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Resistance to *Magnaporthe grisea* in transgenic rice with suppressed expression of genes encoding allene oxide cyclase and phytodienoic acid reductase

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ABSTRACT

Linolenic acid (18:3) and its derivative jasmonic acid (JA) are important molecules in disease resistance in many dicotyledonous plants. We have previously used 18:3- and JA-deficient rice (F78Ri) to investigate the roles of fatty acids and their derivatives in resistance to the blast fungus *Magnaporthe grisea* [A. Yara, T. Yaeno, J.-L. Montillet, M. Hasegawa, S. Seo, K. Kusumi, K. Iba, Enhancement of disease resistance to *Magnaporthe grisea* in rice by accumulation of hydroxy linoleic acid, Biochem. Biophys. Res. Commun. 370 (2008) 344–347; A. Yara, T. Yaeno, M. Hasegawa, H. Seto, J.-L. Montillet, K. Kusumi, S. Seo, K. Iba, Disease resistance against *Magnaporthe grisea* is enhanced in transgenic rice with suppression of ω-3 fatty acid desaturases, Plant Cell Physiol. 48 (2007) 1263–1274]. However, because F78Ri plants are suppressed in the first step of the JA biosynthetic pathway, we could not confirm the specific contribution of JA to disease resistance. In this paper, we generated two JA-deficient rice lines (AOCRi and OPRRi) with suppressed expression of the genes encoding allene oxide cyclase (AOC) and 12-oxo-phytodienoic acid reductase (OPR), which catalyze late steps in the JA biosynthetic pathway. The levels of disease resistance in the AOCRi and OPRRi lines were equal to that in wild-type plants. Our data suggest that resistance to *M. grisea* is not dependent on JA synthesis.

Polyunsaturated fatty acids (PUFAs), including linolenic acid (18:3) and linoleic acid, are acylated in membrane lipids, and are the major sources of the oxidized metabolites called oxylipins. A typical 18:3-derived oxylipin, jasmonic acid (JA), acts as an important signaling molecule in defense responses to pathogen infection and wound stress in many dicotyledonous plants [1]. When a plant is exposed to fungal infection or wound stress, JA is transiently synthesized and then defense responses, such as the expression of pathogenesis-related (*PR*) genes and the biosynthesis of antimicrobial compounds, are activated [1]. The roles of endogenous JA have been confirmed by analyses of the JA-deficient *Arabidopsis* mutants, *fad3 fad7 fad8* and *opr3*. The *fad3 fad7 fad8* mutant is susceptible to the fungal pathogen *Pythium mastophorum* and the dipteran insect *Bradysia impatiens*, and is defective in wound responses and in the induction of *PR* genes [1]. A precursor of JA, 12-oxo-phytodienoic acid (OPDA), can also activate the defense responses via the JA signaling pathways. The *opr3* mutant, which can synthesize OPDA but not JA, is resistant to the fungal pathogen *Alternaria brassicicola* and the insect *B. impatiens*, and is not defective in expression of the JA-responsive genes [2].

The fungal blast caused by *Magnaporthe grisea* is a serious disease in rice (*Oryza sativa*). In a recent study, we found enhanced resistance to *M. grisea* in 18:3- and JA-deficient transgenic rice (F78Ri) plants [3], suggesting that the role of JA in defense responses in rice may be inconsistent with that in *Arabidopsis*. However, the specific contributions of *in vivo* JA in responses to fungal infection have not been clearly elaborated in rice, because mutants or transgenic lines that are defective only in JA production have not yet been analyzed.

The conversion from 18:3 to OPDA and JA consists of several reactions catalyzed by enzymes including lipoxygenase, allene oxide synthase, allene oxide cyclase (AOC) and OPDA reductase (OPR) [1]. OPDA is synthesized by AOC and then is reduced to

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3-oxo-2-(2′(Z)-pentenyl)-cyclopentane-1 octanoic acid (OPC 8:0) by OPR. Finally, JA is formed by three rounds of β-oxidation of OPC 8:0. It is not clear whether OPDA activates the JA signaling pathway in rice, as it does in *Arabidopsis*. Therefore, we studied JA-deficient rice lines with suppressed expression of the genes encoding AOC and OPR, in order to study the roles of these molecules and endogenous JA in defense responses.

