Research Note

Identification of Rice Blast Fungal Elicitor-Responsive Genes by Differential Display Analysis

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In order to study molecular interactions that occur between rice and rice blast fungus upon infection, we isolated fungal elicitor-responsive genes from rice (*Oryza sativa* cv. Milyang 117) suspension-cultured cells treated with fungal elicitor prepared from the rice blast fungus (*Magnaporthe grisea*) employing a method that combined mRNA differential display and cDNA library screening. Data base searches with the isolated cDNA clones revealed that the *OsERG1* and *OsERG2* cDNAs share significant similarities with the mammalian Ca^{2+} -dependent lipid binding (C2) domains. The *OsCPXI* cDNA is highly homologous to peroxidases. The *OsHin1* cDNA exhibits homology to the tobacco *hin1* gene, whose expression is induced by avirulent pathogens. The *OsLPL1* and *OsMEK1* cDNAs share homologies with lysophospholipases and serine/threonine mitogen-activated protein (MAP) kinase kinases, respectively. The *OsWRKY1* and *OsEREBP1* cDNAs are homologous to transcription factors, such as the WRKY protein family and the AP2/EREBP family, respectively. Transcripts of the *OsERG1*, *OsHin1*, and *OsMEK1* genes were specifically elevated only in response to the avirulent race KJ301 of the rice blast fungus. Our study yielded a number of elicitor-responsive genes that will not only provide molecular probes, but also contribute to our understanding of host defense mechanisms against the rice blast fungus.

Plants protect themselves against pathogen attack by launching both localized and systemic defense responses such as the generation of reactive oxygen species, the accumulation of phytoalexins, and the synthesis of pathogenesis-related proteins that include hydrolytic enzymes (Dixon and Lamb 1990; Yang et al. 1997).

When a pathogen penetrates plant tissue, the plant cells perceive signals coming from the pathogen. These signals, known as elicitors, can be compounds such as oligosaccharides, pro-

teins, and glycoproteins that are released from fungal as well as plant cell walls and are transmitted to give activation of defense gene expression via intracellular signaling cascades (Dixon and Lamb 1990; Ryan and Farmer 1991). Elicitors specifically bind to receptors on the plasma membrane and trigger the signaling events necessary for the onset of defense responses (Basse et al. 1993; Nurnberger et al. 1995).

Fungal diseases of rice, such as rice blast and sheath blight, which are caused by *Magnaporthe grisea* and *Rhizoctonia solani*, respectively, remain major factors in limiting rice productivity worldwide (Xu et al. 1996). Among the immediate goals of research is, therefore, the development of appropriate defense systems capable of enhancing resistance against these pathogens. In this endeavor, it is important to understand the molecular mechanisms of the natural defense responses of rice (Masuta et al. 1991). The molecular mechanism of the elicitor-mediated signal transduction pathway in plants is not yet fully understood. Furthermore, though many disease resistance genes of various dicotyledonous plants have been cloned, not much has yet been reported about reliable resistance genes in rice. Suspension-cultured cells, because of their biochemical and physiological homogeneity, offer a better system than whole plants in which to study elicitor-mediated signaling (Masuta et al. 1991). Elicitors emanating from fungal cell walls can provoke many of the defense responses seen in plants and also in cultured cells (Cote and Hahn 1994; Kim et al. 1998). The differential display polymerase chain reaction (PCR) method in combination with polyacrylamide gel electrophoresis allows comparison of PCR-amplified cDNA fragments between two or more mRNA populations, and has been very successful in the identification of differentially expressed genes (Liang and Pardee 1992). The identification of differentially accumulated mRNAs has helped not only in the understanding of gene function but also in the understanding of the underlying molecular mechanisms in a particular biological system.

We set out to study the pathogen signal transduction pathway that occurs in rice (*Oryza sativa* L. 'Milyang 117') upon infection by the fungal pathogen *Magnaporthe grisea*. As a first step, we established optimal experimental conditions for both mRNA differential display and direct cDNA library screening. Then we cloned and sequenced a large number of

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elicitor-responsive genes isolated from a rice cell culture. We treated the embryogenic suspension-cultured rice cells with fungal elicitor (50 µg of glucose equivalents per ml) for 30 and 60 min before isolating total cellular RNA (Ohira et al. 1973). The oligosaccharide fungal elicitor was prepared from the cell wall fraction of the rice blast fungus *Magnaporthe grisea* KJ301, which is the avirulent strain to the rice cultivar Milyang 117, by the method of Doke and Furuichi (1982). One hundred and twenty reverse transcription (RT)-PCRs

