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Characterization of the rice pathogen-related protein Rir1a and regulation of the corresponding gene

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Abstract

In rice (*Oryza sativa* L.), local acquired resistance against *Pyricularia oryzae* (Cav.), the causal agent of rice blast, can be induced by a preinoculation with the non-host pathogen *Pseudomonas syringae* pv. *syringae*. We have cloned a cDNA (*Rir1a*) and a closely related gene (*Rir1b*) corresponding to transcripts that accumulate in leaf tissue upon inoculation with *P. syringae* pv. *syringae*. The cDNA encodes a putative 107 amino acid protein, *Rir1a*, that exhibits a putative signal peptide cleavage site in its hydrophobic N-terminal part and a C-terminal part that is relatively rich in glycine and proline. The *Rir1b* gene contains a *Tourist* and a *Wanderer* miniature transposable element in its single intron and encodes a nearly identical protein. Rir1a is similar in sequence (ca. 35% identical and ca. 60% conservatively changed amino acids) to the putative Wir1 family of proteins that are encoded by pathogen-induced transcripts in wheat. Using antibodies raised against a Rir1a-fusion protein we show that Rir1a is secreted from rice protoplasts transiently expressing a *35S::Rir1a* construct and that the protein accumulates in the cell wall compartment of rice leaves upon inoculation with *P. syringae* pv. *syringae*. Possible roles of Rir1a in pathogen defense are discussed.

Introduction

In response to attacks by potentially pathogenic organisms, plants can activate physiological defense mechanisms that render a subsequent infection by a wide variety of pathogens less successful. This phenomenon is called acquired resistance and has been observed in many plant-pathogen interactions [18, 32]. Apart from being triggered by incompatible pathogens, acquired resistance can also be induced by the application of certain chemical compounds such as salicylic acid (SA) [41], 2,6-dichloroisonicotinic acid (INA) [24], and benzo(1,2,3)thiodiazole-7-carbothioic acid *S*-methyl ester (BTH) [12]. Upon induction, the physiologically resistant state can extend to plant tissue not treated with the inducer (systemic acquired resistance, SAR) [30, 31]. Resistance induction is accompanied by the activation of a set of genes and the accumulation of the corresponding gene products. In tobacco, nine families of coordinately activated genes (*SAR* genes) have been identified, among them the genes encoding the classical pathogenesis-related (PR) proteins [40]. Such genes are likely to have an important role in pathogen defense. Many of them encode proteins with antimicrobial activity [3, 23, 26, 38, 42] and their constitutive expression in transgenic plants can increase the resistance against pathogens [1, 4, 10, 15, 20, 21].

Induced resistance and the associated biochemical and molecular events are presently not as well characterized in monocotyledons as in dicotyledons, in spite of the fact that the former class includes the cereals, with some of the most important crop plants. In cereals, induced resistance has been described in barley

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession numbers Y14824 (*Rir1a*) and Y14825 (*Riri1b*).

[7, 27, 28], wheat [34], and rice [14, 19, 36]. We wanted to identify and isolate genes from rice that are activated upon treatment with a resistance-inducing agent. Here we report the isolation of a cDNA (*Rir1a*) and a closely related gene whose transcripts accumulate upon inoculation with *P. syringae* pv. *syringae.* We argue that the encoded protein is a homologue of the pathogen-induced WIR1 proteins of wheat [5, 11]. We show by immunological methods that these proteins are secreted into the extracellular space and accumulate in the cell wall after contact with potential pathogens.

Materials and methods

Plant growth and treatment

Two-week old rice plants (*Oryza sativa* cv. Norin 29, a japonica rice variety) were grown and infiltrated with *Pseudomonas syringae* pv. *syringae* strain 548 [29] as described [36]. 2,6-dichloroisonicotinic acid (INA) was drench-applied into the soil as a 10 ml suspension of wettable powder in water containing the appropriate concentration of INA. The final concentration was calculated on the asumption that the active ingredient was homogeneously distributed thoughout the pot volume (145 ml). Inoculation of plants with *Pyricularia oryzae* (race 283 from Novartis; the strain is virulent on Norin) was performed by spraying spores on leaves as described [36].

cDNA library construction, screening of libraries and DNA manipulation

Total RNA was extracted from first leaves of rice plants 24 h after they had been infiltrated with a bacterial suspension of *P. syringae* pv. *syringae.* A cDNA library was constructed from poly(A)-containing RNA using the λZAP II cDNA cloning kit (Stratagene, La Jolla, CA) according to the instructions of the manufacturer. The library was differentially screened according to standard procedures [22] with ^{32}P-labelled probes consisting of single-stranded cDNA prepared from the same RNA pool used for the construction of the library, or from RNA of water-infiltrated control leaves. A genomic rice library prepared from the indica rice variety IR36 (*O. sativa* cv. IR36) was purchased from Clontech Laboratories, Palo Alto, CA.

