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OsEIN2 is a Positive Component in Ethylene Signaling in Rice

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EIN2 is a central signal transducer in the ethylene-signaling pathway, and a unique membrane-anchored protein. By screening a cDNA library, we have isolated a cDNA clone (*OsEIN2*) that encodes the rice EIN2 homolog. The full-length ORF clone was obtained by reverse transcriptase-polymerase chain reaction. *OsEIN2* shares significant amino acid sequence similarity with *Arabidopsis EIN2* (57% similarity and 42% identity). Both the numbers and positions of introns and exons in the *OsEIN2* and *AtEIN2* coding regions are also conserved. To address whether this structural similarity is indicative of functional conservation of the corresponding proteins, we also generated transgenic lines expressing the antisense construct of *OsEIN2*. Those plants were stunted and shoot elongation was severely inhibited. Their phenotypes were similar to that found with wild-type rice seedlings that were treated with $AgNO_3$, an ethylene signal inhibitor. In the *OsEIN2* antisense plants, the expression levels of two ethylene-responsive genes, *SC129* and *SC255*, were decreased compared with the wild types. These results suggest that OsEIN2 is a positive component of the ethylene-signaling pathway in rice, just as AtEIN2 is in *Arabidopsis*. Our antisense transgenic plants produced approximately 3.5 times more ethylene than the wild-type plants. Expression analysis of rice *ACS* and *ACO* genes showed that the transcript levels of *OsACS1* and *OsACO1* were elevated in the transgenic plants.

Keywords: Antisense — EIN2 — Ethylene — Rice — Seedling.

Abbreviations: DAI, days after imbibition; EIN2, ETHYLENE INSENSITIVE2; RT-PCR, reverse transcription-PCR.

The nucleotide sequences reported in this paper have been submitted to the GenBank under accession numbers AY396568 (*OsEIN2*), AK104680 (*SC129*), AK061882 (*SC255*).

Introduction

The plant hormone ethylene has profound effects on plant growth and development, including the induction of ripening in climacteric fruits, leaf expansion, promotion of seed germination, promotion or inhibition of flowering, abscission of various organs, and senescence (Abeles et al. 1992). Ethylene production is tightly regulated by internal signals during development and in response to environmental stimuli from biotic (e.g., pathogen attack) and abiotic stresses, such as wounding, hypoxia, ozone, chilling, or freezing (Kende 1993, Johnson and Ecker 1998).

Components of the ethylene signal-transduction pathway have been identified by characterizing ethylene-response mutants in *Arabidopsis thaliana* (for review, see Kieber 1997, Chang and Shockey 1999, Bleecker and Kende 2000, Stepanova and Ecker 2000). Ethylene perception is mediated by a family of membrane receptors, *ETR1*, *ERS1*, *ETR2*, *EIN4*, and *ERS2* (Chang et al. 1993, Hua et al. 1995, Hua et al. 1998, Sakai et al. 1998). Ethylene binds to the receptors via a copper cofactor (Rodriguez et al. 1999, Hirayama et al. 1999) and genetic studies suggest that hormone binding changes the status of receptors to activate ethylene signaling. A recent study using *ers1*;*etr1* loss-of-function double mutants showed that subfamily I members (ETR1 and ERS1) play a unique role in ethylene signal transmission and canonical histidine kinase activity is not essential for the function of the receptors (Wang et al. 2003). In the absence of ethylene, a Raf-like serine/threonine kinase, CTR1, a negative regulator of the pathway is active (Kieber et al. 1993) and the ethylene signaling is blocked. But the mechanism of interaction between ethylene receptors and CTR1 is under investigation (Wang et al. 2003). Biochemical, reverse genetic, and gene expression studies have demonstrated the involvement of a MAPK pathway in ethylene signaling in plants (Ouaked et al. 2003). EIN2, EIN3, EIN5, and EIN6 are predicted to be positive regulators of the ethylene response (Roman et al. 1995). It is not yet known how the ethylene signal is transduced via these molecules at the biochemical level. The product of the *EIN2* gene, which is related to the eukaryotic Nramp family of 12 membrane-spanning, metal ion-transporters (Alonso et al. 1999), is required for ethylene signaling. It acts genetically between *CTR1* and the *EIN3* family of transcriptional regulators (Chao et al. 1997). EIN5 and EIN6, which are proteins of unknown sequence and function, are currently under investigation. The nuclear protein EIN3 and its paralogs, the EIN3-like proteins, are transcription factors that bind to the promoters of ethylene-response genes, e.g., *ETHYLENE RESPONSE FACTOR1* (*ERF1*). They then initiate a transcriptional cascade leading to the regulation of ethylene target genes (Chao et al. 1997, Solano et al. 1998).