Fig. 1. Differential display patterns of total RNA from water-treated (C) and fungal elicitor-treated (30 and 60 min) rice suspension cells using arbitrary 5' primers and 3' anchored oligo-dT primers. To remove contamination of the chromosomal DNA from isolated total RNA, 50 µg of total cellular RNA was incubated for 30 min at 37°C with 10 units of GH-DNase I according to the manufacturer's protocol (Message Clean Kit; GenHunter, Brookline, MA). One hundred nanograms of each total RNA sample was reverse transcribed with the 3' anchored primers (H-T11G, 5'-AAGCTT TTTTTTTTTT-3'; H-T11C-3') and the resultant cDNAs were amplified in the presence of ³²P-dATP with the same 3' primer and either H-AP1 (5'-AAGCTTGATTGCC-3') or H-AP3 (5'-AAGCTTTGGTCAG-3') as the 5' primer with RNA Image Kit (GenHunter, Brookline, MA). The polymerase chain reaction products were separated on a 6% denaturing polyacrylamide gel. The gel was then dried and exposed to X-ray film. Positive fungal elicitor-induced display bands (arrowheads) were excised from the gel and re-amplified according to the manufacturer's specifications. Fungal elicitor (50 µg of glucose equivalents per ml) was added to the growth medium, and incubated for 30 and 60 min.

were performed for each RNA extract with the combinations of 40 5' arbitrary 13-mer-primers (H-AP1-H-AP40) and three different 3' one-base-anchored primers (H-T11G, H-T11A, H-T11C) according to the manufacturer's protocol (RNA Image Kit; GenHunter, Brookline, MA). An example of the mRNA differential display patterns is shown in Figure 1. In each case, RNA from elicitor-treated cells (30 and 60 min) was compared with RNA from water-treated cells as a control. After separation of the cDNAs on a DNA sequencing gel, we were able to identify approximately 800 cDNA fragments up-regulated differentially and ranging from 100 to 350 bp in length. After elution from the gel, these bands were re-amplified with the same set of primers used for the differential display under the same PCR conditions without radioactive nucleotides.

To evaluate the expression patterns of the re-amplified cDNA fragments in response to fungal elicitor, all 800 cDNA

Fig. 2. Northern (RNA) blot analysis of total RNA isolated from water-treated (control) and elicitor-treated (10 and 30 min, 1, 2, 4, 8, 12, and 24 h) cells with the elicitor-responsive genes isolated by both differential display and library screening as probes. Fungal elicitor (50 µg of glucose equivalents per ml) was added to the growth medium and incubations were carried out for the indicated lengths of time. The differential expression patterns of the elicitor-responsive genes observed upon treatment with fungal elicitor were shown. The rice chitinase *Rch2* gene was used as a positive control (Kim et al. 1998). A 20-µg aliquot of total RNA was loaded per lane. RNA equal loading was checked by hybridization of the blot with a probe corresponding to the rice 18S ribosomal RNA (rDNA).

fragments obtained from differential display were tested for transcriptional induction by RNA gel blotting. The majority of cDNA bands were present in equal abundance in the control and elicitor-treated cells. Among them, 70 cDNAs showed differential accumulation of transcript in response to elicitor treatment. All 70 cDNAs were separately used as probes for cDNA library screening. In general, direct cloning of PCR-amplified cDNAs into a plasmid vector is prone to produce two or more unrelated cDNAs of the same size in the re-amplified PCR product. To circumvent this problem, we directly screened a λ ZAP cDNA library constructed from rice suspension-cultured cells treated with fungal elicitor, with the PCR re-amplified cDNA fragments as probes. The probes were 32 P-labeled by random primed labeling.

By analysis of 5'- and 3'-nucleotide sequences of positive cDNA clones isolated from library screening with the BLAST (basic local alignment search tool) program (Altschul et al. 1990), we obtained 115 different cDNA clones. From them we chose 16 cDNA clones for further analysis, because only these clones showed accumulation of transcripts by treatment of the fungal elicitor upon Northern (RNA) blot analysis (Fig. 2). Table 1 summarizes the sequencing results for the cDNAs obtained by the combination of mRNA differential display and library screening.