DNA manipulation and subcloning of fragments into pBluescript SK− (Stratagene, La Jolla, CA) was carried out according to standard procedures [22]. Sequencing was performed on both strands using the chain-termination method [33] with (γ-^{35}S)dATP on double-stranded templates.

RNA and DNA gel blot analysis

Total RNA was extracted from treated or untreated rice leaves (cv. Norin) as described [9]. For gel blot analysis, 10 μg of total RNA was loaded per slot and separated on formaldehyde agarose gels, transferred onto GeneScreen membranes (Dupont NEN, Regensdorf, Switzerland), and cross-linked with a UV crosslinker (Amersham, UK). Loading of the lanes was monitored by ethidium bromide staining of the gel before transfer. As a molecular weight standard, a 0.16–1.77 kb RNA ladder (Gibco-BRL) was used. Filters were hybridized to a ^{32}P-labelled probe in 1 M NaCl, 1% SDS, 10% dextrane sulfate, and 100 μg/ml denatured salmon sperm DNA overnight at 65 °C. Filters were washed in 0.2× SSC (1× SSC is 150 mM NaCl; 15 mM sodium citrate), 0.1% SDS at 65 °C.

Genomic DNA was extracted from rice leaves (cv. Norin) using the CTAB procedure [2]. Digestion with restriction enzymes, electrophoretic separation on agarose gels, and transfer to GeneScreen membranes were performed according to standard procedures [22]. Hybridization and washing conditions as well as the probe were the same as described above.

Preparation of Rir1a*-specific antiserum*

As an antigen for the immunization of rabbits, a fusion protein was produced in *Escherichia coli.* A DNA fragment encoding the putative mature Rir1a was amplified in a PCR reaction from the linearized pRir1a cDNA clone using a forward primer (primer 1) with the sequence 5′-TCCAGATCTGTCTCAGTAGAAGGAGGGAGA and a reverse primer (primer 2) with the sequence 5′-TTAAGATCTTCATGGTTTGGCAGCTGGAGG. The underlined parts of the primers correspond to the underlined sequences in Figure 1A, the additional nucleotides providing *Bgl*II restriction sites at the ends. The amplified fragment was cloned into pGEM-T (Promega, Madison, WI). The insert was cut out with *Bgl*II and cloned in the correct orientation into the *Bgl*II site of the bacterial expression vector p6xHis-DHFRS(0) [37]. This resulted in an isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible gene encoding a fusion protein that consisted of an N-terminal histidine hexamer tag, followed by part of the

cysteine-free mouse dehydrofolate reductase fused to the putative mature Rir1a. The fusion protein was purified from *Escherichia coli* lysates by nickel chelate affinity chromatography using its histidine affinity tag according to Stüber *et al.* [37]. The eluted protein was further subjected to SDS-polyacrylamide gel electrophoresis. After Commassie Brilliant Blue R250 staining, the gel slice containing the fusion protein was cut out, crushed, and directly used to immunize rabbits. Immunization and preparation of antiserum was performed by Eurogentec (Seraing, Belgium).

Extraction and analysis of proteins

Proteins were extracted from 0.5–1 g fresh leaf tissue. After grinding the tissue in liquid nitrogen with mortar and pestle, the powder was transferred to a new mortar and taken up in 2.5 ml extraction buffer (20 mM Tris-HCl pH 6.8). The tissue was further homogenized with a pestle after addition of a spatula of quartz sand. The homogenate was centrifuged at 20 000 $\times g$ for 20 min and the supernatant was collected (fraction 1). The pellet was resuspended in extraction buffer containing 2 M NaCl and 1% Triton X-100 and incubated on a rotator at 4 °C overnight. After centrifugation at 20 000 $\times g$ for 20 min, the supernatant was collected (fraction 2). The pellet was again extracted with a solution containing 1 M NaOH, 5% SDS, and 7 M 2-mercaptoethanol at 80 °C for 1 h. After pelleting the cell debris, the supernatant was again collected (fraction 3). All fractions were extracted with chloroform and desalted using PD-10 columns (Pharmacia, Uppsala, Sweden) in 10 mM Tris-HCl, pH 7.2. Protein concentrations were determined with the BioRad protein assay kit (BioRad, Richmond, CA).