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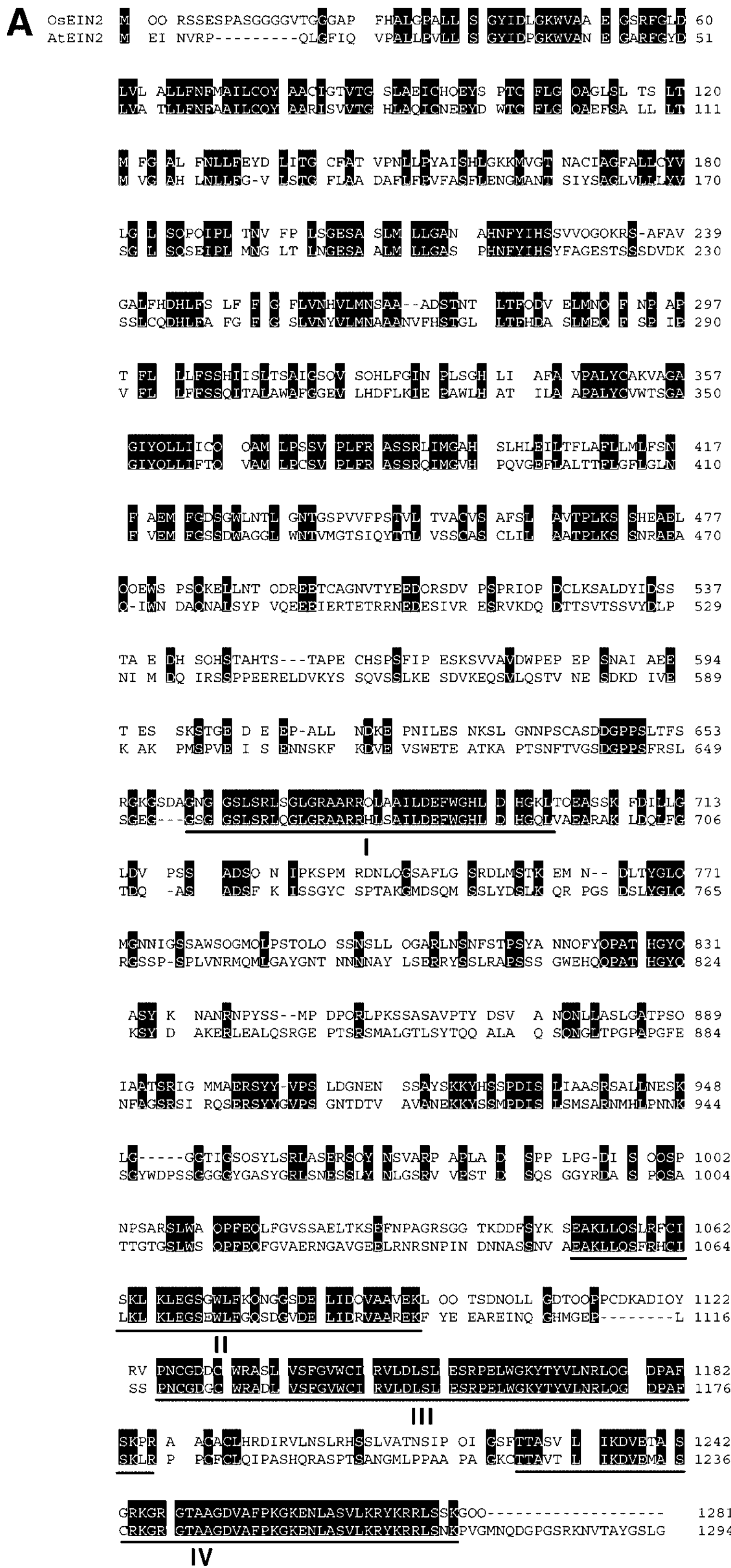

Fig. 1 Sequence analysis of *OsEIN2*. (A) Predicted amino acid sequence alignments of *OsEIN2* (AY396568) and *Arabidopsis EIN2* (AF141203). Identities and similarities between amino acid sequences of *OsEIN2* and *AtEIN2* are indicated by black and gray boxes, respectively. Four sequence island regions (OsEIN2 Gly-661 to Leu-699, Glu-1051 to Lys-1094, Pro-1127 to Arg-1186, and Thr-1225 to Lys-1278) that are highly conserved between OsEIN2 and AtEIN2 proteins in the COOH-terminal are indicated by roman numerals. (B) Comparison of the genomic structure of rice *OsEIN2* (5024.t00011 in TIGR) and the *Arabidopsis AtEIN2* gene (AF141202). Black portions represent the exons; white portions, the introns; and gray portions, the untranslated region. Positions of the exons and introns of *OsEIN2* were confirmed by comparing the cDNA with its genomic DNA sequences in the rice genome sequence database. Lines indicate that each intron of the *OsEIN2* gene corresponds to its counterpart in the *AtEIN2* gene.