The clones F21502 (*OsERG1*) and F21503 (*OsERG2*) exhibited homology to a novel protein from *Zea mays* (U64437). Moreover, the *OsERG1* and *OsERG2* clones also shared significant homology with mammalian Ca^{2+} -dependent lipid binding (CaLB or C2) domains. The *OsERG1* and *OsERG2* cDNAs have open reading frames of 480- and 471-bp encoding putative proteins of 159 and 156 amino acids, respectively, each containing a single C2 domain. In contrast to the large number of C2-containing proteins known in animals, very few C2 domain proteins have been reported in plants. In animal cells, the C2 domain proteins are most likely to be involved in a variety of signal transduction pathways. The C2 domains in

PKC, PLC, cPLA2, synaptotagmin, GAP, and Ras-GTPase play regulatory roles in mediating the Ca^{2+} -dependent recruitment of these enzymes to the phospholipid membrane (Kopka et al. 1998). In contrast, no function has so far been assigned to C2 domains in plant proteins. The presence of a C2 domain within the sequence of *OsERG1* and *OsERG2* suggests that the *OsERG1* and *OsERG2* proteins have an as yet unidentified function that is regulated by $[\text{Ca}^{2+}]$ and/or phospholipid in response to elicitor stimuli. The F20209 (*OsHin1*) clone shares significant homology with *hin1* from tobacco, a gene that is rapidly activated by both harpins and an *avrPto*-mediated signal (Gopalan et al. 1996). The F14201 (*OsCaM3*) clone was 100% identical to the novel rice calmodulin CaM61 with the C-terminal extension of 38 amino acids (U37936). F21711 (*OsWRKY1*) exhibited high homology to the WRKY protein family (Rushton et al. 1996). The WRKY proteins are a novel type of transcription factors with zinc finger domains that bind to the W box (TTGACC motif) located in the promoter sequence of plant defense genes (Rushton et al. 1996). The F12311 (*OsLPL1*) clone has significant homology to the lysophospholipases from Arabidopsis. Lysophospholipases are widely distributed enzymes that hydrolyze lysophospholipids, the detergent-like intermediates in phospholipid metabolism, and therefore regulate the lysophospholipid levels in cells (Wang et al. 1997). The F72101 (*OsCPX1*) clone showed homology to plant peroxidases that are involved in several plant defense responses including lignification and cross-linking of cell wall proteins (Bradley et al. 1992). The F15501 (*OsMEK1*) clone revealed most homology to a novel serine/threonine protein kinase, *PkpA*, from the filamentous fungus *Phycomyces blakesleeana*, whose sequence is related to a number of mitogen-activated protein (MAP) kinase kinase proteins. The *OsMEK1* cDNA encodes a putative MAP kinase kinase. The F12205 and F12212 clones encode an Rab GDP dissociation inhibitor (RabGDI) with more than 50% identity to the RabGDIs of various organisms

Table 1. Sequence analysis of differentially expressed genes isolated from elicitor-treated rice cells by differential display and cDNA library screening^a

Clone	GenBank accession no.	cDNA size ^b	Sequence homology ^c	Data base match (accession no.) ^d	Identity score ^e
F21502	U95135	851	Novel protein (<i>OsERG1</i>)	Maize (U64437)	X53
F21503	U95136	745	Novel protein (<i>OsERG2</i>)	Maize (U64437)	X164
F20209	AF039532	1067	<i>hin1</i> (<i>OsHin1</i>)	Tobacco (Y07563)	X195
F14201	AF090687	926	Calmodulin (<i>OsCaM3</i>)	Rice (U37936)	X412
F21711	AF090699	1714	SPF1-like DNA-binding protein (<i>OsWRKY1</i>)	Tobacco (AF096299)	X216
F14509	AF071063	ND	Unknown protein	Rice (D49093)	X31
F15401	AF061643	687	No match		
F21504	AF090698	710	Novel protein (<i>OsERG3</i>)	Maize (U64437)	X279
F12311	AF039531	1224	Lysophospholipase (<i>OsLPL1</i>)	Arabidopsis (U95973)	X196
F72101	AF019743	1333	Peroxidase (<i>OsCPX1</i>)	Arabidopsis (X98318)	X168
F15501	AF080436	2432	Serine/threonine protein kinase <i>pkpA</i> (<i>OsMEK1</i>)	Fungus (Z46636)	X235
F25105	AF061113	1873	No match		
F12205	AF016896	1770	GDP-associated inhibitor (<i>OsGDI1</i>)	Arabidopsis (Y07961)	X299
F12212	AF016897	1770	GDP-associated inhibitor (<i>OsGDI2</i>)	Arabidopsis (Y07961)	X202
F81201	U95216	1612	EREBP (<i>OsEREBP1</i>)	Arabidopsis (AF003105)	X138
F13839	AF080437	ND	Cinnamoyl-CoA reductase (<i>OsCCR1</i>)	Cider tree (X97433)	X130
F10715	AF092949	ND	Cysteine proteinase (<i>OsCysPro1</i>)	Maize (D45420)	X241
F14609	AF012896	840	ADP-ribosylation factor (<i>OsARF1</i>)	Arabidopsis (P36397)	X197

^a Sequence comparisons were performed with the BLAST search from the National Center for Biotechnology Information (NCBI, USA).

