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Isolation of a cDNA Encoding a Novel Leucine-Rich Repeat Motif from *Sorghum bicolor* Inoculated with Fungi

John D. Hipskind[1], Ralph L. Nicholson[1], and Peter B. Goldsbrough[2]

[1]Department of Botany and Plant Pathology; and [2]Department of Horticulture, Purdue University, West Lafayette, IN 47907, U.S.A.

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A sorghum cDNA clone has been isolated that encodes a protein containing six imperfect leucine-rich repeats (LRRs) of approximately 22 amino acids in length. The putative protein, designated SLRR, also contains a signal peptide, and six potential N-glycosylation sites. Comparisons of SLRR and its LRR consensus sequences found significant homology to the extracellular binding domains of receptor-protein kinases *RLK5* and *TMK1* of *Arabidopsis*, and some plant disease resistance genes. Results from RNA gel blot analyses showed that SLRR mRNA accumulates rapidly in mesocotyls and juvenile leaves by 6 h postinoculation with the fungus *Colletotricum graminicola*. Further experiments suggest that the gene encoding SLRR is neither systemically induced by fungal inoculation, nor transcriptionally activated in a host-fungal-pathogen-specific manner. The presence of LRRs strongly suggests that the SLRR protein is involved in protein-ligand binding and therefore may be a component of a signal transduction pathway.

Additional keywords: Bipolaris maydis, 3-deoxyanthocyanidin, phytoalexins.

To successfully infect a host plant, fungal pathogens must overcome a variety of active host defensive mechanisms that are induced in a rapid and coordinated fashion in an attempt to limit pathogen ingress. Host strategies can include hypersensitive cell death, cell wall modifications, or the synthesis of anti-microbial proteins and phytoalexins (Bowles 1990; Dixon and Harrison 1990; Levine et al. 1994; Nicholson and Hammerschmidt 1992). The successful expression of resistance is therefore dependent upon the host plant's ability to perceive various signals, transmit this information, and activate defense-related responses (Dixon and Harrison 1990; Dixon and Lamb 1990). This signal perception is believed to be mediated by plant cell membrane-associated receptors that interact with either fungal- or plant-derived elicitors (Gabriel and Rolfe 1990). Some elicitors, such as oligogalacturonides, can activate nonspecific host responses (e.g., phytoalexin synthesis), while others are proteins encoded by pathogen avirulence genes that result in a race-cultivar-specific hypersensitive resistance (HR) response (Briggs and Johal 1994; Lamb et al. 1989).

Several investigations have demonstrated that transduction of the signals that lead to activation of defense-related genes is mediated by protein phosphorylation (Despres et al. 1995; Felix et al. 1991; Raz and Fluhr 1993; Suzuki and Shinshi 1995). In recent years, several host disease resistance (R) genes have been cloned that have characteristics similar to receptor-like protein kinases (Martin et al. 1993; Michelmore 1995; Staskawicz et al. 1995). It has been proposed that R genes directly or indirectly recognize specific pathogen-encoded avirulence gene products. For example, the tomato *Cf9* gene confers resistance to the fungus *Cladosporium fulvum* expressing the *Avr9* gene (Jones et al. 1994). The protein encoded by *Cf9* has significant homology to the receptor-like protein kinase encoded by *RLK5* from *Arabidopsis* (Jones et al. 1994; Walker 1993). Other cloned R genes (*RPS2* from *Arabidopsis, N* from tobacco, and *L6* from flax) have features that suggest that these genes are involved in signal perception and transduction (Bent et al. 1994; Lawrence et al. 1995; Whitman et al. 1994).

Among the conserved features of receptor-like protein kinases and some R genes are imperfect leucine-rich repeats (LRRs). In plant-pathogen interactions, these structural domains are believed to function as the site of protein-ligand binding and thereby specify recognition of pathogen-derived elicitors or proteins encoded by avirulence genes (Staskawicz et al. 1995). Thus, LRRs mediate ligand binding in the extracellular receptor domain that subsequently activates signal transduction mechanisms (Kobe and Deisenhofer 1994; Stone and Walker 1995). In this investigation, we have identified a cDNA from sorghum that encodes a putative extracellular LRR-containing glycoprotein. Designated SLRR, the encoded protein has significant homology to the extracellular domain of receptor-like protein kinases from *Arabidopsis* and to proteins encoded by some R genes from tomato and rice. Therefore, this cDNA may represent a gene that is a component in signal perception and subsequent activation of defense-related genes in sorghum. RNA gel blot analyses demonstrate that transcripts of this gene accumulate in sorghum seedlings after

