Contribution of Ethylene Biosynthesis for Resistance to Blast Fungus Infection in Young Rice Plants[1][OA]

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The role of ethylene (ET) in resistance to infection with blast fungus (*Magnaporthe grisea*) in rice (*Oryza sativa*) is poorly understood. To study it, we quantified ET levels after inoculation, using young rice plants at the four-leaf stage of rice cv Nipponbare (wild type) and its isogenic plant (IL7), which contains the *Pi-i* resistance gene to blast fungus race 003. Small necrotic lesions by hypersensitive reaction (HR) were formed at 42 to 72 h postinoculation (hpi) in resistant IL7 leaves, and whitish expanding lesions at 96 hpi in susceptible wild-type leaves. Notable was the enhanced ET emission at 48 hpi accompanied by increased 1-aminocyclopropane-1-carboxylic acid (ACC) levels and highly elevated ACC oxidase (ACO) activity in IL7 leaves, whereas only an enhanced ACC increase at 96 hpi in wild-type leaves. Among six *ACC synthase* (*ACS*) and seven *ACO* genes found in the rice genome, *OsACS2* was transiently expressed at 48 hpi in IL7 and at 96 hpi in wild type, and *OsACO7* was expressed at 48 hpi in IL7. Treatment with an inhibitor for ACS, aminooxyacetic acid, suppressed enhanced ET emission at 48 hpi in IL7, resulting in expanding lesions instead of HR lesions. Exogenously supplied ACC compromised the aminooxyacetic acid-induced breakdown of resistance in IL7, and treatment with 1-methylcyclopropene and silver thiosulfate, inhibitors of ET action, did not suppress resistance. These findings suggest the importance of ET biosynthesis and, consequently, the coproduct, cyanide, for HR-accompanied resistance to blast fungus in young rice plants and the contribution of induced *OsACS2* and *OsACO7* gene expression to it.

In monocot plants, the mechanism of disease resistance, including the roles of defense signal compounds for *resistance* (*R*)-gene-mediated resistance, such as salicylic acid (SA), ethylene (ET), and jasmonic acid (JA), has not been well elucidated. To study the mechanism, the Japonica rice (*Oryza sativa*) cv Nipponbare is an attractive model because of recent developments in genomic and molecular information, such as the Rice Genome Research Program (http//rgp.dna.affrc.go.jp; International Rice Genome Sequencing Project, 2005). Rice blast fungus (*Magnaporthe grisea*) is an extensively studied pathogen whose infection seriously affects rice yields worldwide. Genetic studies have identified 13 major *R* genes to blast fungi in rice plants, and standard rice cultivars with individual *R* genes and corresponding standard blast fungal races have been prepared to identify the race of blast fungus and *R* genes in a rice cultivar (Yamada et al., 1976; Kiyosawa, 1984). In Japan, attempts have been made to generate individual isogenic lines that contain a specific *R* gene with the same genetic background as practical rice cultivars, such as cv Nipponbare (Ise and Horisue, 1988). Actually, multilines containing compatible and incompatible lines were reported to be effective for disease control in the field (Browning and Frey, 1969) and, to our knowledge, there is no information to date on the breakdown of disease resistance by blast fungus races that have newly acquired the ability to infect all lines composing a multiline.

To analyze the resistant mechanism of rice plants to blast fungus infection, we used an isogenic line containing the *R* gene *Pi-i* to blast fungus race 003 in the background of cv Nipponbare and first focused on the levels of defense signal compounds after blast fungus infection. Koga (1994) reported that *R*-gene-mediated resistance to blast fungus infection was accompanied by the hypersensitive reaction or response (HR).

On resistance with the HR in dicot plants such as *Tobacco mosaic virus* (TMV) in tobacco (*Nicotiana tabacum*; De Laat and van Loon, 1983; Malamy et al., 1990) and *Cladosporium fulvum* in tomato (*Lycopersicon esculentum*; Hammond-Kosack et al., 1996), SA accumulation and transient ET emission were accompanied by the formation of HR lesions (HRLs). Many reports indicated the importance of SA on HR-mediated resistance against pathogens such as TMV and *Peronospora parasitica* (Gaffney et al., 1993; Delaney et al., 1994); however, SA is reportedly not required for *Cf-2*- and *Cf-9*-dependent resistance of tomato to *C. fulvum* (Brading et al., 2000). In rice plants, SA levels did not increase in the upper leaves in which the resistance to

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blast fungus was induced by *Pseudomonas syringae* D20 preinoculation to the under leaves (Silverman et al., 1995). We also detected no enhanced SA accumulation on *Pi-i* *R*-gene-mediated resistance to blast fungus in young rice plants (T. Iwai, S. Seo, I. Mitsuhara, and Y. Ohashi, unpublished data). Therefore, SA may not be the critical defense signal for induced or *R*-gene-mediated resistance in young rice plants, whereas SA may play an important role in modulating the redox balance protecting rice plants from oxidative stress (Yang et al., 2004). Although JA is known as another important defense signal compound, blast fungus infection did not alter the endogenous JA level and pretreatment with exogenous JA did not induce local resistance to blast fungus infection in a compatible interaction (Schweizer et al., 1997). Our preliminary experiment also showed that spraying JA solution before blast fungus inoculation did not induce a defensive response in a compatible host, resulting in a similar number of growing lesions in both JA-treated and -untreated leaves. Although JA pretreatment before fungal inoculation was reported to induce systemic resistance to blast fungus infection (Schweizer et al., 1998), JA likely does not contribute to local resistance.

On the other hand, the role of ET emission on resistance with the HR was poorly understood. Therefore, we were especially interested in the ET level after blast fungus inoculation in both susceptible and resistant rice lines. We studied the role of ET emission using susceptible wild-type Nipponbare and its isogenic resistant line, IL7, which contains *R* gene *Pi-i* to blast fungus race 003 (Ise and Horisue, 1988). Because the level of pathogen resistance in adult rice plants is likely different from that in young rice plants (Kim et al., 1987; Yeh et al., 1989; Century et al., 1999), we used the fully developed fourth leaf of young rice plants at the four-leaf stage (about 16 d old) as the material. Our data presented here suggest the involvement of enhanced ET biosynthesis, which accompanies the production of not only ET, but also cyanide, for *R*-gene-mediated resistance to blast fungus and the possible contribution of specific types of genes for 1-aminocyclopropane-1-carboxylic acid (ACC) synthase (ACS) and ACC oxidase (ACO) to increased ET biosynthesis after blast fungus infection at a transcriptional level.

RESULTS

Transient Increase in ET Emission during the Formation of HRLs in IL7

First, we established an experimental system to analyze the mechanism of resistance to pathogen infection in rice plants. We used young rice plants at the four-leaf stage of wild-type Nipponbare and its isogenic line, IL7, which contains the *R* gene *Pi-i* (Ise et al., 1988). Wild type is susceptible and IL7 is resistant to infection by blast fungus race 003 (isolate Kyu89-241). In the fully expanded fourth leaf of IL7 plants, small HRLs were found at 42 h postinoculation (hpi) and gradually developed into dark-brown lesions up to 0.5 mm in diameter by 63 hpi, with no remarkable increase in size thereafter (Fig. 1), exhibiting a resistant response to race 003. In wild-type leaves, no detectable phenotype was found within 63 hpi, and whitish expanding lesions (ELs) 0.5 mm in diameter were first observed at 96 hpi, and the ELs

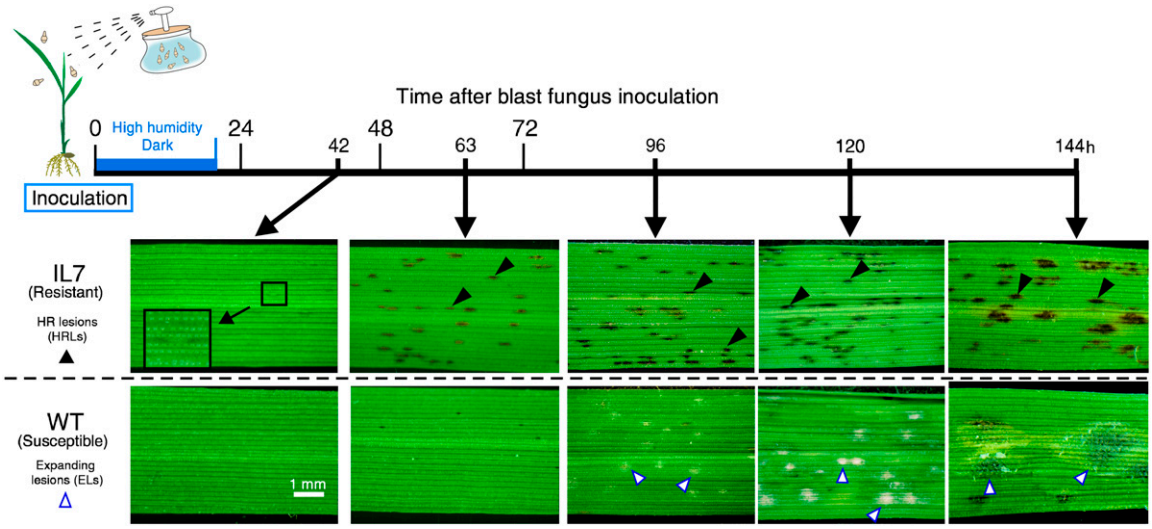

Figure 1. Phenotypes of lesions in young rice leaves inoculated with blast fungus. Photographs of blast fungus-inoculated fourth leaves of IL7 and wild-type (WT) plants at the four-leaf stage. A conidial suspension of blast fungus race 003 was sprayed on an incompatible cultivar IL7 (top), which contains the *R* gene *Pi-i* against blast fungus race 003 in the Nipponbare background, and on wild-type Nipponbare, which is a compatible cultivar (bottom). In inoculated IL7 leaves, HRLs (black arrowhead) appeared at 42 hpi and matured by 63 hpi, turning dark brown. In inoculated wild-type leaves, whitish ELs (white arrowhead) were found at 96 hpi. Bar = 1 mm.

rapidly grew in size thereafter and infected leaves wilted by 144 hpi when inoculated with 1×10^5 conidia mL^{-1}. At lower concentrations, such as 1×10^4 conidia mL^{-1}, ELs grew to about 2×1.5 mm in size, developing conidia at the center of each lesion by 144 hpi (Fig. 1), indicating susceptibility of wild type to infection.

