RESEARCH PAPER

Differential expression of three genes encoding an ethylene receptor in rice during development, and in response to indole-3-acetic acid and silver ions

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Abstract

Five ethylene receptor genes, OS-ERS1, OS-ERS2, OS-ETR2, OS-ETR3, and OS-ETR4 were isolated and characterized from rice. The genomic structure of OS-ERS1 and OS-ERS2 revealed that the introns within the coding sequences occurred in conserved positions to those of At-ETR1 and At-ERS1, whereas each of the OS-ETR2, OS-ETR3, and OS-ETR4 genes contained 1 intron within its coding region located at a position equivalent to those of At-ERS2, At-ETR2, and At-EIN4. Deduced amino acid sequences of OS-ERS1, OS-ERS2, OS-ETR2, OS-ETR3, and OS-ETR4 showed that they exhibited significant homology to the prokaryotic two-component signal transducer and a wide range of ethylene receptors in a variety of plant species. Northern analysis revealed that the level of OS-ETR2 mRNA was markedly elevated either by the exogenous application of IAA or by ethylene treatment in young etiolated rice seedlings, whereas the OS-ERS1 transcript level was only slightly induced under the same experimental conditions. Pretreatment with silver prevented IAA-induced and ethylene-induced accumulation of both mRNAs (OS-ERS1 and OS-ETR2). However, the abundance of OS-ERS2 mRNA was shown to be down-regulated by both IAA and ethylene treatments, indicating that it was not positively regulated by ethylene. Analysis of the expression of the three ethylene receptor genes in different tissues of rice has unravelled their corresponding tissue-specificity in which OS-ERS1 was constitutively expressed in considerable amounts in all tissues studied, while OS-ERS2 and OS-ETR2 exhibited differential expression patterns in different tissues of rice. Moreover, higher levels of these three mRNAs were commonly observed in anthers when compared with their corresponding levels in other tissues, suggesting the important role played by ethylene involved in the regulation of pollen development in rice. Among the five ethylene receptor genes, the expression levels of both OS-ETR3 and OS-ETR4 were too low to be detected by the northern blot analysis. Results from RT-PCR illustrated that both mRNAs were present in young green rice seedlings and anthers.

Key words: Auxin, differential expression, ethylene, ethylene receptors, rice.

Introduction

In contrast to its simple molecular structure, the plant hormone ethylene regulates many aspects of plant growth and development including seed germination, root hair development, senescence, abscission, fruit ripening, and plant defence (Yang and Hoffman, 1984). The ethylene biosynthetic pathway has been well characterized in the past 40 years (reviewed in Kende, 1993) and its production is tightly regulated both by internal signal(s) and environmental stimuli during plant development. However, in order to obtain a clearer picture of the roles played by ethylene in plants, it is of great importance to study how the signal is perceived and transduced. In the last decade, molecular and genetic approaches have contributed a lot to the understanding of the ethylene signalling and transduction pathway. By utilizing the triple response phenotype in Arabidopsis, a number of mutants that were impaired in the ethylene response have been identified (Bleecker et al., 1988; Guzman and Ecker, 1990; Ecker, 1995).
Characterization of the corresponding genes and epistatic analysis have enabled a linear genetic pathway of ethylene hormone signalling to be generated (Johnson and Ecker, 1998; Wang et al., 2002). The first ethylene receptor gene, ETR1 was isolated from an Arabidopsis ethylene-insensitive mutant etr1 by map-based cloning and its recombinant protein expression in yeast confirmed its capability to bind ethylene in vivo (Schaller and Bleecker, 1995). It is now known that in Arabidopsis, ethylene is perceived by a family of five receptors (ETR1, ETR2, ERS1, ERS2, and EIN4) that displayed significant homology to a prokaryotic family of signal transducers known as two-component regulators (Chang et al., 1993; Hua et al., 1995, 1998; Hua and Meyerowitz, 1998; Sakai et al., 1998). The amino-terminal half contains a hydrophobic domain responsible for ethylene binding and membrane-spanning localization whereas the carboxyl-terminal appeared to be involved in signal transmission since it displays similarity to a histidine protein kinase domain and a receiver domain originated from bacterial signal transducers. It is also interesting to note that the ethylene receptor gene family consists of six members in tomato instead of five in Arabidopsis (Klee and Tieman, 2002; Lashbrook et al., 1998; Tieman and Klee, 1999; Wilkinson et al., 1995; Zhou et al., 1996a, b). Moreover, it is now also known that ethylene signal is mediated via a copper transport complex that has been shown to be localized to the endoplasmic reticulum (Chang and Shockey, 1999; Hirayama et al., 1998; Rodriguez et al., 1999). In general, the ethylene receptor gene family in higher plants can be subdivided into two groups (namely, the ETR1-like and ETR2-like subfamilies) based on the structural differences of their deduced amino acid sequences (Hua et al., 1998). In the ETR1-like subfamily, the predicted proteins have three hydrophobic domains at the N-terminal portion whereas ETR2-like proteins have four such domains. A basic framework of the ethylene perception pathway is emerging, but many issues still need to be resolved, such as the presence of multiple isoforms of ethylene receptors. Until now, most of the work related to ethylene perception or signalling processes have mainly focused on dicotyledonous plants. Homologues of ethylene receptor genes have also been isolated from carnation (Shibuya et al., 1998; Nagata et al., 2000), cucumber (Yamasaki et al., 2000), muskmelon (Sato-Nara et al., 1999), passion fruits (Mita et al., 1998), peach (Bassett et al., 2002), and tobacco plants (Xie et al., 2003; Zhang et al., 2001). However, little is known about the involvement of receptor genes in the ethylene perception of monocotyledonous plants such as rice. Rice is a very important cereal crop and also a major food source for more than one-third of the world’s population (Yuan, 1998). The molecular cloning of a genomic sequence of a putative ethylene receptor gene designated as OS-ERS1 from rice is reported here. In addition, the isolation of four other ethylene receptor gene homologues designated as OS-ERS2, OS-ETR2, OS-ETR3, and OS-ETR4 is described. The differential expression patterns of OS-ERS1, OS-ERS2, and OS-ETR2 in different tissues of rice have also been compared, together with their response to exogenous auxin and the ethylene action inhibitor, silver ion.