Corresponding author: John D. Hipskind, The Samuel Roberts Noble Foundation, Box 2180, 2510 Sam Noble Parkway, Ardmore, OK 73402, U.S.A.; Tel: (405) 223-5810; Fax: (405) 221-7380; E-mail: jdhipskind@noble.org

Nucleotide and/or amino acid sequence data have been submitted to GenBank as accession number U62279.

fungal infection but prior to the accumulation of 3-deoxyanthocyanidin phytoalexins. Further experiments suggest that the gene corresponding to this clone is not systemically induced, nor activated in a host-pathogen-specific manner.

RESULTS

Isolation of SLRR by differential screening.

In general, the synthesis of phytoalexins is regarded as a component of plant basic incompatibility (Kúc 1995; Strange 1992). The purpose of the original experiment was to identify defense-related genes in sorghum associated with the synthesis of 3-deoxyanthocyanidin phytoalexins. These anti-microbial compounds accumulate rapidly in a site-specific manner in cells that are either penetrated directly, or are adjacent to the site of fungal penetration (Snyder and Nicholson 1990). Furthermore, sorghum phytoalexins have been shown to accumulate in roots, mesocotyls, and green leaves in response to pathogens, nonpathogens, and fungal elicitors (Hipskind et al. 1990; Nicholson et al. 1987; Ransom et al. 1992; Yamaoka et al. 1990). As flavonoids, the biosynthesis of these phytoalexins is dependent upon chalcone synthase (CHS) (Lue et al. 1989). RNA gel blot analyses indicate that CHS mRNA accumulates to high levels in mesocotyls through 24 h postinoculation with fungi that stimulate phytoalexin biosynthesis (Hipskind et al., in press).

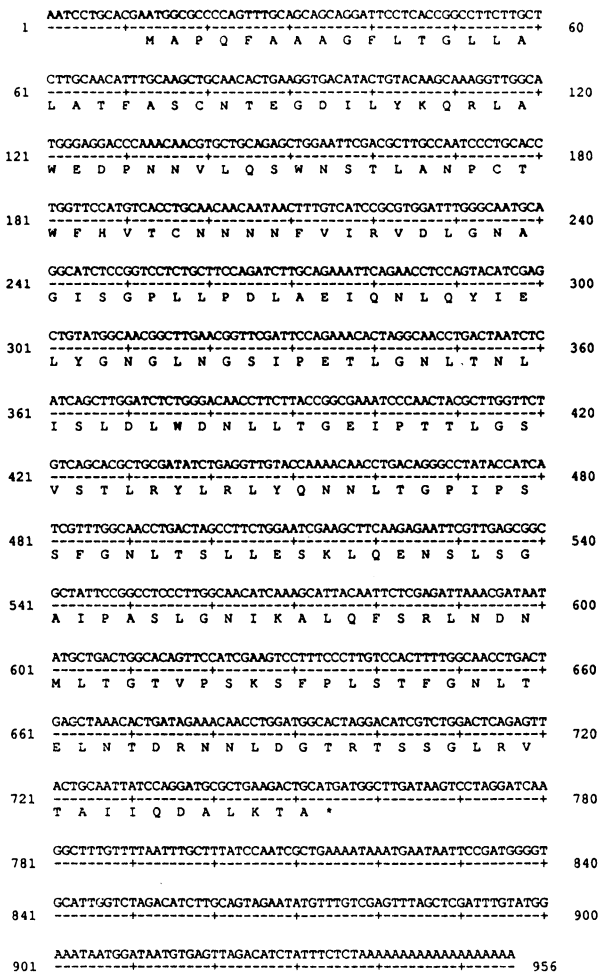

Fig. 1. cDNA sequence data and translation to a putative peptide from the first in-frame ATG codon.