We determined the level of ET emitted from the fourth leaves after blast fungus inoculation, using 16 fourth leaves as one sample (Fig. 2A). One gram of healthy leaf from both IL7 and wild type emitted about 0.9 nL of ET per hour just after detaching. In both mock- and fungus-inoculated IL7 plants, the first peak in ET emission was found at 24 hpi, which was also the case in wild type. This peak by mock inoculation may be caused by the inoculation itself, which involved incubation of plants under high humidity in the dark for 20 h just after spraying the inoculum. The peak at 24 hpi was higher after blast fungus inoculation than mock inoculation in both IL7 and wild type (Fig. 2A), indicating an additive enhancement of ET emission by fungal infection. The second peak of ET emission was detected only in fungus-inoculated IL7 plants at 48 hpi. The enhanced rate of ET emission by fungal inoculation at 48 hpi was 6.4 nL g^{-1} h^{-1} fresh weight in inoculated IL7 and 0.36 nL g^{-1} h^{-1} fresh weight in inoculated wild type, respectively. Because primary HRLs found at 42 hpi (black arrow) were mature by 63 hpi, the significant enhancement of ET emission found in IL7 was thought to be related to the formation of HRLs. During 63 to 96 hpi, emitted ET from inoculated IL7 was maintained at a high level (4 nL g^{-1} h^{-1} fresh

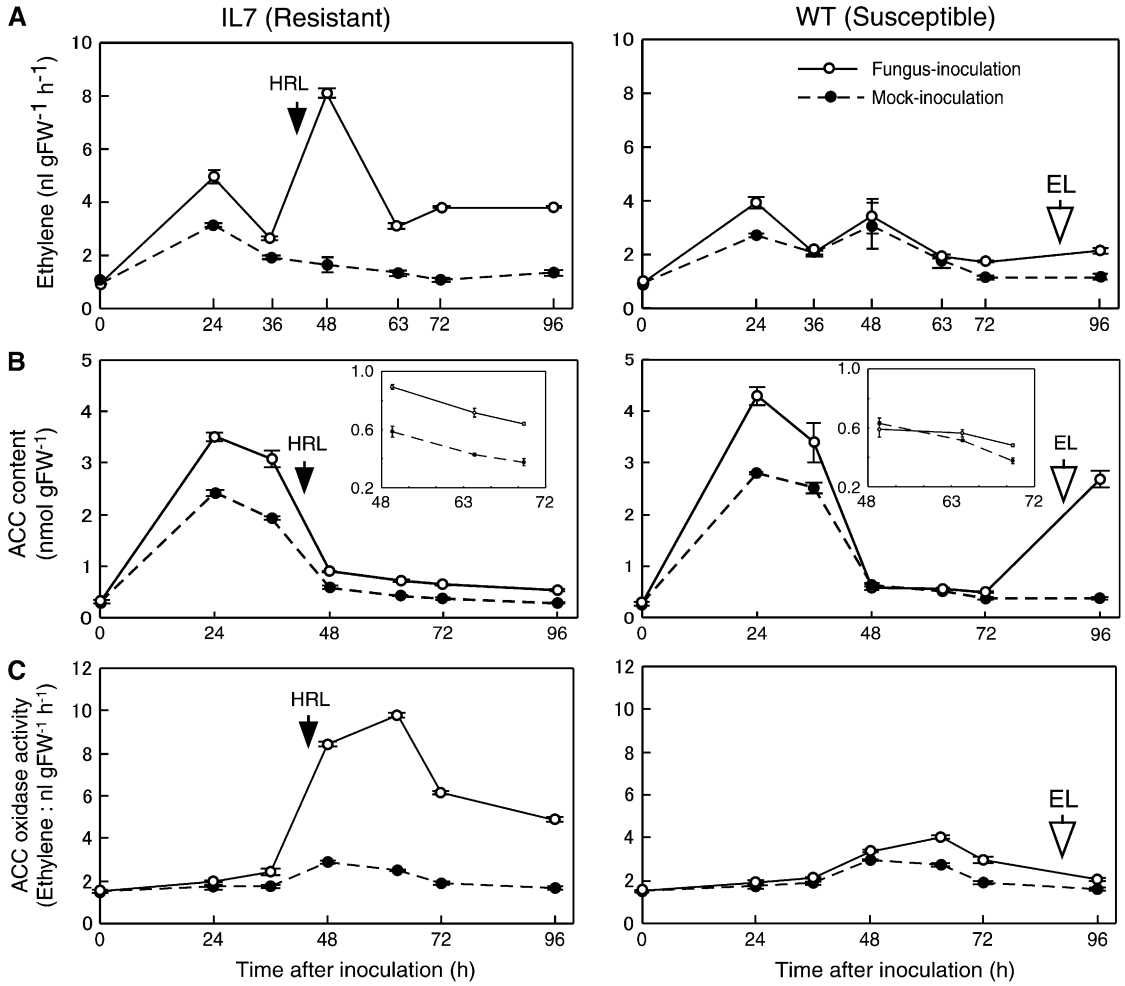

Figure 2. Profiles of ET emission from young rice leaves inoculated with blast fungus. A, ET emission in resistant IL7 leaves and susceptible wild-type leaves determined using 16 fourth leaves at the four-leaf stage for one sample. Solid lines show the level of ET emission after inoculation. Broken lines show the level after mock inoculation. B, ACC content determined using 12 fourth leaves at the four-leaf stage for one sample. Inoculated leaves were homogenized and the ACC in the extract was chemically converted to ET. An expansion at 48 to 72 hpi is shown in frame. C, ACO activity determined as the capacity for converting ACC to ET using 12 fourth leaves at the four-leaf stage for one sample. Values in A, B, and C are shown as the means ± SD based on three independent experiments. The experiment was repeated two times with similar results. The symbol without visible error bars indicates that bars are present inside the symbol.

weight). In inoculated wild-type leaves, the level of ET emission was clearly lower than that in inoculated IL7 and similar to that in mock-inoculated wild-type leaves from 36 to 62 hpi. In wild type, a slight increase was detected at 72 to 96 hpi at which time the development of ELs had started (white arrow). Independent triplicate experiments with similar results confirmed that the data obtained here were reproducible.

The major pathway of ET biosynthesis contains two catalytic steps involving ACS and ACO, producing equal moles of ET and cyanide (Peiser et al., 1984). To study the mechanism of enhanced ET biosynthesis in the resistant response, we determined the levels of emitted ET (Fig. 2A), ACC (Fig. 2B), and ACO activity (Fig. 2C) in blast fungus-inoculated rice leaves at the same time. High levels of ACC were accumulated in both IL7 and wild type at 24 to 36 h after both fungus and mock inoculation. ACC content was 1.5-fold higher after fungus inoculation than mock inoculation, indicating that the fungal infection and/or spraying of the conidial suspension further enhanced ACC accumulation in both rice plants. The peaks at 24 hpi seem to be related to the first peaks in ET emission in Figure 2A. At 48 to 72 hpi during which HRLs were formed and completed, the ACC content of inoculated IL7 leaves was about 1.5- to 1.7-fold that of mock-inoculated leaves, whereas no significant difference in ACC levels was found between mock- and fungus-inoculated wild-type leaves (Fig. 2B). These results suggested that enhanced ACC synthesis and/or suppressed ACC degradation were more strongly induced in resistant IL7 than in susceptible wild type after blast fungus inoculation. In wild type, the ACC level was increased at 96 hpi (Fig. 2B) when EL formation was started. These results indicated that ACC synthesis was accompanied by the formation of both HRLs and ELs.

In inoculated IL7 leaves, ACO activity was dramatically increased at 48 to 63 hpi and remained at a considerable level at 72 to 96 hpi, about 4 nL g^{-1} h^{-1} fresh weight, which was 2.5-fold higher than that in mock-inoculated leaves. In wild-type plants, the profile of ACO activity was quite different from that in IL7. The level was only slightly higher than in mock-inoculated leaves at and after 48 hpi with no clear peak. The time-course profile of ACO activity in inoculated IL7 leaves resembled that of the ET emission profile at 36 to 96 hpi (Fig. 2A), suggesting that increased ACO activity leads to enhanced ET emission in incompatible interaction at and after 48 hpi. At 63 hpi, ACO activity in inoculated IL7 leaves had a peak instead of a rapid decrease in ET emission, probably because of decreased ACC content and/or possible negative regulation by elevated ET.

Characterization of the Rice *ACS* Gene Family

What kinds of ACS and ACO contribute to ET biosynthesis in blast fungus-infected rice plants? We searched for *ACS* and *ACO* genes from expressed sequence tags, full-length cDNAs, and genome databases of rice cv Nipponbare (http://riceblast.dna.affrc.go.jp/; http://cdna01.dna.affrc.go.jp/cDNA).

