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The race-specific elicitor, NIP1, from the barley pathogen, *Rhynchosporium secalis*, determines avirulence on host plants of the *Rrs1* resistance genotype

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NIP1, a small phytotoxic protein secreted by the barley pathogen *Rhynchosporium secalis*, is a race-specific elicitor of defense responses in barley cultivars carrying the resistance gene, *Rrs1*. Co-inoculation employing spores from a virulent fungal race together with the NIP1 protein converted the phenotype of the interaction from compatible to incompatible only on *Rrs1*-containing plants. In addition, transformation of a virulent fungal race with the *nip1* gene yielded avirulent transformants. This demonstrated that the protein is the product of a fungal avirulence gene. The fungal genome was found to contain a single copy of the *nip1* gene. Sequence analysis of *nip1* cDNA and genomic clones revealed that the gene consists of two exons and one intron. The derived amino acid sequence comprised a secretory signal peptide of 22 amino acids and a cysteine-rich mature protein of 60 amino acids. All fungal races that were avirulent on barley cultivars of the *Rrs1* resistance genotype carry and express the *nip1* gene and secrete an elicitor-active NIP1 polypeptide. In contrast, races lacking this gene were virulent. In addition, single nucleotide exchanges were detected in the coding region of the *nip1* alleles in one virulent fungal race and in a race whose interaction with barley is not controlled by the *Rrs1* gene. The resulting exchanges of single amino acids render the gene products elicitor-inactive. Thus, the *R.secalis*–barley interaction provides the first example of a pathosystem conforming to the gene-for-gene hypothesis in which a plant with a particular resistance gene recognizes a pathogen by a virulence factor, i.e. one of its offensive weapons. On the fungal side, in turn, recognition by the host plant is eluded by either deletion of the encoding gene or alteration of the primary structure of the gene product.

Keywords: avirulence gene/gene-for-gene interaction/ *Hordeum vulgare* L.

Introduction

The interaction of physiological races of phytopathogenic microorganisms and cultivars of their host plants is in many cases controlled by a pair of complementary genes, an avirulence gene in the pathogen and a resistance gene in the plant. If either of the two gene partners is missing, the result is the compatible interaction of a virulent pathogen and a susceptible plant (Flor, 1955, 1971). Based on genetic and biochemical data obtained from various plant–pathogen systems conforming to this gene-for-gene concept (Crute, 1985), a common biochemical model was proposed which presumes the involvement of a ligand–receptor interaction (Keen, 1982, 1990, 1992; Gabriel and Rolfe, 1990; De Wit, 1992). The pathogen avirulence gene controls the synthesis of a race-specific elicitor, which binds to a plant receptor. As a consequence of this binding event, a signal transduction pathway is initiated which leads to an often multifaceted plant defense response (Scheel, 1990; Ebel and Cosio, 1994; Lamb, 1994). Pathogen races producing the specific ligand molecule trigger defense reactions only in plants expressing the corresponding resistance gene, but not in plants lacking it. Therefore, resistance gene products are thought to be either specific receptors involved in elicitor perception or molecules involved in early steps of the signal transduction pathway leading to defense gene activation (Keen, 1990; De Wit, 1992, 1995).

The imperfect fungus, *Rhynchosporium secalis*, is the causal agent of scald, a leaf spot disease of barley (Shipton *et al.*, 1974). The interaction of this pathogen and its host plant constitutes a model system for plant–fungus gene-for-gene relationships. Barley cultivars carrying the co-dominant resistance gene, *Rrs1*, are resistant to a number of fungal races that are virulent on plants of the *rrs1* genotype (Lehnackers and Knogge, 1990; Hahn *et al.*, 1993). These fungal races are hence presumed to carry the avirulence gene, *AvrRrs1*, which is lacking in races virulent on *Rrs1* plants.

The development of *R.secalis* on its host plant is unusual in that it is confined predominantly to the subcuticular region of host leaves. After penetration through the cuticle, hyphae grow extracellularly between the cuticle and the outer epidermal cell walls throughout most of the fungal life cycle (Ayesu-Offei and Clare, 1970; Hosemans and Branchard, 1985; Lehnackers and Knogge, 1990; Lyngs Jørgensen *et al.*, 1993). Plant cell walls are not severely damaged and remain intact between the growing hyphae and the plant plasmalemma. The fungus must therefore kill host cells to stimulate the release of nutrients. A small family of necrosis-inducing proteins (NIP1, NIP2, NIP3) was identified in fungal culture filtrates (Wevelsiep *et al.*, 1991). These peptides, with molecular masses <10 kDa, are toxic to leaves of all barley cultivars tested, as well

