A novel lipoxygenase cDNA (3,007 base pairs) was isolated from rice leaves (Oryza sativa cv. Aichiasahi) which had been infected with an incompatible race of the rice blast fungus, Magnaporthe grisea. A single copy of the gene is present in the rice genome and encodes a protein of 923 residues with a molecular weight of 102,714. This gene product shares the least amino acid sequence homology among plant lipoxygenases identified to date. A novel feature of this gene product is a putative transit peptide sequence at the amino terminus, suggesting the enzyme is localized in chloroplasts. An active lipoxygenase was expressed from the cDNA in tobacco leaves and characterized. The lipoxygenase introduces molecular oxygen exclusively into the C-13 position of linoleic and linolenic acids. The gene is expressed at high levels 15 h after inoculation with an incompatible race of M. grisea, at a low level after inoculation with a compatible race of the pathogen, and is not expressed in mock-infected leaves. Gene expression begins at the same time that the pathogen begins to penetrate into leaf tissue. This novel lipoxygenase gene expression is a part of the early response of the host to pathogenic attack.

Lipoxygenase is ubiquitously distributed among plants and animals. Extensive enzymological investigation of lipoxygenases has been carried out (1–3). The enzyme uses molecular oxygen to catalyze the hydroperoxidation of unsaturated fatty acids containing a cis,cis,1,4-pentadiene moiety. The hydroperoxy fatty acids are metabolized into physiologically active compounds. Eicosanoids, one such class of derivatives occurring in mammalian cells, have been investigated extensively with regard to diverse metabolic functions (4). Jasmonic acid, derived from linolenic acid via lipoxygenase in plant cells, is a signal molecule which, among other things, triggers potato tuber formation (5) and gene expression in response to wounding (6). The molecular cloning of a number of lipoxygenases has also been reported from dicot plants (7–12) as well as mammalian species (1). Recently, the cloning of a monocot lipoxygenase, rice lipoxygenase L-2, which is expressed in maturing seeds and seedlings after germination, was reported (13).

Involvement of lipoxygenases in plant defense mechanisms against microbial invasion has been suggested. The production of toxic compounds, phytoalexins, is a typical plant defensive response to microbial invasion. Oxygenated unsaturated fatty acids constitute one such class of phytoalexins; these compounds have been isolated from rice leaves and are effective against the rice blast fungus, Magnaporthe grisea (14, 15). Ohta et al. (16) demonstrated in vitro production of rice phytoalexins using linoleate hydroperoxide, which is generated by a rice lipoxygenase and an enzyme fraction isolated from rice seeds. The same researchers also found increased lipoxygenase activity in rice leaves inoculated with an incompatible race of the blast fungus and an increase in lipid hydroperoxide decomposing activity, which catalyzes the production of antifungal compounds and hydroxylated unsaturated fatty acids (17). Others have suggested that lipoxygenase may be involved in the mechanism of resistance of oats to Puccinia coronata avena (18) and tomato to Cladosporium fulvum (19). These results indicate that the biosynthesis of oxygenated unsaturated fatty acids via the lipoxygenase pathway may be important in plant defense strategies against pathogenic attack. Studies on the role of plant lipoxygenase genes in plant defense have recently begun (20, 21).

Here we report isolation of a novel lipoxygenase cDNA from rice leaves which encodes a lipoxygenase carrying a putative transit peptide sequence, and we propose a second class of plant lipoxygenase genes based upon this putative transit peptide. We also show that this gene is expressed during early stages of an incompatible plant-pathogen interaction.

**EXPERIMENTAL PROCEDURES**

**Plant and Pathogen Growth and Inoculation—**A rice cultivar, Aichiasahi, was used as the host plant. Rice seeds were immersed in running tap water for 2 days and then disinfected by immersing the seeds in a fungicide, 0.2% Benlate (DuPont), for 2 h. The seeds were germinated at 37°C overnight. Seedlings were grown in a 1:2 mixture of commercial soil, Kumiai-Baizo (Tokyo, Japan), and vermiculite. The plants were grown in a phytotron at 25°C with 70% humidity under natural light and watered every other day.