At least five rice *ACS* genes are reported to exist in the rice genome (Zarembinski and Theologis, 1993); corresponding cDNAs are *OsACS1* (AK071011), *OsACS2* (AK064250), *OsACS3* (P0617H07.9 in AC135427), *OsACS4* (OSJNBb0006B22.3 in AC136224), and *OsACS5* (D46839). *OsACS6* (AK065212) was newly identified as the ortholog of *ACS1* (U35779) from wheat (*Triticum aestivum*). The homology in amino acid sequence between OsACS1 and OsACS2 to OsACS6 is 54%, 59%, 56%, 56%, and 46%, respectively. An alignment of the six putative OsACS polypeptides is shown in Figure 3A. All ACS isoforms contain the seven conserved domains (Fig. 3A, boxes), which were found in ACSs from other plant species (Yamagami et al., 2003). The 11 invariant amino acid residues between ACS and aminotransferases in Arabidopsis (*Arabidopsis thaliana*; Yamagami et al., 2003) and tomato (Rottmann et al., 1991), which are shaded in Figure 3A, were also found in the members of the rice *ACS* gene family. In addition, the Tyr residue at 245 in OsACS1 (black inverted triangle), which is a part of the pyridoxal-5′-P-binding site, was conserved among OsACS1 to OsACS5, and it was replaced by Phe in OsACS6.

A phylogenetic analysis with ACS proteins from rice, Arabidopsis (Yamagami et al., 2003), tobacco (Liu and Zhang, 2004), and wheat (Subramaniam et al., 1996) revealed that the genes fall into three groups (Fig. 3B). ACSs from Arabidopsis in groups I and II exhibited ACS activity, but not in group III, which contains AtACS10 and 12 similar to Ala or Asp aminotransferases in Arabidopsis (Yamagami et al., 2003). Thus, OsACS1 to OsACS5 in groups I or II, but not III, might work in rice.

To elucidate the genomic organization of *ACS* in rice, genomic DNA from rice cv Nipponbare (wild type) was subjected to Southern-blot analysis with mixed probes containing the catalytic domain of ACS, which were prepared by PCR amplification using *OsACS1*, *OsACS2*, and *OsACS5* as templates (Fig. 3C). Because the highest homology based on nucleotide sequence between the probe and *OsACS1* to *OsACS6* is 100%, 100%, 76%, 73%, 100%, and 51%, respectively, the mixed probes would detect five signals corresponding to *OsACS1* to *OsACS5* in the digests by *Eco*RI whose internal site was not found in *OsACSs*. These results suggested that five rice ACSs in groups I and II compose a functional *ACS* gene family.

Characterization of the Rice *ACO* Gene Family

As an *ACO* gene in deepwater rice, *OS-ACO1* (X85747) has been reported in relation to submergence (Mekhedov and Kende, 1996) and *OS-ACO2* (AF049888) and *OS-ACO3* (AF049889) in relation to hormonal cross talk (Chae et al., 2000). We searched for their orthologs

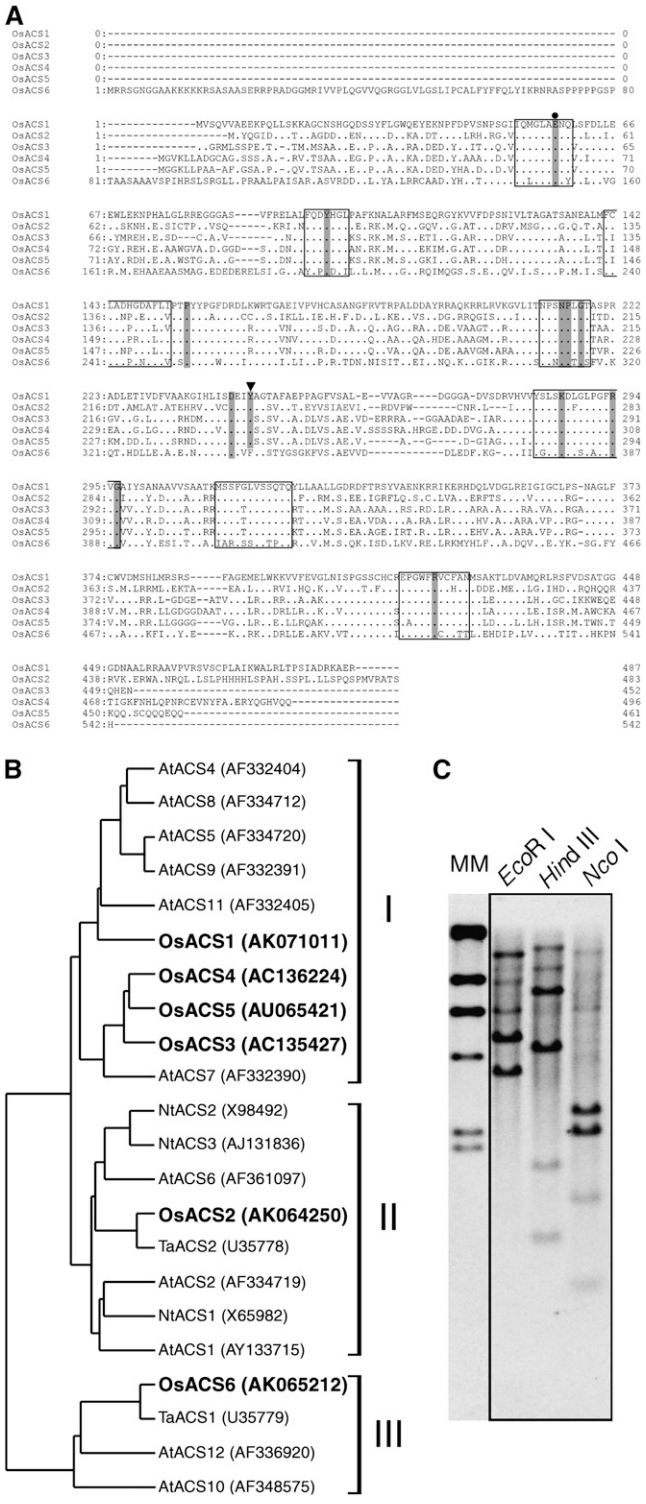

Figure 3. Characterization of the *ACS* gene family in rice plants. A, Amino acid sequence alignment of six putative ACS polypeptides from rice. Shading indicates the 11 invariant amino acids conserved among ACS isozymes and various aminotransferases. The conserved Gln residue (E) marked with the black circle is involved in substrate specificity. The seven conserved domains of the ACS isozymes are marked as boxes. B, Phylogenetic analysis of the putative amino acid sequences of *ACS* genes from rice (Os), Arabidopsis (At), tobacco (Nt), and wheat (Ta) with the unweighted pair group method with arithmetic mean (Sokal and Michener, 1958) using GENETYX. They were classified into three groups (I, II, and III). GenBank accession numbers of each sequence are given in parentheses. C, Genomic Southern-blot analysis of rice *ACS* genes using a common probe for the three rice *ACS* genes. Five micrograms each of rice genomic DNA were digested with *Eco*RI (E), *Hin*dIII (H), and *Nco*I (N), respectively, and applied to each lane. The left lane contains the markers for M_r.

and homologs in expressed sequence tags, full-length cDNAs, and genome databases of rice, and found seven possible *ACO* genes, designated *OsACO1* (AK058296), *OsACO2* (AK071557), *OsACO3* (AK065039), *OsACO4* (AK105491), *OsACO5* (AK061064), *OsACO6* (OJ1504_G04.8 in AC105772), and *OsACO7* (AK102472), respectively. *OsACO6* was mapped next to *OsACO5* in chromosome 5 and we found that *OsACO6* is a pseudogene encoding a truncated ACO peptide. The homology in amino acid sequence between OsACO1 and OsACO2 to OsACO5 and OsACO7 is 93%, 73%, 47%, 48%, and 43%, respectively. An alignment of the six putative ACO polypeptides is shown in Figure 4A. ACO is a member of the Fe(II) ascorbate family of dioxygenases in which the nine amino acid residues shaded in Figure 4A are conserved (Lasserre et al., 1996). Three of these nine residues with an arrowhead contribute to the binding of Fe(II), namely, His at 183, Asp at 185, and His at 240 in OsACO1. OsACO4 lacks two and OsACO5 lacks one of the nine conserved amino acid residues with white and black circles, respectively. Therefore, OsACO4 and OsACO5 might not actually function.

OsACO genes were classified into three major groups in a phylogenic tree based on putative amino acid sequences (Fig. 4B). OsACO1, OsACO2, and OsACO3 are classified in group I, which contains NtACO1 and NtACO2, whose gene expression was accompanied by the formation of HRLs in tobacco (Liu and Zhang, 2004). OsACO7 is classified in group II, which contains StACO3, which was induced in the potato (*Solanum tuberosum*) tuber by inoculation with *Fusarium eumartii* and treatment of SA and indole acetic acid (Zanetti et al., 2002), and LEACO5, whose expression was anaerobically induced (Sell and Hehl, 2005).

Genomic Southern-blot analysis with mixed probes containing the catalytic domain of ACO, which were prepared by PCR amplification using *OsACO1* to *OsACO5* and *OsACO7* as templates, detected six signals in both *Eco*RI and *Hin*dIII digests and seven signals in *Nco*I digests, indicating that seven *OsACO* genes, including the *OsACO6* pseudogene, compose a gene family in rice (Fig. 4C).