A cDNA library was constructed from poly (A^+) RNA extracted from *C. graminicola*–infected mesocotyls 24 h postinoculation, and subsequently screened as described in the Materials and Methods. A number of cDNAs were isolated that appeared to correspond to mRNAs that increased in abundance after inoculation. Some of these cDNAs encode PR-5 and PR-10 pathogenesis-related proteins (J. D. Hipskind and R. L. Nicholson, unpublished). The DNA sequence from one clone, which appeared to be strongly induced, did not detect significant homology with any known plant gene in a search of the GenBank and EMBL data bases (Fig. 1). The DNA sequence of this clone has been submitted to GenBank (accession number U62279).

The cDNA clone contains an apparent single, long, open reading frame initiated by an ATG codon 13 base pairs from the 5′ end and encodes a putative peptide of 247 amino acids (Fig. 1). A subsequent search revealed this peptide has significant homology to the extracellular domain of receptor-like protein kinases from *Arabidopsis*, *RLK5* (38.5% identical; 57.2% similar) (Walker 1993) and *TMK1* (27.6% identical; 49.3% similar) (Chang et al. 1992). Comparisons were also made to putative extracellular ligand binding domains encoded by two disease resistance genes. The encoded protein has homology with the *Cf9* gene from tomato (32.6% identical; 52.4% similar) (Jones et al. 1994) and the recently cloned *Xa21* gene (a putative receptor-like protein kinase) from rice (34.8% identical; 52.7% similar) (Song et al. 1995). Among the most striking features in the extracellular domains of all these genes are the LRRs. This finding suggests that the encoded protein has an alternating β–α structure, and that one function of the protein is to mediate protein-ligand binding (Kobe and Deisenhofer 1994).

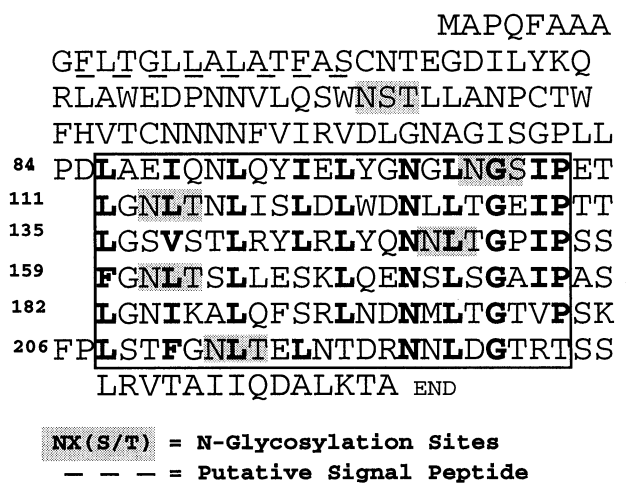

Fig. 2. Putative protein structural motifs. A box encloses six imperfect leucine-rich repeats (bold face) initially identified by conserved isoleucine/proline residues on the carboxy-terminal end of the repeats. Six shaded boxes and a dashed line denote putative N-glycosylation sites and a signal peptide, respectively.

In general, LRRs are difficult to identify because of their highly degenerate nature. In our investigation, the LRRs in the putative peptide were identified by first locating conserved proline residues (Fig. 2). It has been suggested that, although not absolutely conserved, this amino acid, often paired with isoleucine, flanks the carboxy-terminal end of LRRs (Hashimoto et al. 1988). A total of six repeats were identified with five of six repeats consisting of 22 amino acids. We have therefore named the putative protein encoded by this cDNA as SLRR (sorghum leucine-rich repeat). The SLRR peptide also contains a putative signal peptide (von Heijne 1983) and six potential N-glycosylation sites, suggesting that SLRR is an extracellular glycoprotein (Fig. 2). These features are also characteristic of some receptor protein kinases and some plant R genes (Staskawicz et al. 1995; Stone and Walker 1995). A hydrophobicity plot of SLRR revealed that the putative signal peptide is the most hydrophobic domain (data not shown) (Kyte and Doolittle 1982). Further, no apparent transmembrane domain or conserved protein kinase domains could be identified. A consensus of LRR domains shows a striking similarity not only between these proteins but also to proteins encoded by other resistance genes (*RPS2* and *N*) and a polygalacturonase inhibitor (PGI) protein from pear (Fig. 3). However, the number of LRRs within each of these proteins is quite variable.