The leaves of Aichiasahi were inoculated with two races of rice blast fungus, Magnaporthe grisea, 007 and 131, as previously described (22). Briefly, conidia produced synchronously on an agar medium were suspended in a sterile solution of 0.2% Tween 20. The concentration of conidia was adjusted to 5 x 10⁵/ml for inoculation. The fifth leaves of young rice plants were inoculated with the blast fungus by spraying them with the conidial suspension. The inoculated plants were kept in a plastic box (44.5 cm x 38.5 cm x 43.5 cm) at 25°C with 100% humidity for 36 h. Successful inoculation was checked by a method described previously (23).

**Preparation of Poly(A) RNA from Rice Leaves—**The leaves (2 g) were homogenized in 10 ml of 4X sodium thiocyanate, 0.1 X Tris-Cl, 1% 2-mercaptoethanol, pH 7.5 (solution A), using a Polytron homogenizer...
The homogenate was centrifuged at 5,000 x g for 10 min, and the supernatant was recovered. The supernatant (9 ml) was layered onto a cushion of CsCl which was prepared by layering 1.0 ml of solution A containing 2.4 M CsCl on 1.5 ml of 5.7 M CsCl, 25 mM sodium acetate (pH 5.2) in an ultracentrifuge tube (14 x 95 mm). Centrifugation was performed with a swinging bucket rotor, SW41 (Beckman) at 95,000 x g for 2 h. The precipitated RNA was collected by centrifugation (5,000 x g, 10 min, 4°C) and dissolved in 0.5 ml of water for poly(A) RNA isolation. Poly(A) RNA was isolated using a Spin column containing oligo(dT)25-cellulose (Pharmacia) according to the protocol of the supplier.

cDNA Cloning—Poly(A) RNA (2.5 μg) was used to synthesize cDNA either by the method described by Hay nh et al. (54) (for library A) or using a kit (Pharmacia) according to the protocol of the supplier (for library B). The former method used EcoRI linkers and the latter used EcoRI/Nool adapters. The cDNAs synthesized by these methods were introduced into an expression vector, pGtl, and then packaged in phage particles using an in vitro packaging kit (Gigapack Gold, Stratagene). The phages were amplified in Escherichia coli Y1088 (24) and then stored at 4°C. Library A and B consisted of 20 and 3 lots, respectively, the amplification of which were carried out independently. The total number of recombinants in each library was approximately 3 x 106. A mixed oligonucleotide, 5’-TATGCGAARTTNACGNCGR- 3’ (R = A, G, T, C; N, A, C, G, T), 100 μg/ml salmon sperm DNA (pH 7.4). One positive clone, designated ARLL1, was isolated from 2 x 106 recombinants. The cDNA fragment in ARLL1 (1.4 kb) was isolated by EcoRI digestion, subcloned into a Bluescript plasmid pKS- (Stratagene), and the resulting plasmid was designated pRLL1. That plasmid was digested with EcoRI and labeled with 32P-dCTP. The blot was hybridized out by the Sanger procedure with the vector, R408 (Stratagene). DNA sequence data were analyzed with the Phred program.

cDNAs were made using a kit (Erase-a-Base, Promega). Single-stranded DNA was prepared from the deleted constructs using a helper vector, pET3b (27) at a filled in BarnHI site. The plasmid DNA carrying RLUpET3b were collected and suspended in 50 μl of 50 mM sodium phosphate (pH 6.8) and then disrupted by sonication. The particulate material was removed by centrifugation (5,000 x g, 10 min), and the supernatant was used as the source of RNA. A cDNA fragment (1 mg of protein/ml). A control E. coli homogenate was prepared using E. coli carrying the vector, pET3b, in the same manner.