Aliquots containing 20 μg of protein and 0.25 volumes of 5× sample buffer (60 mM Tris-HCl pH 6.8, 10% SDS, 25% glycerol, 5% 2-mercaptoethanol, 0.1% bromophenol blue) were denatured, separated on 15% SDS-polyacrylamide gels and transferred onto nitrocellulose filters (0.45 μm pore size; BioRad) using a semidry blotting apparatus (Pharmacia-LKB, Uppsala, Sweden). For immunodetection of Rir1a, the blots were blocked in TBS (20 mM Tris-HCl pH 7.5, 137 mM NaCl, 0.1% (v/v) Tween-20) containing 7.5% (w/v) non-fat dry milk powder and incubated in a 1:5000 dilution of anti-Rir1a fusion protein antiserum. Blots were washed in TBS, incubated with goat anti-rabbit horseradish peroxidase-conjugated secondary antibody, and developed using a chemoluminescent immunodetection system (ECL, Amersham Interna-

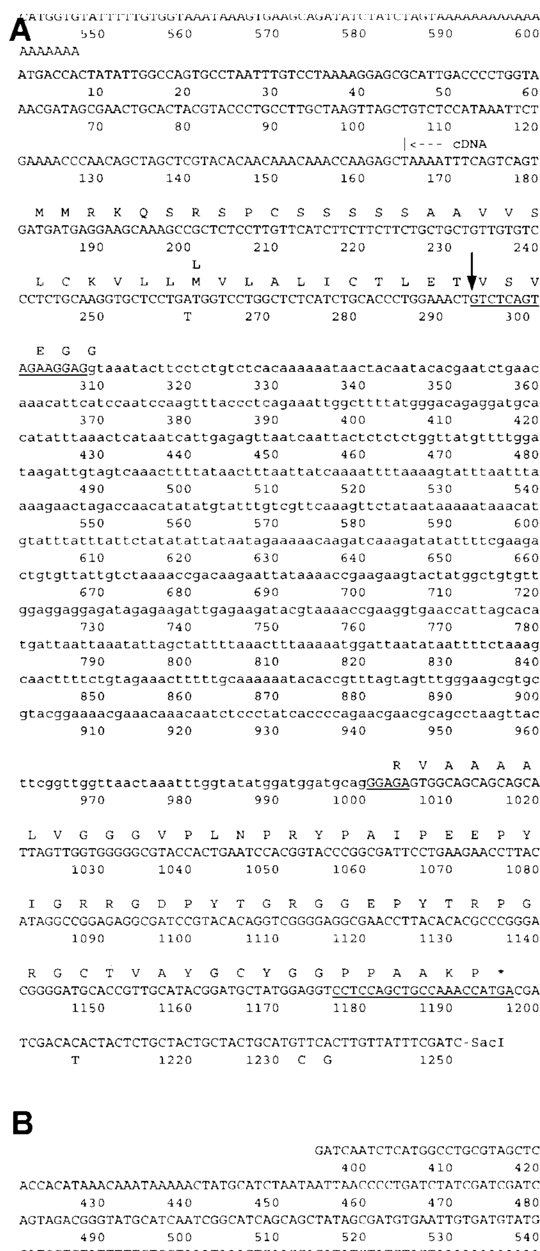

Figure 1. Nucleotide and deduced amino acid sequences of the *Rir1a* transcript and the *Rir1b* gene. A. Sequence of part of the 2.8 kb *Sac*I genomic fragment containing the *Rir1b* gene. The amino acid sequence is given above the nucleotide sequence in the single letter code. The asterisk denotes the termination codon. The intron sequence is given in lower-case letters. The beginning of the cDNA sequence is indicated. The cDNA sequence (*Rir1a*) is identical to the exon sequences of the gene except for the four nucleotide changes indicated below the gene sequence and the single amino acid change indicated above the protein sequence deduced from the gene. The underlined sequences correspond to the oligonucleotides used to prepare a fusion protein (see materials and methods section). B. Sequence of the part of the *Rir1a* cDNA sequence that extends beyond position 1251 of the *Rir1b* gene sequence.

tional) according to the manufacturer's description. To avoid a spotty background staining over the transfer membrane after immunodetection, the primary antibody was pre-adsorbed on crushed polymerized polyacrylamide in TBS overnight at 4 °C. After centrifugation, the supernatant was used for immunodetection.

Transient expression of Rir1a *in protoplasts*

For the construction of a Rir1a expression plasmid, a DNA fragment was amplified from linearized pRIR1a cDNA in a PCR reaction with T3 primer (Stratagene) and primer 2 (see above). This fragment, which contained the 5′-flanking sequence and the complete coding sequence, was cleaved with *Bam*HI and *Bgl*II and cloned in the correct orientation into the *Bam*HI site of the plant expression vector pGY1 that contains the cauliflower mosaic virus (CaMV) 35S promoter [25]. The construct was named pexPIR1a and veryfied by sequencing.