		#Repeats
SLRR	L - - α - - L - - L - L - - N - L - G - IP	6
Xa21	L - - L - - L - - L - L - - N - L - G - IP	23
RLK5	α - - L - - L - - L - L - - N - L - G - IP	21
TMK1	L - - L - - L - L - - N - L - G - IP	11
Cf9	(N/L)- - L - - L - L - - N - L - G - IP	12
PGI	α - - L - - α - - L - α - - N - L - G - IP	8
N	α - - L - - L - - L - L - - - - - - L - - LP	14
RPS2	α - - L - - L - - L - L - - - - - α - - - βP	12

Fig. 3. Consensus derived from imperfect leucine-rich repeat (LRR) domains encoded by receptor-protein kinases and some plant disease resistance genes: SLRR (sorghum LRR encoding cDNA); *Xa21* (rice disease resistance gene; Song et al. 1995), *RLK5* and *TMK1* (*Arabidopsis* receptor-protein kinases; Chang et al. 1992; Walker 1993), *Cf9* (tomato disease resistance gene; Jones et al 1994), PGI (pear polygalacturonase inhibitor protein; Stotz et al. 1993), *N* (tobacco disease resistance gene; Whitham et al. 1994), *RPS2*. (*Arabidopsis* disease resistance gene; Bent et al. 1994); α = variable substitution with either L, I, V, or F, respectively. β = L or I.

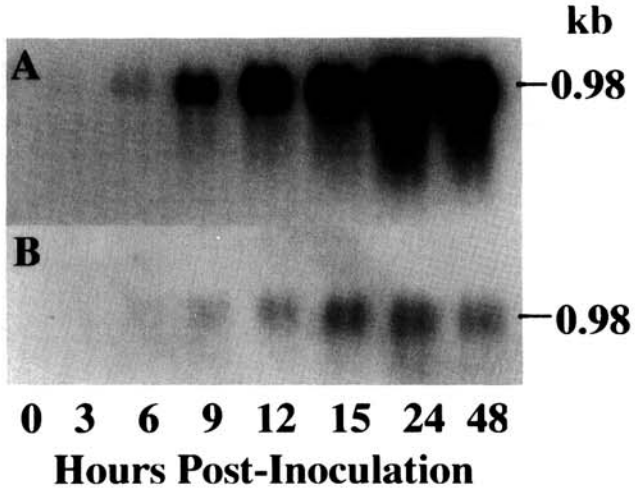

Fig. 4. RNA gel blot analyses of total RNA extracted from **(A)** mesocotyls (5 μg per lane) and **(B)** juvenile green leaves (10 μg per lane) at various times postinoculation with *Colletotrichum graminicola*. Results indicated that the temporal accumulation of transcripts is consistent with attempted fungal penetration and prior to reported phytoalexin accumulation.

RNA gel blot analyses.

To ensure that this cDNA clone was not of fungal origin, DNA from both sorghum and *C. graminicola* was subjected to DNA gel blot analyses under high stringency. Results showed three intense bands of hybridization only in the sorghum DNA digest (data not shown). The temporal accumulation of transcripts with homology to the SLRR cDNA was then analyzed by RNA gel blots. Total RNAs were extracted from both mesocotyls and juvenile leaves at various times after inoculation with the fungus *C. graminicola*. Results from a ^{32}P-labeled probe synthesized from our cDNA clone showed that SLRR mRNA began to accumulate by 6 h postinoculation in both tissue types (Fig. 4A, B). Accumulation of this RNA occurs before CHS enzyme activity is reported to increase and prior to the accumulation of detectable levels of phytoalexins (Hipskind et al. 1990; Lue et al. 1989). In addition, microscopic examinations of mesocotyl and leaf surfaces indicated that by 6 h postinoculation approximately 10% of germinated conidia had formed appressoria (data not shown). These observations are significant because they suggest that fungal penetration is the event that initiates gene activation in mesocotyls and leaves. The apparent lower degree of SLRR mRNA accumulation detected in the juvenile leaves (Fig. 4B) may be due to the fact that, in sorghum leaves, lesions induced by *C. graminicola* and the corresponding host response are restricted to only a few cells adjacent to the site of attempted fungal penetration (Snyder and Nicholson 1990).