The activity of lipoygenase was determined as described elsewhere (29) using a Clark oxygen electrode. The reaction mixture (3 ml) contained E. coli homogenate (0.1 mg of protein), 1.0 mM lipoxyg ene, 0.1% SDS, 0.1% Ficoll (type 400, Pharmacia), 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin (Fraction V, Sigma), 100 μg/ml salmon sperm DNA (pH 7.4). One positive clone, designated ARLL1, was isolated from 2 x 106 recombinants. The cDNA fragment in ARLL1 (1.4 kb) was isolated by EcoRI digestion, subcloned into a Bluescript plasmid pKS- (Stratagene), and the resulting plasmid was designated pRLL1. The cDNA fragment of pRLL1 (isolated by EcoRI digestion) was used as a probe to isolate a longer clone, ARLL2 from library A. The cDNA fragment of ARLL2 (2.867 bp) was digested with EcoRI and subcloned into pUC118 (Takara Shuzo Co., Ltd.) to obtain a plasmid designated pRLL2. That plasmid was digested with PvuII and a fragment (280 bp) located at the 5' end of the cDNA was isolated by HindIII and EcoRI, and then the resulting fragment (containing the downstream sequence of the cDNA beginning with nucleotide 485) was isolated. The fragment was filled in using Klenow enzyme, resulting in additional nucleotides, AG, at the 5’ end derived from the HindIII site. The fragment was then introduced into an expression vector, pET3b (27) at a filled in HindIII site. The plasmid carrying the rice gene in the appropriate orientation was designated RLUpET3b and purified from E. coli HB101. The protein encoded by this construct has two regions: 801 amino acids encoded by the rice lipoygenase cDNA fragment and 15 amino acids at the amino terminus which de- rived from the expression vector as a result of the manipulation. RLL/pET3b was used to transform E. coli BL21(DE3), which carries the gene for T7 RNA polymerase under control of the lac UV5 promoter (27).

An active lipoygenase was expressed from RLL/pET3b by the low temperature cultivation method which was used to express rice lipoygenase gene in E. coli (29) after cultivation at 15°C for 16 h in the presence of 0.4 mM iso C3 isopropyl-β-D-thigalactopyranoside. E. coli cells carrying RLL/pET3b were collected and suspended in 50 ml of 50 mM sodium phosphate (pH 6.8) and then disrupted by sonication. The particulate material was removed by centrifugation (5,000 x g, 10 min), and the supernatant was used as the source of RNA. A cDNA fragment (1 mg of protein/ml). A control E. coli homogenate was prepared using E. coli carrying the vector, pET3b, in the same manner.

To determine the regiospecificity of the enzyme expressed in E. coli, a fraction containing 0.6 nanokatal of lipoygenase activity was added to 1 ml of 0.7 mM linoleic acid or linolenic acid, and 0.25% (v/v) Tween 20 in 0.1 x sodium acetate (pH 5.5). The activity (1 katal) is defined as the amount of enzyme which catalyzes the consumption of 1 mol O2/μs at 30°C. Protein was determined by the Folin method (30).

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RESULTS

Isolation of a Lipoygenase cDNA from Rice Leaves—We prepared cDNA libraries from rice leaves on which an incompatible interaction between rice (cultivar Aichiasahi) and the rice blast fungus (M. grisea) occurred. The fifth leaves of young rice plants were infected with race 131 of the blast fungus, which is incompatible with this rice cultivar. The leaves were harvested 30 h after inoculation of the fungus, the time at which an increased level of lipoygenase activity has been shown (17).
Poly(A) RNA was isolated from the leaves and used to prepare two cDNA libraries (library A and library B) which differed in the type of linkers used to prepare them.

We attempted to screen the cDNA library (library A) using DNA fragments of either soybean lipoxygenase L-1 (7) or rice lipoxygenase L-2 (13) as probes under various hybridization conditions. However, no positive clones were obtained, indicating a low level of nucleotide sequence homology between the rice leaf lipoxygenase cDNA and the previously identified genes.

We utilized a peptide sequence, HAAVNFGQY, conserved in seven plant lipoxygenases (7-13) and animal 5-lipoxygenases (33, 34) to prepare a mixed oligonucleotide probe. Using the probe, ARLL2 (2.8 kb) clone was isolated from library A. Two additional clones, ARLL3 and ARLL4, were isolated using a 5' end fragment of the ARLL2 cDNA from library B (Fig. 1). The nucleotide sequence of the cDNA (3,007 bp) (Fig. 2) contains a long open reading frame beginning with the first ATG codon (119-1,287), and one stop codon (nucleotide 38-40) occurs upstream of the ATG codon in the same frame. The nucleotide sequence at the first ATG codon, ACCATG, is the consensus translation initiation sequence in eucaryotes as shown by Kozak (35). The molecular weight of the gene product is 102,714 based on 923 amino acids. A second ATG codon (nucleotide 350-352) is present in the long open reading frame. For reasons mentioned below, it is unlikely that this codon is the initiation codon, and we assume the first ATG to be the initiation codon.