Materials and methods

Plant materials and experimental treatments

Seeds of indica rice (Oryza sativa L. var. IR36) were soaked in aerated water overnight at room temperature. The seeds were then grown in vermiculite at 30 °C under dark or light conditions. Experimental procedures and treatments were performed in the dark or under safety light. For IAA application, 1 mM IAA solution was sprayed onto intact rice seedlings and the solution was infiltrated into the tissues using a vacuum pump for 1 min. For ethylene treatment, intact rice seedlings were placed into an enclosed rectangular chamber containing either air or indicated ethylene concentrations for 6 h.

Measurement of ethylene production

Leaf segments were placed in a tube sealed with rubber serum cap. 1 ml of gas sample was withdrawn from the headspace of the tube with a syringe, and ethylene was assayed using a gas chromatograph (Hewlett Packard Series II 5890, USA) equipped with an aluminia column and a flame ionization detector (Hewlett Packard, USA).

Genomic library screening

The rice genomic library was purchased from Clontech Laboratories (Clontech, USA) and the DNA source was from 5-d-old etiolated shoots of Oryza sativa (L. indica, var. IR36). Standard procedures of genomic library screening were performed according to Sambrook et al. (1989).

Preparation of gene-specific probes

Gene-specific probes for RNA gel and Southern blot analysis were designed using the less homologous regions at the 3’ end of each gene and labelled with 32P-dCTP using a random primed DNA labelling kit (Roche Diagnostics) according to the manufacturer’s instructions. The regions of the sequences corresponding to the probes are listed as nucleotide numbers of the full length sequences as follows: OS-ERS1, 1546–1977 (431 bp); OS-ERS2, 1703–1909 (206 bp); and OS-ETR2, 1848–2293 (445 bp).

Extraction of total RNA and northern blot analysis

Total RNA was extracted from leaves, tillers, young panicles, leaf sheaths, hulls, and anthers of rice using the hot phenol method as previously described (Shiu et al., 1998). Briefly, the samples were ground in 2 vols of 1:1 ratio of RNA extraction buffer (100 mM LiCl, 1% SDS, 100 mM TRIS-HCl pH 9.0, and 10 mM EDTA) and hot phenol (90 °C) mix, vortexed for 5 min, and centrifuged at 4000 rpm for 30 min. The supernatant was then precipitated twice with 8 M LiCl. Total RNA was separated on a 1.2% agarose/formaldehyde gel in 1× MOPS buffer. Then the gel was soaked in 0.05 M NaCl for 20 min, in 10× SSC for 30 min and transferred to Hybond-N° nylon membrane (Amersham, USA). The membranes were washed, cross-linked with UV light and air-dried. Each membrane was prehybridized at 42 °C for 3 h in a solution containing 50% formamide, 5× SSC, 5× Denhardt’s solution, 0.5% SDS, and 100 µg ml−1 of denatured salmon sperm DNA, and allowed to hybridize with specific probes for further 18 h. After hybridization, membranes were washed once in 2× SSC, 0.1% SDS at room temperature for
10 min and three times in 0.5 × SSC, 0.1% SDS at 65 °C for 30 min. The intensity of the hybridized signal was quantified using a Phosphorimager (Molecular Dynamics, USA) and the data analysed with the Image Quant program.

