

Cultivar-Specific Elicitation of Barley Defense Reactions by the Phytotoxic Peptide NIP1 from *Rhynchosporium secalis*

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Resistance of barley to the phytopathogenic fungus, *Rhynchosporium secalis* race US238.1, was found to be controlled by resistance gene *Rrs1*, which segregated in a manner characteristic for a codominant gene. PRHv-1, a thaumatin-like pathogenesis-related protein, was shown to be encoded by a gene family on chromosome 1. As part of the barley defense response, significant accumulation of PRHv-1 and peroxidase transcripts was induced early during pathogenesis in two *Rrs1* cultivars but not or to a lower level in a near-isogenic, susceptible *rrs1* cultivar or a cultivar lacking known resistance genes. *R. secalis* secretes a small group of necrosis-inducing peptides. One of these, NIP1, which was detected in culture filtrates only of fungal race US238.1, was found to elicit the accumulation of PRHv-1 and peroxidase mRNAs in *Rrs1* cultivars with a time course similar to that upon fungal infection. Therefore, NIP1 is a candidate for the product of fungal avirulence gene *avrRrs1*, which, together with barley resistance gene *Rrs1*, determines incompatibility of the interaction.

Additional keywords: *Hordeum vulgare* L., cultivar-specific elicitor.

Resistance of plants to pathogens is often controlled at the subspecies level by pairs of single genes, a gene for resistance in the plant and a gene for avirulence in the pathogen. The most common biochemical model for this "gene-for-gene" relationship (Flor 1955, 1971) presumes a recognition process that requires a pathogen-derived elicitor of the plant defense and an elicitor receptor in the plant. The gene for avirulence would control elicitor formation, the complementary resistance gene biosynthesis of the receptor (Heath 1981, 1991; Keen 1982). However, only one of the many bacterial avirulence genes that have been cloned appears to fit to this model. *AvrD* from *Pseudomonas syringae* pv. *tomato* was identified upon transfer into the soybean pathogen, *Pseudomonas syringae* pv. *glycinea*. It encodes a protein that appears to catalyze a step in the biosynthesis of syringolide *Ps-I*

(Smith *et al.* 1993), the low molecular weight elicitor of the hypersensitive response in soybean cultivars carrying the *Rpg4* resistance gene (Keen 1990). In fungi, the only avirulence genes cloned thus far are the genes *avr9* and *avr4* from the tomato pathogen, *Cladosporium fulvum*. The products of these genes, themselves, are elicitors of the hypersensitive response on tomato cultivars carrying resistance genes *Cf9* and *Cf4*, respectively (van Kan *et al.* 1991; de Wit 1992; de Wit, personal communication).

The imperfect fungus, *Rhynchosporium secalis* (Oudem.) J. J. Davis, is the causal agent of barley leaf scald, a disease of considerable agronomic importance in cool, semihumid areas of barley production. Studies on the inheritance of resistance to this fungus in barley led to the definition of several major genes for resistance. Barley chromosome 3 appears to have an important role in the resistance to *R. secalis*, because it carries a resistance factor in many of the resistant cultivars (Habgood and Hayes 1971; Shipton *et al.* 1974; Beer 1991). Resistance gene *Rrs1* (symbols for resistance loci as in Moseman 1972 and Sogaard and von Wettstein-Knowles 1987), which was mapped to chromosome 3 (Dyck and Schaller 1961b; Bockelman *et al.* 1977), is regarded to be part of a complex of closely linked genes (*Rrs1-Rrs3-Rrs4*) (Dyck and Schaller 1961a, 1961b; Starling *et al.* 1971). Alternatively, a multiple allelic series has also been suggested for the *Rrs1* locus (Habgood and Hayes 1971).

The availability of near-isogenic barley lines that differ at *Rrs1* and show a differential resistance phenotype upon fungal infection is the prerequisite for any analysis of the mechanisms underlying *Rrs1*-based resistance to *R. secalis*. Barley cultivar Atlas 46, carrying resistance gene *Rrs1* (Dyck and Schaller 1961a; Starling *et al.* 1971), is resistant to *R. secalis* race US238.1, whereas the near-isogenic cultivar, Atlas, which lacks this gene, is susceptible (Lehnackers and Knogge 1990). The resistance phenotype is expressed without macroscopically detectable alterations in the host tissue. In particular, it does not involve a hypersensitive response. Microscopic analyses revealed that penetration of fungal hyphae proceeds in a very similar manner on both cultivars, leading to the early collapse of a few epidermal cells. On the susceptible cultivar, the fungus develops an extensive subcuticular stroma, causes the formation of necrotic lesions, and finally sporulates. In contrast, on the resistant cultivar, fungal growth slows and finally stops (Lehnackers and Knogge 1990).

Recently, a small family of necrosis-inducing peptides (NIPs) was identified in fungal culture filtrates as well as in infected susceptible plants (Wevelsiep *et al.* 1991). Two of these

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Nucleotide and/or amino acid sequence data have been submitted to the EMBL Data Library as accession numbers X58564, X58565, and X58566.

		c	b	
		↓	↓	
PRHv-1abc		AACAAACAGTAAAGCTAACTAGAGCTTGCAGCA		32
PRHv-1abc	ATGTCGACCTCGGCGGTGCTCTTCCTCCTCCTTGTGTTTTTCGCCGCCGGTGCCAGCGCG			92
	MetSerThrSerAlaValLeuPheLeuLeuLeuAlaValPheAlaAlaGlyAlaSerAla			20
PRHv-1abc	GCCACCTTCAACATCAAGAACAACCTGCGGCTCCACAATATGGCCGGCGGGCATCCCGGTG			152
	<u>AlaThrPheAsnIleLysAsnAsnCysGlySerThrIleTrpProAlaGlyIleProVal</u>			40
PRHv-1abc	GGTGGGGGCTTCGAGCTGGGCTCAGGCCAGACGTCGAGCATCAACGTGCCCGCCGGCACC			212
	<u>GlyGlyGlyPheGluLeuGlySerGlyGlnThrSerSerIleAsnValProAlaGlyThr</u>			60
PRHv-1abc	CAAGCCGGAAGGATATGGGCACGCACCGGTGCTCGTTCAATGGCGGTAGCGGGAGCTGC			272
	GlnAlaGlyArgIleTrpAlaArgThrGlyCysSerPheAsnGlyGlySerGlySerCys			80
-1c			G	
PRHv-1ab	CAGACCGGCGACTGCGGCGGCCAGCTCTCATGCTCTCTCTCCGGGCAGCCACCAGCAACG			332
	GlnThrGlyAspCysGlyGlyGlnLeuSerCysSerLeuSerGlyGlnProProAlaThr			100
-1c		T	C	
PRHv-1ab	CTGGCCGAGTTCACCATCGGCGGCGGCAGCACCCAGGATTTCTACGACATCTCGGTTATC			392
	LeuAlaGluPheThrIleGlyGlyGlySerThrGlnAspPheTyrAspIleSerValIle			120
PRHv-1abc	GACGGCTTCAACCTTGCCATGGACTTCTCGTGCAGCACCGGCGACGCGCTCCAGTGCAGG			452
	AspGlyPheAsnLeuAlaMetAspPheSerCysSerThrGlyAspAlaLeuGlnCysArg			140
PRHv-1abc	GATCCAGCTGCCCGCCGCCCAAGCCTACCAACACCCCAACGACGTCGCCACACACGCC			512
	AspProSerCysProProProGlnAlaTyrGlnHisProAsnAspValAlaThrHisAla			160
PRHv-1abc	TGCAGTGGCAATAATAACTACCAGATCACCTTCTGCCCATGA	554		
	CysSerGlyAsnAsnAsnTyrGlnIleThrPheCysPro***	174		