A second experiment was performed with total RNAs that were extracted from mesocotyls after inoculation with a nonpathogen, *B. maydis* race O, the causal agent of southern corn leaf blight. Infection by either *C. graminicola* or *B. maydis* elicits a strong phytoalexin response in sorghum mesocotyls (Nicholson et al. 1987). Results from RNA gel blot analyses confirmed that infection by *B. maydis* also stimulates an increase in the abundance of SLRR mRNA (Fig. 5A). A final

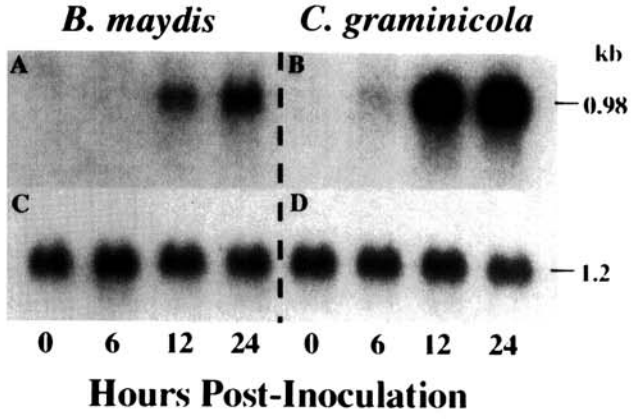

Fig. 5. RNA gel blot analyses of total RNA extracts (5 μg per lane) from mesocotyls inoculated with **(A** and **C)** *Bipolaris maydis* and **(B** and **D)** *Colletotrichum graminicola* extracted at various times postinoculation. Results show that inoculation with either **(A)** *B. maydis* or **(B)** *C. graminicola* can stimulate transcript accumulation. Duplicate blots **(C** and **D)** were probed with a ^{32}P-labeled sorghum ubiquitin carrier protein cDNA as an internal standard.

experiment was performed in which only the bottom half of the mesocotyls were inoculated with *C. graminicola*, leaving the top half uninfected. Under these conditions, only the bottom half of the mesocotyl actively synthesized phytoalexins as determined by high-pressure liquid chromatography analyses (Hipskind et al. 1990). Results clearly show that SLRR mRNA accumulated only in that portion of the mesocotyl where fungal conidia had been applied and where phytoalexins were being produced (Fig. 6A). Taken together, these results strongly suggest that gene activation detected by this cDNA clone is not tissue specific, systemically induced, or activated in a host-fungal-pathogen-specific manner, but is associated only with a defense-related (phytoalexin) response.

DISCUSSION

A new member of the LRR super family.

Proteins that contain LRRs are believed to be involved in protein-ligand or protein-protein binding. As many as half of the known proteins containing LRRs are believed to be involved in signal transduction, although in most cases the ligands are unknown (Kobe and Deisenhofer 1994). Further, the degenerative nature of LRRs precludes accurate prediction of the nature of a potential ligand or the LRR-ligand site of action. Identification of this binding motif in plant R genes and in related receptor-protein kinases is significant because the activation of the HR response is believed to be determined by specific avirulence genes in the pathogens. However, the exact nature of these interactions has yet to be demonstrated. For example, in tomato both the R gene *Cf9* and its corresponding fungal pathogen avirulence gene, *Avr9,* have been cloned (Jones et al. 1994), but the direct interaction between the two encoded proteins has not been reported.

It has been proposed that the accumulation of LRRs within a specific gene arose by unequal cross-over and duplication events, perhaps at exon-intron junctions (Kobe and Deisenhofer 1994). Such a mechanism has also been proposed for the evolution of race-specific resistance conferred by the R genes, such as *Rp1* of maize (Michelmore 1995; Sudapak et al. 1993). Thus, this process might result in the accumulation of several related genes that encode LRR proteins. Indeed, resistance genes that have been isolated are members of multigene families (Staskawicz et al. 1995). Likewise, the *Arabidopsis RLK5* gene is also a member of a multigene family (Walker 1993). A recently cloned resistance gene from rice, designated *Xa21*, confers specific resistance to *Xanthomonas oryzae* pv. *oryzae* race 6 (Song et al. 1995). The *Xa21* gene encodes a putative receptor-like protein kinase homologous to the *RLK5* protein, shares similar homology to our cDNA encoded protein, and contains LRRs with a nearly identical consensus (Fig. 3).