Cloning of ethylene receptor gene homologues in rice

Degenerate PCR primers of CY4F (sense: 5'-GCCGGATCCGT-GAAYCAYGARA-TGAGRAC-3') and CY4R (antisense: 5'-GCCGAAGCTTGCTAAAYTNTGTRAADA-C-3') were designed against two conserved motifs within the histidine kinase domain and 1 µg of rice genomic DNA was used as template for running PCR. 5'-RACE and 3'-RACE was performed using the SMART cDNA synthesis kit (Clontech, USA) in accordance with the manufacturer’s instructions. The sequence of the gene-specific PCR primers was as follows: CY12F: 5'-ATGGATGGATGTGA-TACCTGAACAGCCC-GGTAAAAGAATCC-3' (sense); CY12R: 5'-TCATATACTTCTCTGAT-AAGCGTGCA-AGA-3' (antisense); CY21F: 5'-TGATTAAGCCAAGTAGCAGCCGCGTGCA-AGA-3' (sense); CY21R: 5'-TACCTGAACAGCCC-GGTAAAAGAATCC-3' (antisense).

Results

Isolation of an homologous probe and screening of the rice genomic library

Using a pair of PCR degenerate primers (CY4F and CY4R) designed from the two conserved motif within the histidine kinase domain of both Arabidopsis ETR1 (At-ETR1) and tomato NR proteins (MNHEMRT and VFTKFA), a PCR fragment of 550 bp was obtained and designated as pBr21 (Chang et al., 1993; Wilkinson et al., 1995). Its deduced amino acid sequences displayed high homology to various plant ethylene receptor homologues after searching through the GenBank database. pBr21 was then used as a probe to screen a commercially available rice genomic library (Clontech, USA). Finally, ten putative rice genomic sequences were used to obtain the full-length cDNA sequences of (accession number AY136816).

Cloning of five ethylene receptor gene homologues in rice

In an attempt to isolate the full length cDNA sequence of OS-ERS1, specific primers (CY12F and CY12R) designed from the OS-ERS1 genomic sequence were used to perform RT-PCR using total RNA extracted from 7-d-old etiolated rice seedlings. A nearly full length cDNA sequence was obtained and a 3'-RACE end amplification method was employed to generate the sequence information at the 3' end. Finally, a full length cDNA sequence of 2234 bp long was obtained which contains an open reading frame of 636 amino acids (accession number AF013979). Based on the studies of the ethylene-response pathway in Arabidopsis and tomatoes, multiple ethylene receptors have been shown to be present in both plant species (Hua and Meyerowitz, 1998; Klee and Tieman, 2002). Therefore it is possible that rice may also consist of different ethylene receptor isoforms. During the progress of this study, the rice genome draft sequence had not yet been published and by searching through the GenBank rice EST database, it was possible to identify three additional EST sequences that are not identical to OS-ERS1, but are highly homologous to other plant ethylene receptor genes. The EST sequence of accession number C72213 is 64.7% homologous to the coding sequence of OS-ERS1 and named as OS-ERS2. Specific PCR primers (CY21F and CY21R) were designed to amplify this partial cDNA by RT-PCR using the same pool of total RNA as described above. 5'-RACE and 3'-RACE strategies were employed to obtain the full-length cDNA sequences of OS-ERS2. Eventually, the full length cDNA sequence of OS-ERS2 was obtained. It is 2541 bp long encoding a predicted polypeptide of 635 amino acids (accession number AF460181).

The translated protein sequences of accession numbers D47766 and AU093433 are 83% and 87% similar to Zea mays ethylene receptor-like protein (accession number AB040406), respectively. Similarly, 5'-RACE and 3'-RACE methods were carried out to generate the full-length cDNA sequences of OS-ETR2 using the same pool of total RNA. OS-ETR2 is 2595 bp long and its open reading frame encoded protein of 763 amino acids (accession number AY136816).

