Note

Molecular Characterization of the Gene Encoding Rice Allene Oxide Synthase and Its Expression

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The gene encoding rice allene oxide synthase, OsAOS, was intronless and had nucleotide sequences with the high GC content of 67%. Deduced amino acid sequences had very high similarity with other AOS proteins, in particular 74% similarity to barley, characterized by the conserved motifs of P450 cytochrome of the CYP74A family. Purified recombinant rice AOS protein expressed in Escherichia coli converted 13-hydroperoxylinolenic acid to allene oxide. Several restriction enzyme digestions and Southern analysis showed that OsAOS was likely to have two copies in its genome. The basal level of OsAOS expression was detected in various tissues and the transcription level was increased by jasmonate treatment.

Key words: allene oxide synthase; jasmonate; rice

AOS enzyme is a key step in the synthesis of jasmonate by the octadecanoid pathway. Many studies have examined lipoxygenase, which synthesizes 13-hydroperoxylinolenic acid (13-POT) from linolenic acid (18:3), an unsaturated fatty acid that is freed from membranes, and involved in plant defense response. Two kinds of LOX in rice have been identified. Cytosol-targeted LOX shows pH-dependent regiospecificity and is found in maturing seeds and seedlings. However, chloroplast-targeted LOXs are pathogen-inducible or induced by chemical inducers of acquired resistance. LOX is thought to be essential for jasmonate biosynthesis because much jasmonate is produced when plants are treated with linolenic acid, and the treatment with LOX inhibitor or decreased LOX expression in Arabidopsis thaliana reduces jasmonate biosynthesis. At first, AOS has been identified in flaxseed, and shown to produce allene oxides from fatty acid hydroperoxides. It is a heme protein and belongs to the cytochrome P450 family. The same gene was also found in A. thaliana, tomato, spinach, and barley. AOS genes in spinach, tomato, and A. thaliana have transit peptide sequences, which were not found in rubber and barley, although AOS proteins in barley have been found in chloroplasts by immunocytochemical analysis. The spatial expression of AOS is different from one organism to another. AOS responds to external and internal molecules and to mechanical damage. The expression level is increased by wounding, phytodienoic acid (PDA), jasmonate, and ethylene, and decreased by aspirin, but not changed by abscisic acid, NaCl, or pathogens. AOS is involved in the intermediate step of jasmonate biosynthesis, so AOS expression is related to the amount of jasmonate and intermediates in jasmonate synthesis.

The enzyme step mediated by AOS is a regulatory point in jasmonate biosynthesis. Investigations of AOS genes and their expressions are important to understand not only jasmonate biosynthesis, but also jasmonate-mediated effects, such as defense mechanisms. Until now, no reports on rice AOS have been published. We report the characterization of the OsAOS gene, its expression in various tissues, and the regulation by jasmonate.

A. thaliana AOS (AtAOS) cDNA was used for a partial cDNA sequence database search (http://bank.dna.affrc.go.jp/) to find similar genes in rice. The most highly matched clone (S13809) was obtained in order to identify and characterize the similar gene. Its sequence analysis showed that the clone had 1809 bp, including 1437 bp of open reading frame, 126 bp of 5′ untranslated sequences and 246 bp of 3′ untranslated sequences. The ORF en-

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Abbreviations: PDA, phytodienoic acid; RT, reverse transcriptase; 13-POT, 13-hydroperoxylinolenic acid; 18:3, linolenic acid; 12-OPDA, 12-oxophytodienoic acid; TMSCl, trimethylsilylchloride; IPTG, isopropylthio-β-D-galactoside
coded 478 amino acid residues (52.3 kDa). The GC content in this cDNA clone was higher (67%) than the mean (40%) amount of plant cDNA. In contrast to flax, spinach, tomato, and *A. thaliana*, there were no chloroplast transit peptide sequences, which are analogous to barley AOS genes.

Deduced amino acid sequences were aligned with AOS protein amino acid sequences from other species (Fig. 1). All sequences were highly conserved regardless of N- or C-terminal. This sequence had the highest similarity with barley (74%), and less with flax (56%), *A. thaliana* (57%), and tomato (55%). The heme-binding domain in P450 cytochrome of the CYP74A family, P-V-NKQCAG sequences, were conserved among AOS amino acids except a change A to P in rice, but had identical sequences with barley AOS. When another highly conserved motif, G-KIL was compared, the rice sequence had L instead of I in *A. thaliana* while in barley I was changed to V.

To verify that the rice AOS cDNA encodes a protein with AOS activity, a histidine-tagged recombinant form of rice AOS was synthesized in *Escherichia coli*, purified using affinity chromatography, and characterized (Fig. 2(A)). The AOS expression construct was prepared by inserting the full-length cDNA without a stop codon into the PET28b bacterial expression vector. The insert was inserted in frame so that a hexa-histidine tag was added to the carboxyl-terminus of the AOS protein. Cell culture and purification procedures were previ-
Fig. 2. Affinity Purification of His-tagged AOS Protein (A) and Coupled Assay of the Purified Rice AOS (B).