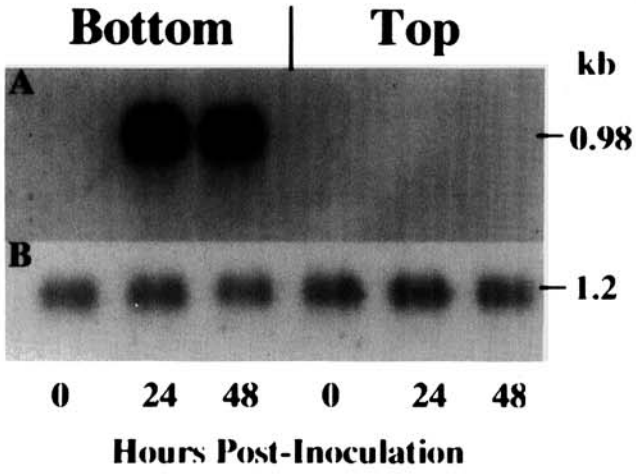

Fig. 6. RNA gel blot analyses of total RNA extracts (5 μg per lane) from the bottom of mesocotyls inoculated with *Colletotrichum graminicola* and top of the same mesocoyls that were not inoculated. Results in **(A)** show that the corresponding transcripts only accumulate where fungal inoculum has stimulated a subsequent phytoalexin response (bottom), and that transcripts do not accumulate systemically (top). A duplicate blot **(B)** was probed with a ^{32}P-labeled sorghum ubiquitin carrier protein cDNA as an internal standard.

N-glycosylation and truncated transcripts.

A significant question that has not been addressed in investigations of either receptor protein kinases or R genes is the function and specificity of post-translational modifications. The putative SLRR protein contains at least six potential N-glycolsylation sites (Fig. 2). It has been reported that a single nucleotide change in the *RPS2* resistance gene of *Arabidopsis,* which led to the loss of one putative N-glycosylation site in an LRR domain, resulted in a loss of race-specific HR (Bent et al. 1994). Recently, 3-D models of β–α helix structures conferred by LRRs have been presented, but the details of how these structures or their ligand-binding specificity might be affected by glycosylation were not presented (Kobe and Deisenhofer 1994). Studies of self-incompatibility (SI) in members of the *Brassicaceae* have identified polymorphic extracellular glycoproteins that have significant homology (about 90%) to the N-terminal (extracellular) protein-binding domains of a putative receptor-like protein kinase (*SRK*) (Stein et al. 1991; Umbach et al. 1990). Interestingly, truncated *SRK*-encoded glycoproteins corresponding to this extracellular domain have also been identified (Giranton et al. 1995). These glycoproteins may act together in receptor-ligand interactions to facilitate SI or other receptor-kinase mediated events (Lamb 1994; Walker and Zhang 1990). Truncated cDNAs encoded by alternative splicing mechanisms from the tobacco *N* and flax *L6* disease resistance genes have been isolated (Lawrence et al. 1995; Whitham et al. 1994). However, unlike the case with SLRR mRNA, there is no report of these transcripts accumulating in response to pathogen attack.

Models for encoded peptide function.

Based upon studies of other related proteins, we can only speculate regarding a possible function for the putative SLRR protein. Studies of animal receptor protein kinases have demonstrated that extracellular receptor oligomerization is essential for ligand binding (Williams 1989; Yarden and Schlessinger 1987). Therefore, the SLRR protein may function by forming protein-protein duplexes (hetero- or homodimers) with receptor or receptor-like protein kinases. Bifunctional receptor-like pairs have recently been proposed as models for the function of proteins encoded by *SRK*, *Cf9,* and *Pto*. Interactions between two homologous (or truncated) proteins are proposed to facilitate ligand binding and subsequent signal transduction (DeWit 1995).

An alternative hypothesis is that the SLRR protein may act

as a metabolic inhibitor by binding any ligand that would otherwise activate a homologous receptor-like protein kinase. Evidence for this comes from two studies of animal receptor kinase genes (Duan et al. 1991; Flickinger et al. 1992). Like the S-locus *SRK* gene, the *PDGFr* and *c-erbB* genes utilize alternative splicing to produce truncated messages corresponding to their extracellular domains. These truncated proteins bind their respective ligands with high affinity, thus blocking receptor-kinase-mediated events. Therefore, in sorghum, the putative SLRR protein may block constitutive signal transduction events mediated by protein kinase/phosphatase activity, thus allowing for sustained host-defense-related responses. Identification of the putative SLRR protein-ligand will be necessary in order to test these theories.

Conclusion.

