Inducible Overexpression of a Rice Allene Oxide Synthase Gene Increases the Endogenous Jasmonic Acid Level, *PR* Gene Expression, and Host Resistance to Fungal Infection

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Many studies in dicotyledonous plants have shown that jasmonates, including jasmonic acid (JA) and methyl jasmonate, are important signal molecules involved in induced resistance to pathogen infection and insect herbivory. However, very little genetic and molecular evidence is available to demonstrate their role in host defense response of rice and other economically important monocot plants. In this study, we have shown that exogenous application of JA was able to activate defense gene expression and local induced resistance in rice seedlings against the rice blast fungus (*Magnaporthe grisea*). Furthermore, we have characterized a pathogen-inducible rice *OsAOS2* gene (which encodes allene oxide synthase, a key enzyme in the JA biosynthetic pathway) and examined the role of endogenous JA in rice defense response through transgenic manipulation of the JA biosynthesis. Sequence analysis indicated that *OsAOS2* contains four common domains of the cytochrome P450 enzyme, but does not have the signal peptide for chloroplast targeting. The basal level of *OsAOS2* expression is very low in leaves but relatively high in the sheath, culm, and flower of rice plants. Interestingly, the expression of *OsAOS2* in rice leaves can be induced significantly upon *M. grisea* infection. Transgenic rice lines carrying the *OsAOS2* transgene under the control of a strong, pathogen-inducible *PBZ1* promoter accumulated abundant *OsAOS2* transcripts and higher levels of JA, especially after the pathogen infection. These transgenic lines also exhibited enhanced activation of pathogenesis-related (*PR*) genes such as *PR1a*, *PR3*, and *PR5* and increased resistance to *M. grisea* infection. Our results suggest that JA plays a significant role in *PR* gene induction and blast resistance in rice plants.

Additional keywords: *Oryza sativa*

Jasmonates are lipid-derived regulators that mediate biotic and abiotic stress responses as well as plant growth, development, and senescence (Creelman and Mullet 1997; He et al. 2002; Howe and Schilmiller 2002; Turner et al. 2002; Weber 2002). A large body of genetic and molecular evidence has shown that jasmonates are key endogenous signals involved in plant defense response to pathogen infection and insect herbivory (Farmer et al. 2003; Kessler et al. 2004). For example, *Arabidopsis* mutants impaired in jasmonic acid (JA) perception (e.g., *coi1*, *jar1*, and *jin1*) or biosynthesis (e.g., *aos*, *fad3/7/8*, *opr3/dde1*, and *dad1*) are unable to deploy effective defense responses against pathogen infection (Feys et al. 1994; Park et al. 2002; Penninckx et al. 1996; Sanders et al. 2000; Stintzi and Browse 2000; Thomma et al. 1998; Turner et al. 2002; Vijayan et al. 1998; Xie et al. 1998). By contrast, *Arabidopsis* mutants (e.g., *cev1*) with constitutive activation of the JA pathway exhibit enhanced resistance to bacterial and fungal pathogens (Ellis et al. 2002; Ellis and Turner 2001). In addition to *Arabidopsis*, the importance of jasmonates in defense gene expression and disease resistance also has been demonstrated in tomato, tobacco, and potato plants through mutant or transgenic plant analysis (Cohen et al. 1993; Harms et al. 1995; Howe et al. 2000; Howe and Ryan 1999; Laudert et al. 2000; Li et al. 2001; Sivasanker et al. 2000; Wang et al. 1999).

Although the role of JA in disease resistance has been well established in dicotyledonous plants, little is known about its function in host defense response of rice and other economically important monocot plants. To date, most of the information comes from the studies of exogenous JA or methyl jasmonate (MeJA) treatments on the induction of rice defense genes or phytoalexins. Exogenous JA treatment was shown to induce a number of pathogenesis-related (*PR*) genes in rice, including *PR1a* (Agrawal et al. 2000a), *PR1b* (Agrawal et al. 2000b), *PR2* (Simmons et al. 1992), *PR3* (Xu et al. 1996), *PR5* (Rakwal and Komatsu 2000), and *PR10* (Jwa et al. 2001). JA also induces many other stress or defense-related rice genes (Lee et al. 2001; Moons et al. 1997; Rakwal and Komatsu 2000; Sasaki et al. 2001; Xiong et al. 2001). In addition, exogenous application of JA was reported to increase the production of phytoalexins sakuranetin and momilactone A in rice leaves (Nojiri et al. 1996; Tamogami et al. 1997). Reduction of endogenous JA levels with tetcyclacis, an inhibitor of jasmonate biosynthesis, significantly inhibits the pathogen-induced *PR1* accumulation (Schweizer et al. 1997a). These results indirectly demonstrate that JA may play an important role in defense

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gene activation and disease resistance responses in rice plants. However, Schweizer and associates (1997b) showed that inoculation of rice seedlings with *Magnaporthe grisea* or *Pseudomonas syringae* pv. *syringae* (a nonpathogen) did not significantly increase the endogenous levels of JA despite the induction of *PR* genes. Paradoxically, exogenous JA application or wounding (which induces transient accumulation of JA) only led to induced blast resistance in systemic leaves but not in local, treated leaves (Schewizer et al. 1998). Therefore, the role of JA in rice defense response is not clear and needs to be further elucidated through more robust genetic and molecular analysis using rice mutants or transgenic lines with altered JA production or signal perception.