There are four *AOC* genes, *AtAOC1*, *AtAOC2, AtAOC3* and *AtAOC4* in *Arabidopsis* [4]. The OPR isoenzymes are biochemically classified into two groups, designated OPRI and OPRII [5]. In *Arabidopsis*, two OPRI isozymes encoded by *AtOPR1* and *AtOPR2* and one OPRII isozyme encoded by *AtOPR3* have been characterized [6,7]. In rice, one *AOC* gene (*OsAOC*) and one *OPR* gene (*OsOPR1*) have been identified [8], however, until this study no OPR3-type homolog had been isolated. In this study, we isolated the rice gene *OsOPR3*. We generated and analyzed two transgenic rice lines, one with suppressed expression of *OsAOC* and one with suppressed expression of both *OsOPR1* and *OsOPR3*.

Materials and methods

RNA isolation and expression analysis. Total RNA was isolated using the TRIZOL reagent (Invitrogen Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. Expression analyses were performed by semi-quantitative reverse transcription-PCR as described previously [3]. The primers used to amplify each fragment were as follows: 5′-GCAGGAGATGTTCGTGTACG and 5′-GTTGCGCTCCGGCACGTGCT for *OsAOC*; 5′-TCCACCGCAAGGGCGCCCTC and 5′-GATGGCGTTCCGCGCGGCTC for *OsOPR1*; 5′-AGGCAGAGCTTCTCACCAAG and 5′-CGGTCATTGATTCCATCCTT for *OsOPR3*; 5′-ACCCGGTGCCCGGGGAGCAGC and 5′-GTGCTATCTTGGACCATCGGTTG for *JAmyb*; 5′-GCGTTCAACAACGACATGGACG and 5′-GTTGGCGTTCAGCAGCAGGTTG for *OsMAPK5*; 5′-CTACAGGCATCAGTGGTCAGTA and 5′-TCATCTTAGGCGTATTCGGCAG for *PBZ1*; 5′-TGGAGGTATCCAAGCTGGCC and 5′-TTAGTAAGGCCTCTGTCCGA for *PR1b*.

Wound and JA treatment. The wound treatment and JA application of rice plants were described previously [3].

Gene constructs and rice transformation. RNAi suppression vectors, targeted to *OsAOC* and to both *OsOPR3* and *OsOPR1*, were constructed as described previously [3,9]. Antisense and sense fragments were amplified from the cDNAs of each gene, and designated antisense (ASA) and sense (SA) fragments for *OsAOC*, antisense (AS3) and sense (S3) fragments for *OsOPR3*, and antisense (AS1) and sense (S1) fragments for *OsOPR1*. The primers used to amplify each fragment were as follows: 5′-CTTCTAGAAGGTGTAGAAGATCTTGAA (XbaI site underlined) and 5′-GAGGATCCTCGGCGATCTCGTCCCCTTC (BamHI site underlined) for ASA; 5′-GAGGCGCGCCTCGGCGATCTCGTCCCCTTC (AscI site underlined) and 5′-TCGAGCTCGAAGGTGTAGAAGATCTTGAAG (SacI site underlined) for SA; 5′-TATCTAGATACCCGCCAGCAGCGATGAAAG (XbaI site underlined) and 5′-CTGGATCCAGCCTCGAGAACCGGTGCCG (BamHI site underlined) for AS1; 5′-CTGGCGCGCCAGCCTCGAGAACCGGTGCCG (AscI site underlined) and 5′-TAGGCCTATACCCGCCAGCAGCGATGAAAG for S1; 5′-GCTCTAGAGCCCCGGAGGGTGCGCATCAG (XbaI site underlined) and 5′-GAGGATCCTCACTTTCCAACCGCTGCCG (BamHI site underlined) for AS3; 5′-GAGGCGCGCCTCACTTTCCAACCGCTGCCG (AscI site underlined) and 5′-GCGAGCTCGCCCCGGAGGGTGCGCATCAG (SacI site underlined) for S3. To create restriction sites at both ends of the S1 fragment, the amplified fragment was ligated into the pGEM-T easy vector (Promega, Madison, Wisconsin, USA) and was then cleaved out using the prepared AscI site and the ApaI site that is in the multiple cloning site of the vector. As a linker fragment, the third intron of the rice actin gene (*RAc1*; GenBank Accession No. X16280) was amplified from rice genomic DNA (*Oryza sativa* cv. Taichung 65) using the primers 5′-ACGGATCCGTGAGCACATTCGACACTGAAC (BamHI site underlined) and 5′-CAGGCGCGCCTGGGAAAAGGAAATTCAG (AscI site underlined). The CaMV35S promoter (35S) fragment was amplified from the vector pIG121Hm using the primers 5′-GGTTTAAACCTGCA GGTCCCCAGATTAGCC (PmeI site underlined) and 5′-TCTAGAGTCCCCCGTGTTCTC (XbaI site underlined).