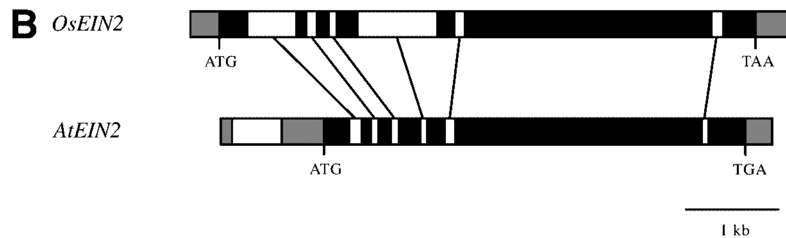

Fig. 1 (B)

The physiological responses of rice to ethylene have long been studied, such as submergence-induced shoot elongation and adventitious root formation (Kende et al. 1998, Vriezen et al. 2003, Mergemann and Sauter 2000). Deep-water rice plants respond to submergence by sensing the altered composition of their internal gas atmosphere. Lowered O_2 and increased CO_2 tensions promote the synthesis of ethylene and also enhance its growth-promoting effect (Raskin and Kende 1984a). Ethylene induces the elongation of deep-water rice internodes by increasing their responsiveness to GA (Raskin and Kende 1984b). Similarly, low-oxygen stress activates the ethylene-signaling pathway and induces epidermal cell death at the site of adventitious root emergence (Mergemann and Sauter 2000) in deep-water rice plants.

However, far less research has been done on identifying the mechanism for the ethylene-signaling pathway and its mode of action in rice plants. Here, we report the isolation of a cDNA encoding the putative rice EIN2 protein and analyses of its molecular properties and function in rice.

Results

Isolation of OsEIN2 and sequence analysis

During our EST analysis, the clone SC636 showed similarity to the C-terminal region of *Arabidopsis* EIN2 (AtEIN2). Because it was a partial clone (638 bp), a rice cDNA library was screened with SC636 as a probe, so that we obtained a longer cDNA (2,836 bp). Using RT-PCR and 5′-end RACE, we isolated a cDNA containing a full-length ORF, and designated this gene *OsEIN2* (*Oryza sativa EIN2*). The *OsEIN2* cDNA (AY396568) is 4,646 bp long and carries an ORF that putatively encodes a polypeptide of 1,281 amino acids with a predicted molecular mass of 138 kDa. This deduced polypeptide shows 42% amino acid identity with AtEIN2 over the entire sequence (Fig. 1A). In comparison, *AtEIN2* encodes a polypeptide of 1,294 amino acids with a molecular mass of 141 kDa and a dimorphic structure (Alonso et al. 1999). The NH_2-terminal 461 amino acids of AtEIN2 contain regions of extreme hydrophobicity. Computer analysis predicts 12 membrane-spanning domains in the N-terminal half. This domain shows similarity to the Nramp family of proteins and may be involved in the regulation of the AtEIN2 protein. The similarity between the amino acid sequences of OsEIN2 and AtEIN2 in the NH2-terminal domain (63% similarity and 48% identity) is higher than for the entire sequence (57% similarity and 42% identity). In contrast, the COOH-terminal 833 amino acids of AtEIN2 are predominately hydrophilic, and show no sequence similarity with any other proteins in the databases (Alonso et al. 1999). The COOH-terminal domain is thought to be essential for signal transduction to downstream. The similarity between the amino acid sequences of OsEIN2 and AtEIN2 in the COOH-terminal domain (55% similarity and 39% identity) is lower than for the entire sequence (57% similarity and 42% identity).

The structure of the AtEIN2 protein is reminiscent that of the glucose sensors in budding yeast (Coons et al. 1997, Ozcan et al. 1998, Hirayama and Alonso 2000). The C-terminal end of the glucose sensor was also known to function as a signal transmitter by interacting with downstream components (Schmidt et al. 1999). Four sequence island regions (OsEIN2 Gly-661 to Leu-699, Glu-1051 to Lys-1094, Pro-1127 to Arg-1186, and Thr-1225 to Lys-1278) are highly conserved between the OsEIN2 and AtEIN2 proteins in the COOH-terminal (I, II, III, and IV in Fig. 1A). Some of these domains may be involved in protein–protein interactions with downstream components for ethylene signal transduction.

To identify the genomic sequences of *OsEIN2*, we searched both the Beijing Genomics Institute database (Yu et al. 2002) and The Institute for Genome Research rice genome sequences. A comparison of the *OsEIN2* cDNA and the genomic sequences revealed that the coding region of *OsEIN2* is interrupted by six introns. This genomic structure is identical to that of *AtEIN2* (Alonso et al. 1999), with the position and size of the exons being perfectly conserved between species (Fig. 1B). Taken together, these structural conservations between *OsEIN2* and *AtEIN2* in their predicted amino acid sequences and genomic organizations suggest that OsEIN2 plays a role in the ethylene-signaling pathway in rice plants.