Specific *ACS* and *ACO* Genes Are Induced Transiently at 48 hpi in IL7

As described, our results suggested the importance of ET biosynthesis for resistance to blast fungus infection. When the data on ACC content and ACO activity

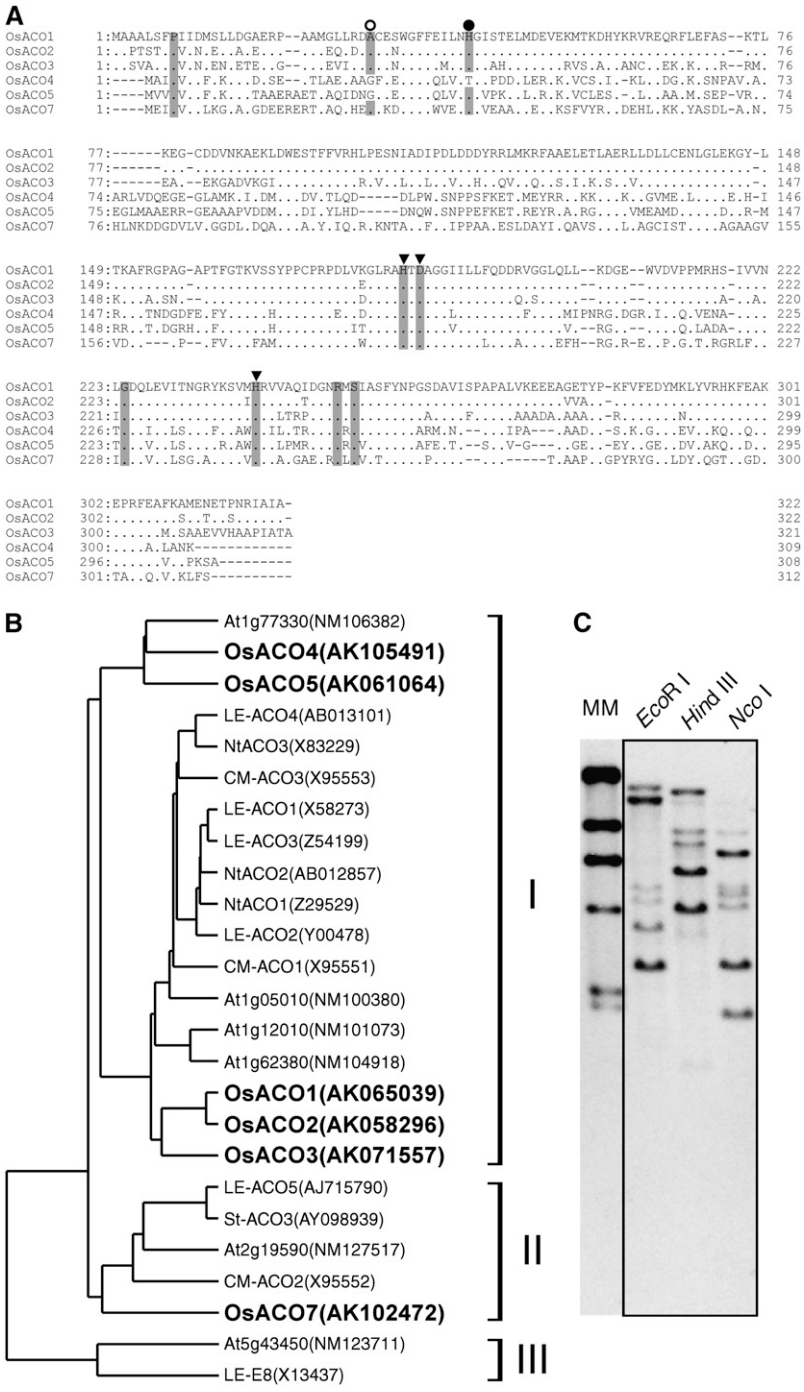

Figure 4. Characterization of the *ACO* gene family in rice plants. A, Amino acid sequence alignment of six putative ACO polypeptides, except OsACO6, which is encoded by a pseudogene. Nine shaded amino acid residues were conserved in members of the Fe(II) ascorbate family of dioxygenases (Lasserre et al., 1996). Three conserved amino acid residues found in ACOs from other plant species for the binding of iron (II), namely, His (H) at 183, Asp (D) at 185, and H at 240, are shown by arrowheads. Amino acid residues with white or black circles were not conserved in OsACO4 or OsACO5. B, Phylogenetic analysis of the putative ACO proteins from rice (Os), Arabidopsis (At), tomato (LE), tobacco (Nt), and melon (*Cucumis melo*; CM) with the unweighted pair group method with arithmetic mean using GENETYX. The proteins were classified into three groups. GenBank accession numbers of the sequences are given in parentheses. C, Genomic Southern-blot analysis using a common probe derived from six rice *ACO* genes. Five micrograms each of rice genomic DNA were digested with *Eco*RI (E), *Hin*dIII (H), and *Nco*I (N), respectively, and applied to each lane. The left lane contains the markers for M_r.

after blast fungus inoculation in Figure 2 were reconstructed in Figure 5A, it became clearer that the levels before and during the formation of HRLs (36–63 hpi) were significantly higher in IL7 than those in wild type, suggesting an additive effect on ET biosynthesis in IL7 leaves. Thus, we studied the expression profiles of *ACS* and *ACO* genes in inoculated IL7 and wild-type plants.

The time-course expression profiles of six *ACS* genes were studied by one-step reverse transcription (RT)-PCR with specific primers for each *ACS* gene (Fig. 5B). Transient *OsACS1* expression was found in mock-inoculated leaves at a low level and in blast fungus-inoculated leaves at a high level at 24 hpi in both plants, indicating *OsACS1* may function for the first peak of ET emission at 24 hpi (Fig. 2A). *OsACS2* was transiently induced at 48 hpi in fungus-inoculated IL7 plants, which is likely related to increased ACC content and dramatic ET emission at 48 hpi (Fig. 2A). A considerable level of *OsACS2* transcript was found at

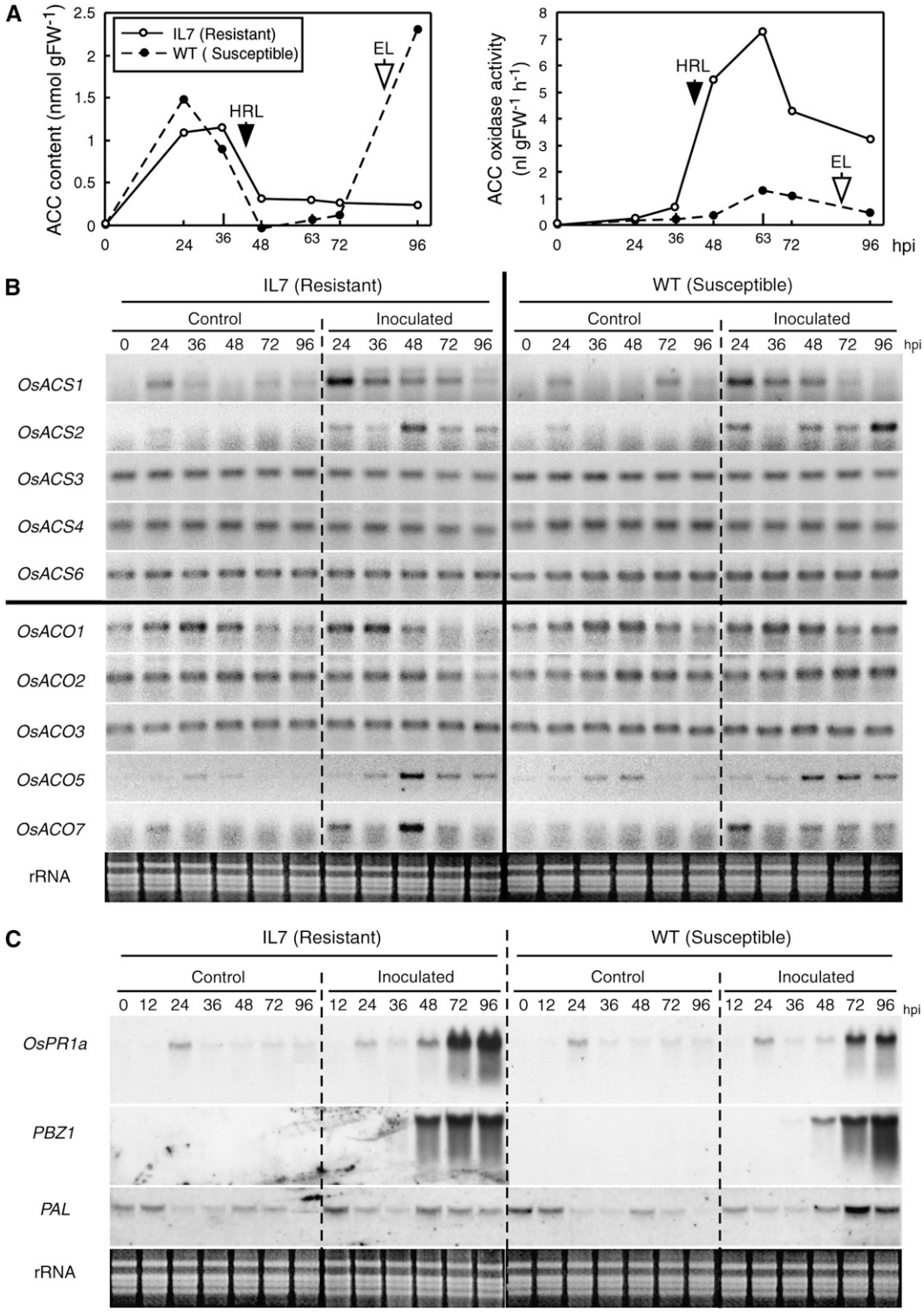

Figure 5. Expression profiles of *ACS, ACO,* and pathogenesis-related (*PR*) genes in young rice leaves infected with blast fungus. A, Comparison of blast fungus-induced increase in ACC content and ACO activity in IL7 (black circle) and wild type (white circle). ACC content and ACO activity of fungus-inoculated leaves were subtracted by those in mock-inoculated leaves from the data in Figure 2, B and C, respectively, and new figures were reconstructed. B, Expression profiles of rice *ACS* and *ACO* genes by one-step RT-PCR using 0.5 μg of total RNA prepared from mock- and fungus-inoculated rice leaves at indicated time points. The sequences of the specific primers for each *OsACS* and *OsACO* gene and the length of amplified products, respectively, are shown in Table I. C, Induced expression of rice PR protein genes. Total RNA extracted from inoculated rice leaves was used for RNA-blot analysis. Specific probes for *OsPR1a*, *PBZ1*, and *PAL* were prepared by PCR using the 3′-untranslated region of each clone. Sequences of the specific primers are given in "Materials and Methods."