PRHv-1a	552	TGAAGCCTCTTTATGTGCGCACCGCCAACCAATAATAGGCGTACGT.....ATAT		611
-1b		-----C-----GTGTGTGTGTCT---		
-1c		-----C-----GTATAT.....		
PRHv-1a		ATATATATATGTGGTGATATAAAATATAAAATATAAAATATAAAATAAGAGCCGTACT		671
-1b		-----A-ATAT-----T---T--GGTC-----A----		
-1c	-----T-----		
PRHv-1a		GCTTAATACTCCCTTCGTTCCATAAATACAGTATATATCTTTTAAGAGATTTTACTAATGG		731
-1b		-----A-----GGAAAAAAAAAAAAAAAAAAAA		
-1c		-----C-----A-----G---		
PRHv-1a		ACAACATACGAAACAAAATAAGTGAATGTATACTCTAAAGTATATCTATAAACATCTATA		791
-1b		AA		
-1c		--T-----T-----AAAAAAAAAAAAAAAAAAAA		
PRHv-1a		TGTAGTTTCCTAAAAAAAAAAAAAAAAAAAA		820
-1c		AAAAAAA		

Fig. 1. Nucleotide and deduced amino acid sequences of three PRHv-1 cDNA clones. **Top**, 5' untranslated and coding regions of the three cDNA clones. Underlined amino acid sequence positions 21-48 are identical to the sequenced 28 N-terminal amino acids of PR protein Hv-1 from barley. Underlined amino acid 96 (glutamine) is changed to arginine in PRHv-1c. Arrows indicate the 5' ends of pcPRHv-1c and -1b. **Bottom**, 3' untranslated regions. -, nucleotide identity; ..., sequence gaps. The nucleotide sequences have been submitted to the EMBL Data Library as accession numbers X58564, X58565, and X58566.

peptides, NIP1 and NIP3, act as stimulators of the plant plasma membrane ATPase (Wevelsiep *et al.* 1993). One of them, NIP1, was detected only in culture filtrates of fungal race US238.1 (Wevelsiep *et al.* 1991). Since the occurrence of these phyto-toxins correlated with lesion development (Wevelsiep *et al.*

1991), they are thought to be involved in the killing of plant cells for the purpose of releasing plant nutrients.

The first goal of the present study was to identify resistance-related plant reactions that reflect the *Rrs1*-based host defense response at the molecular level. Utilizing these

PRHv-1 (barley)	MSTSA	VFLLLAVFA	AGASAATFNI	KNNCGSTIWP	AGIPV.....	20	
WIR232 (wheat)	-A--P	-----	-----	-----	-----	20	
zeamatin (maize)			. . V-TV	V-Q-PF-V-A	SV--.....	20	
PR 5 (tobacco)	MNFLKSFPFF	AFLYFGQY-V	-VTH	-D-	V-K-TY-V-A	AS-.....	19
PR P23 (tomato)		. . V-FFLLCV	TYTY	IEV	R- -PY-V-A	ST-I.....	20
Osmotin (tob.)	MGNLRS	SFVFF-LALV	TYTY	IEV	R- -PY-V-A	ST-I.....	20
Thaumatococcus	MAAT-CF	FFLFPFLLLL	TLSR	-E-	V-R-SY-V-A	ASKGDAALD	25
PRHv-1 (barley)	GGFELGSGQ	TSSINVPAGT	QAGRIWARTG	CSFNG.GSGS	COTGDCGGQL	69	
WIR232 (wheat)	-A-	-----	-----	-----	-----	69	
zeamatin (maize)	-RQ-NR-E	SWR-TA-	T-A-	-C-DAS-R-	R-----VV	70	
PR 5 (tobacco)	-RR-D-	SW- -NP-	VQA- -G-N	-N-D-S-R-N	E-----N-M	69	
PR P23 (tomato)	-RR-NR-	-WV- -A-R-	KMA- -G-	-N-AA-R-T	-----V-	70	
Osmotin (tob.)	-RR-DR-	-WV- -A-R-	KMA-V-G-N	-N-AA-R-T	-----V-	70	
Thaumatococcus	A-RQ-N-E	SWT- -EP-	NG-K-	-M-DDS-	-I-----L-	75	
PRHv-1 (barley)	SCSLSGOPPA	TLAEFTIGGG	STQDFYDISV	IDGFNLAMDF	S.....	110	
WIR232 (wheat)	-R-	-Y-	-----	-----	-----	110	
zeamatin (maize)	Q-TGY-RA-N	-----YALKQF	NNL- -F- -I	L- -VPYS-	LPDGGSG..C	118	
PR 5 (tobacco)	E-QGY-KA-N	-----ALNQF	N. - -V- -L	V- -IP-E-	-PTN..GG.C	115	
PR P23 (tomato)	Q-TGW-K-N	-----YALDQF	-NL- -W- -L	V- -IP-T-	APTTPSGGKC	120	
Osmotin (tob.)	Q-TGW-K-N	-----YALDQF	-GL- -W- -L	L- -IPIT-	.PTNTPSGGKC	119	
Thaumatococcus	R-KRF-R-T	-----SLNQ.	YGR-YI- -N	-K- -VP-N-	-PT..TRG.C	121	
PRHv-1 (barley)	CS	TGDALQ..C	RDP...SCPP.	126
WIR232 (wheat)	126
zeamatin (maize)	SRGPRCAVDV	NARCPAELR.	QDGVCNNA	P	VFKKDEYC-V	GSAANN-H-T	167
PR 5 (tobacco)	.RNLRCTAPI	NEQCPAQLKT	Q.GGCNNP	T	VIKTNEYC-T	NG-G.-G-T	162
PR P23 (tomato)	.HAIHCTANI	NGECPRALKV	P.GGCNNP	T	-FGGQ-YC-T	QG-...-G-T	165
Osmotin (tob.)	.HAL.CTA.I	NGECPAELRV	P.GGCNNP	T	-FGGQ-YC-T	QR-...-G-T	162
Thaumatococcus	.RGVRCADI	VGQCPAKLKA	PGGGCNDA	T	VFQTSYEC-T	...TGK-G-T	167
PRHv-1 (barley)	PQAYQHPNDV	ATHA..C.SG	NNNYQITFCP			153
WIR232 (wheat)	-----	-----	-----	-----	-----	153
zeamatin (maize)	NYSRYFKGQC	-D- -SY-K-D	-STFT-PA	.T- -KVV-	-----	-----	206
PR 5 (tobacco)	DLSRFFKERC	-D- -SY-Q-D	P-SLFT-P	.T- -RVV-	-----	-----	201
PR P23 (tomato)	ELSKFFKKRC	-D- -SY-Q-D	P-STFT-PG	ST- -RVV-	-----	NGVADPNFPL	205
Osmotin (tob.)	FFSKFFKQRC	-D- -SY-Q-D	P-STFT-PG	ST- -RVI-	-----	NGQAHPNFPL	202
Thaumatococcus	EYSRFFKRLC	-D- -FSYVI-K	P- -TVT-P-	SS- -RV-	-----	TA	205
PR P23 (tomato)	EMPASTDEVA	K	216				
Osmotin (tob.)	EMPGS.DEVA	K	212				