RT-PCR analysis of OsEIN2 transcript accumulation

RT-PCR analysis of the spatial expression of *OsEIN2* indicated that its transcripts were present in all organs tested (Fig. 2A). The expression level was higher in leaf sheaths than in leaf blades and roots. *OsEIN2* transcript was weakly present from the first day after imbibition (1 DAI), and steady expression of the gene was observed from 2 DAI embryos to 5 DAI seedlings (Fig. 2B).

It has been reported that the expression of genes involved in the ethylene signal-transduction pathway is often affected by ethylene (Leclercq et al. 2002). To investigate this possible

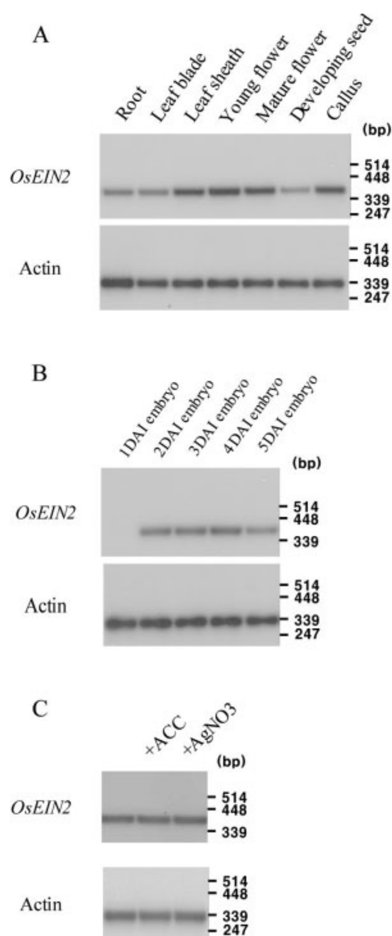

Fig. 2 RT-PCR analysis of the *OsEIN2* transcript accumulation. (A) Transcript levels of *OsEIN2* in roots, leaf blades, leaf sheaths, flowers, developing seeds, and calli. (B) Expression of *OsEIN2* during seed germination. (C) Effect of ACC or $AgNO_3$ on expression of *OsEIN2*. Six-day-old seedlings treated with ACC or $AgNO_3$ were used for RNA isolation. In each experiment, the internal reference *actin* transcript was co-amplified. Amplification of the *actin* genomic DNA could be distinguished from the cDNA because the amplified region contains an intron. Results are representative of three separate experiments.

relationship with *OsEIN2*, we extracted RNA from rice seedlings germinated under constant light on an MS/2 medium containing no additional chemical, 20 μM ACC or 10 μM $AgNO_3$. Our results showed that *OsEIN2* expression is not affected by exogenous application of either ACC or $AgNO_3$ (Fig. 2C).

The OsEIN2 antisense phenotype

To address the functional significance of *OsEIN2* and explore its physiological role, we inserted the gene in the antisense orientation under the ubiquitine promoter. This construct was then placed in the binary Ti plasmid vector. The chimeric molecule was transferred into rice via the *Agrobacterium*-mediated transformation procedure. Two independent lines, Line 471 and Line 456, which displayed strong antisense expression and considerable down regulation of the endogenous *OsEIN2* transcript, were selected for further molecular and physiological characterizations.

Fig. 3 Morphology of the 10-DAI seedlings expressing the *OsEIN2* antisense transcript. Seeds were germinated and grown on MS/2 media at 25°C under continuous light. AS1, antisense plant from Line 471; AS2, antisense plant from Line 456. Scale bar = 1 cm.

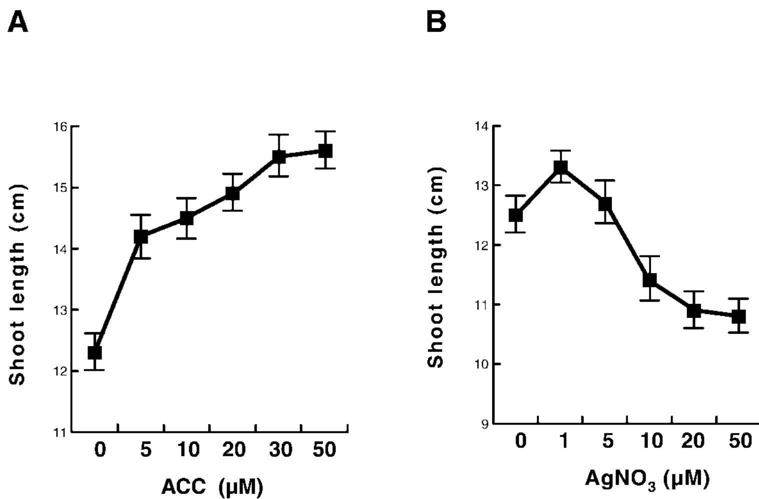

Fig. 4 Shoot elongation of wild-type seedlings in response to ACC (A) and $AgNO_3$ (B). Rice seedlings were grown from seed on MS/2 media at 25°C for 7 d in the light. Data are averages from 35 to 40 seedlings.