In 2002, the draft genome sequences of both indica and japonica rice were published and through the database searching, it was possible to find out that, altogether, there are five ethylene receptor-like proteins in the rice genome, including the three members mentioned above (Goff et al., 2002; Yu et al., 2002). The deduced polypeptide of the additional two putative ethylene receptors also exhibit high homology to various ethylene receptors from different plant species, therefore, these two homologues were named as OS-ETR3 and OS-ETR4 (see the detailed comparisons and descriptions in Fig. 2 further on in the Results). Specific primers were designed to isolate the full-length cDNA sequences using total RNA extracted from young green rice seedlings by 5'-RACE and 3'-RACE methods. OS-ETR3 (accession number AY434735) is 3294 bp long encoding a predicted polypeptide of 836 amino acids, whereas OS-ETR4 (accession number AY434734) is 2711 bp long and its open reading frame encoded protein of 836 amino acids.

Genomic organization of the rice ethylene receptor gene family

The genomic sequence of OS-ERS1 revealed that its coding sequence is interrupted by four introns occurring in a conserved position when compared with that of the At-ETR1 and At-ERS1 genomic sequences. However, the first
and third introns of OS-ERS1 are about five and nine times longer than those found in the corresponding positions in At-ETR1 and At-ERS1. Although draft sequences of both indica and japonica rice have been released (Goff et al., 2002; Yu et al., 2002), it is still of crucial importance to have a detailed physical map for each of the rice chromosomes. With the help of the web resources from the Rice Genome Research Program (http://rgp.dna.affrc.go.jp) in Japan, it was possible to identify the chromosomal position of all five ethylene receptor genes as well as the exon–intron boundaries within each genomic sequence (Fig. 1). In the rice genome, each ethylene receptor gene is located on a different chromosome. Similar to OS-ERS1, the coding sequence of OS-ERS2 is interrupted by four introns which also occur in conserved positions to those of At-ETR1 and At-ERS1. There is only one intron present within the coding region of each of the OS-ETR2, OS-ETR3, and OS-ETR4 located at the conserved positions equivalent to those of At-ERS2, At-ETR2, and At-EIN4.

Comparison of the structural characteristics of OS-ERS1, OS-ERS2, OS-ETR2, OS-ETR3, and OS-ETR4 proteins

Comparison with ethylene receptor homologues from other plants revealed that OS-ERS1 and OS-ERS2 proteins show the highest level of 79% and 71.3% identity with the
orchid ethylene receptors (*Phalaenopsis* sp, ‘Kc butterfly’, AF113541), respectively, instead of the rice ethylene receptor family. OS-ERS1 and OS-ERS2 proteins only share 69.2% identity with each other, whereas OS-ETR2 displays highest homology to Zm-ETR2 (*Zea mays*, AB040406) with 80.8% amino acid identity in the database. Although OS-ETR3 and OS-ETR4 exhibited the strongest homology to OS-ETR2 with 49.1% and 42.7% identity, respectively, they are more structurally diverse in that they are only 33.8% identical to each other. All the five members exhibit sequence similarity to receptor histidine kinases of the prokaryotic two-component signal transducer system (Stock *et al*., 1990). They all contain the putative N-terminal ethylene binding region with three or four transmembrane domains as shown in Fig. 2. Moreover, they also include a histidine kinase domain in which OS-ERS1 and OS-ERS2 have all five consensus motifs, while OS-ETR4 only possesses two of them including the putative histidine autophosphorylation residue. Notably, OS-ETR2 and OS-ETR3 lack all these motifs, raising the question whether they exhibit any histidine kinase activity. In between the ethylene binding and histidine kinase domains, a region of unknown function, which has sequence similarity to GAF domains, is present in all three of these receptors (Fig. 2). Although the functional significance of the GAF domain has not been established, it is thought to play a role in the binding of cGMP (Aravind and Ponting, 1997). Another main structural difference from other rice ethylene receptors, lying between OS-ERS1 and OS-ERS2, is that they both lack the response regulator domain at the C-terminal end. Interestingly, OS-ETR3 has an extraordinary stretch of 80 amino acid residues located at the N-terminus when compared with other plant ethylene receptors. By searching through iPSORT WWW server (http://hypothesiscreator.net/iPSORT/), it was predicted that OS-ETR3 consists of a putative chloroplast transit peptide which may act as a sorting signal.