The jasmonate biosynthetic pathway, which begins with fatty acid linolenic acid, has been fairly well characterized in plants (Liechti and Farmer 2003; Turner et al. 2002). Allene oxide synthase (AOS, EC 4.2.1.92, hydroperoxide dehydratase) is a key enzyme in the pathway and catalyzes the dehydration of 13-hydroperoxy octadecatrienoic acid to an unstable epoxide, which then is converted by allene oxide cyclase to the cyclopentenone ring-containing 12-oxo-phytodienoic acid (OPDA), the direct precursor of JA. AOS first was identified as a cytochrome P450 enzyme from flax (Song and Brash 1991) and subsequently was cloned and characterized in flax (*Linum usitatissimum*; Song et al. 1993), guayule (*Parthenium argentatum*; Pan et al. 1995), *Arabidopsis* (Laudert et al. 1996), tobacco (*Nicotiana attenuata*; Ziegler et al. 2001), tomato (*Lycopersicon esculentum*; Howe et al. 2000; Sivasankar et al. 2000), barley (*Hordeum vulgare*; Maucher et al. 2000), and rice (*Oryza sativa*; Agrawal et al. 2002; Ha et al. 2002; Haga and Iino 2004). In *Arabidopsis*, the expression of AOS is activated by wounding, jasmonate, and salicylate and occurs in anthers and pollens (Kubigsteltig et al. 1999; Laudert and Weiler 1998). However, overexpression of *Arabidopsis* AOS using constitutive CaMV 35S promoter did not alter the basal level of JA in transgenic *Arabidopsis* and tobacco (Laudert et al. 2000). However, when the transgenic plants were wounded, they produced two- to threefold higher levels of JA than did wounded control plants. In contrast, overexpression of flax AOS in transgenic potato led to a 6- to 12-fold higher level of endogenous JA than nontransgenic plants (Harms et al. 1995). Recently, *Arabidopsis* AOS mutants have been obtained through transposon or T-DNA insertional mutagenesis (Park et al. 2002; von Malek et al. 2002). These mutants are defective in JA biosynthesis and exhibit male sterility that can be rescued by exogenous application of MeJA, confirming that AOS is essential for the biosynthesis of biologically active jasmonates.

In contrast to *Arabidopsis*, which carries a single AOS gene, the rice genome appears to contain four AOS genes (Agrawal et al. 2004; Haga and Iino 2004). Although some rice AOS (*OsAOS*) genes have been isolated and analyzed for their mRNA expression patterns during the course of our study (Agrawal et al. 2002; Ha et al. 2002; Haga and Iino 2004), transgenic analysis and functional characterization of AOS have not been reported in rice or any other monocotyledonous plants. In this study, we first examined the effect of exogenous JA treatment on the basic *PR1* gene (*PR1b*) expression and induced resistance against *M. grisea* infection. We then identified a pathogen-inducible rice AOS gene (*OsAOS2*). Furthermore, transgenic rice lines were generated, which carried the *OsAOS2* transgene under the control of a strong, pathogen-inducible promoter of rice *PBZ1* gene (which encodes an intracellular PR10 protein) (Midoh and Iwata 1996). Molecular, biochemical, and pathological analyses of the first (T0), second (T1), and third (T2) generations of transgenic rice lines revealed that *OsAOS2* plays a significant role in pathogen-induced JA biosynthesis, defense gene activation, and host resistance to the rice blast fungus.

RESULTS

Activation of rice *PR1b* gene and induced resistance by exogenous JA treatment.

To examine the effect of exogenous JA on induced resistance, 2-week-old rice seedlings were treated with JA, along with Salicylic acid (SA), benzothiadiazole (BTH, a synthetic activator of induced resistance in many plants), or probenazole (PBZ, a fungicide and activator of induced resistance in rice). To verify the effectiveness of chemical sprays, the rice *PR1b* expression was monitored at 0, 1, 2, and 3 days after the treatments. RNA blot analysis showed that rice *PR1b* gene was induced only weakly, if at all, by SA, but was strongly activated by JA (Fig. 1A). In addition, *PR1b* also was induced significantly in BTH- or PBZ-treated leaves. However, induction of *PR1b* was not detected in upper, systemic leaves (data not shown).

Three days after chemical treatments, rice seedlings were inoculated with *M. grisea*. Induced resistance against *M. grisea* infection was evaluated at 6 days postinoculation. The pretreatment with SA, a poor inducer of basic *PR1* genes, failed to activate induced resistance against blast infection (Fig. 1B). In contrast, pretreatments with JA, BTH, and PBZ, which are effective inducers of the *PR1b* gene, conferred significant levels of induced resistance, as shown by reduced lesion size and number in pretreated leaves. Compared with the H₂O control, pretreatments with JA, BTH, and PBZ resulted in 65, 66, and 47% reduction in lesion number and 51, 47, and 43% reduction in lesion size, respectively. In addition to visual assessment of disease severity, the growth of *M. grisea* in rice seedlings was quantified by the real-time polymerase chain reaction (PCR) using a specific pair of primers designed according to the 3' untranslated region of 28S rDNA of *M. grisea* (Qi and Yang 2002). The pretreatment of rice seedlings with JA, BTH, and PBZ resulted in 84, 80, and 66% reduction of the fungal growth, respectively. These data demonstrate that JA is an effective activator of induced resistance as well as basic *PR1* expression in rice plants.

Sequence analysis of *OsAOS2* cDNA.