Table I. Interaction of *R.secalis* races and barley cultivars

Race	US		AU					CV		UK		
Cultivar	238.1	262	1	2	3	4	5	1	3	5	7	8
Turk (*Rrs1*)	–[a]	+	–	+	–	–	–	+	–	+	–	+
Atlas 46 (*Rrs1*)	–	+	–	+	–	–	–	+	–	+	–	+
Atlas (*rrs1*)	+	+	+	+	–	+	+	++	++	+	+	+
Hannchen (*rrs1*)	++	++	++	+	+	++	++	++	++	++	++	++

[a]–: incompatible interaction; +, ++: compatible interaction with varying extents of disease expression.

as to leaves of other cereals and some dicotyledonous plants such as bean (*Phaseolus vulgaris* L.). Two of these peptides, NIP1 and NIP3, were detected in inoculated leaves of a susceptible barley cultivar and their presence correlated with the development of disease symptoms. Furthermore, these two peptides were found to stimulate the K^+-stimulated, Mg^{2+}-dependent plasmalemma H^+-ATPase of barley (Wevelsiep *et al.*, 1993) and of bean (unpublished data).

Resistance of barley to *R.secalis* is not associated with rapid local cell death at penetration sites (hypersensitive response). However, rapid and transient accumulation of mRNAs encoding peroxidase and pathogenesis-related proteins of the PR-5 type is induced by an avirulent fungal race in two barley cultivars (Turk and Atlas 46) carrying the same resistance gene, *Rrs1* (Hahn *et al.*, 1993). This response was not observed in the near-isogenic cultivar, Atlas (*rrs1*), in a number of barley cultivars bearing other defined resistance genes, or in a universal susceptible cultivar lacking any resistance genes. NIP1, in addition to its function as a non-specific phytotoxin, was found to induce this barley response in a very similar manner on *Rrs1* plants only. This suggested that the race-specific elicitor, NIP1, is the product of the avirulence gene, *AvrRrs1*, which is complementary to barley resistance gene *Rrs1*. In the present paper, the avirulence-determining function of NIP1 is demonstrated. Structural properties and expression modes of the *nip1* gene in different fungal races are correlated with the fungal avirulence phenotype on different host cultivars.

Results

Determination of avirulence by NIP1

The product of an avirulence gene, by definition, is active only in genotypically defined plant–pathogen interactions. The NIP1 protein from *R.secalis*, race US238.1, is presumed to be the product of such an avirulence gene that triggers the plant defense response only in combination with barley resistance gene *Rrs1*. The purified protein should therefore confer avirulence on a virulent fungal race on *Rrs1*-containing plants in co-inoculation experiments. In order to identify a suitable race for this type of complementation study, the interactions of 12 fungal races with the near-isogenic barley cultivars, Atlas 46 (*Rrs1*) and Atlas (*rrs1*), were analyzed (Table I; cf. Hahn *et al.*, 1993). In addition, the barley cultivar, Turk, from which the *Rrs1* gene was originally introgressed into the genetic background of Atlas (Riddle and Briggs, 1950; Habgood and Hayes, 1971) and a universal susceptible, Hannchen, were included in these infection experiments. Six races were found to be avirulent on both *Rrs1* cultivars, but virulent on the cultivars lacking the gene, while five races were virulent on all four cultivars. One race, AU3, was virulent only on the universal susceptible cultivar, Hannchen, indicating that its interaction with barley is not controlled by resistance gene *Rrs1*. The degree of virulence varied substantially, with the strongest disease symptoms usually developing on cultivar Hannchen. On the other cultivars, race AU2 was the most virulent and caused the strongest disease symptoms on *Rrs1* as well as *rrs1* plants (Table I). In comparison, race CV1 displayed the weakest virulence on *Rrs1* plants. Therefore, race AU2, which apparently does not possess the functional complementary avirulence gene, *AvrRrs1*, was chosen as the virulent race for NIP1 co-inoculation experiments.

Near-isogenic barley cultivars Atlas 46 (*Rrs1*) and Atlas (*rrs1*) were inoculated either with suspensions of spores from races US238.1 (*AvrRrs1*) or AU2 (*avrRrs1*) or with mixtures of these spore suspensions and purified NIP1 protein from race US238.1. Disease symptoms developed within 2 weeks post-inoculation on both cultivars with race AU2, and on cultivar Atlas upon inoculation with race US238.1 (Figure 1A). Co-inoculation with NIP1 had no effect on interactions involving race US238.1. However, race AU2 was no longer able to colonize the *Rrs1* cultivar, Atlas 46, while its interaction with the *rrs1* cultivar, Atlas, remained unaffected (Figure 1A). In addition to the phenotype of the interactions, the accumulation of PR*Hv-1* mRNA encoding PR5-type proteins (Hahn *et al.*, 1993) was monitored (Figure 1B). In all incompatible interactions, a high level of PR*Hv-1* transcripts was detected 24 h post-inoculation. Only the interaction of the *Rrs1* cultivar Atlas 46 with the fungal race AU2 was influenced by the presence or absence of the NIP1 protein, with high PR*Hv-1* mRNA levels accumulating when NIP1 was present. This result supports the conversion of the interaction to incompatibility. In all other interactions, PR*Hv-1* transcript levels remained unchanged. The observed avirulence-determining function of NIP1 implies that it is indeed the product of an avirulence gene.