A phylogenetic tree, generated from the ethylene receptor gene families from rice and various plant species, demonstrated that OS-ERS1 and OS-ERS1 are more closely related to each other and to orchid Ph-ETR1 and banana MA-ERS1, while OS-ETR2 is more closely related to maize Zm-ETR2, OS-ETR3, and OS-ETR4 (Fig. 3). Although there is limited number of ethylene receptor genes isolated from monocotyledonous species so far, our results illustrate that there is a presence of a clade containing ethylene receptor members from monocotyledonous species.

**Expression levels of OS-ERS1, OS-ERS2, and OS-ETR2 in various tissues of rice**

In order to understand the possible roles played by OS-ERS1, OS-ERS2, and OS-ETR2 in rice, RNA gel blot was performed to analyse their spatial gene expression patterns in various tissues. Individual gene-specific probes corresponding to OS-ERS1, OS-ERS1, and OS-ETR2 were used for hybridization. Results indicated that the expression level of OS-ERS1 is the highest among the three receptor genes and it is constitutively expressed in all the tissues studied (Fig. 4). The mRNA level of OS-ERS2 is very low, but still detectable if the blot was exposed for a longer period of time of 48 h. Interestingly, the etiolated condition seemed to inhibit the accumulation of OS-ERS2 transcript where the ethylene production from young green seedlings...
is much lower (around 6-fold) than that from etiolated seedlings (data not shown). On the other hand, the amount of OS-ETR2 transcript is higher in etiolated seedlings than in green seedlings. Although the three receptor genes displayed differential expression patterns in different tissues, it was commonly observed that the overall level of these transcripts is highest in the developmental stages of rice anthers. However, a substantial amount of OS-ERS1 mRNA was accumulated during pollen development in rice when compared with its transcript level in other tissues studied or to both OS-ERS2 and OS-ETR2 mRNA levels in anther tissues. Among the five rice ethylene receptor genes, the expression levels of both OS-ETR3 and OS-ETR4 are too low to be detected by conventional RNA gel blot analysis. Thus RT-PCR was performed to measure their mRNA levels. Results showed that both OS-ETR3 and OS-ETR4 mRNAs were present in young green rice seedlings and anthers (CP Yau, unpublished results).

**Effects of auxin, ethylene and silver ions on the expression levels of OS-ERS1, OS-ERS2, and OS-ETR2**

Next, the effect of ethylene on the transcript levels of the three ethylene receptor genes was analysed. The experiments were done in the dark using 7-d-old etiolated seedlings. Different concentrations of ethylene (10 ppm and 50 ppm) were injected into rectangular containers in which intact rice plants were placed and incubated for 6 h at room temperature. Results indicated that ethylene could markedly stimulate the accumulation of OS-ETR2 mRNA. A higher concentration of ethylene (50 ppm) appeared to enhance more OS-ETR2 mRNA expression when compared with that of a lower concentration (10 ppm) (Fig. 5), whereas ethylene only mildly increased the amount of OS-ERS1 mRNA (Fig. 5). On the other hand, ethylene treatment appeared to exhibit an inhibitory effect (although to a lesser extent) on OS-ERS2 to lower its transcript level only slightly. It is known that silver is an effective and specific inhibitor of ethylene action in all the systems studied (Veen, 1987). The effect of silver ions on the expression of these three ethylene receptor genes in rice was examined here. Intact rice plants were pretreated with 5 mM AgNO₃ solution for 2 h and then ethylene was added and allowed to incubate for another 6 h. Results showed that silver ions could effectively inhibit the stimulatory effect of ethylene on its induced expression of OS-ETR2 and OS-ERS1 mRNA (Fig. 5). It is also worth noting that silver ions could even exert a suppressing effect on the expression levels of OS-ERS1, OS-ERS2, and OS-ETR2 transcripts (Fig. 5, lane 2) below the corresponding basal values in etiolated rice seedlings.

**Fig. 4.** Expression levels of OS-ERS1, OS-ERS2, and OS-ETR2 in different tissues of rice. Total RNA (25 μg lane⁻¹) from 7-d-old etiolated seedlings (ES), 7-d-old green seedlings (GS), roots (R), old leaves (OL), hulls (H), immature anthers (IA), and mature anthers (MA) were used for RNA gel blot analysis and hybridized with the corresponding gene-specific probe (also used in Southern blot analysis). The same blot was hybridized, washed, and stripped during the experiment. A direct comparison of the signal intensities was not possible among the different probes because of the different exposure time required for each probe. The exposure time for the blot of OS-ERS1 was 6 h, OS-ERS2 for 40 h, and OS-ETR2 for 24 h. The equivalence of RNA loading was demonstrated by ethidium bromide staining of RNA on the gel.