96 hpi in wild type, but not IL7. It may be accompanied by the formation of ELs in wild-type plants. *OsACS3*, *OsACS4*, and *OsACS6* were almost constitutively expressed in mock- and blast fungus-inoculated IL7 and wild-type leaves. No signal for the *OsACS5* transcript was found in fourth leaves under the same conditions (data not shown). These results suggest that *OsACS1* contributes to dark- and high humidity-induced ET emission found at 24 hpi, and *OsACS2* mainly acts to increase the ACC level during HRL formation at 48 hpi in IL7 and EL formation at 96 hpi in wild type.

Next, expression profiles of the six *ACO* genes, except for the pseudogene *OsACO6*, were determined using one-step RT-PCR with specific primers for each gene (Fig. 5B). *OsACO1* was detected at 24 to 48 hpi in mock- and fungus-inoculated IL7 and wild-type plants. *OsACO1* may be expressed under dark and high humidity conditions during inoculation, probably contributing to the first peak of ET emission at 24 hpi (Fig. 2A). *OsACO2* was constitutively expressed in both plants, but down-regulated in blast fungus-inoculated IL7 at 72 and 96 hpi. *OsACO3* was constitutively expressed in both plants not affected by the treatments. No signal for the *OsACO4* transcript was found in either plant (data not shown). Expression of both *OsACO5* and *OsACO7* was inducible and enhanced by infection in both plants. Notably, expression of *OsACO7* was very transient and strong at 48 hpi in IL7, but not wild-type plants. Whereas *OsACO5* was also transiently enhanced to express at 48 hpi in IL7, the transcript was found in wild type at 48 to 96 hpi as well. From these results, transient expression of *OsACO7* is thought to be most important for the increase of ACO activity at 48 to 72 hpi in infected IL7 leaves, possibly in cooperation with *OsACO5* expression.

Blast fungus-induced *OsACSs* and *OsACOs* expression profiles were compared with control defense-related genes, such as *OsPR1a* (AJ278436; Agrawal et al., 2000) and *PBZ1* (D38170; Midoh and Iwata, 1996) using each specific probe (Fig. 5C). The transcript for *OsPR1a*, but not *PBZ1*, was transiently and slightly accumulated at 24 h after mock and blast fungus inoculation in both host plants. In addition, in IL7, the transcript of *OsPR1a* was expressed again at 48 hpi, increasing to greater amounts thereafter, at which time the formation of HRLs was completed. In wild type, the *OsPR1a* transcript accumulated at 72 and 96 hpi accompanied by the formation of ELs. *PBZ1* expression was found to be accompanied by the formation of lesions, but not mock inoculation. The transcript accumulated at 36 hpi and was almost saturated at 72 hpi in IL7. In wild type, it was found at 48 hpi, increasing at 96 hpi. Thus, expression of the two defense marker genes was found during and after the formation of lesions in both blast fungus-infected IL7 and wild-type plants. In IL7, these expression levels were remarkably higher after lesions had formed (72–96 hpi) than during their formation (48 hpi), indicating the downstream genes of defense signaling in rice. These expression profiles were clearly different from those of *OsACS2* and *OsACO7*, which are very transient at 48 d postinfection in IL7. Expression of the Phe ammonia lyase (PAL) gene (X16099; Minami et al., 1989) was slightly down-regulated by the inoculation procedure and the level of expression induced by fungus inoculation was higher in wild type than in IL7.

Inhibition of ET Biosynthesis, But Not ET Signaling, Results in Suppressed Resistance to Fungal Infection

Enhanced ET emission during the formation of HRLs in IL7 depends on ET biosynthesis. To confirm the contribution of ET biosynthesis to resistance, the effect of aminooxyacetic acid (AOA), which is an inhibitor of pyridoxal-5′-P-related enzymes, such as ACS (Yu et al., 1979; Yang and Hoffman, 1984), was studied. The fourth leaves of 16 independent blast fungus-inoculated rice plants were detached at 24 hpi and a solution of AOA at 0.1, 0.5, or 1.0 mM was fed through the base of the detached leaf blades for 24 h, respectively. Then the leaves were put in an airtight vial and the level of ET released from the leaf was determined for 3 h (Fig. 6, A and B). Mock-inoculated leaves from IL7 and wild-type plants, which had been detached at 24 hpi and fed with water for 24 h, emitted ET at the rate of 7.5 and 8.5 nL g^{-1} h^{-1} fresh weight, respectively, whereas those from intact IL7 and wild-type plants were 1.7 and 3.1 nL g^{-1} h^{-1} fresh weight, respectively. These results indicate that detaching leaves, which is a kind of wounding, enhanced ET emission from the leaf blade. When mock-inoculated leaves were treated with 0.1 mM AOA solution at 24 hpi, ET emission was diminished to about 1 nL g^{-1} h^{-1} fresh weight in both IL7 and wild type, which is similar to that at time 0 (see Fig. 2B), indicating that wound-induced ET emission was completely suppressed by 0.1 mM AOA. In fungus-inoculated leaves fed water at 24 hpi, the highest increase in ET emission (i.e. 14.8 nL g^{-1} h^{-1} fresh weight in IL7 and 10.6 nL g^{-1} h^{-1} fresh weight in wild type) was observed. When fed with 0.1, 0.5, or 1.0 mM AOA at 24 hpi, fungus-inoculated IL7 leaves emitted ET at the rate of 5.4, 1.1, and 1.0 nL g^{-1} h^{-1} fresh weight, which corresponded to a 64%, 92%, and 93% decrease of that emitted from water-treated control leaves, respectively. In fungus-inoculated wild type, AOA treatment at 0.1, 0.5, or 1.0 mM resulted in an 86%, 91%, and 92% decrease in the level of ET compared with the control, respectively. These results indicate that, for strong inhibition of fungus-induced ET emission, 0.1 mM AOA was not enough and 0.5 mM was required for IL7, whereas 0.1 mM was enough for wild type. Therefore, a higher level of ACS activity, which could not be inhibited by 0.1 mM AOA, seems to be induced in fungus-inoculated IL7, but not in wild type.

To confirm the effect of AOA on resistance to blast fungus in rice, infected leaf pieces were treated at 96 hpi with lactophenol-trypan blue, which stains the mycelium blue. In 0.1 mM AOA-treated IL7 leaf pieces, HRLs remained dark brown and were not stained blue

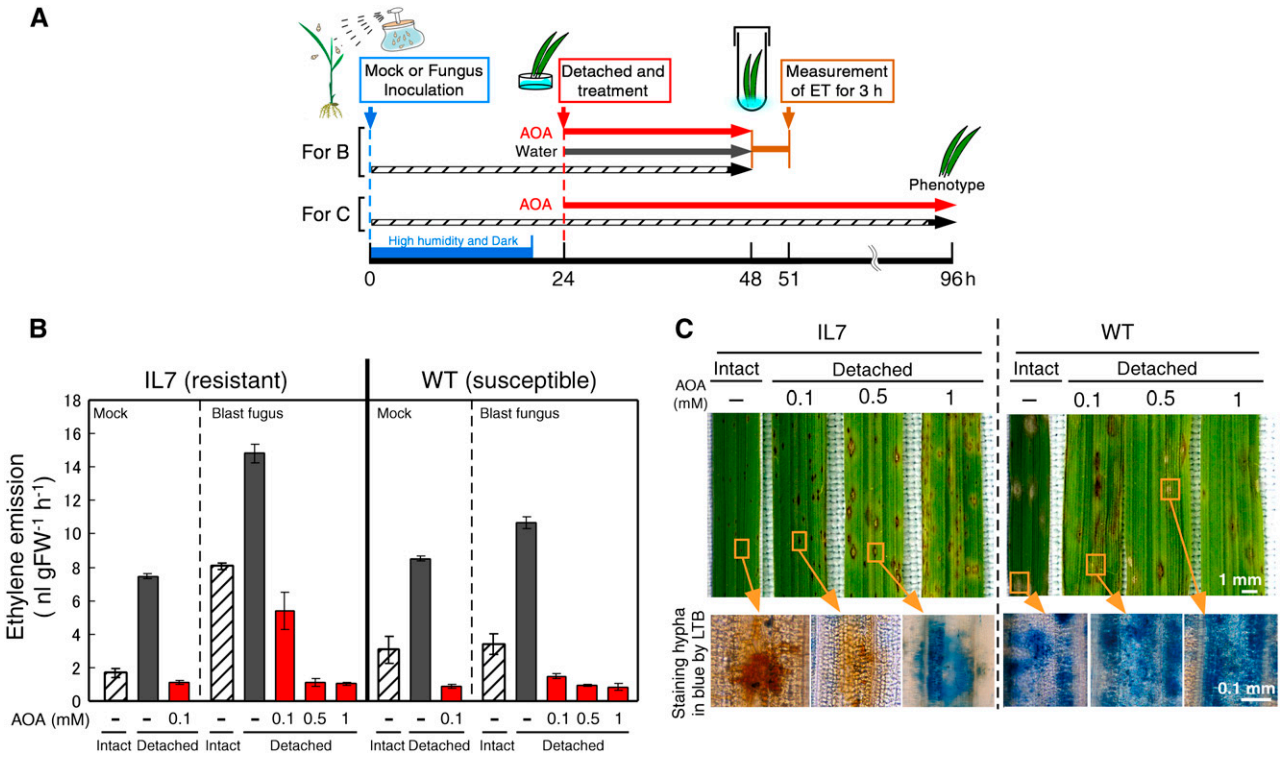

Figure 6. Treatment with AOA results in suppressed ET emission accompanied by breakdown of resistance to blast fungus infection. A, Illustrated procedure of the experiments with AOA. An AOA solution (0.1, 0.5, or 1 mM) or water (control) was supplied from the cut base of the freshly detached leaf at 24 hpi and incubated for a further 24 h to determine the level of ET emission (for B), or for a further 72 h to observe the phenotype of treated leaves (for C). The leaves were transferred into a sealed vial at 48 hpi and the ET emitted from these leaves was determined after 3 h. Sixteen leaves from 16 individual plants at the four-leaf stage were used for one sample. For observation of lesion phenotypes in treated leaves, leaf sections of IL7 and wild type were treated with lactophenol-trypan blue at 96 hpi for staining fungal mycelium in blue. B, Levels of ET emission after AOA treatment. Levels of ET emission are shown as the mean ± SD based on three independent experiments. C, Phenotypes of AOA-treated and fungus-inoculated rice leaves at 96 hpi. Nondetached control leaves are shown at the far left.