The nucleotide sequence of the cDNA shows a strong bias toward G and C in the selection of nucleotides in the open reading frame (66% G/C), especially at the third codon position (91% G/C), while the 5' and 3' noncoding nucleotides (nucleotides 1-118 and 2888-2999) contain 51 and 31% G/C, respectively. A similarly strong G/C bias was observed in the coding region of rice lipoxygenase L-2 cDNA, but not in the noncoding regions (13). The strong G/C bias (65% G/C) in the nucleotide sequence between the first ATG and the second ATG (nucleotide 118-249) of the long open reading frame suggests that the first ATG codon is the initiation codon.

Protein Structure of the Novel Lipoxygenase—Several striking features of the lipoxygenase gene product were found by comparing the deduced amino acid sequence with those of previously identified lipoxygenases from rice (13), soybean (7-10), and pea (11, 12). The gene product contains two regions: (i) a putative signal sequence which is limited in composition to several amino acid residues; (ii) an amino terminal sequence which is limited in composition to several amino acid residues. It is unlikely that the amino terminus coding region of the cDNA clone is an artifact of the cloning process since the cDNA clones ARLL2, ARLL3 and ARLL4 all contain regions which overlap one another (Fig. 1). In addition to this, ARLL3 and ARLL4 were isolated from a different cDNA library than the one from which ARLL2 was isolated. Finally, Northern blot analysis using DNA fragments from the amino terminus coding region and from the LHR showed that both probes hybridized to the same band on a Northern blot (data not shown).

The gene product shares approximately 43% amino acid sequence identity in the LHR with plant lipoxygenases, including rice lipoxygenase L-2 (Table I). The LHR is located between Gly111 and Arg112, residues which are completely conserved among the plant lipoxygenases and the carboxyl terminus. Despite the fact that the homology of this protein with other plant lipoxygenases is low, the conserved amino acids found among plant and mammalian lipoxygenases, which are thought to be
The carboxyl-terminal sequence, GIPNS(T)SI, is also conserved in lipoygenase function (1, 3), are present (Fig. 2). The amino-terminal amino acid sequence (Met1 to Ile110) found upstream of the LHR (Gly111 to Ile293) demonstrates conserved secondary structure between this protein and rice lipoxygenase L-2 (data not shown). Taken together, these results suggest that the gene product is a plant lipoygenase.

The amino-terminal amino acid sequence (Met1 to Ile110) is also conserved among plant lipoygenases. The hydrophathy profile of the LHR (Fig. 3) demonstrates conserved secondary structure between this protein and rice lipoygenase L-2 (data not shown). Taken together, these results suggest that the gene product is a plant lipoygenase.

The amino-terminal amino acid sequence (Met1 to Ile110) found upstream of the LHR consists of hydroxyl amino acids, Ser and Thr (33%), and small hydrophobic residues, Val and Ala (24%), positively charged amino acids, Arg, Lys, and His (13%), negatively charged amino acids, Glu and Asp (5%), and other amino acids (25%). Clusters of these hydroxyl amino acids and dispersion of hydrophobic amino acids are seen throughout the sequence. These features of the amino acid sequence are consistent with the structure of transit peptides for translocation of proteins into chloroplasts (36). It is unlikely that the protein is targeted into mitochondria because the amphiphilic structure prerequisite for such targeting (36) was not found in the sequence.

Characterization of the Leaf Lipoygenase—To determine whether the gene product has lipoygenase activity, we expressed the gene in E. coli from a fragment of the cDNA (nucleotides 485–5007) which encodes 99.8% of the LHR and the 3‘-noncoding region but not the putative transit peptide sequence (Fig. 3). We took advantage of the low temperature cultivation method previously described for expression of rice lipoygenase L-2 in E. coli (28). Cultivation of E. coli carrying the cDNA fragment at 15 °C produced an active lipoygenase. The E. coli homogenate prepared from the cDNA fragment at 15 °C was most active from pH 5 to 6. Activities at pH 5.5 in the homogenate were 2 nanokatal/dmg of protein toward linolenic acid and 0.5 nanokatal/dmg of protein toward linoleic acid. The observed activity was heat labile, and no activity was observed using a control homogenate prepared from E. coli cells carrying the vector without the cDNA fragment.