Fig. 2. Comparison of amino acid sequences of thaumatin-like proteins from different plant species. WIR232 is a clone from wheat that was isolated using the original WIR2 clone (Rebmann *et al.* 1991b). It is 97% identical to PRHv-1. Zeamatin, antifungal protein from maize seeds (Richardson *et al.* 1987, Roberts and Selitrennikoff 1990; Huynh *et al.* 1992); PR 5, virus-induced protein from tobacco (Cornelissen *et al.* 1986; Payne *et al.* 1988); PR P23, viroid-induced PR protein from tomato (Rodrigo *et al.* 1991); osmotin, salt-induced protein from tobacco (Singh *et al.* 1987; 1989). Sequence identity of PRHv-1 with thaumatin is about 45% excluding gaps. The arrow indicates the N-terminal amino acid after cleavage of the signal peptide, where the numbering of the sequences starts. Boxes show amino acids identical in all seven sequences. Dots indicate 10 cysteine positions which are identical in all sequences, while asterisks identify those six cysteines positioned in gaps of the barley and wheat protein sequences.

reactions as a monitoring system, the subsequent goal was to detect molecules of fungal origin that elicit the host defense.

RESULTS

Phenotypic segregation analysis.

Prior to molecular studies aimed at the elucidation of the *Rrs1*-based resistance mechanism, the *Rrs1*-mediated resistance phenotype was characterized in barley primary leaves upon inoculation with spores of *R. secalis* race US238.1. The near-isogenic cultivars, Atlas 46 (*Rrs1*) and Atlas (*rrs1*), were therefore crossed, and the segregation of the resistance phenotype was followed in the offspring. On primary leaves of the susceptible cultivar, Atlas, necrotic lesions became visible 10–14 days after inoculation. Leaves of the resistant cultivar, Atlas 46, did not show symptoms after 4 wk or later. In comparison, heterozygous plants of the F₁ generation showed an intermediate phenotype; lesions appeared 2–4 wk postinoculation. Phenotypic variation was much greater than in the susceptible parent, with lesions differing in size and time of appearance. Among 575 F₂ individuals, these phenotypes were found to segregate at a ratio of 151 resistant to 299 intermediate to 125 susceptible. These data fit well to the 1:2:1 ratio expected of the segregation of a codominant allele for resistance and a recessive allele for susceptibility ($\chi^2 = 3.349$, $P = 0.1150$ – 0.25).

Identification of pathogen-induced mRNAs.

Upon inoculation of wheat with barley powdery mildew (*Erysiphe graminis* f. sp. *hordei*), a fungus nonpathogenic on wheat, different mRNAs have been described to be induced or enhanced, and corresponding cDNAs (WIR1 to WIR6) have been cloned (Schweizer *et al.* 1989). WIR2, a chimeric clone, was used to isolate another cDNA clone, pWIR232 (Rebmann *et al.* 1991b), and WIR3, representing a truncated mRNA, was used to isolate cDNA clone pPOX381 (Rebmann *et al.* 1991a). Sequence comparison of these cDNAs suggested that pWIR232 encodes a thaumatin-like (TL) protein and that pPOX381 encodes a peroxidase. The original WIR2 and WIR3 cDNAs from wheat were used as probes on gel blots with RNA from barley primary leaves inoculated with *R. secalis* race US238.1, and transcripts with sizes similar to those of the wheat mRNAs (Schweizer *et al.* 1989) were detected. More importantly, homologous mRNAs accumulated to significantly higher levels in the resistant cultivar Atlas 46 than in the susceptible cultivar Atlas (data not shown). Therefore, the accumulation of TL proteins and peroxidase was assumed to be part of the barley defense response and barley cDNAs homologous to the wheat clones to be suitable tools to monitor this response.

Isolation of barley TL protein cDNA clones.

The TL protein cDNA clone (WIR2) from wheat was used to isolate homologous clones from a cDNA library of the inoculated, resistant cultivar, Atlas 46. Hybridization with radiolabeled wheat cDNA revealed that about 0.1% of the cDNA clones in this library were homologous to the wheat probe. This supported the RNA gel blot hybridization data, which indicated a high level of these transcripts in inoculated barley leaves. Three cDNA clones containing the longest inserts among a total of 20 randomly selected clones were chosen for sequence analysis. Figure 1 shows that all three were nearly

full-length, containing one open reading frame of 519 bp beginning at the ATG at position 33, which was preceded by two in-frame stop codons (TAA) at positions 9 and 15. Complete identity was found between part of the deduced amino acid sequence of the cDNA clones and the sequenced 28 N-terminal amino acids of a pathogenesis-related (PR) protein, Hv-1, induced in barley upon infection with an avirulent race of powdery mildew (Fig. 1) (Bryngelsson and Gréen 1989). The clones were consequently termed pcPRHv-1a, pcPRHv-1b, and pcPRHv-1c. In addition, the amino acid sequences were highly homologous to thaumatin, the sweet-tasting protein of the tropical shrub, *Thaumatococcus daniellii* (Iyengar *et al.* 1979), and to TL proteins from different mono- and dicotyledonous plants, as shown in Figure 2.