as in water-treated leaves (Fig. 6C), indicating that 0.1 mM AOA could not entirely inhibit the formation of HRLs, whereas 63% of ET emission at 48 to 51 hpi was inhibited. However, treatment with 0.5 and 1.0 mM AOA, which almost completely suppressed ET emission, resulted in larger lesions with blue-stained mycelia in IL7 plants. Thus, reduced ET emission possibly led to a breakdown of HRL formation, permitting enhanced mycelial growth in the inoculated IL7 leaves. In fungus-inoculated wild-type leaves treated with 0.1 to 1.0 mM AOA, similar ELs were formed in the water-treated control wild type and 0.5 to 1 mM AOA-treated IL7 leaves.

AOA is known as an inhibitor of ACS, but also that of deaminases and transaminases containing PAL (Amrhein and Gerhardt, 1979). To confirm that the effect of AOA treatment depended on the inhibition of ACS activity, we conducted the same AOA treatment in the presence of ACC, which is the product of ACS. Inoculated or mock-inoculated IL7 leaves were treated with a solution containing 0 or 0.5 mM AOA or both 0.5 mM AOA and 1 mM ACC at 24 hpi, and the rates of ET emission at 48 hpi and development of lesions at 96 hpi were determined (Fig. 7A). In fungus-inoculated control IL7 leaves fed with water, the rate of ET emission was 16.1 nL g^{-1} h^{-1} fresh weight and the formation of HRLs was observed by 96 hpi. In fungus-inoculated IL7 leaves fed with 1 mM ACC, the rate of ET emission was increased to 25.6 nL g^{-1} h^{-1} fresh weight (i.e. 1.6-fold higher compared with water-treated leaves), and similar HRLs were found. Similar to Figure 6, B and C, in the leaves fed with 0.5 mM AOA, the rate of ET emission was decreased to 1.73 nL g^{-1} h^{-1} fresh weight, and the formation of whitish ELs instead of HRLs was observed at 96 hpi. However, treatment with a solution containing both 0.5 mM AOA and 1 mM ACC compromised the breakdown of the resistance, recovering the rate of ET emission to 16.7 nL g^{-1} h^{-1} fresh weight and the formation of HRLs (Fig. 7). These results indicate that the reason for the reduced ACC level would be via AOA-originated inhibition of ACS activity; the main reason of the breakdown of the resistance is inhibition of ACS activity by AOA because exogenously added ACC complemented the resistance.

Does the necessity of ET biosynthesis, which accompanies the production of equal moles of cyanide for

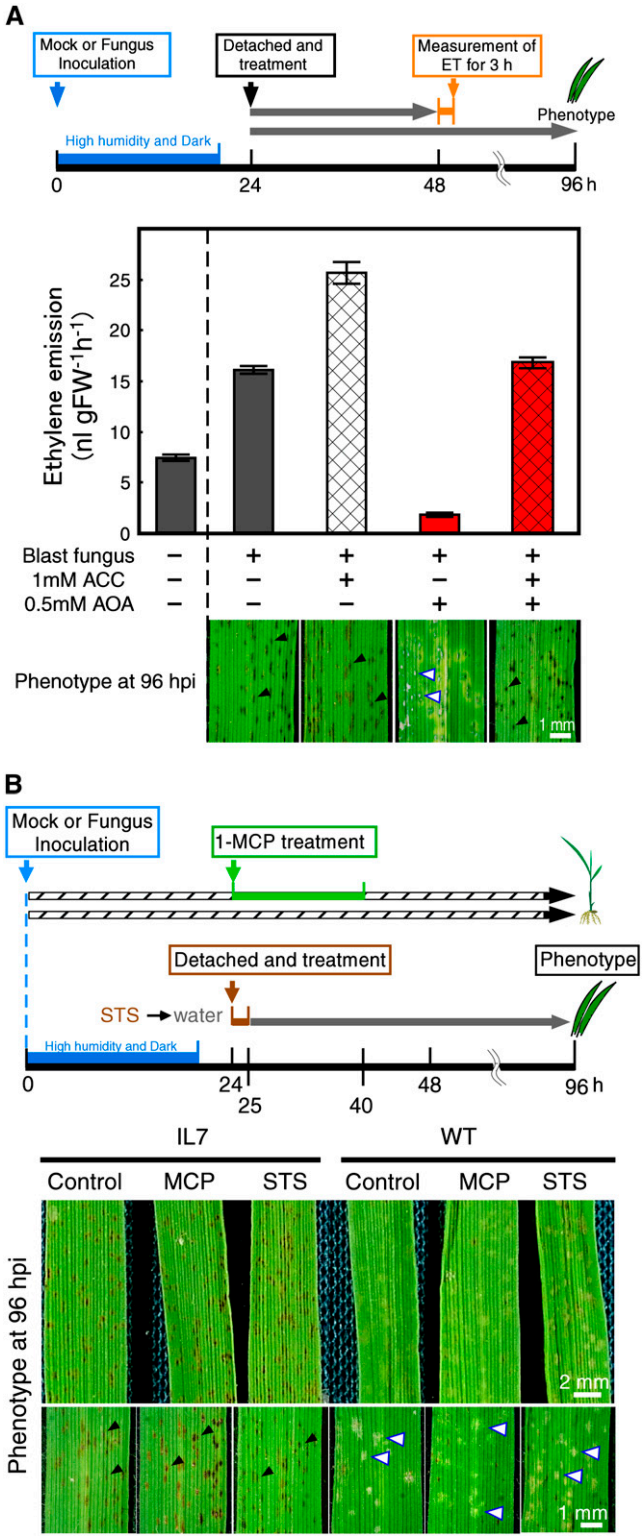

Figure 7. Effects of exogenously supplied ACC and inhibitors of ET action on resistance. A, Exogenously supplied ACC canceled the suppressed resistance and reduced ET emission by AOA treatment in IL7. A solution containing 1 mM ACC, 0.5 mM AOA, or both 0.5 mM AOA and 1 mM ACC was supplied from the cut base of the freshly detached IL7 leaf at 24 hpi and incubated for a further 24 h. Emitted ET from these leaves was quantified at 48 hpi for 3 h using 16 leaves from 16 individual plants at the four-leaf stage. Phenotypes of HRLs in IL7 leaves were observed at 96 hpi. The levels of ET emission are shown as the mean ± SD based on three independent experiments. B, Inhibitors for ET action did not alter the formation and phenotypes of HRLs and ELs in IL7 and wild-type leaves, respectively. Treatments with gaseous 1-MCP at 2 μL L^{-1} and 2 mM STS solution resulted in similar HRL and EL formation in IL7 and wild type at 96 hpi, respectively.

resistance, mean the importance of ET signaling? To elucidate, we examined the effect of the inhibitors for ET action, 1-methylcyclopropene (1-MCP) and silver thiosulfate (STS). Fungus-inoculated rice plants at the four-leaf stage were treated with gaseous 1-MCP 24 hpi for 16 h at 2 μL L^{-1}, which is enough concentration to disturb accelerated ripening and softening of climacteric fruit or accelerated senescence of cut flowers (Serek et al., 1995). Phenotypes of formed HRLs and ELs in the treated IL7 and wild-type leaves were very similar to these in nontreated control leaves at 96 hpi, respectively (Fig. 7B). Treatment with 2 mM STS solution through the base of detached leaves at 24 hpi for 1 h resulted in very similar phenotypes of HRLs and ELs compared with nontreated IL7 and wild-type leaves, respectively (Fig. 7B). These results indicated that inhibitors for ET action would not affect resistance to blast fungus infection, at least in our experimental condition, and the importance of cyanide production via ET biosynthesis.

DISCUSSION

We demonstrated here that ET biosynthesis, but not ET itself, is necessary for resistance to blast fungus infection in young rice plants. In *Pi-i*-mediated resistance in IL7 plants, enhanced ET emission at 48 hpi is distinctive because the phenomenon was not found in fungus-infected wild-type plants or mock-inoculated IL7 and wild-type plants. AOA treatment, which induced remarkable inhibition of ET emission, broke down resistance in IL7, preventing the formation of HRLs. Addition of ACC compromised the AOA-induced breakdown of resistance, indicating ACC synthesis by ACS is critical for resistance. Treatment of 1-MCP and STS, the inhibitors of ET action, did not induce the breakdown of resistance. From the characterization of the rice *ACS* and *ACO* gene families, we found that enhanced ET emission during the formation of HRLs would be supported by specific *ACS* and *ACO* genes at the transcriptional level.