The *OsAOS2* gene is 1,775 bp long, including the 1,437-bp open reading frame region encoding a 52.3-kDa protein with 478 amino acids and a theoretical pI of 8.21. The result from sequence alignment indicated that *OsAOS2* has four conserved domains of cytochrome P450 proteins (Fig. 2A, domains A–D), which are common for all AOS enzymes. Similarly to the AOSs from barley and wheat, *OsAOS2* lacks an amino-terminal transit peptide for targeting to the chloroplast, which is different from many AOSs of dicotyledonous species such as *Arabidopsis*, tomato, tobacco, and flax. The gene we reported here is the same as the *OsAOS* gene reported by Agrawal and associates (2002) and Ha and associates (2002), and the *OsAOS2* gene reported by Haga and Iino (2004). Southern blot analysis revealed a single, strong hybridizing band in the *EcoRI* or *HindIII* digestion after probing with the full-length *OsAOS2* cDNA (Fig. 2B), indicating that *OsAOS2* is a single-copy gene. The weakly hybridizing bands suggest the presence of other related genes. It was reported recently that the rice genome contains four members of the small AOS gene family (Agrawal et al. 2004; Haga and Iino 2004).

The AOS phylogenetic analysis showed that *OsAOS2* was very closely related to those from other monocotyledonous species, such as barley and wheat (Fig. 2C). *OsAOS2* was 73.5, 72.6, and 69.9% identical at the amino acid sequence level with barley AOS1, barley AOS2, and wheat AOS, respectively, but only 50.6, 49.6, 48.1, 48.0, 47.8, 45.9, 45.6, 45.5, and 43.6% identical with the AOS proteins from guayule, potato, tobacco, *Arabidopsis*, tomato, *Medicago truncatula*, flax,

muskmelon, and *Physcomitrella patens*, respectively. OsAOS2 shared 55% sequence identity with OsAOS1, the only AOS enzyme identified so far to contain a chloroplast transit peptide in monocotyledonous species (Haga and Iino 2004).

Tissue-specific expression of *OsAOS2*.

In rice plants, the expression level of *OsAOS2* is very low and hardly detectable by RNA gel blot analysis. Therefore, real-time reverse-transcription (RT)-PCR was used to determine the expression pattern of *OsAOS2*. The total RNA was extracted from 2-week-old and 2-month-old leaves, sheath, and roots at vegetative stages and from culm, young panicle, panicle, and florets at reproductive stage. The expression analysis revealed the highest level of *OsAOS2* expression in the 2-week-old sheath, which was 90-fold higher than the lowest expression of *OsAOS2* in the 2-week-old leaf (Fig. 3). Culm and panicle also had relatively high levels of *OsAOS2* expression.

Induction of *OsAOS2* during *Magnaporthe grisea* infection.

In order to examine the expression of *OsAOS2* in response to pathogen infection, 2-week-old rice seedlings were spray-inoculated with the blast fungus. Leaf samples were taken daily from day 0 to day 4 for RNA isolation and subsequent quantitative RT-PCR assays. Upon *Magnaporthe grisea* infection, the level of *OsAOS2* expression was induced dramatically at day 1 and reached the highest level at day 2, when small blast lesions started to appear on the infected leaves (Fig. 4). The expression of *OsAOS2* slightly decreased but remained high at 3 and 4 days post infection. In the mock-inoculated

leaves, the *OsAOS2* expression increased only at day 1 and then went back to the basal level. The transient increase of *OsAOS2* expression in the mock treatment at day 1 likely was due to general stress effect in the dew chamber. In contrast to the *OsAOS2* induction by *M. grisea* infection, wounding or defense signal molecules such as JA, SA, abscisic acid, BTH (a functional analog of SA), and ethephon (an ethylene releaser) did not significantly activate the expression of *OsAOS2* in rice leave (data not shown).

Inducible overexpression of *OsAOS2* transgene in rice.

To further clarify the function of *OsAOS2* in the JA biosynthesis pathway and determine the role of JA in *PR* gene induction and defense response in rice, the full-length *OsAOS2* cDNA was fused with the rice *PBZ1* gene promoter that is inducible strongly by pathogen infection. The *PBZ1::OsAOS2* construct then was introduced into rice cv. Nipponbare via the *Agrobacterium*-mediated transformation. In all, 21 independent transgenic lines were generated and confirmed by Southern blot analysis. As indicated in the genomic Southern blot (Fig. 5A), all transgenic lines carried an additional 2.5-kb *EcoRI* fragment hybridizing with the *OsAOS2* cDNA probe, whereas the wild-type control plant contained only a 3.0-kb hybridizing fragment. Furthermore, RNA blot showed a strongly induced expression of *OsAOS2* in the transgenic lines compared with a very low level of *OsAOS2* in the untransformed control plant (Fig. 5B). The pathogen-inducible *PBZ1* promoter (rather than a constitutive promoter) was used to control the expression of *OsAOS2* transgene; therefore, considerable variation was ob-

Fig. 1. Activation of *PR1b* and induced resistance in chemical-treated rice seedlings. **A**, Expression of rice *PR1b* gene in response to chemical treatments. Total RNAs were isolated from rice leaves sprayed with H₂O, salicylic acid (SA, 1.5 mM), jasmonic acid (JA, 0.1 mM), probenazole (PBZ, 0.25 mM), and benzothiadiazole (BTH, 0.25 mM) and probed with the rice *PR1b* or 25S rDNA fragment. **B**, Activation of local induced resistance against the infection of *Magnaporthe grisea*. At least 125 seedlings were scored and analyzed for each experiment. Statistical analysis (*t* test) has shown a significant reduction (<0.05) of lesion number, lesion size, and the fungal growth in JA-, PBZ- and BTH-treated leaves. The standard errors were indicated in the figure. Similar results were obtained in three experiments.

served in the level of *OsAOS2* transcripts in individual transgenic plants following the *M. grisea* infection.