Maintenance of a rice suspension culture derived from *O. sativa* cv. Nipponbare and preparation of protoplasts was carried out according to Datta *et al.* [8]. 5×10^6 protoplasts were transfected with 150 μg plasmid DNA by the polyethylene glycol method as described [8]. Protoplasts were incubated for 20 h and collected by centrifugation. Proteins were extracted and separated into fraction 1 (soluble proteins) and fraction 2 (insoluble proteins) as described in the previous section. To collect proteins secreted into the medium (fraction 3), the medium was concentrated in dialysis bags (cut-off value 3500 Da) by placing them onto solid polyethylene glycol 35000 overnight at 4 °C. Proteins were rinsed from dialysis bags with 10 mM ammonium acetate, lyophilized, and redissolved in 1.5 ml distilled water. The protein concentration was determined with the BioRad protein assay kit and samples containing 20 μg of proteins were subjected to gel blot analysis as described above.

Results

cDNA cloning of Rir1a*, a new pathogen-induced gene transcript from rice*

Inoculation of 2-week old rice plants with the bacterial non-host pathogen *P. syringae* pv. *syringae* leads to acquired resistance against a challenge infection by the rice blast fungus *Pyricularia oryzae* occurring 2–6 days later [36]. To identify genes whose transcripts accumulated after the resistance-inducing treatment, a λZAPII cDNA library representing an mRNA population of primary rice leaves 24 h after inoculation with *P. syringae* pv. *syringae* was prepared [29]. Differential screening of this library resulted in the isolation of a clone named λ29 that hybridized to radioactively labelled cDNA transcribed from mRNA of inoculated plants but not to a probe prepared from uninoculated control plants. The insert of λ29 was subcloned into pBluescript SK− and used as a hybridization probe to isolate a corresponding clone from a rice genomic library. The genomic λ clone contained a 2.8 kb *Sac*I fragment hybridizing to the λ29 cDNA insert. Sequence analysis of the cDNA and the 2.8 kb genomic fragment revealed that both contained nearly identical sequences (Figure 1A and 1B). The longest open reading frame of the cDNA encodes a putative protein of 107 amino acids. Although there is no in-frame stop codon in the cDNA upstream of the methionine codon at nucleotide position 17 of the cDNA, it is likely that this codon represents the translation initiation site because it is the first methionine codon in the open reading frame in the gene sequence.

The gene sequence is interrupted by a single intron and differs from the cDNA sequence at 3 nucleotide positions in the 3′-untranslated flanking region and at one position in the coding region, the latter resulting in a conservative amino acid change (Leu/Met at amino acid position 27; Figure 1A). Interestingly, more than half of the 692 bp intron exhibits about 80% sequence similarity to members of two different families of miniature inverted-repeat transposable elements (MITEs) [6]. A 233 bp sequence (nucleotide position 474–707; Figure 1A) is homologous to *Tourist* elements, while the 190 bp between nucleotide position 740 and 930 (Figure 1A) are homologous to *Wanderer* elements [6] (data not shown).

Initially, sequence comparison of the putative 107 amino acid protein encoded by the cDNA to entries in the SwissProt (release 34) and EMBL (release 50) sequence databases using the FastA and TFastA programs (Genetics Computer Group, Madison, WI) revealed no obvious similarities. However, closer analysis suggested that the 107 amino acid protein belonged to the same class of proteins as the products of the pathogen-induced *Wir1* gene family of wheat [5, 11]. To reflect this, the rice gene corresponding to the cDNA was named *Rir1a* (for *r*ice *i*nduced *r*esistance gene *1a*) and the encoded protein Rir1a. Because the gene we cloned is not completely identical in sequence to the cDNA sequence, we refer to

it as *Rir1b.* Figure 2A shows an alignment of Rir1a with the putative proteins encoded by the *Wir1* gene family. Rir1a shares 35–36% identical and 57–60% conserved amino acids with the Wir1 variants, including two conserved cysteine and tyrosine residues. The encoded Rir1a sequence is slightly larger than the sequences of the Wir1 variants, which are from 81 to 88 amino acids in length. To optimize the alignment, several gaps were introduced into the Wir1 sequences, the largest of which corresponds to an insertion in Rir1a of 11 amino acids. This insertion contains an imperfect 8 amino acid repeat of a sequence found twice immediately adjacent to it on the C-terminal side in Rir1a as well in the Wir1 variants (arrows, Figure 2A). Thus, Rir1a contains three imperfect repeats of this sequence, while the Wir1 variants contain olny two. As evident from the hydropathy profiles shown in Figure 2B, Rir1a and the Wir1 proteins both consist of a hydrophobic N-terminal half and a more hydrophilic C-terminal half. The latter part of the molecule is relatively rich in glycine and proline. Within the C-terminal 56 amino acids of Rir1a, 20% and 23% of the residues are glycine and proline, respectively. Similar values between 20–25% apply for the corresponding regions of the Wir1 proteins [5, 11].