Mechanism of ET Biosynthesis in Resistant Response

In our experimental system, the resistant cultivar IL7 exhibits strong resistance to blast fungus race 003; saturated numbers of solid HRLs small in size and dark brown in color were detected at about 63 hpi, with no remarkable enlargement thereafter. On the other hand, typical susceptibility is found in wild type, which lacks the *Pi-i* gene in almost the same genetic

Table I. *DNA sequence of primers for RT-PCR*

Target Gene	Accession No.	Forward	Reverse	Product Length
				bp
OsACS1	AK071011	TCGGCCAAGACCCTCGACG	CGAAAGGAATCTGCTACTGCTGC	300
OsACS2	AK064250	ACCTGCGGCACAATCGCGGG	GAAGGCGAGCGTCTCCTGGG	293
OsACS3	AC135427	GCTGCTTCATCAAGAAATGGGAGC	GATGGAGCCATAGAGGGAGC	255
OsACS4	AC136224	CATGGACGCGTGGTGCAAAGC	CCTCCCTCTCTTCTCCCAATCTCG	271
OsACS5	AU065421	CACGGTCAGGCGCAAGATGC	CGTGTCCATGAAGCGGCTTATCCTG	670
OsACS6	AK065212	GAGCGAACATGATATCCCTGTC	GCTCCTACTGGTTGTTAGGAG	217
OsACO1	AK065039	GATAGCGTGTGTACCACAGCGACC	CACGGTACAGCACGCCGCAC	254
OsACO2	AK058296	AGCAACCCCGGCCTCGCTC	AGGGACTTGCTATGACACGG	242
OsACO3	AK071557	CGCCGCCGAGGTCGTCCACG	GCCCGTTACACACACTTGAG	242
OsACO4	AK105491	CCCGCAGTGAAGGCTCTCGC	GTTCGCCAGGGCTGCGAACC	723
OsACO5	AK061064	CCGAAGGAGCTTCTTGATCGG	ATTTTGGCGCCTTGACGGCC	793
OsACO7	AK102472	GTGATCGCGCCGGCGACGGC	GGGGAACCCTGCGTACTAC	257

background; whitish ELs were first visualized at 96 hpi, and they rapidly develop in size with vigorous conidiation in the center. In this system, the time point around 48 hpi is crucial to detect the difference in resistance to blast fungus because, at this time, HRLs have just started to form in IL7 leaves, whereas no clear phenotype is found in inoculated wild-type leaves (Fig. 1). Two peaks of ET emission were observed in IL7 and wild-type plants infected with blast fungus race 003. The first peak at 24 hpi was likely a result of the darkness and high humidity in both host plants, and the second peak at 48 hpi was specific for the formation of HRLs in infected IL7 plants (Fig. 2A).

The level of the second peak in ET emission was almost proportional to the number and size of developing HRLs. When the conidial suspension (1×10^5 conidia mL^{-1}) was sprayed onto IL7 plants at the four-leaf stage, about 150 HRLs were detected on the fourth leaf accompanied by ET emission at the rate of 7 nL g^{-1} h^{-1} fresh weight at 48 hpi. This level of ET emission is similar to the formation of HRLs mediated by the *N* gene (18 nL g^{-1} h^{-1}fresh weight) in TMV-infected tobacco plants (De Laat and Van Loon, 1981). The sharp peak in ET emission similar to the second peak in infected IL7 plants was observed during the formation of HRLs in *N*-gene-mediated resistance in TMV-infected tobacco plants (De Laat and Van Loon, 1981, 1983) as well as *Cf*-gene-mediated resistance in *C. fulvum*-infected tomato plants (Hammond-Kosack et al., 1996). Enhanced ET emission at 48 to 72 hpi, when HRLs developed and matured in infected IL7 plants, was accompanied by infection-enhanced ACC accumulation and ACO activity (Figs. 2 and 5A). The amount of ACC at 36 to 72 hpi was slightly, but significantly, higher in infected IL7 than infected wild-type leaves, and it reversed at 96 hpi, at which time formation of ELs is going on, indicating that ACC synthesis was accompanied by the formation of not only HRLs but also ELs in rice leaves infected with blast fungus (Fig. 5A). In *N*-gene-mediated resistance, accumulation of increased ACC was reportedly restricted to the area of HRLs (De Laat and Van Loon, 1983), suggesting localization of increased ACC at the area of HRLs and ELs in rice plants. ACO activity was dramatically and specifically elevated during the formation of HRLs in infected IL7 plants (Fig. 2C), suggesting enhanced ACO activity and increased levels of ACC at 36 to 63 hpi are important for ET emission from HRLs.

In deepwater rice, *OS-ACS1*, whose product shares 99% homology with OsACS1 from Nipponbare wild type, was induced by partial submergence at the uppermost elongating internode and involved in stem elongation (Zarembinski and Theologis, 1993, 1997). Lowland rice plants, such as Nipponbare wild type and IL7, possibly recognized the dark and high humidity conditions as partial submergence, and ET emission might be enhanced at 24 hpi in both mock- and blast fungus-inoculated leaves. OsACS1 was classified into group I, which contains auxin-responsive AtACS4 and AtACS5 (Tsuchisaka and Theologis, 2004), suggesting involvement in plant hormone-mediated responses. OsACS2 was classified into group II. Group II contains *AtACS6*, which was rapidly induced by ozone exposure-induced cell death (Overmyer et al., 2000) and NtACS1, NtACS2, and NtACS3, whose gene expression was induced during an *N*-gene-mediated TMV-resistant response in tobacco (Kim et al., 2003). Thus, ACS proteins in group II would function in response to biotic stresses, including pathogen infection. Phosphorylation was also reported to be important for ACS proteins belonging to group II, such as AtACS2 and AtACS6, which are very similar to OsACS2. These AtACS proteins are stabilized by phosphorylation at their C-terminal regions by Arabidopsis mitogen-activated protein kinase 6, the ortholog of tobacco SA-induced protein kinase, whose activation confers TMV resistance (Liu and Zhang, 2004). Thus, studies on posttranscriptional and posttranslational regulation of OsACS2 would also be important to elucidate the dynamic regulation of ET biosynthesis for disease resistance.

Maintenance of a high level of ACO activity at 48 to 96 hpi may guarantee a considerable level of ET biosynthesis during this period. Two newly characterized rice *ACO* genes, *OsACO5* and *OsACO7*, out of seven *ACO* members, were transiently induced by blast fungus in IL7 plants (Fig. 5B). Because OsACO5 lacks one of nine conserved amino acid residues, it might confer weaker or no ACO activity (Fig. 4A). *OsACO5*

expression was induced by blast fungus infection in both IL7 and wild type in a similar manner, suggesting no relation to resistance. Interestingly, the maximal level of the *OsACO7* transcript was found at 48 hpi in infected IL7, but not wild type, indicating a transcriptional contribution of *OsACO7* to ET emission during the formation of HRLs in IL7. It was also increased at 24 hpi in both IL7 and wild type, possibly indicating a contribution to the first peak of ET emission at 24 hpi. OsACO7 belongs to group II, as well as *StACO3*, which was induced by infection with *F. eumartii* in potato (Fig. 4B). On the other hand, information on transcriptional regulation of plant *ACO* genes is limited and we could find no evidence of posttranscriptional regulation of ACO in plants. Thus, the mechanism on the regulation of ACO for disease resistance remains to be solved.

Role of ET Biosynthesis in the Resistance Response

In this article, we proposed the involvement of ET biosynthesis, but not ET itself, in resistance to blast fungus infection in rice plants. This indicates the positive role of ET biosynthesis in blast fungus-dependent HRL formation and suppression of subsequent fungal growth. AOA treatment at 24 hpi clearly inhibited ACC synthesis and subsequent ET emission in IL7 at 48 hpi, inducing EL-like lesions with vigorous hyphal growth instead of HRLs, which are observed in the absence of AOA (Fig. 6, B and C). Addition of ACC canceled the inhibitory effect of AOA, recovering enhanced ET emission and formation of HRLs in IL7 (Fig. 7A). These results indicate that ET biosynthesis is essential for *Pi-i* (*R*-gene)-mediated resistance. In the cytological studies reported by Koga (1994), hypersensitive cell death occurred during fungal penetration and little growth of invading hyphae was permitted after the host cell had died. At the final step of ET biosynthesis, endogenous ACC is converted by ACO to equal moles of ET and cyanide, which is an inhibitor for the mitochondrial respiratory chain (by blocking cytochrome oxidase in complex IV). Thus, the role of ET itself for disease resistance should be separately elucidated from the role of ET biosynthesis, which accompanies cyanide production. Using inhibitors for ET action, such as 1-MCP and STS, we evaluated the role of ET itself for resistance to blast fungal infection. Treatment of gaseous 1-MCP (2 μL L^{-1}) for 16 h or STS (2 mM) for 1 h at 24 hpi could not significantly affect the formation and the phenotypes of HRLs in inoculated IL7 leaves and ELs in inoculated wild-type leaves (Fig. 7B). These results indicate the importance of cyanide production for resistance rather than ET production. This evidence is coincident with the information that HRLs were generated in response to avirulent fungi and bacteria such as *C. fulvum*, *P. parasitica*, and *P. syringae* pv *glicinea* in ET-insensitive mutants or transgenic plants with modified ET receptors (Brading, 1997; Van Loon et al., 2006). The contribution of cyanide for blast fungus protection was also suggested by the studies using an effective fungicide metominostrobin (SSF126) in rice plants (Mizutani et al., 1996). The agrochemical is a derivative of strobilurin A, which covers a wide range of antifungal spectra. SSF126 strongly inhibited mycelial growth of blast fungus in vitro by prohibiting the mitochondrial oxidative respiration chain at the earlier period. However, 20 min after the treatment, the mycelia again began to respire, inducing cyanide-resistant respiration, which is sensitive to salicyl hydroxamic acid (Mizutani et al., 1995). On the other hand, cyanide-resistant respiration of the fungus was inhibited by flavonoid compounds such as flavone, flavanone, and naringenin, which widely exist in the plant kingdom, proposing the following mechanism that the inhibition of cyanide-sensitive respiration by SSF-126 and inhibition of cyanide-resistant respiration by flavonoids cooperatively suppress fungal growth (Mizutani et al., 1996). Actually, blast fungus-induced accumulation of a flavanone phytoalexin, sakuranetin, was detected at 40 hpi, and the level was increased thereafter only in resistant rice plants (Kodama et al., 1992). The importance of cyanide production via ET biosynthesis to resistance was emphasized by our results presented here; however, the possible cooperation of ET, which is produced from ACC at the same time, could not be excluded for the resistance mechanism.