**Fig. 5.** The effect of ethylene and silver ions on the expression levels of OS-ERS1, OS-ERS2, and OS-ETR2 mRNAs. 7-d-old etiolated seedlings were treated with or without ethylene, or with or without 5 mM AgNO₃. Each lane contains 25 μg of total RNA which was used for RNA gel blot analysis. The same blot was hybridized, washed, and stripped during the experimental procedures. Exposure time for the blot of OS-ERS1 was 16 h, OS-ERS2 for 40 h and OS-ETR2 for 24 h. The equivalence of RNA loading was demonstrated by ethidium bromide staining of RNA on the gel.
The regulatory effect of auxin on ethylene biosynthesis has been extensively studied in various plant species (Abeles et al., 1992; Yang and Hoffman, 1984). It has been reported that auxin could stimulate ethylene production in various plant tissues (Abel and Theologis, 1996; Chae et al., 2000; Yang and Hoffmann, 1984). The effect of IAA, as well as silver ions, on the expression of the three rice receptor genes has been investigated here. Intact rice plants were treated with 1 mM IAA in the absence or presence of silver ions. Ethylene production was measured from the rice leaf segments at 6 h, at which its maximal value was reached (data not shown). Results indicated that the exogenous application of IAA could promote ethylene production to nearly 7-fold at around 155 nl g⁻¹ h⁻¹ when compared with the control (23 nl g⁻¹ h⁻¹) (Fig. 6, lower panel). The expression pattern of OS-ETR2 was parallel with those of ethylene production and the abundance of its mRNA was increased to around 1.5-fold (Fig. 6, third panel). However, the extent of the stimulatory effect of IAA-induced expression of OS-ETR2 was lower than that treated with ethylene directly. The effect of IAA resembled that of ethylene in inducing OS-ERS1 transcript accumulation in the way that IAA could only slightly increase the abundance of OS-ERS1 mRNA. By contrast with the positive regulatory effect on OS-ERS1 and OS-ETR2 mRNA abundances, IAA (similar to ethylene treatment) could also slightly decrease the OS-ERS2 transcript level (Fig. 6). Pretreatment with silver ions considerably reduced the IAA-induced ethylene production to around 45 nl g⁻¹ h⁻¹, which was still higher than the basal value of ethylene produced by the control. On the other hand, silver ions completely blocked IAA-induced OS-ERS1 and OS-ETR2 mRNA accumulation. These data suggest that silver ions have an antagonizing effect on the IAA-dependent positive regulatory role on both OS-ERS1 and OS-ETR2 transcript levels. To a lesser extent, silver ions could also decrease the amount of OS-ERS2 mRNA either in the presence or absence of IAA (Fig. 6).

**Discussion**

Three putative ethylene receptor genes OS-ERS1, OS-ERS2, and OS-ETR2 were isolated from rice that exhibit sequence similarity to ethylene receptors from various plant species such as Arabidopsis, tomato, and maize. In Arabidopsis, there are altogether five ethylene receptors and each of them has been shown to be capable of binding ethylene when expressed in yeast. Interestingly, there are six ethylene receptors in tomato instead of five in Arabidopsis. Hua et al. (1998) classified the ethylene receptor gene family in Arabidopsis into two categories, namely, the ETR1-like and ETR2-like subfamilies. The ETR1-like proteins consist of three hydrophobic domains at the N-terminus, while the ETR2-like proteins contain four hydrophobic domains at the N-terminus. Within each subfamily, ETR (with the receiver domain) and ERS homologues (without the receiver domain) are present. A phylogenetic tree, constructed from various ethylene receptors, indicates that, similar to Arabidopsis sequences (Bleecker, 1999), the five ethylene receptor proteins in rice can be divided into two subfamilies. One subfamily consists of OS-ERS1 and OS-ERS2 (only ERS type homologues are present) in which they do not have the receiver domain, whereas the second group contains OS-ETR2, OS-ETR3, and OS-ETR4. The three members (OS-ETR2, OS-ETR3, and OS-ETR4) are more structurally divergent from each other, and OS-ETR3 and OS-ETR4 only contain three N-terminal hydrophobic regions. The putative histidine kinase domains in these three members are very different from the canonical bacterial histidine kinase sequences. Notably, the autophosphorylated histidine residue is absent in both OS-ETR2 and OS-ETR3 suggesting that they may not be active histidine kinases. Recently, rather different results were obtained when