^b Determined by sequencing predicted full-length cDNA inserts except the F21711, F14509, F13839, and F10715 clones.

^c Sequence homology according to the NCBI BLAST search results (gene designations in brackets refer to gene names isolated from elicitor-treated rice cells).

^d GenBank/EMBL/SwissProt accession number for known sequence with the closest match according to the NCBI BLAST search.

^e Identity score of each positive clone with known sequences according to the NCBI BLAST search (BLASTX program).

(Garrett et al. 1994). The F81201 (*OsEREBP1*) clone showed high similarity to AP2/EREBP transcription factors of plants. These are DNA binding proteins specific for the GCC box (TAAGAGCCGCC motif) that is required for ethylene responsiveness of several basic pathogenesis-related (PR) protein genes (Okamuro et al. 1997). Clone F13839 (*OsCCR1*) encodes a putative cinnamoyl-CoA reductase (CCR). CCR catalyzes the reduction of CoA esters of hydroxycinnamic acids to cinnamyl alcohols derived from the phenylpropanoid metabolism during lignin biosynthesis. Lignin plays an important role in structural support and in defense responses (Douglas 1996). Clones F14509, F15401, F21504, and F25105 did not reveal any significant sequence similarities to known genes in the data bases and are thus considered novel.

To determine the expression patterns of the fungal elicitor-responsive genes isolated from rice, the time course of their expression in response to fungal elicitor was examined by Northern blot analysis (Fig. 2). The transcript levels of *OsERG1*, *OsHin1*, *OsCaM3*, *OsWRKY1*, and F14509, F15401, F21504, and *OsLPL1* rose rapidly but transiently within 0.5 to 2 h of the elicitor addition, decreasing 8 to 12 h after the treatment. Thus, these elicitor-responsive genes could be used as molecular probes for the analysis of early events in the pathogen-plant cell interaction. In contrast, the transcripts of *OsCPX1*, *OsMEK1*, and F25105 were observed at increased levels at a late time after elicitation. A third pattern of expres-

sion in response to fungal elicitor was seen for the *OsGDI1*, *OsEREBP1*, *OsCCR1*, *OsCysPro1*, and *OsARF1* transcripts. Induction of these transcripts occurred to a lesser extent but was sustained for up to 24 h after treatment. As a positive control, we monitored the expression of rice class II chitinase (*Rcht2*) in response to fungal elicitor (Fig. 2), since treatment with the fungal elicitor effectively induced the accumulation of *Rcht2* transcripts in the rice suspension-cultured cells (Kim et al. 1998).

We have established the optimal condition for the use of cultured rice cells to study *avr/vir* interactions that occur between rice and rice blast fungus upon infection. Rice cells in suspension culture were infected with fungal spores (5×10^5 spores per ml). The viability of suspension cells after inoculation was checked by TTC (2,3,5-triphenyl tetrazolium chloride) staining assay (Steponkus and Lanphear 1967). To examine whether the transcript of the elicitor-responsive genes correlated with virulence, the expression kinetics of *OsERG1*, *OsHin1*, *OsMEK1*, and *Rcht2* (positive control) were examined in cultured cells inoculated with either *M. grisea* race KJ301 (avirulent) or race KJ101 (virulent). As seen in Figure 3, transcripts of the *OsERG1*, *OsHin1*, and *OsMEK1* genes accumulated specifically in cells inoculated with the avirulent race, but not with the virulent race. The *Rcht2* chitinase expression pattern was similar to that of these three elicitor-responsive genes.

We conclude that the elicitor-responsive genes isolated from rice make good candidates for genes that are involved in plant defense responses. They can therefore be used as molecular probes to study elicitor-mediated signal transduction pathways.

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Fig. 3. Avirulence-specific induction of transcripts of elicitor-responsive genes in response to rice blast fungus infection. A nearly isogenic rice line (NIL15-3) was used to monitor the expression of *OsERG1*, *OsHin1*, and *OsMEK1* in response to infection with *Magnaporthe grisea* race KJ301 (avirulent) or race KJ101 (virulent). Rice cells in suspension culture were infected with fungal spores (5×10^5 spores per ml) and incubated for the lengths of time indicated. The rice chitinase *Rcht2* gene was used as a positive control (Kim et al. 1998). Twenty micrograms of total RNA from each culture was used for Northern (RNA) blot hybridization with each of the cloned cDNAs for *OsERG1*, *OsHin1*, and *OsMEK1*. RNA equal loading was checked by hybridization of the blot with a probe corresponding to the rice 18S ribosomal RNA (rDNA).

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