Accumulation and pathogen-induced elevation of endogenous JA levels in *OsAOS2* transgenic rice.

To correlate the *OsAOS2* overexpression with increased JA biosynthesis, gas chromatography mass spectrometry (GC-MS) was used to determine JA levels in transgenic rice lines (see Materials and Methods). The mass spectra of endogenous JA from rice leaves matched very well with that of authentic JA standard, as indicated by the similar relative abundance of major ions between the JA standard and endogenous JA from rice leaf tissues (Fig. 6A). For JA quantification, an internal standard 9,10-dihydro jasmonic acid (DHJA), which was not detected in rice leaves under the same conditions) was included for adjusting the JA extraction efficiency. The area of *m/z* 224 of methyl JA and *m/z* 153 of methyl DHJA was used to calculate JA and DHJA amounts, respectively. The standard curve of area ratio of methyl JA and methyl DHJA versus amount ratio of JA and DHJA was plotted and showed a linear regression with $R^2 = 0.99$ (Fig. 6B).

Under normal growth conditions (in the absence of pathogen infection), the *OsAOS2* overexpression lines had relatively higher basal levels of JA (12 to 21 ng/g of fresh weight [FW]) than that of the control plant (7.5 ng/g of FW) (Fig. 6C). Upon infection by *M. grisea*, a drastic increase of endogenous JA (51 to 282 ng/g of FW) was observed in the transgenic lines carrying the pathogen-inducible *PBZ1::OsAOS2* transgene (Fig. 6D). In contrast, only a modest increase of endogenous JA (36 ng/g of FW) was found in the *M. grisea*-infected control plant. Therefore, inducible overexpression of *OsAOS2* indeed contributed to the increased level of endogenous JA in transgenic rice, especially in the presence of pathogen infection.

Enhanced activation of *PR* genes in *OsAOS2* transgenic rice.

It has been well documented that exogenous application of JA could induce the expression of various *PR* genes in rice and other plants. To determine whether elevated levels of endogenous JA could activate rice *PR* genes in the *OsAOS2* transgenic lines, the expression of six *PR* genes was examined in the detached leaves of the control and transgenic plants. In general, the transgenic lines had a much higher level of *PR* gene expression than that of the control plants (Fig. 7), especially for *PR1a*, *PR3*, and *PR5*. In the OX3 lines, for instance, the levels of *PR1a*, *PR3*, and *PR5* expression were 554, 204, and 1,524 higher, respectively, than those in the untransformed control plant. The expression of *PR1b*, *PR2*, and *PBZ1* genes also was higher in most of transgenic lines compared with that of the control plant. These data demonstrated that inducible overexpression of *OsAOS2* transgene not only increased the level of endogenous JA but also significantly activated the expression of many *PR* genes in transgenic rice lines.

Increased resistance of *OsAOS2* transgenic lines to *M. grisea* infection.

The inducible overexpression of *OsAOS2* transgene was found to increase the levels of endogenous JA and *PR* gene expression; therefore, the transgenic rice lines were evaluated further for disease resistance to the blast fungus. Following infection with a moderately virulent *M. grisea* isolate, the disease severity was significantly lower in the transgenic lines than that of the control plant (Fig. 8A). In addition, a 32 to 83% reduction in *M. grisea* growth was observed in the transgenic lines compared with the control plant (Fig. 8B), indicating a significant increase of disease resistance to *M. grisea* infection.

Fig. 2. *Oryza sativa* allene oxide synthase (*OsAOS2*) and its relationship with other plant allene oxide synthase AOS proteins. **A**, Deduced amino acid sequence of *OsAOS2* with four conserved domains (A to D) of cytochrome P450 in C-terminal region. **B**, Southern blot analysis of *OsAOS2* in rice genome. Total DNA from rice cv. Nipponbare was digested individually with *EcoRI* and *HindIII* and hybridized with the full-length cDNA probe of *OsAOS2*. **C**, Relationship between *OsAOS2* and other plant AOS proteins. The dendrogram was generated based on the multiple sequence alignment of the following AOS proteins: AtAOS (*Arabidopsis thaliana* Y12636), CmAOS (muskmelon, *Cucumis melo* AAM66138), HvAOS1 and 2 (barley, *Hordeum vulgare* 1, AJ250864 and 2, AJ251304), LeAOS (tomato, *Lycopersicon esculentum* AF230371), LuAOS (flax, *Linum usitatissimum* U00428), MtAOS (*Medicago truncatula* AJ316561), NaAOS (tobacco, *Nicotiana attenuata* CAC82911), PaAOS (guayule, *Parthenium argentatum* X78166), PpAOS (moss, *Physcomitrella patens* AJ316566), *OsAOS1-4* (rice, *Oryza sativa*, AY062258, AY310358, AK105964, and AK068620), StAOS (potato, *Solanum tuberosum* AAN37417), and TaAOS (wheat, *Triticum aestivum* AY196004).