The ASA, linker and SA fragments were inserted together into the XbaI-SacI site of the pIG121Hm Ti-vector [10], to produce pBIAOCRi, using the prepared restriction sites. The 35S, AS1, linker and S1 fragments, and the AS3, linker and AS3 fragments were inserted together into the PmeI–ApaI site and the XbaI–SacI site of the pIG121Hm Ti-vector, respectively, to produce pBIOPRRi. Wild-type rice was transformed with pBIAOCRi and pBIOPRRi by *Agrobacterium*-mediated transformation [10].

Quantification of JA and OPDA. Tissue samples were ground in liquid nitrogen with a mortar and pestle and then mixed with 70% methanol. [$^2H_2$](±)-JA was added to the extract as an internal standard. The extracts were centrifuged at 10,000*g* for 10 min and the supernatant was passed through a Strata C8 (100 mg/ml^{-1}) cartridge (Phenomenex, CA). Then, 20 μl of the eluate were injected into an Alliance 2695 separations module (Waters, Tokyo, Japan) coupled with a Quattro Ultima Pt mass spectrometer (Micromass, Inc., Manchester, UK). Fractions were separated on an L-column ODS column (2.1 × 150 mm, Chemicals Evaluation and Research Institute, Tokyo, Japan). The elution was performed using the solvent systems A, 0.1% (v/v) formic acid in water and B, 0.1% (v/v) formic acid in acetonitrile as follows: 0 min, 5% B in A; 0–5 min, linear gradient from 5% B to 50% B in A; 5–15 min, linear gradient from 50% B to 100% B in A; 15–20 min, 100% B. The flow rate was 0.2 ml min^{-1}. The MS/MS analysis was carried out in the negative-ion mode with an electrospray ionization interface. JA, [$^2H_2$](±)-JA and OPDA were detected in combination at m/z 209/59, 211/59 and 291/165, respectively, in the multiple reaction-monitoring mode.

Inoculation of plants with the blast fungus Magnaporthe grisea. The preparation of fungi, and inoculation of plants with the incompatible race 102 (84–107B) and the compatible race 001 (kyu91–107) of *M. grisea* were described previously [3]. The inoculated leaves were harvested at the indicated time points for expression analysis. Seven days after inoculation, the leaf lesions were classified into four levels of disease severity as follows. Level 1: when *M. grisea* infection had failed, the inoculation spot became a small white scar, similar to that resulting from a mock-inoculation. Level 2: a small dark-brown lesion had formed at the inoculation spot. Level 3: the dark-brown lesion had spread and surrounded the inoculation spot. Level 4: chlorotic and stunted regions had spread across the leaves.

Results

Characterization of rice OPR3-homologous genes

To identify rice *OPR3*-homologous genes, we performed a BLAST search of the Rice Annotation Project database (RAP-DB, http://rapdb.dna.affrc.go.jp) using the *AtOPR3* gene as a probe, and found a single locus (RAP-DB Locus ID. Os08g0459600). The corresponding cDNA clone was isolated from rice plants (cv. Taichung 65) by PCR using the sequence of this fragment to design primers. The sequence of the cDNA clone is similar to that of a known cDNA clone reported as *OsOPR13* [8]. The deduced ORF is 1185 bp in length and encode 394 amino acid residues. The amino acid sequence is more homologous with that of AtOPR3 (72% identity) than with those of AtOPR1 and AtOPR2 (50% and 50% identity, respectively, Table 1). Ten more *OPR* homologous genes have been reported for rice [8]. In the RAP-DB, we also found 2 novel genes