The open reading frames found in the three cDNAs were identical with the exception of three nucleotide positions (Fig. 1). The deduced peptides consisted of 173 amino acids and were identical in PRHv-1a and PRHv-1b, whereas one amino acid difference was found in PRHv-1c (arginine instead of glutamine at position 96, Fig. 1). Sequence comparison with the N-terminus of the mature Hv-1 protein (Bryngelsson and Gréen 1989) confirmed that the clones identified in this paper encode precursor proteins as indicated by the presence of N-terminal hydrophobic signal peptides (von Heijne 1986) of 20 amino acids (Fig. 1). A molecular mass of 15,626 Da and an isoelectric point of pH 4.19 were calculated for the proteins encoded by pcPRHv-1a and pcPRHv-1b, whereas the respective data for the PRHv-1c protein were 15,654 Da and pH 4.38. These data deviated somewhat from those estimated experimentally for the Hv-1 protein (19 kDa, pH 3.5) (Bryngelsson and Gréen 1989).

The three PRHv-1 clones displayed a high degree of homology over their entire nucleotide sequences but differed in length due to variable 3'-untranslated regions (Fig. 1). Assuming identical 5' ends of the corresponding mRNAs and equal sizes of their poly(A)⁺ tails, the sequences differ by 89 (pcPRHv-1a vs. pcPRHv-1b) and 30 bp (pcPRHv-1a vs. pcPRHv-1c). These data may explain the size heterogeneity of PRHv-1 transcripts observed on RNA gel blots ranging from 0.75 to 0.85 kb, and suggesting the existence of a gene family. This was confirmed by DNA gel blot analysis, as shown in Figure 3. Several prominent bands were detected in a wheat-barley chromosome addition line and the barley chromosome donor cultivar, Betzes. Moreover, these bands were detected only in the addition line carrying chromosome 1, indicating the chromosomal location of the gene family.

Accumulation of TL protein and peroxidase mRNAs in infected leaves.

The PRHv-1a clone was used as a probe to follow the accumulation of TL protein mRNA upon inoculation of the resistant cultivar, Atlas 46, and the near-isogenic susceptible cultivar, Atlas, with spores of *R. secalis* race US238.1. Figure 4A shows a comparison with cultivar Turk, from which *Rrs1* had originally been introgressed into the Atlas genetic background, and with the universal susceptible, Hannchen. Two phases in the plant response were apparent: the first, extending to about 48 hr postinoculation; the second, beginning at 48 hr. During the earlier phase, both resistant *Rrs1* cultivars, Turk and Atlas 46, displayed a strong, transient accumulation of PRHv-1 mRNA. For cultivar Atlas 46, the peak in PRHv-1

mRNA accumulation 18–24 hr postinoculation was clearly delayed relative to that of cultivar Turk. The amounts of transcript found in the susceptible cultivars, Atlas and Hannchen, remained much lower during this time. In the second phase, Atlas 46 showed another transient increase, while the susceptible cultivars Atlas and Hannchen also responded with a steady increase in PRHv-1 mRNA levels. Finally, at very late stages of pathogenesis 2–3 wk postinoculation, transcript levels remained high in the susceptible cultivars (data not shown). Except for quantitative differences, the accumulation kinetics of cultivars Turk and Atlas 46 were highly reproducible in 10 independent inoculation experiments. A higher degree of variation was, however, observed with cultivars Atlas and Hannchen. In two experiments, significant PRHv-1 mRNA levels were detected in the early phase, whereas levels after 72 hr postinoculation were lower (data not shown).

The barley homologue of the putative wheat peroxidase pPOX381 (Rebmann *et al.* 1991a), pBH6-301, was cloned from a cDNA library of RNA from barley leaves inoculated with an avirulent race of *E. graminis* f. sp. *hordei* using cDNA probes enriched by subtractive hybridization against RNA from noninoculated leaves (Thordal-Christensen *et al.* 1992). The amino acid sequence deduced from the nucleotide se-

quence of clone pBH6-301 showed 89% identity with the wheat protein represented by pPOX381 (Thordal-Christensen *et al.* 1992). Using the cDNA, pBH6-301, as a probe revealed an mRNA accumulation pattern very similar to that of PRHv-1 in the resistant cultivars Turk and Atlas 46, as shown in Figure 4B. Little peroxidase mRNA was detected in the susceptible near-isogenic cultivar Atlas, whereas a weak induction was found in the universal susceptible, Hannchen. The RNA used for this hybridization experiment showed also a relatively strong signal with the PRHv-1 probe during the early stage (data not shown). Unlike PRHv-1 mRNA, a late accumulation of peroxidase mRNA was not observed.

When barley primary leaves were wounded by compression of the tissue with forceps or by punching holes in the leaf blade with a syringe needle (20 per leaf), TL protein mRNA was not significantly induced 24 hr later. Also, when compression-wounded leaves of Atlas 46 were inoculated with heat-killed fungal spores (50° C, 10 min), the accumulation of TL protein mRNA was not detected. The same was true for peroxidase mRNA. However, both mRNA species were induced, although to a lesser extent than upon fungal infection, after application of 1 M KCl to the leaf surface (data not shown).

Elicitation of PRHv-1 mRNA accumulation by NIP1.

The strong differential accumulation of PRHv-1 mRNA was utilized to identify compounds of fungal origin that are able to elicit this response in the plant. Different fractions were prepared from *R. secalis* race US238.1 cell walls and culture filtrates and tested for elicitor activity without success. However, necrosis-inducing peptide 1 (NIP1) (Wevelsiep *et al.* 1991) strongly induced the accumulation of PRHv-1 mRNA in a dose-dependent manner in primary leaves of barley cultivar Atlas 46 but did not in the near-isogenic cultivar Atlas (Fig. 5A). F₁ plants displayed intermediate PRHv-1 mRNA levels (Fig. 5B). Protease treatment of NIP1 completely destroyed elicitor activity, whereas heat treatment (10 min, 100° C) slightly reduced it (Fig. 5B).

The accumulation of PRHv-1 mRNA upon NIP1 treatment was analyzed via RNA dot blots in different barley cultivars with known resistance genotypes (Fig. 6). NIP1 proved to be highly active on the *Rrs1* cultivars, Atlas 46 and Turk, but not on the *rrs1* cultivars, Atlas and Hannchen. Two cultivars unrelated to Turk and Atlas 46 but reported to carry the *Rrs1* gene, Hudson and Brier (Habgood and Hayes 1971), showed low PRHv-1 mRNA levels comparable to cultivar Atlas in response to NIP1 treatment. Three cultivars with different alleles at the *Rrs1* locus, Modoc (*Rrs1*²), La Mesita (*Rrs1*⁴), and Jet (*rrs1*⁵) (Habgood and Hayes 1971), as well as one cultivar with a different resistance gene, Nigrinudum (*rrs8*) (Habgood and Hayes 1971), failed to respond detectably. Only cultivar Kitchin, reported to carry resistance gene *Rrs9* (Baker and Larter 1963) and resistant to race US238.1 (Lehnackers and Knogge 1990), accumulated intermediate levels of PRHv-1 mRNA.