DISCUSSION

During the past decades, JA was found to play an important role in plant disease resistance in addition to its well-documented involvement in induced resistance against insect herbivory (Dong 1998; Farmer et al. 2003; Reymond and Farmer 1998). JA was suggested to mediate induced resistance through a distinct, SA-independent signaling pathway or interact with SA, ethylene, reactive oxygen intermediates, and other signaling molecules to modulate host defense response (Pieterse and van Loon 1999). In rice, an increasing number of studies have shown that JA is able to induce phytoalexin production and expression of many defense-related genes (Agrawal et al. 2000a,b; Jwa et al. 2001; Lee et al. 2001; Moons et al. 1997; Rakwal and Komatsu 2000; Schweizer et al. 1997a,b, 1998; Tamogami et al. 1997; Xiong et al. 2001). For example, both exogenous JA treatment and *M. grisea* strongly induced rice

PR1 proteins as detected by using antisera raised against the major basic PR1 of tomato (Schweizer et al. 1997a). Furthermore, jasmonate biosynthesis inhibitors such as tetcyclacis significantly reduce endogenous JA levels in rice and inhibit *M. grisea*-induced PR1 accumulation, suggesting that endogenous JA is required for PR1 induction. Compared with mock treatments, paradoxically, infection of rice seedlings with *M. grisea* or *Pseudomonas syringae* pv. *syringae* (a nonpathogen) did not significantly enhance the endogenous levels of JA within 48 h after inoculation (Schweizer et al. 1997b). It is possible that other octadecanoid derivatives that are functionally similar to JA may act as major inducible molecules for induced resistance in rice. Previously, a couple of octadecanoid derivatives have been demonstrated to play a role in host defense against rice blast fungus (Kato et al. 1984; Song et al. 1994). In this study, however, a modest increase of endogenous JA (from 7.5 to 36 ng/g of FW) was observed at 5 days after *M. grisea* infection. More extensive infection and lesion development at the late stage of infection might have contributed to the increased level of endogenous JA.

Schweizer and associates (1998) found that exogenous application of JA or wounding was able to induce disease resistance in systemic leaves but not in local, treated leaves. In contrast, we observed clear induction of *PR1* gene and induced resistance in local, JA-treated leaves, rather than in systemic, untreated leaves. Such discrepancy may result from a number of experimental variables. Schweizer and associates (1997a,b, 1998) inoculated rice seedlings with a highly virulent isolate of *M. grisea*, which might have overcome JA-induced resistance. In our experiments, a moderately virulent isolate of *M. grisea* was used, which may allow the detection of local induced resistance. In addition to phenotypic measurement of lesion size and number, which often show considerable variations, we have

Fig. 3. Differential expression of *Oryza sativa* allene oxide synthase (*OsAOS2*) in various parts of rice plants. The relative expression of *OsAOS2* was determined by quantitative reverse-transcription polymerase chain reaction. L = leaves; S = sheath; R = roots; YP = young panicle; P = panicle; C = culm; and F = florets. Experiments were conducted twice with similar results.

Fig. 4. Induction of *Oryza sativa* allene oxide synthase (*OsAOS2*) following the infection of *Magnaporthe grisea*. The quantitative reverse-transcription polymerase chain reaction data were averaged from three independent samples. Experiments were conducted three times with similar results.

Fig. 5. Molecular analysis of *Oryza sativa* allene oxide synthase (*OsAOS2*) overexpression lines. A, Southern blot analysis of representative transgenic lines. The genomic DNAs from leaves of control and transgenic plants were digested with *EcoRI* and probed with *OsAOS2* cDNA. OX = overexpression line and CK = control plant. B, RNA blot analysis of the *OsAOS2* inducible overexpression in transgenic lines 5 days after *Magnaporthe grisea* infection (dpi = days postinoculation).

quantified the relative growth of *M. grisea* in rice seedlings for more accurate assessment of disease severity.

There is strong evidence demonstrating that AOS is a key enzyme and a control point of the JA biosynthetic pathway in *Arabidopsis*, a model dicotyledonous species. For instance, AOS knock-out mutant is male sterile and the male sterility could be rescued by exogenous application of MeJA or by constitutive expression of functional AOS in a complementation experiment (Park et al. 2002). The same was true for another *Arabidopsis* mutant called *delayed-dehiscence2-2* (*dde2-2*) that has a frameshift mutation in the AOS gene (von Malek et al. 2002). Based on sequence analysis in this study, the OsAOS2 is very close to the AOS proteins from barley and wheat (approximately 70% identity in amino acid level) (Fig. 1B). A common feature of these AOSs is lack of a signal peptide for targeting to the chloroplasts. In contrast, all AOSs from dicotyledonous species contain the chloroplast signal peptide, except rubber tree. OsAOS2 shares relatively low homology with AOSs of dicotyledonous species (approximately 50%). However, like all other AOS genes, the OsAOS2 encodes four common domains (Fig. 2A, domains A–D) that are distinctive for the CYP74A family of cytochrome P450 proteins, which does not require oxygen, NADPH, and P450 reductase for enzymatic activity (Schaller 2001). Although four members (*OsAOS1* to *OsAOS4*) of the small AOS gene family were

found in the rice genome (Haga and Iino 2004), *OsAOS2* appears to be a single-copy gene based on our Southern blot analysis (Fig. 2B). This is consistent with the result of Agrawal and associates (2002) but differs from that of Ha and associates (2002), who reported two *OsAOS2* copies due to the erroneous explanation of their Southern blot data. The sequence identity between *OsAOS1* and *OsAOS2* was only 55% at the amino acid level. *OsAOS1* was isolated by map-based cloning of the gene responsible for rice *cpm1* mutant (which is impaired in the phytochrome-mediated inhibition of coleoptile growth and in the process of anthesis) and shown to be involved in phytochrome-mediated regulation of seedling growth (Haga and Iino 2004).