Fig. 3 shows the phenotype of the *OsEIN2* antisense plant at 10 DAI. The transgenic plants were stunted compared with the wild types, and shoot elongation was severely inhibited. Smalle et al. (1997) and Alonso et al. (1999) have suggested that ethylene stimulates the elongation of *Arabidopsis* hypocotyls under light. The action of ethylene in submergence-induced shoot elongation has also been long studied in deep-water rice (Kende et al. 1998). Thus, the phenotypes of our antisense *OsEIN2* plants were consistent with that expected from the hypothesis that OsEIN2 is a positive signal component in ethylene signaling, and that inhibited gene expression reduces the ethylene response.

Ethylene promotes shoot elongation in rice seedlings

To examine whether the stunted phenotype was due to reduced ability of ethylene signaling, we examined the role of ethylene in wild-type seedlings. Here, the addition of the ethylene precursor ACC into the growth medium significantly promoted shoot elongation (Fig. 4A), in a dose-dependent manner. In contrast, the application of $AgNO_3$, an ethylene-signaling inhibitor, inhibited shoot elongation, again being dose-dependent (Fig. 4B). These results support that the phenotypes observed in the *OsEIN2* antisense construct were due to a disturbance of the ethylene-signaling pathway.

Inhibition of OsEIN2 represses the expression of ethylene-inducible genes

To further investigate whether the phenotypes of the *OsEIN2* antisense plants were indeed due to inhibition of the ethylene-signaling pathway, we monitored the expression patterns of various ethylene-regulated genes in transgenic plants. To this end, we isolated putative ethylene-responsive genes

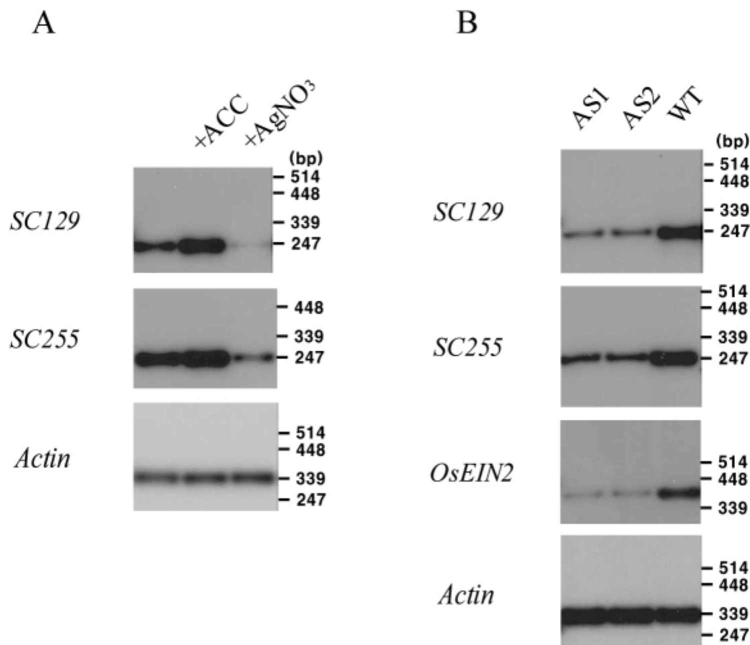

Fig. 5 Expression patterns of ethylene-responsive genes in *OsEIN2* antisense plants. (A) Effects of ACC and $AgNO_3$ on expression of *SC129* (AK104680) and *SC255* (AK061882). Seedlings (6 DAI) treated with ACC or $AgNO_3$ were used for RNA isolation. (B) Transcript levels of *SC129*, *SC255*, and *OsEIN2* in *OsEIN2* antisense plants. RNA was isolated from the 10-DAI seedlings. The internal reference *actin* transcript was co-amplified. AS1, antisense plant Line 471; AS2, antisense plant Line 456.

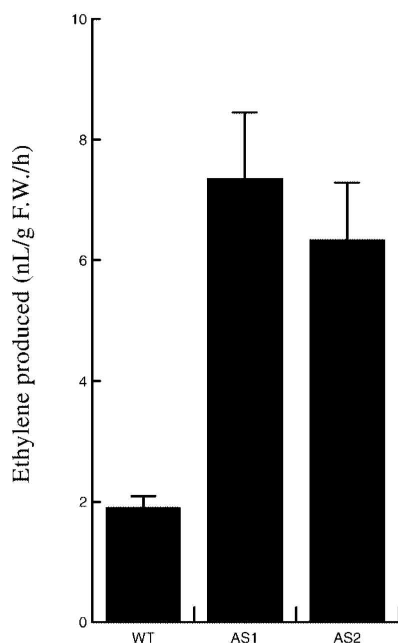

Fig. 6 Ethylene biosynthesis by wild type (WT) and *OsEIN2* antisense seedlings. Seeds were germinated and grown in airtight 12 ml glass bottles. These bottles were then flushed with hydrocarbon-free air and capped. After incubation at 25°C for 6 h, ethylene was measured. Values for AS1 and AS2 are means of three replicates from Line 471 and Line 456, respectively.