When time courses of PRHv-1 transcript accumulation upon NIP1 treatment were compared to those upon inoculation with fungal spores, a very similar response was observed with the *Rrs1* cultivars, Turk and Atlas 46, as shown in Figure 7. The fastest increase in PRHv-1 mRNA levels was obtained with Turk. The susceptible cultivars, Atlas and

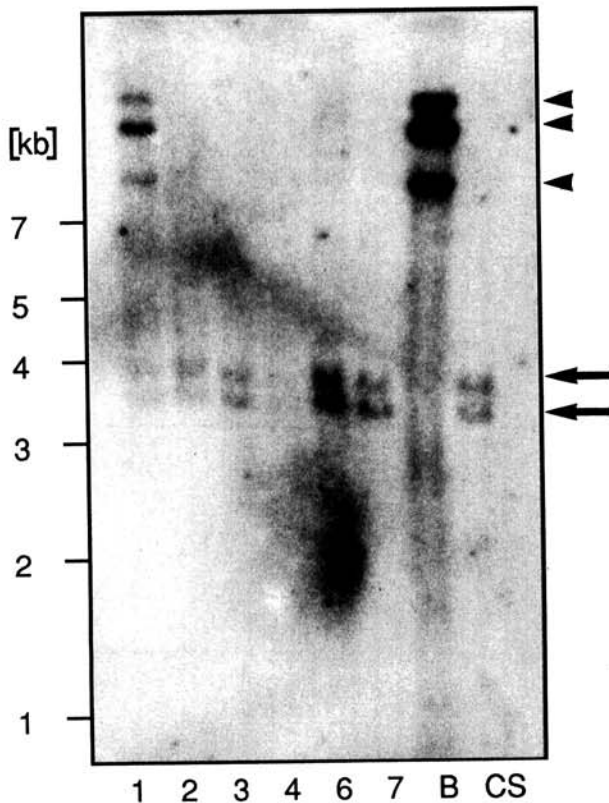


Fig. 3. Chromosomal location of the PRHv-1 gene family. Gel blot of DNA from 6 wheat/barley chromosome addition lines, digested with *Hind*III and hybridized with PRHv-1a cDNA. Numbers indicate the number of the barley chromosome present in the wheat background. An additional line carrying chromosome 5 does not exist. For comparison, DNA from the chromosome donor, barley cultivar Betzes (B) and the chromosome acceptor, wheat cultivar Chinese Spring (CS) was also included. Arrowheads indicate positions of PRHv-1-encoding barley DNA fragments; arrows mark positions of WIR2-encoding wheat DNA fragments.

Hannchen, responded to an even lesser extent than upon fungal infection; the latter cultivar showing no significant amounts of PRHv-1 mRNA. Using the peroxidase cDNA, pBH6-301, as a probe revealed a differential induction pattern very similar to that of PRHv-mRNA (data not shown).

DISCUSSION

The simplest possible biochemical model for the gene-for-gene hypothesis (Flor 1955, 1971) assumes a triggering of the plant defense response by the interaction of a pathogen-secreted molecule, the avirulence gene product, with a host receptor, the product of the complementary resistance gene. Cultivars of a host plant that carry a particular resistance gene are therefore capable of recognizing a complementary race-specific fungal feature. The nature of this fungal signal may be very different in different pathosystems and will probably depend on the particular fungal strategy to colonize a plant. A resistance-specific response of barley that occurs only if *Rrs1* is present in the plant is the prerequisite for an approach to identify the complementary avirulence gene, *avrRrs1*, in *R. secalis* (Knogge 1991).

Resistance-specific response of barley.

Many, if not all, higher plants respond to attempted infection by pathogenic organisms with the accumulation of a set of pathogenesis-related (PR) proteins that are believed to be part of the defense response (van Loon 1985; Rigden and Coutts 1988; Bol *et al.* 1990; Bowles 1990; Linthorst 1991). Although PR proteins of monocotyledonous plants have not yet been extensively studied, the available data indicate that they are similar to those of dicotyledonous plants and include β -1,3-glucanases and chitinases (Fink *et al.* 1988; Kragh *et al.* 1990), peroxidases (Kerby and Somerville 1989; Schweizer *et al.* 1989; Rebmann *et al.* 1991a; Thordal-Christensen *et al.* 1992), glutathione S-transferase (Dudler *et al.* 1991), and TL proteins (Bryngelsson and Gr en 1989; Rebmann *et al.* 1991b).

The accumulation of mRNAs encoding peroxidase and TL proteins (PRHv-1) beginning 9–12 hr after inoculation is the earliest induced defense response of barley to *R. secalis* infection detected thus far. Since significant penetration of the fungus was not observed until 24–48 hr postinoculation (Lehnackers and Knogge 1990), the accumulation of these mRNA species can be regarded as a rapid response of infected plant