Isolation and characterization of genes such as the SLRR cDNA will be essential to unravel the many different levels of specific and nonspecific host-defense-related responses. The biochemical basis for disease resistance and the control of these responses mediated by receptor proteins or by R genes is likely to involve many other host genes. For example, a locus identified as *Prf* has also been found to be essential for *Pto*-mediated resistance to *Pseudomonas syringae* pv. *tomato* (Salmeron et al. 1994). Interestingly, the *Prf* gene has been isolated and appears to encode a protein that contains 18 LRRs, membrane-spanning domains, and a P-loop (Chasan 1994; B. J. Staskawicz, personal communication).

MATERIALS AND METHODS

Plant materials and pathogens.

Seed of the sorghum (*Sorghum bicolor* (L.) Moench) hybrid DK18 (Dekalb Pfizer Genetics, Dekalb, IL) were imbibed in water for 12 h at 25°C and incubated between layers of moist germination paper in the dark for 96 h (Nicholson et al. 1987). Phytoalexin synthesis in either mesocotyls or juvenile leaves was induced by inoculation with the fungus *Bipolaris maydis* (Nisikado & Miyake) Shoemaker race O, a nonpathogen of sorghum, or by inoculation with *Colletotricum graminicola* (Ces.) G. W. Wils., as previously described (Hipskind et al. 1990; Nicholson et al. 1987). After inoculation, seedlings were incubated at 100% relative humidity under cool-white fluorescent bulbs (60 $\mu E\ s^{-1}\ m^{-2}$) at room temperature for periods up to 48 h as required for that particular experiment.

cDNA library construction and screening.

Total RNA was isolated from mesocotyls 24 h postinoculation with *C. graminicola* by the method of Zhou and Goldsbrough (1993). Poly (A^+) RNA was purified from the total RNA by hybridization to oligo (dT) cellulose resin (Gibco-BRL, Grand Island, NY) under conditions described by Sambrook et al. (1989). The poly (A^+) RNA then served as the template for cDNA synthesis and construction of a lambda ZAP-cDNA library according to the manufacturer's instructions (Stratagene, La Jolla, CA).

For the primary differential screen, the library was plated on NZY agar (0.5% NaCl, 0.2% $MgSO_4 \cdot 7H_2O$, 0.5% yeast extract, 1% NZ amine, 1.5% agar, pH 7.5) in *Escherichia coli* cell line XL1-Blue MRF′ and incubated for 18 h at 37°C. Duplicate plaque lifts were performed with BA-S 85 Optitran membranes (Schleicher and Schuell, Keene, NH) according to the manufacturer's instructions. The phage DNA was covalently cross-linked to the membranes (UV Crosslinker, Fisher Scientific, Chicago, IL) and prehybridized for 2 h at 60°C in a mixture of 5× SSPE (0.75 M NaCl, 0.05 M $NaH_2PO_4 \cdot H_2O$, 0.05 M EDTA, pH 7.4), 5× Denhardt's solution (US Biochemical, [Amersham], Arlington Heights, IL), 0.125% sodium dodecyl sulfate (SDS), and 50 µg of denatured salmon sperm DNA (Sigma Chemical, St. Louis, MO) per ml. As previously described, poly (A^+) RNA isolated from uninoculated (control) and inoculated mesocotyls was converted to first-strand cDNA (US Biochemical). Individual membranes were then probed with ^{32}P-labeled cDNAs in a hybridization solution consisting of 5× SSPE, 2.5× Denhardt's, 0.5% SDS, and 50 µg of denatured salmon sperm DNA per ml at 60°C for 18 h (Deca-Prime Labeling Kit, Ambion, Austin, TX). The membranes were washed once in a solution of 5× SSPE and 0.5% SDS at room temperature for 30 min followed by a second wash with 0.1× SSPE and 0.5% SDS at 60°C for 20 min. The membranes were then exposed to X-ray film (X-OMAT AR, Kodak, New Haven, CT) with an intensifying screen (Cronex Lightning Plus, Sigma) for 3 to 6 days at –80°C prior to development.