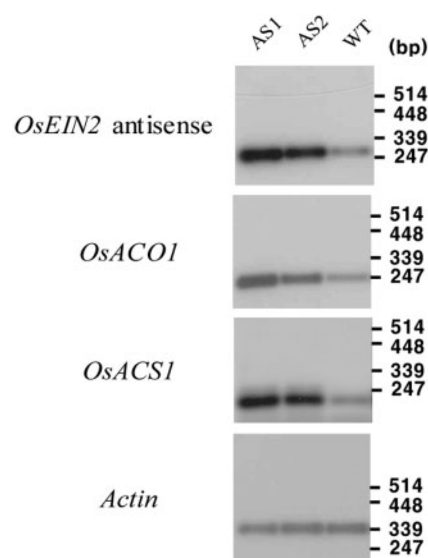

Fig. 7 RT-PCR analysis of *OsACS1* and *OsACO1* in *OsEIN2* antisense plants. Transcript levels of *OsEIN2* antisense transcript, *OsACS1*, and *OsACO1* in *OsEIN2* antisense plants. RNA was isolated from 10-DAI seedlings. The internal reference *actin* transcript was co-amplified. AS1, antisense plant Line 471; AS2, antisense plant Line 456.

from ESTs. The *SC129* (AK104680) clone is similar to glutathione *S*-transferase (Itzhaki and Woodson 1993) and the *SC255* (AK061882) clone shares similarity with an ethylene-responsive transcription factor (Zegzouti et al. 1999). Their responsiveness to ethylene was tested in 6-DAI seedlings. Here, the expression of both genes was induced by 20 μM ACC and repressed by 10 μM $AgNO_3$ (Fig. 5A). Analysis of the *OsEIN2* antisense transgenic plants showed that expression levels of *SC129* and *SC255* were decreased compared with the wild types (Fig. 5B), indicating that suppression of *OsEIN2* causes reduced expression of ethylene-responsive genes.

Ethylene biosynthesis in OsEIN2 antisense plants

We analyzed ethylene production from both *OsEIN2* antisense seedlings and wild-type plants. The former produced approximately 3.5 times more ethylene than the latter (Fig. 6), indicating that a reduction in the positive regulator of the signal pathway results in up-regulation of the ethylene biosynthesis pathway. Guzman and Ecker (1990) have demonstrated in *Arabidopsis* that blocking the ethylene-signaling pathway enhances the ethylene biosynthesis rate. Therefore, to examine whether the greater amount of ethylene in our transgenic rice plants was caused by increased biosynthesis, we measured the expression levels of the ACC synthase (*ACS*) genes and the ACC oxidase (*ACO*) genes. At least five *ACS* genes and three copies of the *ACO* genes have been reported in rice (Zarembinski and Theologis 1993, Mekhedov and Kende 1996, van der Straeten et al. 1997, Chae et al. 2000). In our study, transcript levels of *OsACS1* and *OsACO1* were correlated with that of *OsEIN2* antisense transcript (Fig. 7), whereas the expression of other *ACS* and *ACO* genes was not affected (data not shown). Both *OsACS1* and *OsACO1* are known to be submergence-induced and also involved in submergence-induced shoot elongation. Therefore, ethylene produced by *OsACS1* and *OsACO1* may play a major role in shoot growth.

Discussion

The molecular characterization presented here demonstrates in several ways that *OsEIN2* is a positive component of ethylene signaling in rice. First, the antisense plants of *OsEIN2* manifested a reduced-shoot elongation phenotype, a response similar to that found with wild-type rice seedlings that are treated with $AgNO_3$, an ethylene signal inhibitor. Second, the expression levels of two ethylene-responsive genes, *SC129* and *SC255*, were lower in the antisense plants than in the wild types. Third, the antisense transgenics produced approximately 3.5 times more ethylene than did the wild-type plants.

Furthermore, our comparison of gene structures and expression patterns suggest that *OsEIN2* is an ortholog of *AtEIN2*. First, significant similarity was found between the amino acid sequences of OsEIN2 and AtEIN2. *AtEIN2* was shown to be a single-copy gene in *Arabidopsis* (Alonso et al. 1999) and there is no other gene showing considerable similarity to *AtEIN2* in *Arabidopsis* genome. *OsEIN2* is the rice gene

with the highest similarity to *AtEIN2* in both the indica and the japonica rice genomic sequence databases. Second, the genomic structure of *OsEIN2* was identical to that of *AtEIN2*. A genome-wide comparison of the P-type ATPase ion pumps in *Arabidopsis* and rice has suggested that the gene structure has been relatively stable since the split from their common angiosperm ancestor (Baumberger et al. 2003, Baxter et al. 2003). Also, there is a striking conservation of intron numbers and positions among orthologs or paralogs. Finally, *OsEIN2* was expressed ubiquitously. *AtEIN2* is known to be expressed in the roots, leaves, inflorescence stems, and flowers and the steady-state mRNA level was unaffected by treatment with ethylene.