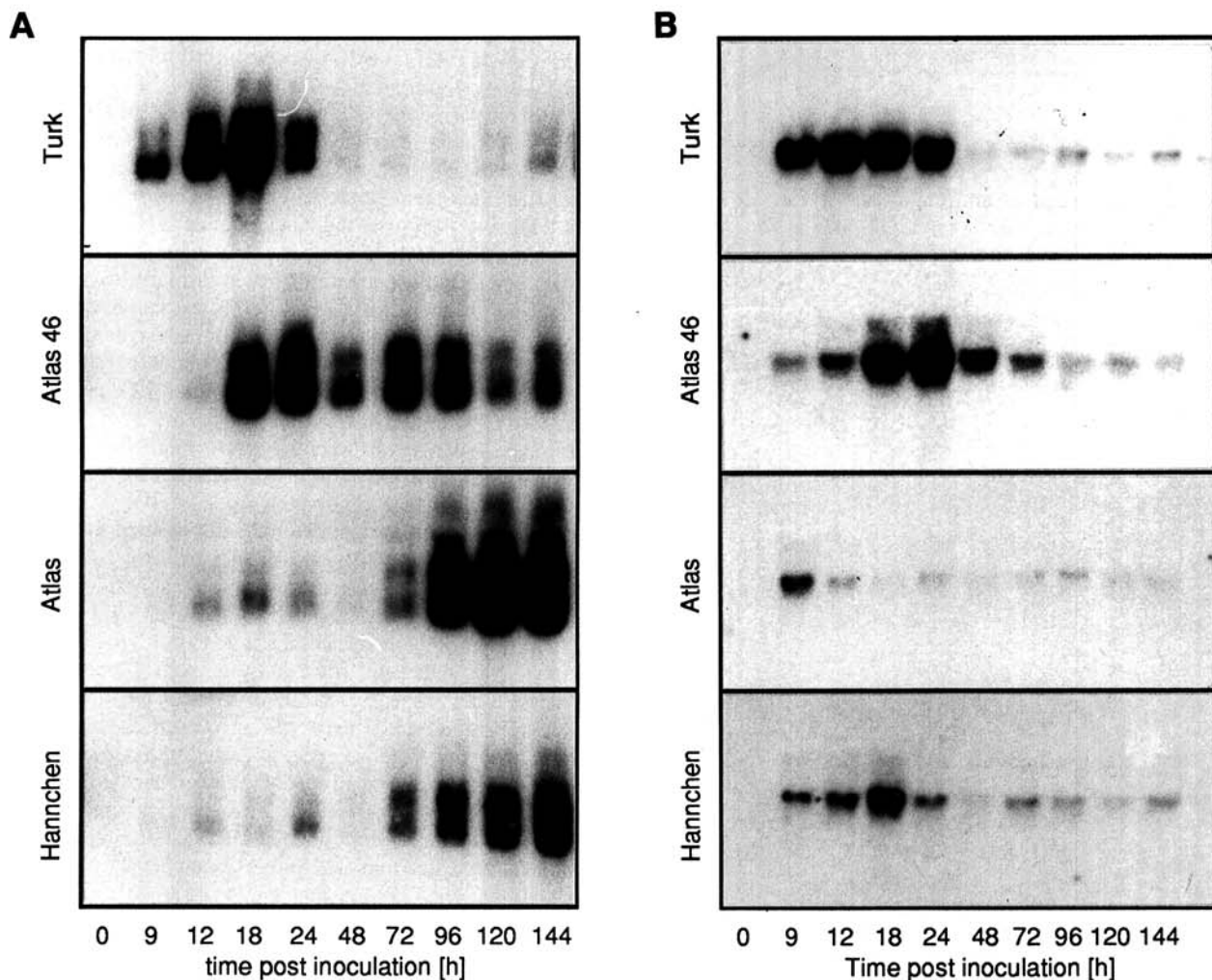


Fig. 4. Accumulation of TL protein and peroxidase mRNAs in resistant and susceptible barley cultivars upon fungal infection. Gel blot of RNA extracted at the indicated times post inoculation with spores from *R. secalis* race US238.1 and probed with (A) PRHv-1a cDNA and (B) peroxidase cDNA, pBH6-301. Double hybridizing bands in A indicate size heterogeneity of PRHv-1 transcripts which is likely to be caused by the presence of different TL protein encoding genes in the barley genome.

tissue in this pathosystem, possibly even preceding fungal penetration.

PRHv-1 was found to be encoded by a gene family located on barley chromosome 1. The coding regions of the PRHv-1 cDNAs characterized were almost identical. The deduced amino acid sequences revealed that the encoded proteins are synthesized as precursors containing an N-terminal 20 amino acid signal peptide. In analogy to PR R (= PR 5) from tobacco (Cornelissen *et al.* 1986), which is located in the extracellular space of infected tobacco leaves (Pierpoint *et al.* 1987, 1992), it is likely that the mature PRHv-1 protein is secreted as well.

PRHv-1 is a member of the tobacco PR-5 type class of proteins (Linthorst 1991), all of which share extensive homology to the sweet-tasting protein thaumatin (Iyengar *et al.* 1979). These proteins may be involved in diverse processes, since they are present in tissues of untreated plants (Naele *et al.* 1990; Pierpoint *et al.* 1990) as well as in plants exposed to osmotic (Singh *et al.* 1987, 1989; King *et al.* 1988) or biotic stress (Cornelissen *et al.* 1986; Pierpoint *et al.* 1987; Bryngelsson and Gréen 1989; Rodrigo *et al.* 1991). Recently, zeamatin, a TL protein from maize, was described (Roberts and Selitrennikoff 1990; Huynh *et al.* 1992; compare also Richardson *et al.* 1987); it showed antifungal properties, the mode of action possibly being the permeabilization of fungal plasma membranes (Roberts and Selitrennikoff 1990). Similar proteins were also found in other cereals including barley and were termed "permatins" (Vigers *et al.* 1991).

The occurrence of TL proteins with highly homologous sequences in a variety of plant species may imply similar functions of these proteins, such as antifungal activity, as an integral part of basic plant defense (Vigers *et al.* 1991). The accumulation of PRHv-1 mRNA in the resistant, *Rrs1*-carrying bar-

ley cultivars Atlas 46 and Turk may therefore reflect part of the plant's basic defense, which is either not triggered or delayed in the susceptible cultivars Atlas and Hannchen. Consequently, the function of *Rrs1* may be to link cultivar-specific recognition to the basic defense response of the host-susceptible species, barley, upon attack by *R. secalis* (Heath 1981; Knogge 1991).

Cultivar-specific elicitor activity of a toxic fungal peptide, NIP1.

The early occurrence of detectable levels of PRHv-1 and peroxidase mRNAs in contrast to the much later observed significant fungal penetration suggests that the elicitor of this plant defense response is produced and secreted as early as spore germination on the leaf surface. This was further substantiated by the observation that heat-treated spores lost the ability not only to germinate but also to elicit the plant response. Alternatively, the putative elicitor could be extremely heat-labile. During the interaction of barley with *R. secalis*, the plant cell wall persists between host plasmalemma and pathogen. Therefore, the putative elicitor may diffuse through the stomata into the leaf and across the cell wall to reach its plant cell targets. Recently, necrosis-inducing peptides of *R. secalis* were identified (Wevelsiep *et al.* 1991). The phytotoxic activity of two of these peptides, NIP1 and NIP3, appears to be based

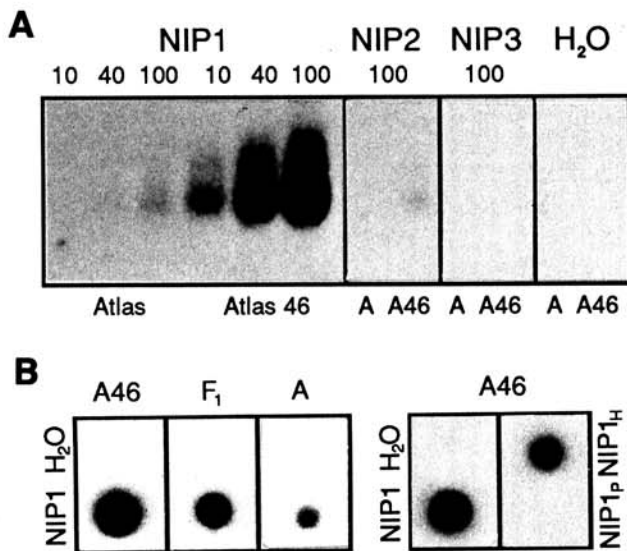


Fig. 5. Induction of PRHv-1 mRNA by NIP1 from *R. secalis*. **A**, Gel blot of RNA from NIP1-treated barley primary leaves. Fifteen- microliter samples of the three peptides at the concentrations indicated ($\mu\text{g/ml}$) were applied to the leaf surface. Total RNA was isolated 24 hr after treatment. A concentration of 40 $\mu\text{g/ml}$ was used for subsequent experiments. **B**, Dot blots of RNA from NIP1-treated primary leaves. **A**, cultivar Atlas; **A46**, cultivar Atlas 46; **F₁**, individuals from a cross Atlas 46 \times Atlas; **NIP1_H**, heat-treated NIP1 (10 min, 100°C); **NIP1_p**, proteinase K-treated NIP1.