Individual plaques that appeared to increase in intensity were isolated and stored in 0.5 ml of SM buffer (0.58% NaCl, 0.2% $MgSO_4$, 0.01% gelatin, 50 mM Tris-HCl, pH 7.5) at 4°C. The cDNA inserts contained within the lambda phage were released by three cycles of freezing in liquid nitrogen and thawing followed by PCR. The PCR conditions were as follows: 5 ng of SK (5′ CGCTCTAGAACTAGTGGATC 3′) and T7 (3′ CGGGATATCACTAGCATAATG 5′) primers, 1× polymerase chain reaction (PCR) buffer, 10 mM dNTPs, and 2 units of *Taq* DNA polymerase (Promega, Madison, WI). All reagents were obtained from Sigma. Thermocycler (Perkin-Elmer, Norwalk, CT) conditions were as follows: denature at 95°C for 2 min, anneal at 50° for 1 min, elongate at 72°C for 2 min, for a total of 30 cycles. Five microliters of the amplified cDNA was then analyzed by standard 1% agarose gel electrophoresis in 1× TPE (10 mM Tris-HCl, 0.25% phosphoric acid, 2 mM EDTA, ph 7.4) buffer to determine the size and number of cDNA inserts (Sambrook et al. 1989). Secondary and tertiary screens of individual plaques were then performed whereby the PCR-amplified cDNA was diluted 100-fold, separated by 1% agarose gel in TPE buffer, denatured, neutralized, and transferred to nylon membrane (Zeta-Probe GT, Bio-Rad Laboratories, Hercules, CA) by the method described by Sambrook et al. (1989).

Plaque rescue and DNA sequencing.

Positive phagmid clones from the tertiary screen were excised from the Uni-Zap vectors with the ExAssist/SOLR system (Stratagene). DNA from the plasmid containing the approximately 1.0-kb cDNA insert was isolated from a 100-ml overnight culture by the method of Birnboim and Doly (1979). Both strands of the cDNA insert were sequenced at the Purdue Center for DNA Sequencing by a modification of Sanger dideoxy sequencing (Sanger et al. 1977). The sequence data were obtained with a Dupont Genesis 2000 DNA Analysis System with fluorescent chain terminating reactions (Prober et al. 1987) and submitted to GenBank (accession number U62279). The sequence data and subsequent translation from

the first ATG were used in a computer-aided search of various data bases through the National Center for Biotechnology Information (NCBI) network (Altschul et al. 1990).

RNA gel blot analyses.

Total RNAs from infected green leaves or mesocotyls were extracted from 4-g tissue samples taken at various times post-inoculation depending upon the individual experiment. In all experiments, RNA was extracted by the method of Zhou and Goldsbrough (1993). The concentrations and integrity of total RNAs in individual extracts were determined spectrophotometrically (A_{260}) and by staining of the ribosomal RNAs with ethidium bromide after agarose gel electrophoresis, respectively. For RNA gel blot analyses, 5 or 10 μg of total RNA was denatured and separated in a formaldehyde–1% agarose gel in MOPS buffer (0.20 M [3-(N-morpholino)propanesulfonic acid], 0.50 M sodium acetate, 0.10 M EDTA, pH 7), and subsequently transferred by capillary action onto a nylon membrane (Nytran Plus, Schleicher and Schuell) (Sambrook et al. 1989). The RNA was covalently cross-linked to the membrane by UV irradiation (Model Fb-UVXL-1000 UV Crosslinker, Fisher Scientific) followed by 2 h of prehybridization in a mixture of 5× SSPE, 5× Denhardt's (US Biochemical), 0.125% SDS, and 50 μg of denatured salmon sperm DNA (Sigma) per ml. Individual membranes were then probed with ^{32}P-labeled SLRR cDNA clone or sorghum ubiquitin carrier protein (UBC) cDNAs in a hybridization solution consisting of 5× SSPE, 2.5× Denhardt's, 0.5% SDS, and 50 μg of denatured salmon sperm DNA per ml at 60°C for 18 h. Probes were synthesized via random primers (Deca-Prime Labeling Kit, Ambion). Membranes were washed twice with 5× SSPE, 0.5% SDS at room temperature, followed by a single wash with 0.2× SSPE, 0.5% SDS at 60°C. The membranes were then exposed to X-ray film (X-OMAT AR, Kodak) with an intensifying screen (Cronex Lightning Plus, Sigma) for 3 to 6 days at –80°C prior to development. The size of mRNA species detected was estimated by use of a 0.24- to 9.5-kb RNA ladder (Gibco-BRL).

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