The expression level of plant AOS genes often varies in different organs and tissues. For example, the AOS transcripts were more abundant in the leaf and flower but much less in the root, stem, and silique of *Arabidopsis* (Laudert and Weiler 1998). High-level expression of AOS also was detected in the achenes of flax (Song and Brash 1991), in flower (Sivasankar et al. 2000) and stem of tomato (Howe et al. 2000), and in scutellar nodule and leaf base of barley (Maucher et al. 2000). Compared with *Arabidopsis* and several other plant AOS genes, the overall expression of *OsAOS2* is rather low (even after the induction by *M. grisea*) and difficult to detect by RNA blot analysis. However, it does exhibit preferential expres-

Fig. 6. Endogenous jasmonic acid (JA) levels in control and *Oryza sativa* allene oxide synthase (*OsAOS2*) transgenic plants. **A**, Mass spectra of commercial JA standard and endogenous JA from rice leaf tissues. **B**, Standard curve derived from amount ratio versus area ratio of JA/9,10-dihydro jasmonic acid (DHJA). **C**, Endogenous JA levels in T2 plants of three overexpression lines. **D**, Endogenous JA levels in T2 transgenic plants 5 days after *Magnaporthe grisea* infection. The data were averaged from three independent samples with standard error.

sion in sheath, culm, and flower tissues of rice plants. Quantitative RT-PCR assays also revealed that *OsAOS2* was significantly activated in rice leaves by the infection of *M. grisea*, suggesting its potential involvement in the rice-pathogen interaction and defense response. However, our study indicated that *OsAOS2* was not induced by wounding or JA. In contrast, the expression of most plant AOS genes are positively regulated by wounding or JA (Harms et al. 1995; Park et al. 2002; Ziegler et al. 2001). It is possible that a high basal level of SA in rice plants may have inhibited the *OsAOS2* expression and reduced the endogenous JA level in rice (5 to 10 ng/g of FW for the basal level and 10 to 40 ng/g of FW after wounding) (this study; Schweizer et al. 1997a,b, 1998) that is much lower than that of *Arabidopsis* (10 to 100 ng/g of FW for the basal level and 1,000 to 2,000 ng/g of FW after wounding) (Kubigsteltig and Weiler 2003; Park et al. 2000) and flax (approximately 100 ng/g of FW for the basal level and approximately 1,000 ng after wounding) (Harms et al. 1998). Tomato plants that contain a relatively high level of endogenous SA also appear to have relatively low levels of JA (approximately 2.5 ng/g of FW for the basal level and approximately 50 ng/g of FW after wounding) (Li et al. 2002). This is consistent with previous reports in flax and tomato that exogenous SA can significantly inhibit JA- or wounding-induced AOS expression (Harms et al. 1998; Sivasankar et al. 2000). Indeed, in SA-deficient rice plants (Yang et al. 2004), we observed a significant increase of *OsAOS2* expression in the absence or presence of *M. grisea* infection (Y. Yang, unpublished data).

Previously, overexpression of AOS genes under the control of constitutive CaMV 35S promoter has been reported in potato, tobacco, and *Arabidopsis* transgenic plants (Harms et al. 1995; Laudert et al. 2000; Wang et al. 1999). Constitutive overexpression of AOS in *Arabidopsis* and tobacco did not increase the basal level of JA; however, the transgenic lines exhibited

Fig. 8. Increased tolerance of *Oryza sativa* allene oxide synthase (*OsAOS*)2 overexpression lines to *Magnaporthe grisea* infection. **A**, Blast disease evaluation according to the rating scale of Marchetti and associates (1976) and presented as relative disease severity. **B**, Disease severity measurement based on the relative fungal growth of *M. grisea*.

Fig. 7. Enhanced expression of rice pathogenesis-related (PR) genes in *Oryza sativa* allene oxide synthase (*OsAOS*)2 overexpression lines. The stress-inducible *PBZ1* promoter::*OsAOS2* in transgenic plants was moderately activated by placing leaf segments in deionized water under continuous light at 25°C for 24 h. Total RNAs were isolated from control and transgenic leaf segments for **A**, Northern analysis and **B**, phosphoimaging quantification.

an earlier JA peak and higher levels of JA than the control plant after wounding (Laudert et al. 2000; Wang et al. 1999). In contrast, transgenic potato overexpressing *AOS* showed a higher level of endogenous JA but did not activate the expression of JA-responsive genes such as the proteinase inhibitor gene (Harms et al. 1995). It was suggested that the constitutive and a higher basal level of JA might not act as an effective signal, but that the relative increase of JA in a short period of time is more critical for the induction of JA-responsive genes (Schaller 2001). From this point of view, overexpression of *AOS* under the control of an inducible promoter might be better than the use of a constitutive promoter. In this study, we have generated and characterized transgenic rice lines with overexpression of *OsAOS2* under the control of a strong, pathogen-inducible *PBZ1* promoter. Compared with the control plant, transgenic rice lines exhibited a significant increase of endogenous JA level, especially after the infection with *M. grisea* (Fig. 6C and D). More importantly, we were able to demonstrate that such an increase in *OsAOS2* transcripts and endogenous JA resulted in the activation of many JA-responsive *PR* genes (Fig. 7). Furthermore, the increase of endogenous JA and stronger induction of *PR* genes appear to be translated into the enhanced resistance of transgenic rice to the blast fungus. As a result, our study not only shows for the first time the function of an *AOS* gene in the biosynthesis of JA in monocotyledonous species, but also demonstrates the importance of JA in mediating rice *PR* gene expression and disease resistance.