DNA gel blot analysis (Figure 3) using the cDNA insert as a probe revealed a strong and a weak band in most of the lanes containing genomic DNA digested with various restriction enzymes for which both the gene and the cDNA sequence contain no recognition sites. The most likely interpretation of this pattern is that the strong band represents the gene corresponding the cloned cDNA, while the weak band represents a cross-hybridizing related gene.

Figure 4A shows the time course of *Rir1a* transcript accumulation in rice leaves that have been infiltrated with the resistance-inducing non-host pathogen *P. syringae* pv. *syringae.* The hybridization signal corresponded to an RNA of ca. 600 to 700 bp in length, indicating that the cloned cDNA is not far from full-length. RNA levels started to increase 16 h after inoculation (p. i.) and reached a plateau 48 h p. i. No *Rir1a* mRNA accumulation was observed in systemic leaves, i.e. in uninoculated upper leaves of plants whose lower leaves have been infiltrated (data not shown). Drench application into the soil of the resistance-inducing compound 2,6-dichloroisonicotinic acid (INA) [24] also lead to an increase of *Rir1a* transcript levels, as did the infection with *Pyricularia oryzae* (Figure 4B and C).

A

```
                                             *
WIR1a   1 ....MASLGSSAGGRRPTVLLQIALFVVVAAIIINSSV
WIR1b   1 ....MAS..HSAAGRRPTALVHIALFVAIAAVIINSSV
WIR1c   1 ....MAS.....AGRRPTVLMQIALFFVVAAVIMNSSV
RIR1a   1 MMRKQSRSPCSSSSSAAVVSLCKVLLLVLALICTLETV

WIR1a  35 CLGATAVHDAAASGTG.ALDPNVPAVPT..........
WIR1b  33 CLGA.AVHDAATSGTG.ALDPNVPAVPT..........
WIR1c  30 CLGAAA...DATAGSG.AFDPNRPAVPS..........
RIR1a  39 SVEGGRVAAAALVGGGVPLNPRYPAIPEEPYIGRRGDP
                *

WIR1a  62 .PGGAGQPYT..GRGCRTVYGCR.PPAGGQP
WIR1b  59 .PGGAGQPYT..GRGCRTVYGCK.PPAGSQP
WIR1c  54 .TGGAGKPYT..GRRCLPKYRCY.PPAGGQP
RIR1a  77 YTGRGGEPYTRPGRGCTVAYGCYGGPPAAKP
```

B

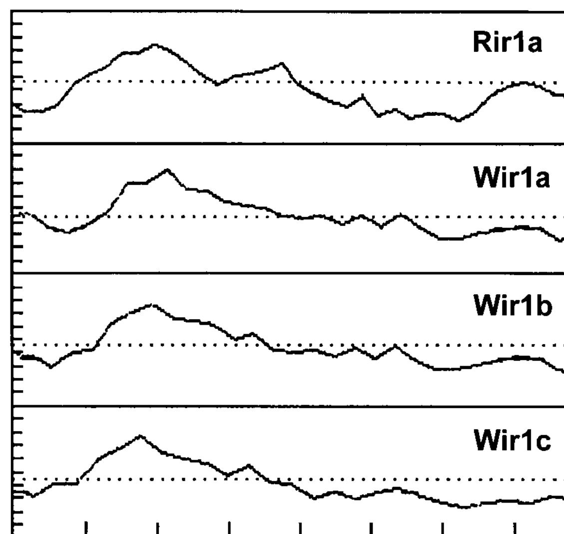

Figure 2. A. Multiple sequence alignment of Rir1a and the Wir1 variants from wheat. The intron positions in the *Wir1a* and the *Rir1b* genes relative to the encoded amino acid sequence are denoted by an asterisk above the wheat Wir1a and below the rice PPIR1a sequence (both introns are of type 1). Arrows indicate three imperfect repeats in the Rir1a sequence. B. Hydropathy profiles of Rir1a and the putative Wir1 variants from wheat.