(A) Protein samples were separated by 12.5% SDS-PAGE and stained for protein with Coomassie blue: M, Molecular standards; lane 1, total protein in 15-μl portions of bacterial cells without IPTG; lane 2, total protein after IPTG treatment; lane 3, 20 μg of 100,000 x g supernatant protein; lane 4, AOS proteins (5 μg) purified with a Ni-NTA column. (B) A substrate of AOS was prepared by hydroperoxidation of α-linolenic acid catalyzed by soybean lipoygenase purchased from Sigma (St. Louis, MO) as described previously. The reaction mixture containing 2 mM linolenic acid, 0.25% Tween-20, 50 mM sodium phosphate (pH 7.0), and 10 units of soybean lipoygenase in a total volume of 3 ml was incubated at room temperature and the formation of conjugated diene was monitored by following the increase in absorbance at 234 nm. The purified AOS enzyme (2 μg) was immediately added when the formation of 13-hydroperoxylinolenic acid reached its saturation level after 20 min. The decrease in hydroperoxylinolenic acid was monitored by following the absorbance at 234 nm.

Fig. 3. Southern Analysis of Rice AOS Genomic DNA.

Isolated rice genomic DNA (5 μg) was digested with XbaI (X), HindIII (H), EcoRI (E), or BamHI (B). After separation on the 0.8% agarose gel, it was probed with the full length of OsAOS cDNA.

As for AOS activity measurement, the coupled assay method was used with a soybean lipoygenase enzyme and the purified rice AOS enzyme in the presence of the substrate, linolenic acid, as described in the legend of Fig. 2(B). The formation of 13-hydroperoxylinolenic acid from linolenic acid by the catalysis of soybean LOX was observed with a gradual increase in absorbance at 234 nm. However, this 13-hydroperoxylinolenic acid was rapidly catalyzed into corresponding allene oxides which were monitored by the decrease in A234 when the purified rice AOS was added to the reaction. In addition, AOS reaction products were further identified by GC-MS to be α-ketol and 12-oxophytodienoic acid (12-OPDA), which were derived nonenzymatically from the AOS product allene oxide. AOS reaction products were extracted with dichloromethane and dried under reduced pressure. The extraction products were mixed with TMSCl, kept at 85°C for 5 min, and immediately subjected GC-MS in splitless mode with a 30 m × 0.32 mm I.D., 0.25-μm film thickness Rtx-1 column with 5% phenyl methyl silicone stationary phase. Temperature programming was 80–180°C at 20°C/min, 180–200°C at 8°C/min, 200°C for 27.5 min, 200–270°C at 14°C/min, and 270°C for 10 min. Both products had the same retention time with standards showing 17.4 min for α-ketol and 25.1 min for 12-OPDA and the identical mass spectra with [M]+ ion at m/z 526, 511 [M-CH3]+, and 457 [M-CH3CH2CH2CH3]+ for α-ketol and [MH]+ ion at m/z 437, 365 [MH-Si(CH3)]+, 349 [MH-Si(CH3)3]+ for 12-OPDA. This indicates that the rice AOS may be involved in the biosynthesis of jasmonic acid.

Southern analysis was done (Fig. 3) to find out how many copies were in rice genomic DNA, and to determine the exon/intron structure. Large amounts of restriction enzymes and long digestion times were necessary to eliminate partial digestion. Because two strong bands were detected with the use of five different enzymes, the copy number of the AOS gene in the rice genome was likely to be two. The AOS gene did not have any introns like those reported in A. thaliana. Only the cDNA size (1437) of PCR products in genomic DNA PCR reaction were observed with primers (forward primer, 5'-d(CTAG-GCGCCATGGAGCTA)-3'; reverse primer, 5'-d(G-AAGTGATGCGCGGCTTA)-3'), which included...
Fig. 4. Expression of Rice AOS in Various Tissues and by Jasmonate.

Total RNA (10 μg) was separated on a 1.5% formaldehyde gel and probed with the full length of rice AOS cDNA (1437 bp). Root, scutellum, and shoot tissue were obtained from 4-day-old rice seedlings (cv. Nakdong). Flower and leaf samples were prepared when rice plants were at flowering stage, grown in a paddy field. For the 6th and 7th lanes, leaf segments (about 10 mm) floating in 20 mM Hepes-KOH, 0.1% Tween-20, pH 7.0 were treated with 50 μM jasmonate for 48 h before total RNA isolation. Equal amount of RNA was confirmed by ethidium bromide staining.

start and stop codon sequences. Additionally, one intronless gene in the rice genomic sequencing clone (CLC9174) was identical to AOS cDNA (www.tmri.org). These results confirmed that OsAOS genomic DNA did not have any introns.

OsAOS expression by Northern analysis was not particularly highly induced, but a basal level of expression was detected in all of the examined tissues, such as root, shoot, and leaves, suggesting various physiological roles of jasmonate (Fig. 4). Noticeably, although high expression of OsAOS was reported in a certain type of tissues, such as the scutellum node and flower in barley and tomato, respectively, there was no detection in rice plants. We have not yet measured pathogen-induced the OsAOS expression but the OsAOS expression in leaves was increased by jasmonic acid, an end product in jasmonate biosynthesis, indicating that this product, itself served as an internal signal molecule.

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References