We noticed that the *OsAOS2* transcript level, JA content, and *PR* gene expression were not proportionally correlated with one another among the overexpression lines. Due to the use of the pathogen-inducible *PBZ1* promoter (rather than a constitutive promoter), induction of *OsAOS2* transgene by *M. grisea* might vary considerably among different leaves and plants of a particular transgenic line. A relatively large quantity of leaf samples from different seedlings of each transgenic line were required for JA quantification (Fig. 6D); however, a single seedling of a particular line was used for the RNA blot (Fig. 5B), which might skew the correlation between *OsAOS2* expression and JA level. In addition, the potential activation of other *AOS* genes and post-transcriptional events (e.g., efficiency of protein translation, protein stability, and enzyme activity) might account for the discrepancy between the *OsAOS2* transcript level and JA content.

To further determine the role of *OsAOS2* and octadecanoid pathway in rice defense gene expression and induced resistance, we also have generated *OsAOS2* suppression lines using double-stranded RNA interference (C. Mei and Y. Yang, *unpublished*). However, suppression of *OsAOS2* (which already has a low level of basal expression in wild-type rice plants) did not significantly reduce the JA level and disease resistance of the transgenic rice RNAi lines. Because the rice genome contains four *AOS* genes, it is likely that other *AOS* members may have compensated for the defect of *OsAOS2* in the RNAi lines. We are now in the process of generating rice RNAi lines with the suppression of two or more *AOS* genes. We have also generated rice RNAi lines that are insensitive to JA and other octadecanoid derivatives. Hopefully, these RNAi lines will further aid in the elucidation of JA- or octadecanoid-mediated resistance response in rice plants in the near future.

MATERIALS AND METHODS

Sequence analysis of *OsAOS2* cDNA.

The *OsAOS2* gene was provided as an expressed sequence tag clone by the Japanese Rice Genomics Program. The full-length of *OsAOS2* cDNA was sequenced completely with the primer-walking approach in the DNA core facility at the Uni-

versity of Arkansas. Sequence analysis was conducted using the Vector NTI 6.0 software (InforMax Inc., MD, U.S.A.) and the BLAST program from the National Center for Biotechnology Information.

OsAOS2 overexpression construct and rice transformation.

The overexpression construct, *PBZ1::OsAOS2*, was made by directly inserting the full-length *OsAOS2* cDNA into the pCAMBIA1300 vector containing the pathogen-inducible rice *PBZ1* promoter (Lee 2002). The *PBZ1::OsAOS2* construct was introduced into *Agrobacterium tumefaciens* strain EHA105 by electroporation. Subsequent transformation was conducted using rice (*Oryza sativa* L.) cv. Nipponbare according to a modified method of Hiei and associates (1994) as described previously (Xiong and Yang 2003).

Plant materials and fungal isolates.

The wild-type Nipponbare cultivar as well as the *OsAOS2* transgenic plants were grown in Redi Earth soil mix (Hummert International, Springfield, MO, U.S.A.) in a 28°C greenhouse with a light-and-dark cycle of 14 and 10 h, respectively. Plants were fertilized with 0.5% ammonium sulfate every 2 weeks until flowering. For transgenic plants confirmed with Southern blot analysis, self-pollinated seed from independent transgenic lines were harvested individually. T1 and T2 plants carrying the transgene were selected based on hygromycin resistance and maintained as described previously (Xiong and Yang 2003). A moderately virulent isolate (18-1, IC-17 pathotype) of *M. grisea* was used to inoculate rice seedlings (Harp and Correll 1998).

Pathogen inoculation and chemical treatments.

For *M. grisea* infection, the 2-week-old plants were spray inoculated with the fungal conidial spores at a concentration of 2.5×10^5 spores/ml (Lee et al. 2001). After incubation in a dew chamber at 22°C for 24 h, rice seedlings were moved to a 28°C growth chamber with 14 h of light. For mock treatment, rice seedlings were sprayed with water. Leaf samples were taken at 0, 1, 2, 3, and 4 days after fungal inoculation.

Chemical activation of induced resistance in rice was conducted by spraying rice seedlings with H₂O, SA (1.5 mM), JA (0.1 mM), PBZ (0.25 mM), or BTH (0.25 mM). Approximately 125 to 500 2-week-old seedlings with a near-fully expanded third leaf were used for each experiment. Three days later, chemical-treated rice plants were spray inoculated with *M. grisea* at the concentration of 2.5×10^5 spores/ml. Disease severity on the third leaves of each plant was measured at 6 days after inoculation. Each leaf was scored for the number of necrotic lesions and size of the three largest lesions. Subsequently, the same leaves were collected for total DNA extraction and quantification of *M. grisea* growth based on its *rDNA* content in rice tissues (Qi and Yang 2002).

DNA and RNA blot analyses.