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**Fig. 6.** Effects of IAA and Ag⁺ on the expression levels of OS-ERS1, OS-ERS2, and OS-ETR2 mRNA. Intact etiolated rice seedlings were incubated for 6 h in the presence or absence of 100 μM IAA. Each lane consists of 25 μg of total RNA which was used for RNA gel blot analysis. The same blot was hybridized, washed, and stripped during the experimental procedures. Exposure time for the blot of OS-ERS1 was 16 h, OS-ERS2 for 48 h, and OS-ETR2 for 24 h. The equivalence of RNA loading was demonstrated by ethidium staining of RNA on the gel.
studying the kinase activities of both At-ETR1 in Arabidopsis and NT-HK1 in tobacco. Gamble et al. (1998) reported that At-ETR1, like its bacterial counterparts, exhibits histidine kinase activity; whereas Xie et al. (2003) indicated that tobacco NT-HK1 has serine/threonine kinase activity but no histidine kinase activity in in vitro studies (Gamble et al., 1998; Xie et al., 2003). Although it was demonstrated recently that the histidine kinase domain of At-ETR1 is not required for ethylene signalling in Arabidopsis (Wang et al., 2003), further research has to be performed to investigate the function(s) of individual kinase activity (whether it has histidine or serine/threonine kinase activity) of all the rice ethylene receptors. Questions like the significance of the existence of multiple forms of ethylene receptors and their interactions to form homodimers or heterodimers in the ethylene receptor complex still remain to be addressed.

The analysis of the expression of the three ethylene receptor genes in rice has demonstrated their tissue or developmental stage specificity. Both in Arabidopsis and tomato, the expression patterns alter among different ethylene receptor homologues. For instance, At-ERS1 was ubiquitously expressed in Arabidopsis, whereas stronger expression of At-EIN4 was observed in pollen and tapetum cells when compared with the At-EIN4 mRNA level in leaves and roots. Furthermore, in tomato, LE-ETR1 was expressed constitutively in all the tissues examined, whereas LE-ETR2 was expressed at a low level in vegetative tissues but up-regulated in seeds before germination. In rice, the three ethylene receptor genes (OS-ERS1, OS-ERS2, and OS-ETR2) also behave similarly in that their corresponding mRNA levels vary in different tissues. OS-ERS1 was constitutively expressed in all the tissues studied, whereas OS-ETR2 was expressed at lower levels in green vegetative tissues than in young etiolated seedlings. By contrast, the transcript levels of OS-ERS2 (when compared with that of OS-ERS1 and OS-ETR2) are very low, but still detectable, and present at a higher amount in green vegetative tissues than in etiolated seedlings (Fig. 4). Notably, the expression levels of these three mRNAs are strongest in reproductive tissues, such as anthers, when compared with their corresponding abundance in other tissues. It is also worth noting that only anthers isolated from two different developmental stages (meiotic and microspore stage) of pollen were used in this study. Therefore, the active involvement of the ethylene receptor gene family during early pollen development implies that ethylene may play an important role in regulating the initial formation stages of anther.