The regiospecificity of hydroperoxidation on linolenic and linoleic acids by the lipoygenase expressed in E. coli was analyzed using reverse-phase HPLC (Fig. 4). The lipoygenase introduced molecular oxygen exclusively into the C-13 position of these fatty acids. The ratio of 9-hydroperoxy to 13-hydroperoxy fatty acid reaction products was 2% for linolenic acid and 12% for linoleic acid. It is noteworthy that formation of 13-hydroperoxy linolenic acid is a prerequisite for biosynthesis of jasmonate, a signal molecule for a variety of physiological events, including plant defense against insects (6).

Expression of the Lipoygenase Gene after Pathogen Attack—We examined expression of the gene in the leaves of a rice cultivar, Aichiasahi which carries a resistance gene, Pi-a, for the rice blast fungus. The fifth leaves of young plants were inoculated with either an incompatible race, 007, or a compatible race, 131, of the blast fungus, M. grisea, and the amount of the lipoygenase protein and RNA was measured at various time points. We detected the lipoygenase protein in rice leaves using antibodies raised against the protein expressed in E. coli.

The lipoygenase protein (approximately 100 kDa) was detectable by Western blot analysis of leaf samples by 15 h after inoculation with the compatible race, 007, of the blast fungus, M. grisea, and the amount of the lipoygenase protein and RNA was measured at various time points. We detected the lipoygenase protein in rice leaves using antibodies raised against the protein expressed in E. coli.

TABLE 1
Comparison of a lipoygenase homologous region (Gly111–Ile293) of the pathogen inducible lipoygenase from rice with the corresponding regions of other plant lipoygenases

|       | R2a | S1b | S2c | S2′ | S514′ | P2′ | P3′ | At′ |
|-------|-----|-----|-----|-----|-------|-----|-----|-----|
| RLL'  | 41.8| 43.4| 43.6| 44.5| 44.5  | 43.5| 43.5| 43.4|
| R2    | 52.8| 53.4| 53.7| 51.6| 52.4  | 53.3| 53.3| 55.7|
| S1    | 84.4| 71.6| 69.8| 69.8| 78.8  | 78.8| 56.4| 56.4|
| S2    | 75.0| 72.8| 84.8| 74.5| 58.2  | 58.2| 58.2| 58.2|
| S3    | 71.4| 72.3| 71.4| 71.4| 61.3  | 61.3| 61.3| 61.3|
| S514  |     |     |     |     |       |     |     |     |
| P2    |     |     |     |     |       |     |     |     |
| P3    |     |     |     |     |       |     |     |     |

* Rice L-2 (13).
* Soybean L-1 (7).
* Soybean L-2 (8).
* Soybean L-3 (9).
* Soybean SC514 (10).
* Pea sequence (11).
* Pea sequence (12).
* Arabidopsis thaliana sequence (21).
* Rice sequence reported here.

Expression of the cloned rice lipoygenase cDNA in E. coli. E. coli cells carrying RLL/pET3b (lanes 2 and 3) or pET3b (lane 1) were cultivated at 37 °C for 3 h (lanes 1 and 2) or at 15 °C for 16 h (lane 3). Protein from these cells were separated on a 16% polyacrylamide gel containing 1% SDS. The lipoygenase purified from E. coli cells carrying RLL/pET3b was also electrophoresed (lane 4). The position of lipoygenase on the gel is indicated by an arrow.