Genomic DNA was extracted from rice leaves according to the previously described method (Qi and Yang 2002) and digested with appropriate restriction enzymes. Digested DNA samples were separated in a 0.7% agarose gel at 30 V overnight and blotted onto a nylon membrane using the standard protocol (Sambrook et al. 1989). The total RNA was extracted from rice tissues using the TRIzol reagent (Invitrogen, Carlsbad, CA, U.S.A.). Total RNAs (10 µg each) were fractionated on a 1.2% agarose gel containing formaldehyde and blotted onto a nylon membrane. For Southern and Northern hybridization, gene-specific probes were labeled with [α -³²P] dCTP using the random-priming kit (Promega Corp., Madison, WI, U.S.A.). Hybridization of DNA and RNA blots was carried out overnight

with PerfectHyb Plus hybridization buffer (Sigma-Aldrich, St. Louis) at 62°C. Subsequently, blots were washed with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) twice for 10 min, then 0.5× SSC and 0.5% sodium dodecyl sulfate twice for 20 min at 62°C. Relative levels of gene expression were quantified with a phosphorimaging system (Molecular Dynamics, Sunnyvale, CA, U.S.A.).

To prepare the *PR* gene probe, gene-specific fragments were amplified by PCR using the following specific pairs of primers: *PR1a* forward primer, 5'-TCG AGC AGG TTA TCC TGC TGC TTG-3' and reverse primer, 5'-GAG TAG TTG CAG GTG ATG AAG ACG-3' (accession number: AJ278436; Agrawal et al. 2000a); *PR2* forward primer, 5'-ACA TCA AGG TGA CGA CGT CG-3' and reverse primer, 5'-ATC AGA AGC TGA TGG GGT AG -3' (accession number: X58877; Simmons et al. 1992); *PR3* forward primer, 5'-TAC TGT GTC CAG AGC TCG CAG TGG-3' and reverse primer, 5'-TCT GGT TGT AGC AGT CCA AGT TGG-3' (accession number: X87109; Xu et al. 1996); and *PR5* forward primer, 5'-ACC TCT TCC GCT GTC CTC-3' and reverse primer, 5'-GAA GAC GAC TTG GTA GTT GC-3' (accession number: X68197; Agrawal et al. 2003). All PCR-amplified fragments were first cloned into pGEM-T Easy vector (Promega Corp.) and verified by DNA sequencing. The *PR1b* and *PBZ1* genes were provided by the Japanese Rice Genomics Program and described previously (Yang et al. 2004).

Quantitative RT-PCR for *OsAOS2* expression.

The total RNA was isolated from rice tissues and treated with RNase-free DNase I (Promega Corp.) to remove the trace DNA in RNA samples. Reverse transcription was carried with M-MuLV reverse transcriptase (New England BioLab, Beverly, MA, U.S.A.) according to the manufacturer's instructions. Quantitative real-time PCR was conducted in the Mx3000P real-time PCR system (Stratagene, La Jolla, CA, U.S.A.) with the QuantiTect SYBR Green PCR kit (Qiagen, Valencia, CA, U.S.A.). The following specific pair of primers was used to detect the *OsAOS2* expression: forward primer, 5'-AGC TAG CTA GAA GAG AGT TAG C-3' and reverse primer, 5'-ACT CGA AGT ACT TGT CCT GC-3'. The quantitative RT-PCR and relative expression of *OsAOS2* was normalized using the rice ubiquitin 1 gene (amplified with forward primer 5'-GTG GCC AGT AAG TCC TCA GC-3' and reverse primer 5'-GAA ACG GGA CAC GAC CAA GG-3').

Analysis of endogenous JA levels by GC-MS.

Endogenous JA levels in rice tissues were determined by GC-MS following the methods of Weber and associates (1997), Li and associates (2002), and Gundlach and associates (1992), with several major modifications. Briefly, approximately 1 g of leaf tissues was ground to fine powder with liquid nitrogen. Ice-cold methanol (10 ml) and 150 ng of DHJA (as an internal standard, made by Olchemim, Olomouc, Czech Republic) were added to the powder. After rotary shaking in a 4°C cold room overnight, ice-cold water was added (to the final methanol concentration of 70%) and mixed with the sample. The mixture was centrifuged at 5,000 rpm for 10 min at 4°C and supernatant was further filtered through a 0.45-μm membrane. The filtrate was adjusted to pH 8.5 with 0.1 M of NH₄OH and loaded to a reverse phase C18 column (J. T. Baker) which had been conditioned with methanol and then 70% methanol, and then washed with an additional 75% methanol. All of the run-through solution, added with water to make a methanol concentration less than 20%, was adjusted to pH 3.5 with 10% formic acid. Jasmonates were partitioned three times with chloroform. The chloroform phase was dehydrated with Na₂SO₄ and dried down with nitrogen gas at 35°C. For

derivatization of JA and DHJA, we found that boron trifluoride (BF₃)/methanol (14% wt/vol) (Alltech Associates, Inc., Deerfield, IL, U.S.A.) was a good derivatization agent for JA quantification of rice leaves. Therefore, 75 μl of BF₃/methanol were added to the above dried-down samples for derivatization at 70°C for 1 h, following by the addition of 450 μl of saturated NaCl and 1 ml of hexane. The JA and DHJA derivatives were partitioned into hexane phase by mixing and spinning and hexane phase was dried down with nitrogen gas at 35°C. Hexane (30 μl) was added to dissolve the derivatives and 2 μl were used for injection. JA detection was carried in GC-MS (Varian 3400, Saturn 4D GC/MS with FactorFour capillary column VF-5MS, 0.25 mm by 0.25 μm by 30 m) under the condition of 77°C for 1.2 min, 10°C/min for 10 min, 180°C for 4 min, 40°C/min to 280°C, and finally 280°C for 6 min. JA quantification was carried out using a standard curve of area ratio versus amount ratio of JA and DHJA.

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