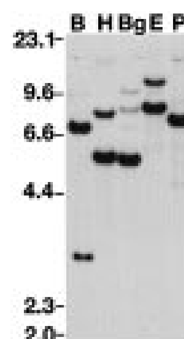

Figure 3. Gel blot hybridization of rice (var. Norin) genomic DNA. Ten μg DNA digested with *Bam*HI (B), *Hin*dIII (H), *Bgl*II (Bg), *Eco*RI (E), or *Pst*I (P) was loaded per slot and probed with the ^{32}P-labelled *Rir1a* cDNA insert. The numbers on the right indicate the size in kb of DNA marker fragments.

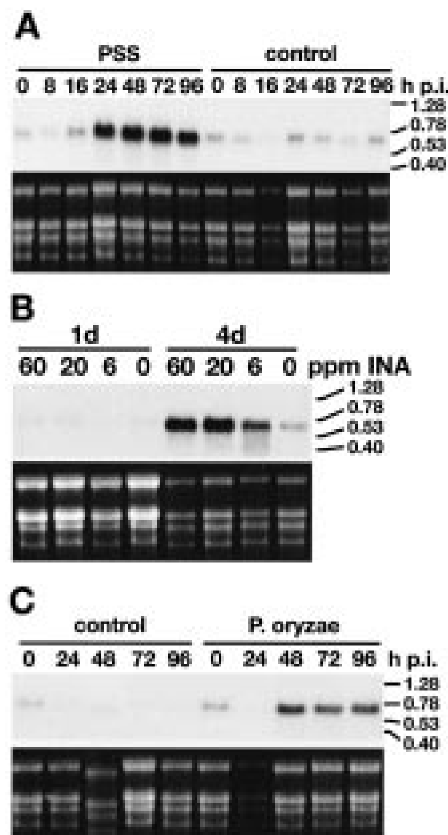

Figure 4. Time course of *Rir1a* mRNA accumulation in response to different treatments. Total RNA was extracted at the indicated time points (h.p.i, hours after treatment; d, days after treatment). Per slot 10 μg was loaded. A. Plants were infiltrated with *P. syringae* pv. *syringae* (PSS) or with H_2O (control). B. Plants were treated with INA by drench application into the soil. The concentrations given were calculated assuming homogeneous distribution of the compound in the pot volume. C. Plants were inoculated with *P. oryzae* or water (control). The hybridization probe consisted of radiolabelled *Rir1a* cDNA insert. The upper panels show the autoradiogram, the lower ones the corresponding ethidium bromide-stained gel before blotting. On the right the position and size in kb of RNA size markers is indicated.

Rir1a *is secreted*

Computer analysis of the Rir1a sequence according to the method of von Heijne [39] predicted Rir1a to contain an N-terminal signal peptide (amino acids 1 to 37; Figure 1A and 2A), and, consequently, to enter the secretory pathway. In contrast, a similar analysis of the wheat homologs Wir1a and Wir1b originally did not clearly reveal a signal peptide, but rather suggested these proteins to be integrated into the membrane, with the hydrophilic C-terminal half facing the extracytoplasmic side [5]. In order to localize Rir1a, rabbit antisera were raised against a fusion protein produced in *Escherichia coli* that contained the putative mature Rir1a (amino acids 38–107). Gel blots were prepared with protein fractions extracted from rice leaves that were inoculated with *P. syringae* pv. *syringae* 48 h p.i. and from uninoculated control leaves. As depicted in Figure 5A, the anti-Rir1a antibody recognized a protein of an apparent molecular mass of about 11 kDa in NaCl extracts of cell wall preparations from inoculated plants but not in extracts from control plants. In addition, the antibody also reacted with two constitutively expressed soluble cytoplasmic proteins of about 35 and 31 kDa. It is likely that the ca. 11 kDa protein represents the product of the *Rir1a* gene, while the soluble cytoplasmic proteins do not, because they are larger than the coding capacity of the *Rir1a* transcript and they are not accumulating upon inoculation.