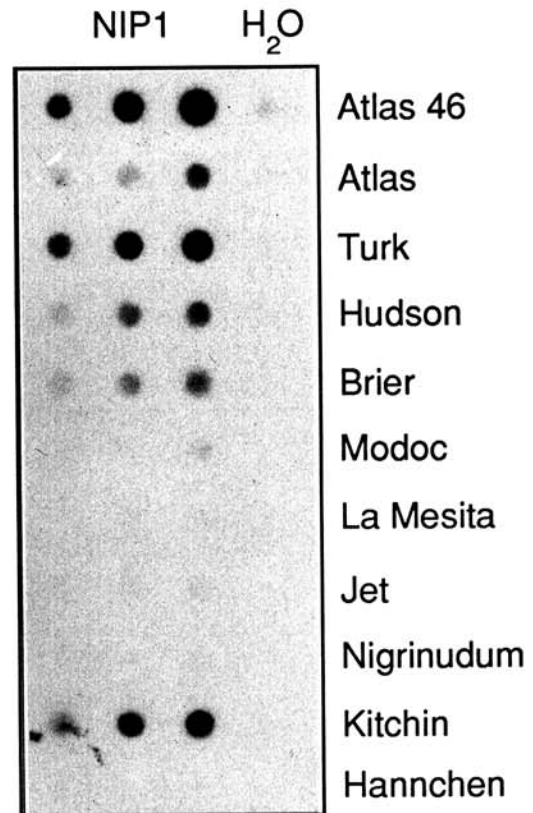


Fig. 6. NIP1-induced accumulation of PRHv-1 mRNA in different barley cultivars. Dot blot of total RNA (0.1, 0.3, 1.0 μg) extracted from five leaves 24 hr after application of NIP1 to the leaf surface. Control leaves were treated with water. All RNA blots were hybridized with PRHv-1a cDNA. Resistance genes described to be present in the cultivars are: Atlas 46, *Rrs1*, *Rrs2*; Atlas, *Rrs2*; Turk, *Rrs1*, *Rrs5*, *rrs6*; Hudson, *Rrs1*; Brier, *Rrs1*, *rrs6*; Modoc, *Rrs1²*, *rrs6*; La Mesita, *Rrs1⁴*, *Rrs10*; Jet, *rrs1⁵*, *rrs6*; Nigrinudum, *rrs8*; Kitchin, *Rrs9*.

on their stimulatory effect on the plant plasmalemma ATPase (Wevelsiep *et al.* 1993). Whether these toxic peptides may represent the fungal features that are recognized by the plant and used to elicit the defense response is the question addressed in the present work (compare also Knogge 1991).

When the NIPs and other *R. secalis*-derived fractions were applied to the surface of barley primary leaves, NIP1 was the only fungal compound that exhibited elicitor activity, as measured by the accumulation of PRHv-1 mRNA. NIP3, the other ATPase-stimulating peptide, had no such effect. This indicates that the elicitor activity of NIP1 is not causally related to its effect on the membrane enzyme. More importantly, NIP1, which was detected in culture filtrates only of *R. secalis* race US238.1 (Wevelsiep *et al.* 1991), acted as a strong inducer of PRHv-1 mRNA accumulation only in cultivars that carry the Turk-derived *Rrs1* gene (Turk, Atlas 46), but not in eight other cultivars including two reported to carry the *Rrs1* gene, and to a lesser extent in one cultivar, Kitchin, which is not expected to carry the gene. In addition, timing of transcript accumulation after application of NIP1 to the leaf surface of cultivars Atlas 46 and Turk was very similar to that upon infection. This indicates that NIP1 may indeed be the putative early elicitor. If this is the case, the heat stability of NIP1 activity would imply that the inability of heat-treated spores to induce PRHv-1 transcript accumulation was due to an effect on NIP1 synthesis or secretion.

Many barley cultivars are reported to carry the resistance factor *Rrs1*, originally termed *Rh* (Habgood and Hayes 1971; Shipton *et al.* 1971; Beer 1991), on chromosome 3. It has not been proven, however, that *Rrs1* is the same gene in all of these cultivars. It may also be a locus that carries different closely linked genes in different cultivars or even closely linked loci with a high complexity of genes and alleles. An example of such a complex situation is the *Rpl* locus of maize, which determines race-specific resistance to the rust fungus, *Puccinia sorghi*. Originally, this locus was believed to contain 14 alleles. Recent results indicate that at least some of these alleles are closely linked genes (Bennetzen *et al.* 1991). Due

to the breeding history of cultivar Atlas 46 (Riddle and Briggs 1950), the genetic character called *Rrs1* in the present paper, which confers resistance to fungal race US238.1 and which segregates as a single codominant gene, must be identical to one of the resistance genes in cultivar Turk, but not necessarily to the *Rrs1* gene reported in cultivars unrelated to Turk. This may explain why two purportedly *Rrs1*-carrying cultivars, Hudson and Brier, responded only weakly to NIP1 treatment. The intermediate PRHv-1 transcript levels induced by NIP1 in cultivar Kitchin, reported to carry the codominant gene *Rrs9* (Baker and Larter 1963), may indicate that this cultivar also has the *Rrs1* gene or that the *Rrs9* gene may be an *Rrs1* allele.

Low but detectable levels of PRHv-1 mRNA were observed in the susceptible cultivar Atlas after several days of NIP1 treatment. Assuming that binding of NIP1 to a particular target molecule, possibly the product of resistance gene *Rrs1*, leads to gene activation, the response of *rrs1* plants could result from a target that is less effective in coupling elicitor binding via a signal transduction chain to gene activation. Therefore, much higher concentrations of NIP1 and/or longer incubation times may be necessary to trigger mRNA accumulation in Atlas to levels that are induced in Atlas 46 at low elicitor doses. This could explain the high levels of PRHv-1 mRNA found in Atlas 2–3 wk after inoculation with fungal spores. At these late stages of pathogenesis, after lesions are visible, significant amounts of the phytotoxin NIP1 are detected in protein extracts from infected leaves (Wevelsiep *et al.* 1991). Alternatively, different signals could be involved in the induction of PRHv-1 mRNA at early and late stages.