The completion of two shotgun sequences of the rice genome (Goff et al. 2002, Yu et al. 2002) provides an opportunity for comparing the components of any biological process in a monocot with that of a dicot, *Arabidopsis*. Nearly all the genes known to control disease-resistance responses in *Arabidopsis* have putative orthologs in rice, suggesting an extensive conservation of such signaling between monocots and dicots (Goff et al. 2002). Likewise, several components of the gibberellin-signaling pathway have been identified from rice as well as *Arabidopsis* (Peng et al. 1997, Silverstone et al. 1998, Silverstone et al. 2001, Ikeda et al. 2001, Itoh et al. 2002, Sasaki et al. 2003). In both species, the GA signal is transduced via the same mechanism, and the components of the processes are highly conserved. Nonetheless, in the case of flowering, a putative ortholog of *Arabidopsis FLC* has not been identified in rice (Tadege et al. 2003).

Penninckx et al. (1998) have reported in *Arabidopsis* that the ethylene and jasmonic acid pathways interact with each other, co-regulating the expression of some of the genes involved in plant defense. To test whether this is true for rice, we monitored the expression patterns of three jasmonic acid-regulated rice genes – *JAMyb*, *OsAOS*, and *JIOsPR10* – in our *OsEIN2* antisense transgenic plants (Jwa et al. 2001, Lee et al. 2001, Ha et al. 2002). RNA was isolated from the 6-DAI seedlings that were germinated on the agar media containing 50 μM MeJA and RT-PCR analysis was performed. Transcript levels of these genes were unaffected in the transgenics compared with the performance of the wild-type plants (data not shown). Therefore, we postulate that the induction of jasmonic acid-inducible genes does not require an active ethylene-signaling pathway in rice. However, more careful studies using jasmonic acid-response mutants, more ethylene-sensitive mutants, and other jasmonic acid-responsive genes are required to confirm this hypothesis.

Our current work is the first functional study of the components of ethylene signaling in a monocot species. Based on these results, we suggest the possibility of a conserved ethylene-signaling pathway existing between monocot and dicot plant systems.

Materials and Methods

Isolation of cDNA clones containing full-length ORF

A cDNA library was constructed in a λ-ZAPII (Stratagene, La Jolla, CA, U.S.A.) vector, with poly (A+) RNAs using a cDNA synthesis kit (ZAP cDNA kit, Stratagene) and an in vitro packaging mix (Gigapack III Gold, Stratagene). The cDNA library was made with mRNA from seed coats at 6–10 DAP (days after pollination) of rice (*Oryza sativa* L. var. japonica cv. Dongjin). pBluescript plasmids containing cDNA inserts were rescued via mass in vivo excision, and were sequenced with a T3 primer. The plaque hybridization experiment was performed as described by Kang et al. (1999). To identify the genomic sequences of OsEIN2, we searched the Rice Annotated Protein Database at The Institute for Genome Research (including all the sequences predicted from the International Rice Genome Sequencing Project) (http://www.tigr.org and http://rgp.dna.affrc.go.jp) and Rice GD (http://btn.genomics.org.cn/rice/index.php) (Yu et al. 2002). Putative exons of the 5′-region of *OsEIN2* were identified by TblastN (Altschul and Lipman 1990) searches using AtEIN2. Alignments of amino acid sequences were performed using the ClustalW program (http://www.clustalw.genome.ad.jp/).

Seedling growth

To grow plants under sterile conditions, seeds of rice (*O. sativa* L. var. japonica cv. Dongjin) were surface sterilized for 30 min in 2.5% sodium hypochlorite, then distributed evenly on bottles containing half-strength Murashige and Skoog (MS/2) media supplemented with 0.2% phytagel. Various concentrations of ACC (Sigma, St. Louis, MO, U.S.A.) and $AgNO_3$ (Merck) were added to the media to determine their effects on seedling growth. The plates were stored at 4°C in the dark for 2 d, and then placed in a growth chamber at 28°C under continuous light.

Generation of antisense plants

The partial cDNA clone of *OsEIN2* (SC636) was inserted in the antisense orientation into the multiple cloning site of pGA1611 (Kang et al. 1998). In the constructs, *OsEIN2* was placed under the control of the maize *ubiquitin* (*ubi*) promoter in the antisense direction. The fusion between the CaMV 35S promoter and the *hygromycin phosphotransferase* (*hph*) gene was used as a plant selectable marker. Scutellum-driven embryonic calli of *Oryza sativa* var. japonica cv. Dongjin were then transformed using the *Agrobacterium*-mediated co-cultivation method described by Lee et al. (1999).