To confirm identity and localization of the ca. 11 kDa protein, the coding sequence of the *Rir1a* cDNA was placed under the control of the cauliflower mosaic virus (CaMV) 35S promoter and transfected into protoplasts prepared from rice tissue culture cells. Northern blot analysis revealed that protoplasting itself did not lead to the accumulation of *Rir1a* transcripts (data not shown). Twenty hours after transfection, proteins were extracted from protoplasts and separated into soluble and insoluble fractions. Secreted proteins were concentrated from the medium. Figure 5C shows the results of a gel blot analysis of electrophoretically separated protein fractions using the anti-Rir1a fusion protein antiserum. As can be seen, the anti-Rir1a antibody recognized a band corresponding to a protein of a size similar to the ca. 11 kDa protein extracted from the cell wall fraction of *P. syringae* pv. *syringae*-inoculated rice leaves in the medium of protoplasts transfected with the *35S::Rir1a* construct, but not in the medium of protoplasts transfected with a control plasmid without insert. This band was not visible in the lanes loaded with the soluble or insoluble fractions of protoplasts. We conclude from these experiments that Rir1a is secreted into the extracellular space. Rice protoplasts were also transfected with a *35S::Wir1b* construct. However, the anti-Rir1a antiserum did not recognize a protein in any fraction of the transfected protoplasts (data not shown).

Discussion

We have isolated a cDNA clone representing an mRNA that accumulates in rice leaves infiltrated with the resistance-inducing non-host pathogen *P. syringae*

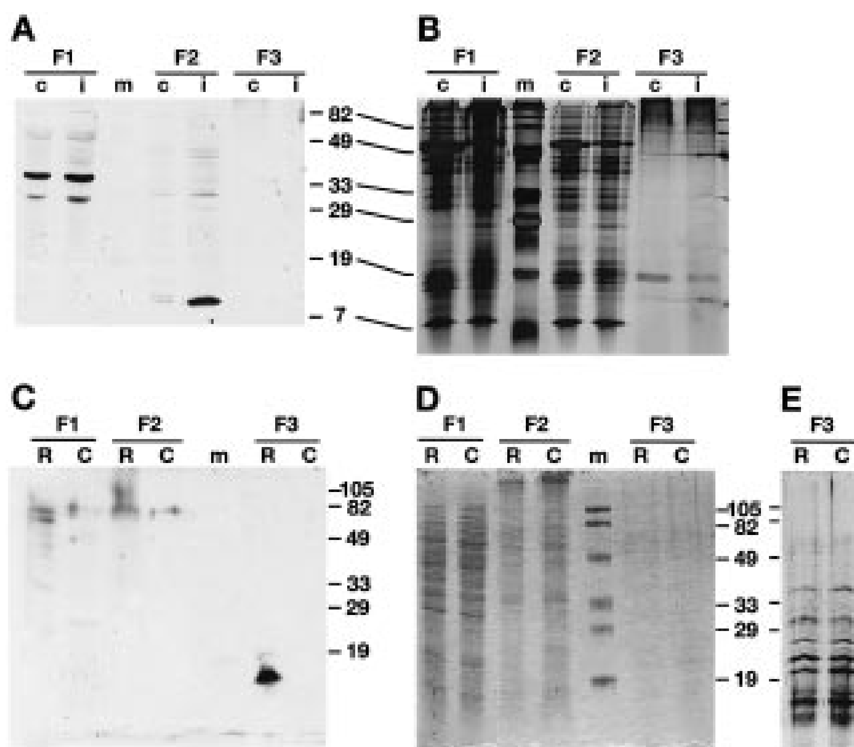

Figure 5. Protein gel blot analysis of Rir1a. A. Proteins extracted from control (c) or inoculated (i) rice leaves were separated into soluble proteins (F1), salt-extracted proteins (F2), and proteins extracted under reducing alkaline conditions (F3). B. Silver-stained gel with the same protein fractions. C. Protoplasts were transformed with a *35S::Rir1a* construct (R) and with a control plasmid without insert (C). After 20 h, proteins were extracted from protoplasts and separated into fraction 1 (F1; soluble proteins) and fraction 2 (F2; insoluble proteins). Secreted proteins (F3) were collected and concentrated from the medium. D. Coommassie-stained gel identical to the one used to prepare the blot shown in C. E. Part of a similar gel with F3 fractions stained with silver. Numbers on the side of the panels indicate the size in kDa of molecular weight markers loaded on the lanes labelled with m.

pv. *syringae*. The encoded protein Rir1 shares about 35% identical and 60% conserved amino acids as well as a similar hydropathy profile with the putative Wir1 protein variants described in wheat [5, 11], suggesting that these proteins comprise a family of defense-related proteins thus far identified only in cereals. The members of this family are characterized by a relatively small size, a hydrophobic N-terminal part and a hydrophilic C-terminal half that is relatively rich in glycine and proline. The latter part contains also two cysteine and two tyrosine residues that are conserved in the four sequences known. This family is also represented in barley, as the Wir1b cDNA hybridizes to barley transcripts accumulating after powdery mildew infection (unpublished observation). Computer analysis suggested Rir1a to contain a signal peptide, and we have shown that Rir1a is indeed secreted. The exact size of the mature protein, estimated to be ca. 11 kDa by comparison to molecular size markers, can only be determined by its isolation and N-terminal sequencing. However, we repeatedly failed to isolate enough Rir1a from cell walls of pathogen-inoculated rice leaves by immunoaffinity chromatography and other separation methods to obtain an unambigous N-terminal sequence.