The analysis of rice *ACS* and *ACO* gene families pointed out that specific members, such as *OsACS2* and *OsACO7*, are transcriptionally activated during the formation of HRLs in fungus-infected IL7 leaves. The expression profile of a gene often suggests its function, but the studies about modification of the gene products such as dynamic activation/inhibition or stabilization/unstabilization would also be important. Loss- or gain-of-function studies about OsACS2 and OsACO7 would clearly indicate the roles of these genes in the defense against blast fungus in relation to ET biosynthesis. Such experiments have been started in our laboratory.

MATERIALS AND METHODS

Plant Materials

Rice (*Oryza sativa* cv Nipponbare) and the isogenic line IL7 (Ise and Horisue, 1988), which carries the *R* gene *Pi-i* against blast fungus (*Magnaporthe grisea*) race 003 (isolate, Kyu89-241; Yamada et al., 1976), were grown for about 3 weeks in a greenhouse at 25°C. The fourth leaf of 3-week-old young plants at the four-leaf stage was used as the material in all experiments. For ET analysis, 24 and 16 fourth leaves at the four-leaf stage were used as one sample, respectively.

Inoculation with Rice Blast Fungus

Blast fungus race 003 was grown on oatmeal medium (Difco) for 2 weeks at 26°C in the dark, and then spores were induced to form under a 20-W BLB light (FL20S BLB; Toshiba) for 2 to 3 d at 24°C. A spore suspension (1×10^5 conidia mL^{-1}) containing 0.05% (w/v) Tween 20 was sprayed onto rice plants. The inoculated plants were incubated at 25°C with high humidity in the dark for 20 h and then moved to a greenhouse.

Light Microscopy

Blast fungus-inoculated leaves, cut 0.5 cm in length, were vacuum infiltrated with water and then stained with a lactophenol-trypan blue

solution containing 10 mL of lactic acid, 10 mL of glycerol, 10 g of phenol, and 10 mg of trypan blue dissolved in 10 mL of distilled water (Koch and Slusarenko, 1980). Leaf segments were boiled for 3 min in the stain solution and decolorized in a chloral hydrate solution containing 2.5 g of chloral hydrate dissolved in 1 mL of distilled water for at least 24 h. They were mounted in the chloral hydrate solution and viewed under a microscope.

Chemical Treatments

AOA was purchased from Sigma-Aldrich. AOA was dissolved in water and the pH adjusted to 7.0 with NaOH solution. 1-MCP was provided by Rohm and Haas. Two millimolar STS solution was prepared by adding 20 mL of 0.01 M silver nitrate (Sigma-Aldrich) solution to 80 mL of 0.01 M STS (Sigma-Aldrich) solution. Inoculated fourth leaves were detached at 24 hpi and fed with solutions of 0.1, 0.5, and 1.0 mM AOA, 1 mM ACC, both 0.5 mM AOA and 1 mM ACC, or 2 mM STS, respectively, from the cut base of freshly detached leaf blades. Inoculated rice plants at the four-leaf stage were treated with gaseous 1-MCP in airtight containers at 24 hpi for 16 h, following the manufacturer's instructions.

Measurement of ET Emission

At 0, 24, 36, 48, 63, 72, and 96 h after the inoculation, fourth leaves were detached from the base of the leaf blade. Sixteen leaves were put into 52-mL glass vials with 5 mL of water, sealed with a gas-proof septum, and left in a growth cabinet at 24°C for 3 h under light. One milliliter of gas was withdrawn from the airspace of each tube using a gas-tight syringe (Hamilton) and injected into a gas chromatograph (Shimadzu GC-14B) equipped with an aluminum column (Shumpak-A; Shimazu) and a flame-ionization detector for ET determination.

Determination of ACC Content

Leaf material frozen in liquid nitrogen was ground with a mortar and pestle and stirred with a 5% (w/v) sulfosalicylic acid solution (2 mL g^{-1} fresh weight) for 30 min at room temperature. The concentration of ACC in the supernatant after centrifugation at 30,000*g* for 30 min was determined directly by chemical conversion to ET according to Lizada and Yang (1979), with modifications by De Laat and Van Loon (1983).

Determination of ACO Activity

The ACO assay was performed as described by Mekhedov and Kende (1996). Twelve mock-inoculated or blast fungus-inoculated fourth leaves from 12 individual plants (about 0.5 g fresh weight) were homogenized with 1.0 mL of extraction buffer (100 mM Tris-HCl, pH 7.2, containing 30 mM sodium ascorbate and 10% [v/v] glycerol) in triplicate. ACO activity was analyzed by incubation of a total of 2.0 mL of reaction mixture (1.7 mL of extraction buffer, 50 μL of 40 mM ACC solution, 50 μL of 2 mM $FeSO_4$ solution, and 200 μL of leaf extract) at 30°C for 3 h in a sealed 9-mL glass vial. One milliliter of the headspace was withdrawn and analyzed for ET in a gas chromatograph as described above.

DNA- and RNA-Blot Analyses

DNA- and RNA-blot analyses were performed using the digoxigenin nonradioactive nucleic acid labeling and detection system (Roche), following the manufacturer's instructions.

Genomic DNA was isolated from rice seedlings as described by Murray and Thompson (1980). DNA-blot analysis was performed using 5 μg of genomic DNA from rice cv Nipponbare after digestion with *Eco*RI, *Hin*dIII, and *Nco*I and a common probe for rice *ACS* or *ACO* genes under low stringency conditions (two washes with 1 × SSC and 0.1% SDS at 68°C for 20 min). Sequences of the primers for the probe for rice *ACS* in Figure 4C are 5′-CAG(A/C)T(C/G)GG(C/T)CTCGCCGAGAAC-3′ and 5′-GTC(A/C/G)(A/C)A(A/C)(C/G)C(A/G/T)GGGTAGTA(A/T)GG-3′. Those for the probe for rice *ACO* in Figure 5C are 5′-CTCCGCGCCCACACCGAC-3′ and 5′-GGGTTGTAGAA(C/G)G(A/T)(C/G)GCG-3′.

Total RNA was extracted from rice leaves using the auxin tricarboxylic acid method described by Nagy et al. (1988). Twenty micrograms of total RNA were subjected to RNA-blot analysis using a specific probe under high stringency conditions (two washes with 0.1 × SSC and 0.1% SDS at 68°C for 20 min). Sequences of the primers for the *OsPR1a* (accession no. AJ278436) probe in Figure 6C are 5′-TACGGCGAGAACATCTTCTGG-3′ and 5′-GTAGTTG-CAGGTGATGA-3′. Those for the *PBZ1* (accession no. D38170) probe are 5′-AAGGTGGAGTACGAGCTCGAGG-3′ and 5′-GGTGGGATATACTGGAT-AGAGGC-3′. Those for the *PAL* (accession no. X16099) probe are 5′-GCA-GAAGCTCCGCGCCGTGC-3′ and 5′-TGATGGGTGTATGGCAATGG-3′.

One-Step RT-PCR

Total RNA was extracted from inoculated rice leaves using TRIzol Reagent (Invitrogen). Also, 0.5 μg of total RNA as template was supplied for amplification of rice *ACO* and *ACS* genes using the SuperScript One-Step RT-PCR system with Platium *Taq* (Invitrogen). Specific primers were designed based on DNA sequences reported to GenBank and displayed the primer DNA sequence listed in Table I. The specificity of the primers was checked by excising the RT-PCR products after electrophoresis. Among 0.01 to 1 μg of total RNA as a template for RT-PCR, amplification products for each gene increased linearly. PCR amplification conditions were 50°C for 30 min, 96°C for 2 min, followed by 30 cycles of 96°C for 30 s, 56°C for 30 s, and 72°C for 1 min, and then one cycle of 72°C for 5 min.

The relative transcript amounts were visualized by using a luminescent image analyzer LAS-1000plus (Fujifilm) from the images of agarose gels after electrophoresis.

Accession numbers of each gene are described in parentheses: *OsACS1* (AK071011), *OsACS2* (AK064250), *OsACS3* (P0617H07.9 in AC135427), *OsACS4* (OSJNBb0006B22.3 in AC136224), *OsACS5* (D46839), *OsACS6* (AK065212), *OsACO1* (AK058296), *OsACO2* (AK071557), *OsACO3* (AK065039), *OsACO4* (AK105491), *OsACO5* (AK061064), *OsACO6* (OJ1504_G04.8 in AC105772), *OsACO7* (AK102472), *OsPR1a* (AJ278436; Agrawal et al., 2000; these cDNA clones, except *OsACS4* and *OsACO6*, were provided from the Rice Genome Resource Center in the National Institute of Abrobiological Sciences [Kikuchi et al., 2003]), *PBZ1* (D38170; Midoh and Iwata, 1996), and *PAL* (X16099; Minami et al., 1989).

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