Accession No. X85747) had no remarkable change (Fig. 5). However, the overexpression of *OsARD* had no significant effects on the expression of these genes (Fig. 5).

3.4. OsARD is invovled in ethylene signal transduction

To assess the effects of RNAi and overexpression of *OsARD* on ethylene signal transduction pathway, we measured the expression of three rice homologs of ethylene signal transducers including *ETR2* (ethylene receptor 2, AY136816), *EIN3* (ethylene-insensitive 3, AB074971) and *ERF3* (ethylene-responsive element binding factor 3, AB036883) in RNAi transgenic plants under flood conditions and in overexpression transgenic plants under water deficit. Compared with the wild-type plants, the transcript levels of *ETR2* and *EIN3* increased, whereas the transcript level of *ERF3* decreased in RNAi transgenic lines (Fig. 6). The expression of these genes in the wild-type plants under water deficit was similar to those in RNAi transgenic plants under flood conditions (Fig. 6). The overexpression of *OsARD* had no significant effects on the expression of these genes with the exception of *ERF3*, which was suppressed more seriously in overexpression transgenic plants than in the wild-type plants (Fig. 6).

4. Discussion

Two aci-reductone dioxygenase (ARD and ARD′) have been identified from *K. pneumoniae* that catalyze different oxidative decomposition reactions of an advanced aci-reductone intermediate in the methionine salvage pathway (Dai et al., 2001). Although ARD and ARD′ are encoded by the same gene, they bind different metal ions and are involved in two different biochemical pathways: the methionine salvage pathway (ARD′) and an offpathway with unknown function (ARD). A novel water-dificit-suppressed gene encoding an ARD-like protein was cloned from rice in this study, and designated *OsARD*. The putative polypeptide of *OsARD* shows high homology to the ARD domain and contains three histidine

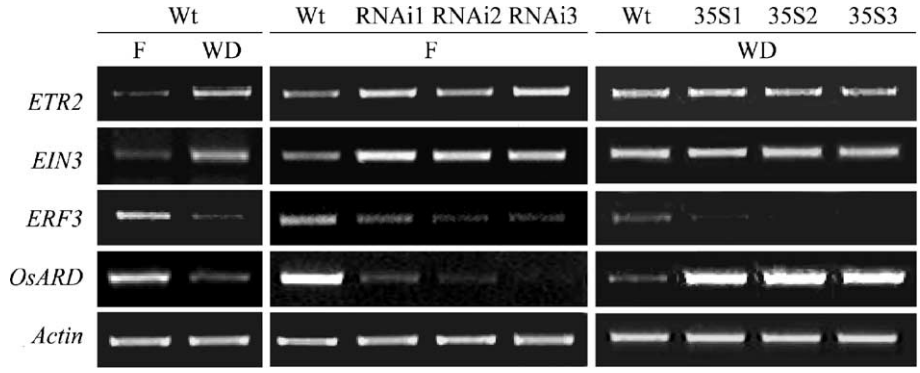

Fig. 6. RT-PCR analysis of three genes involved in ethylene signal transduction in the wild-type and transgenic plants. Seven-day-old seedling roots of the wild-type (Wt), three independent RNAi transgenic lines (RNAi1, RNAi2 and RNAi3) and three independent overexpression transgenic lines (35S1, 35S2 and 35S3) were harvested at 0 h (Flooding, F) and 72 h (Water Deficit, WD) after water deficit treatment. Expression of three genes, *ETR2*, *EIN3* and *ERF3*, are shown. The lower panel shows loading control of *Actin* transcripts in each sample.

residues and one glutamic acid residue implicated in metal ion ligation, as in all other members of the ARD family (Fig. 2A) (Pochapsky et al., 2002).

During submergence, ethylene accumulates rapidly in deepwater rice to induce a reduction in ABA level and an increase in GA level. Both changes together result in the internodal elongation induced by the activity of GA (Kende et al., 1998). Ethylene not only mediates internodal elongation, but also stimulates some additional adaptations to hypoxia, such as programmed cell death during root aerenchyma formation (Drew et al., 2000), the growth of adventitious roots at the nodes of the stem (Lorbiecke and Sauter, 1999) and adventitious root emergence (Mergemann and Sauter, 2000). Ethylene synthesis in submerged roots enhances the requirement for methionine, whereas the concentration of methionine is extremely low in plant roots (Ma et al., 1995). Based on the observation that *OsARD* is mainly expressed in roots under flood conditions, is induced by ethylene and GA, and is suppressed by water deficit (Fig. 3), we proposes that the *OsARD* may act as an ARD′ enzyme to accelerate the recycling of methionine from MTA through the methionine salvage pathway, and then to promote ethylene synthesis.

ABA is a key regulator during drought, salt and cold stresses, and its level increases to promote stress tolerance under such abiotic stress conditions (Thomashow, 1999; Finkelstein et al., 2002; Xiong et al., 2002; Zhu, 2002). It has been demonstrated that ethylene synthesis is restricted by ABA under water deficit to maintain the shoot and root growth (Sharp, 2002). In our study, the expression of *OsARD* was downregulated by exogenous ABA (Fig. 3D). It may suggest that *OsARD* is involved in the interaction between ABA and ethylene. Ethylene synthesis is known to be induced during salt and cold stresses (Morgan and Drew, 1997; Bleecker and Kende, 2000). In our study, however, the *OsARD* expression was suppressed by these stresses (Fig. 3C). Since ABA represses the expression of *OsARD*, and accumulates during salt and cold stresses, the decrease of *OsARD* expression under these stresses may be attributed to the effect of ABA. The expression of *OsARD* in response to abiotic stresses and phytohormones suggests that the methionine salvage pathway is regulated by phytohormones in plants in order to adjust the methionine metabolism to increase tolerance to abiotic stresses.

The rate-limiting step for ethylene synthesis is the conversion of SAM to ACC by ACC synthase (Wang et al., 2002). The formation of SAM from methionine and ATP is catalyzed by SAM synthase. Our study revealed that transcript levels of *OsSAMS* and *OsACS1* increased in RNAi transgenic plants with reduction of *OsARD* expression (Fig. 5). This may suggest that these genes are upregulated to maintain the levels of SAM and ethylene since the methionine salvage pathway is likely to be interrupted due to the reduction of *OsARD* expression in RNAi transgenic plants. Reduction of *OsARD* in RNAi transgenic plants also affected the expression of some components of the ethylene signal transduction pathway in a similar way to the effects caused by water deficit in the wild-type plants (Fig. 6). However the RNAi of *OsARD* did not cause any detectable change in the growth and development of rice in either normal or water deficit conditions. Thus we propose that plants may accelerate the methionine synthesis from cysteine to maintain the metabolic balance of methionine within the RNAi of transgenic plants with suppression of *OsARD* expression. It is also possible that some unknown bypathways might exist in the plant, which could recycle the methionine from MTA. Overexpression of *OsARD* had no significant effects on the expression of those genes involved in ethylene synthesis and signal transduction (Figs. 5 and 6), and also did not cause a detectable change in growth and development of rice in either normal or water deficit conditions. This implies that the activation of only one part of the methionine salvage pathway, such as the overexpression of *OsARD*, could not accelerate the whole pathway to affect the balance of the methionine metabolism.

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