NIP1 occurs race-specifically only in culture filtrates of *R. secalis* race US238.1 and has elicitor activity only in barley cultivars carrying resistance gene *Rrs1*. Therefore, NIP1 is a candidate for the product of the fungal avirulence gene, *avrRrs1*, complementary to resistance gene *Rrs1*. Future research, therefore, will focus on the isolation of the NIP1 gene in order to perform complementation analysis; transfer into a virulent fungal race should render the transformants

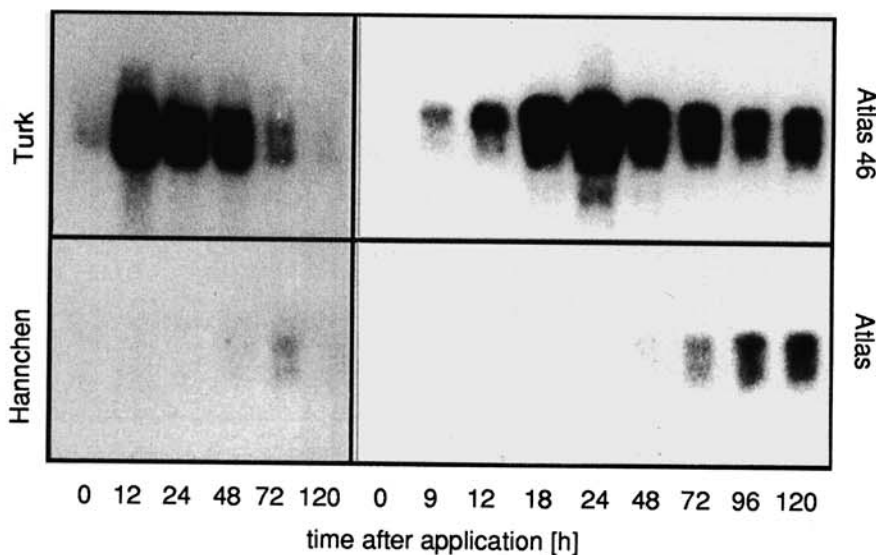


Fig. 7. Accumulation of PRHv-1 mRNA in barley cultivars after treatment with NIP1. Gel blot of RNA extracted at the indicated times after NIP1 treatment of the leaves and probed with PRHv-1a cDNA. Double hybridizing bands originate from the size heterogeneity of PRHv-1 mRNAs which is likely to be caused by activation of different PRHv-1 genes.

avirulent on *Rrs1* plants. The only fungal avirulence genes cloned thus far, *avr9* and *avr4* from the tomato pathogen *Cladosporium fulvum*, were verified in this way (van Kan *et al.* 1991; de Wit 1992, personal communication).

MATERIALS AND METHODS

Plant and fungal material.

Origin and culture conditions of barley cultivars used and of *Rhynchosporium secalis* race US238.1, as well as inoculation techniques, have been described previously (Lehnackers and Knogge 1990). Wheat-barley chromosome addition lines (Islam *et al.* 1981), chromosome donor barley cultivar Betzes, and chromosome acceptor wheat cultivar Chinese Spring were available at the institute.

NIP1 treatment of leaves.

NIP1 was isolated from culture filtrates of *R. secalis* race US238.1 as described elsewhere (Wevelsiep *et al.* 1991). Fifteen microliters of a solution containing 0.05% (w/v) Tween 20 and 40 µg of NIP1 per microliter, unless otherwise stated, was applied to the distal 3 cm of primary leaves of 7-day-old barley seedlings. Control plants were treated with 0.05% (w/v) Tween 20 (water controls). The plantlets were incubated in the same manner as after inoculation with fungal spores (Lehnackers and Knogge 1990).

RNA gel and dot blot analyses.

Total RNA was extracted by the guanidinium hydrochloride procedure (Logemann *et al.* 1987) from the distal halves of five primary leaf blades per treatment. Amounts were quantitated spectrophotometrically (Maniatis *et al.* 1982). For gel blot analysis, 15-µg RNA samples were subjected to electrophoresis on 1.3% (w/v) agarose gels containing 0.65 M formaldehyde (Maniatis *et al.* 1982) and transferred to nylon membranes (Hybond-N; Amersham-Buchler, Braunschweig, Germany). For dot blots, 0.1-, 0.3-, and 1-µg RNA samples were directly applied to the membrane. cDNA probes were radioactively labeled using random hexamer primers (Pharmacia, Freiburg, Germany) (Feinberg and Vogelstein 1983) or nonradioactively labeled using the digoxigenin system (Boehringer GmbH, Mannheim, Germany) according to the manufacturer's protocol. Hybridizations were performed at 60° C (WIR cDNAs) or 80° C (PRHv-1 or peroxidase cDNAs) overnight in 5 mM sodium phosphate buffer, pH 7.4, containing 0.75 M NaCl, 5 mM EDTA, 0.1% (w/v) Ficoll, 0.1% (w/v) polyvinylpyrrolidone, 0.1% (w/v) BSA, 10% (w/v) dextran sulfate, 0.5% (w/v) sodium lauryl sulfate (SDS), and 100 µg/ml of sonicated salmon sperm DNA. After hybridization, gel blot membranes were washed at 60 or 80° C (see above) in 30 mM sodium citrate buffer, pH 7.0, containing 0.3 M NaCl and 0.2% (w/v) SDS, and autoradiographed for 24 hr. Dot blot membranes were washed in 3 mM sodium citrate buffer, pH 7.0, containing 0.03 M NaCl and 0.2% (w/v) SDS.

DNA gel blot analysis.

DNA was isolated (Dellaporta *et al.* 1983) from leaves of wheat-barley chromosome addition lines, from barley cultivar Betzes, and wheat cultivar Chinese Spring. Fifteen-microgram samples were digested with *HindIII*, separated on a 0.8% (w/v) agarose gel, and transferred to a nylon membrane (Hy-

bond-N) using standard conditions. Hybridization was performed at 70° C using the same buffer as for RNA gel blots.

Construction of a barley cDNA library and sequence analysis.

For cDNA synthesis, poly(A)⁺-RNA from barley cultivar Atlas 46, 4 days postinoculation with *R. secalis* race US238.1, was used as a template. cDNAs were cloned in the λ Uni-ZAP vector system (Stratagene, La Jolla, CA), and the library was screened according to the manufacturer's instructions with WIR2 cDNA. Three of the hybridizing clones (pcPRHv-1) were selected for sequencing. DNA excised as pBluescript II SK(-) phagemids was sequenced by the dideoxy chain termination method (Sanger *et al.* 1977; Chen and Seeburg 1985) using T7 DNA polymerase (Pharmacia, Freiburg, Germany).

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