Computer analysis of the Wir1 sequences also revealed weak potential signal peptide cleavage sites, but it more convincingly predicted these proteins to contain membrane-spanning domains, and they were hypothesized to be integrated into the membrane, their C-terminal part facing the extracytoplasmic side [5]. Because the Rir1a-specific antiserum did not recognize the *Wir1b* gene product, we could not determine whether Wir1b is also secreted or associated with the membrane. However, in the light of the fact that Rir1a is secreted, we consider it likely that the Wir1 proteins are also secreted.

Recently, the sequence of a barley cDNA (pBH72-Q3) corresponding to a transcript that accumulated after powdery mildew infection has been reported

[13]. The encoded protein of 65 amino acids in length was predicted to contain a signal peptide and the putative mature peptide consists of 27% proline and 16% glycine, respectively, arranged in short imperfect repeats [13]. Although no significant sequence similarity to the Wir1 and Rir1 proteins could be detected, in its amino acid composition and hydropathy profile it is reminiscent of the latter proteins and may have similar properties. Thus, a family of functionally related proteins may exist in cereals that is larger than revealed by sequence similarity alone, the detection of which is also hampered by the small size of these proteins.

Genomic DNA gel blot analysis resulted in one strongly and one weakly hybridizing band with each of three different restriction enzymes. The simplest interpretation of this pattern is that besides *Rir1a*, there is only one other cross-hybridizing gene in the haploid genome of rice, which is, however, more distantly related to *Rir1a* than *Rir1b* is, as judged from the hybridization intensities. We consider the four-nucleotide difference between *Rir1a* and *Rir1b* likely to represent polymorphic changes, as the two clones originated from two different cultivars. As mentioned above, it is possible that more genes exist in the rice genome encoding functionally similar proteins that are not easily detected by cross-hybridization.

Rir1a transcripts accumulate locally, but not systemically in rice leaves after inoculation with *P. syringae* pv. *syringae*, while this treatment was reported to induce resistance systemically [36]. This discrepancy would argue that *Rir1a* is not involved in systemic acquired resistance of rice towards rice blast. However, in our laboratory, inoculation of rice leaves with the same strain of *P. syringae* pv. *syringae* as used in the study of Smith and Métraux [36] in all cases lead only to local acquired resistance, i.e. to resistance of the inoculated leaves. We never were able to observe enhanced resistance of the untreated leaves of inoculated plants (unpublished results). Whatever the reason for this discrepancy with the published literature, in our experiments the expression domain of the *Rir1a* gene is in agreement with the local acquired resistance observed. Thus, it is possible that Rir1a plays a role in local acquired resistance. Drench application of INA into the soil leads to the accumulation of *Rir1a* transcripts in leaves, which are themselves not treated. However, this is not necessarily indicative of the generation of a systemic signal, as INA itself is taken up and transported throughout the plant [24].

The relative richness in glycine and proline of Rir1a and the putative Wir1 proteins is reminiscent of cell wall structural proteins like hydroxyproline-rich glycoproteins, proline-rich proteins, and glycine-rich proteins. However, in contrast to the former, the latter proteins are characterized by their extensively repetitive sequence motifs [16, 17, 35]. Nevertheless, the small size and the relative proline/glycine richness of Rir1a and its homologs may indicate a structural role. Rir1a appears to be ionically bound to cell wall components as it can only be eluted with buffer containing high salt concentrations. Although quantification is difficult, Rir1a appears to be synthesized in low amounts as it can only be detected on protein gel blots with very sensitive chemoluminescent immunodetection methods (unpublished observation). However, it is possible that these analyses underestimate the true abundance of Rir1a, as it cannot be excluded that only a small fraction of the total amount present in the cell wall can be eluted, while the rest may be covalently linked to other cell wall components, perhaps via its tyrosine residues. Rir1a may also be post-translationally modified and contain hydroxyproline, which may interfere with antibody binding. In any case, Rir1a may reinforce the physical barrier the cell wall presents to invading pathogens. Alternatively, Rir1a may have a direct antifungal effect. Due to the difficulty to extract sufficient amounts of Rir1 from cell walls, this hypothesis is difficult to test directly. We are producing transgenic rice plants that constitutively express Rir1a. These plants should help to determine the roleRir1a plays in pathogen defense.

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