RT-PCR Analysis

We used Tri-reagent (MRC Inc., Cincinnati, OH, U.S.A.), to isolate total RNA from the roots, leaves, sheaths, flowers, developing seeds, calli, and seedlings at various stages. For the first-strand cDNA synthesis, 2 μg of total RNA was reverse-transcribed in a volume of 25 μl containing 10 ng of oligo(dT) 12–18 primer, 2.5 mM dNTPs, and 200 units of moloney murine leukemia virus RT (Promega, Madison, WI, U.S.A.) in a reaction buffer. PCR was performed in a 30 μl solution that consisted of a 1 μl aliquot of the cDNA reaction, 0.2 μM gene-specific primers, 10 mM dNTPs, 1 unit of rTaq DNA polymerase (Takara Shuzo, Shiga, Japan), and 10× reaction buffer. The reaction included an initial 5-min denaturation at 94°C, followed by 18–24 cycles of PCR (94°C, 1 min; 56°C, 1 min; and 72°C, 1 min), and a final 10 min at 72°C. Afterward, we analyzed 10 μl of the reaction mixture on a 1.2% (w/v) agarose gel, and transferred it to a Hybond N+ nylon membrane (Amersham, Buckinghamshire, U.K.). The blot was hybridized at 60°C for 12 h in Church buffer (Church and Gilbert 1984), using a ^{32}P-labeled probe. Membranes were washed once for 10 min with 0.2× SSC and 0.1% (w/v) SDS at 25°C, and twice, for

10 min each, with 0.2× SSC and 0.1% (w/v) SDS at 58°C. These washed membranes were then exposed at RT for 2 h on X-ray film with intensifying screens. The RT-PCR was repeated at least three times for separately harvested samples. Primers included: for *actin*, 5′-TCC ATC TTG GCA TCT CTC AG-3′ and 5′-GTA CCC GCA TCA GGC ATC TG-3′; *EIN2* sense transcripts, 5′-CGG ATA GGT ACT ATG ATG GC-3′ and 5′-GCA CTC GAC ACA CCA AAC AG-3′; *EIN2* antisense transcripts, 5′-CAA CAG CCT GCG CCA CAG-3′ and 5′-TAA AAC GCG ACA GGA TCA CG-3′; *OSACO-1*, 5′- GTC CAT GGA AAC CGA GAC CT-3′ and 5′-GAG CTC GTC GCG AGT AGT AA-3′; *OSACO-2*, 5′-GGC GAG ACG TAT CCC AAG TT-3′ and 5′-AAC GCG AGC TGA GTA GCT GA-3′; *OSACO-3*, 5′-ACG CTT CGA GGC CAT GAA GT-3′ and 5′-GGT AGC CAT TGA AGC ACA GA-3′; *SC129*, 5′-TGA CGG TGT ACG GTC CGA T-3′ and 5′-TCG GCG TAC TGG TCA CAG AT-3′; *SC255*, 5′-AAG GAT GAG AAG GCC GTC AA-3′ and 5′-CAA GCT GTG CCT GGG TCA T-3′; JAmyb, 5′-CTG GTG TAA CAA TGG CCA CT-3′ and 5′-AGC TGG TTG GTC CTG AAA CT-3′; AOS, 5′-ACT CAA CCA GCT CCG TCC TA-3′ and 5′-GAT CCG ATA CAT ATT AGA GGT-3′; JIOsPR10, 5′-CAG TTC AAC TTC ACC TCA GCC AT-3′ and 5′-GCA AAA CCA ACA GGT AGA TGC T-3′; OsACS1, 5′-GAA TTC GAT GGT GAG CCA AGT-3′and 5′-AGC GCG TGG GGG TTC TTC T-3′; OsACS2, 5′-AAT GGC CGT GCA GGG CAT C-3′ and 5′-TTG GCG ATC CTC TTG AAC TG-3′; OsACS3, 5′-ATC CAG ATG GGC CTT GCA GA-3′ and 5′-GTC TCC ATG AAG CTC GCC A-3′; OsACS4, 5′-GTC TCC ATC GAC CTC CTC GA-3′ and 5′-AGC GCA TTG TCC CTG AAG CT-3′; OsACS5, 5′-CTC ACC TTC ATC CTC GCC AA-3′ and 5′-GTA GAT CTC GTC GGA GAT GA-3′.

Ethylene measurements

The seeds were germinated and grown on in airtight 12 ml glass bottles. Seven days after imbibition, these bottles were flushed with hydrocarbon-free air and capped. After incubation at 25°C for 6 h, a 1 ml gas sample was withdrawn by syringe from the headspace of each bottle and its concentration was measured by gas chromatography (Hewlett-Packard 5890 Series II). 0.01–5 ppm of standard ethylene was used to calibrate gas chromatography. Separations were carried out at 50°C, using N_2 as the carrier gas, and the ethylene peak was detected with a flame ionization detector. The peak area was integrated with a Hewlett Packard 3396 Series II integrator and compared with an 8-point standard ethylene curve. The quantified data, divided by fresh weight and time were converted to specific activities.

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