It has been reported that either environmental stimuli or developmental cues could up-regulate the transcript levels of ethylene receptor genes in various plant species. For example, RP-ERS1 mRNA level could be up-regulated by flooding (Voeseenek et al., 1997) in Rumex palustris leaves, PpETR1b mRNA level was raised by wounding in peach fruits, Cm-ERS1 and NR transcripts level were increased during muskmelon and tomato fruit ripening, respectively (Lashbrook et al., 1998; Sato-Nara et al., 1999; Wilkinson et al., 1995). Furthermore, ethylene could induce the accumulation of mRNA levels of NR, LE-ETR4, and LE-ETR5 in tomato leaves (Ciardi et al., 2000; Wilkinson et al., 1995); At-ERS1, At-ERS2, and At-ETR2 in Arabidopsis leaves (Hua et al., 1998); RP-ERS1 in Rumex leaves (Voeseenek et al., 1997); CS-ETR1, CS-ETR2, and CS-ERS1 in cucumber shoots (Yamasaki et al., 2000); Pp-ERS1 in peach fruits (Rasori et al., 2002); and Pe-ERS2 in arils of passion fruits (Mita et al., 2002). Recently, it has also been demonstrated that flooding could also stimulate the accumulation of OS-ETR2 mRNA in rice seedlings (CP Yau, L Wang, M Yu, SY Zee, WK Yip, unpublished results). Similar to these findings, the results presented here illustrated that an exogenous stimulus such as IAA or ethylene could also up-regulate the expression levels of both OS-ETR2 and OS-ERS1 (although to a much lesser extent) in etiolated rice seedlings (Figs 5, 6). However, the response of OS-ETR2 to either ethylene or IAA is more sensitive than that of OS-ERS1. It has been reported that auxin could stimulate ethylene production in various plant tissues by promoting de novo synthesis of ACC synthase (Abel and Theologis, 1996; Yang and Hoffman, 1984). It is proposed that the effect of IAA on the positive regulation of both OS-ERS1 and OS-ETR2 mRNA levels is mediated through an ethylene-dependent pathway. This hypothesis is further supported by the evidence that this up-regulation process could be completely blocked by pretreatment with silver ions. It was proposed by Veen (1987) that silver ions exert their effect by replacing another metal ion, such as copper, which may be a part of the ethylene complex, and, thereby, preventing the ethylene signal to be transduced (Veen, 1987). These results suggest that the inhibitory effect of silver ions may be propagated by reducing the number of functional ethylene receptors (at least in the case of OS-ERS1 and OS-ETR2). Although OS-ERS1 and OS-ERS2 are more closely related to each other in rice (69.2% amino acids identity), their gene expression characteristics are remarkably different. The OS-ERS2 transcript level was shown to be down-regulated by both IAA and ethylene since its level decreased inversely with the increase in ethylene production applied or produced (Figs 5, 6). This result indicates that OS-ERS2 is not positively regulated by ethylene. A similar phenomenon has also been observed in DC-ERS2 and DC-ETR1 of carnations, where their corresponding mRNA level was inversely proportional to ethylene production in flowers (Shibuya et al., 2002). The accumulated evidence indicated that ethylene receptors actually act as negative regulators of the ethylene response pathway in Arabidopsis and tomato (Ciardi et al., 2000; Hua and Meyerowitz, 1998; Tieman et al., 2000). Loss-of-function of four ethylene receptors in Arabidopsis resulted
in a constitutive triple-response phenotype (Hua and Meyerowitz, 1998). In addition, a reduction of LE-ETR4 mRNA in transgenic tomatoes also resulted in hypersensitive responses to ethylene. Moreover, transgenic tomato plants with a several-fold increase in NR expression are less sensitive to ethylene (Ciardi et al., 2000). These results illustrate the existence of the inverse relationship between ethylene sensitivity and levels of ethylene receptors. More ethylene receptors can actually desensitize tissue to ethylene while fewer ethylene receptors increase sensitivity. Therefore it is possible that the reduction of OS-ERS2 transcript levels can ultimately increase the ethylene sensitivity of rice in response to IAA- or ethylene-treated etiolated rice seedlings. Considering the up-regulation of OS-ETR2 mRNA levels by ethylene and the down-regulatory effect of ethylene on OS-ERS2 expression would seem paradoxical. To address this question, further studies are needed to establish the correlation between ethylene receptor levels and the sensitivity to ethylene, as well as their individual threshold levels to initiate ethylene responses.

Although subcellular localization of ethylene receptors has been studied in both Arabidopsis and tobacco, rather different results were reported between these two systems (Chen et al., 2002; Xie et al., 2003). In Arabidopsis, ETR1 was shown to be predominantly localized to the endoplasmic reticulum in planta, whereas NT-HK1 was mainly localized to the plasma membrane in a transient system. This difference suggests that they may have different roles in perceiving and transducing ethylene signals from different locations. By using the PSORT WWW server (available at http://psort.nibb.ac.jp), it was predicted that OS-ERS1, OS-ERS2, and OS-ETR2 were localized to the plasma membrane. However, approaches to visualize the localization of OS-ERS1, OS-ERS2, and OS-ETR2 by utilizing green fluorescent protein (GFP) were inconclusive and these results are similar to the results reported by Chen et al. (2002) who also failed to observe clear fluorescent signals at the subcellular level. Six constructs were made by fusing GFP to the C-terminus of each of the three rice receptor genes and their corresponding truncated protein without the signal peptide and the transmembrane domains. The expression of these fusion proteins was driven by the cauliflower mosaic virus 35S promoter. Transient expression assays performed with these constructs in onion cells were not conclusive because large intracellular aggregates of fluorescent signal formed within the cytoplasm of the transformed onion cells which made interpretation difficult (CP Yau, unpublished results). Efforts are now being made to investigate the subcellular localization of these five ethylene receptors in rice, by stable transformation of each of the members, using an inducible expression system that may offer a much higher level of expression for visualization purposes.

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