FIG. 3. Expression of the cloned rice lipoygenase cDNA in E. coli. E. coli cells carrying RLL/pET3b (lanes 2 and 3) or pET3b (lane 1) were cultivated at 37 °C for 3 h (lanes 1 and 2) or at 15 °C for 16 h (lane 3). Protein from these cells were separated on a 16% polyacrylamide gel containing 1% SDS. The lipoygenase purified from E. coli cells carrying RLL/pET3b was also electrophoresed (lane 4). The position of lipoygenase on the gel is indicated by an arrow.
A Novel Lipoxygenase from Rice

FIG. 4. Reverse-phase HPLC of hydroperoxides produced by the lipoxygenase expressed from the cDNA in E. coli. Linoleic (A, B) or linolenic (C, D) acid (0.7 mM) was incubated at 20 °C for 1 h with E. coli homogenate from cells expressing the lipoxygenase cDNA (A, C) or cells carrying the empty expression vector (B, D). The reaction products were extracted with chloroform and subjected to HPLC. Retention times of 9-hydroperoxy fatty acids and 13-hydroperoxy fatty acids were determined using authentic standards. Their elution positions are indicated in the figure as C9 and C13, respectively.

FIG. 5. Expression of the rice lipoxygenase protein in leaves after inoculation with M. grisea. Proteins were extracted from the fifth leaves of the rice cultivar, Aichiasahi, after inoculation with race 131 of the rice blast fungus (B), a compatible race 007 (C), or mock-infected (A). The proteins (40 μg/slot) were separated on a 10% polyacrylamide gel containing 1% SDS and then transferred to a nitrocellulose membrane. Lipoxygenase on the membrane was visualized using an antibody raised against lipoxygenase expressed from cDNA in E. coli. The positions of the lipoxygenase in (B) and (C) and an additional faint higher molecular weight band at 15 h in (B) were shown by arrowhead and dot (.), respectively.

tute a precursor form of the lipoxygenase, but no further examination was carried out.

Expression of the mRNA was observed 15 h after inoculation with the incompatible pathogen, and the level of this mRNA increased until 33 h after inoculation (Fig. 6). In contrast, the expression of the mRNA was weak in leaves infected with the compatible pathogen and very weak in mock-infected leaves 24 h after treatment (Fig. 6). The amount of RNA used in these experiments was normalized using expression of a rice actin gene as an internal control.

These results indicate that expression of the lipoxygenase gene is specific to the incompatible interaction between rice and the pathogen. It is noteworthy that the start of lipoxygenase gene expression coincides with penetration of the rice leaf by the pathogen (23), indicating that gene expression is an early event in the host plant response against the pathogen.
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Genomic Southern Analysis—Genomic DNA was isolated from leaves of the rice cultivar, Aichiasahi, and digested with restriction enzymes for Southern analysis (Fig. 7). The result of this experiment indicates the presence of a single copy of the lipoxygenase gene in the rice genome.

Discussion

We isolated a novel lipoxygenase cDNA from rice leaves after inoculation with the incompatible blast fungus. The gene product is a novel type of plant lipoxygenase and contains two regions: an amino-terminal sequence (Met↑ to Ile↑↑↑↑) and an LHR (Gly↑↑↑↑ to Ile↑↑↑↑↑). The LHR has the lowest amino acid identity among lipoxygenases studied to date; similarity even with other rice lipoxygenases such as L-2 is quite low (13) (Table I). In spite of low homology, amino acids generally conserved among plant and mammalian lipoxygenases, especially histidine residues which have been suggested to be putative iron-binding sites (1, 3), are present in the protein sequence (Fig. 2). More recently, from the electron density map of the crystallized soybean lipoxygenase L-1, Minor et al. (37) identified five ligands of the active site iron atom in the enzyme, His↑, His↑↑↓, Asp↑↓, and Ile↑↑, the carboxyl-terminal residue which are also found in the sequence. The hydropathy profile of the LHR is similar to those of rice lipoxygenase L-2 and dicot lipoxygenases. These results and expression of an active lipoxygenase in E. coli from part of the LHR indicate that the gene encodes an active plant lipoxygenase.

We propose to classify this gene into a new category of plant lipoxygenase gene. The previously identified lipoxygenase genes including rice lipoxygenase L-2 can be grouped into a single type of gene category (Lox1) based upon their mutual sequence similarity extending from amino to carboxyl termini and their lack of obvious structural differences (41). The gene isolated in this study can be classified into a second gene category (Lox2) based on the novel feature of the amino-terminal sequence upstream of the LHR and the relatively low level of coded amino acid sequence conservation with members of the Lox1 family. In conformation with the guidelines issued by the Commission on Plant Gene Nomenclature (41), we designate the first member of the Lox2 family from rice (Oryza sativa) Lox2:Os:1, with Os indicating the species origin.

The gene isolated in this study is expressed in rice leaves during an incompatible interaction between rice and a fungal pathogen, M. grisea. Southern analysis showed that the gene is derived from the rice genome (Fig. 7), indicating that the gene expression is a host reaction against the incompatible race of the pathogen. The gene expression (mRNA and protein) was observed 15 h after inoculation with the incompatible race of the pathogen (Figs. 5 and 6), the time at which the pathogen begins to penetrate into the leaves (23). This coincidence indicates that Lox2:Os:1 expression is an early response of the plant against pathogen attack. In contrast, expression of the gene was weak when the plant was inoculated with a compatible race of the pathogen, although the compatible race penetrated into the leaves at the same time as the incompatible race (23). These results suggest that Lox2:Os:1 gene expression is involved in plant defense against the invading pathogen. Recently, Koch et al. (20) found an increase in lipoxygenase mRNA in tomato leaves upon inoculation with plant pathogenic bacteria. They detected the lipoxygenase mRNA using a heterologous probe derived from a pea lipoxygenase cDNA (76% identity at the nucleotide level to soybean lipoxygenase L-3) under medium stringent conditions (50 °C, 2 x SSC). It seems likely that the tomato lipoxygenase mRNA belongs to Lox1. However, this question must be investigated further by nucleotide sequencing of the tomato lipoxygenase mRNA. More recently, Melan et al. (21) reported cloning of an Arabidopsis thaliana lipoxygenase cDNA and showed induction of the gene by pathogens. That gene can be classified into Lox1. It will be of considerable interest to determine whether a counterpart of the Lox2 gene studied here also exists among dicots.

Using DEAE-Tylosep chromatography, Ohta et al. (17) purified three lipoxygenases, leaf lipoxygenase-1, leaf lipoxygenase-2, and leaf lipoxygenase-3, from leaves both inoculated and uninoculated with race 131. They found that the increase in overall lipoxygenase activity was attributable mainly to leaf lipoxygenase-3, although activities of leaf lipoxygenase-1 and leaf lipoxygenase-2 also increased slightly. The similar induction pattern we observed using antibodies prepared against the product of Lox2:Os:1 suggests its identity as leaf lipoxygenase-3. However, further studies will be required to confirm its identity.

It seems likely that the amino-terminal sequence, Met↑ to Ile↑↑↑↑ of the pathogen inducible lipoxygenase is a transit peptide for targeting the lipoxygenase into chloroplasts. However, we have not yet confirmed its intracellular location for the mature protein. Chloroplasts contain both substrates, oxygen and unsaturated fatty acids, required for the lipoxygenase reaction. A rapid disappearance of chloroplast glycolipids accompanied by increases in other lipid species was observed in leaves subjected to rust infection (38). It is possible that lipoxygenase localized in the same compartment produces hydroperoxides in combination with the release of fatty acids during conversion of glycolipids to other lipids.

It remains to be determined whether lipoxygenase gene expression in rice leaves is involved in the production of antifungal compounds, oxygenated fatty acids, or in the production of other physiological active compounds. Li et al. (39) showed that 13-hydroperoxides and 13-hydroxides of both linoleic and linolenic acids rapidly increase in areas of rice leaves which have been inoculated with the rice blast fungus. The highest concentrations of these compounds were reached within 24 h after inoculation. These results are consistent with our observation of lipoxygenase gene expression in rice leaves. Croft et al. (40) proposed a role for lipoxygenase in initiating processes that lead to hypersensitive cell death by showing that increases in lipoxygenase activity precede increases in superoxide dismutase and peroxidase activity in Phaseolus vulgaris (L) cv. Red Mexican, in response to infection with an incompatible race of Pseudomonas syringae, pv. Phaseolicola race 1. It will be useful to introduce antisense lipoxygenase messages into rice for assessment of lipoxygenase function in plant defense mechanisms.

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