Isolation of nip1 genomic and cDNA clones

Sequencing of the purified NIP1 protein from race US238.1 resulted in an N-terminal sequence of 52 amino acids (Figure 2). Based on the derived nucleotide sequence, degenerate oligonucleotides (primers 1 and 2) were synthesized and used as primers for PCR using fungal cDNA as a template. After cloning, the amplification products were probed with degenerate internal oligonucleotide 3. One positive clone, pSR-NIP1, was obtained and confirmed by sequence analysis (Figure 3A).

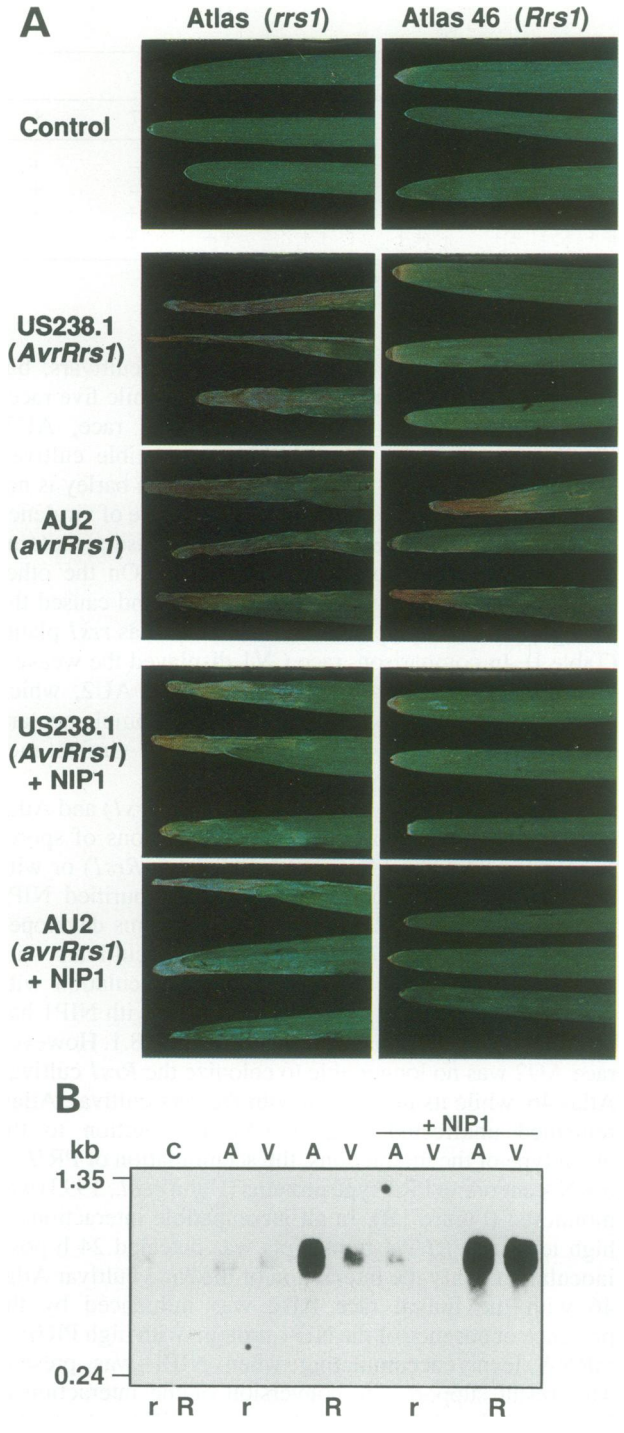

Fig. 1. Co-inoculation of near isogenic barley cultivars with spores of *R.secalis* and NIP1 polypeptide. (**A**) The phenotype of interactions of races US238.1 (*AvrRrs1*) and AU2 (*avrRrs1*) with barley cultivars Atlas (*rrs1*) and Atlas 46 (*Rrs1*) was analyzed in the absence (upper panels) and presence (lower panels) of purified NIP1 from race US238.1 during inoculation. Only the normally compatible interaction of race AU2 with the *Rrs1* cultivar, Atlas 46, was converted to incompatibility by the protein. Reduction in symptom severity 2 weeks post-inoculation of Atlas in the co-inoculation experiments relative to fungal inoculations lies within the variation observed in different experiments. (**B**) Induction of a barley defense reaction, the accumulation of PR*Hv-1* mRNA, 24 h post-inoculation and co-inoculation (+NIP1), respectively, with the avirulent race US238.1 (A) or the virulent race AU2 (V). C, uninoculated control plants; r, cv. Atlas (*rrs1*); R, cv. Atlas 46 (*Rrs1*).

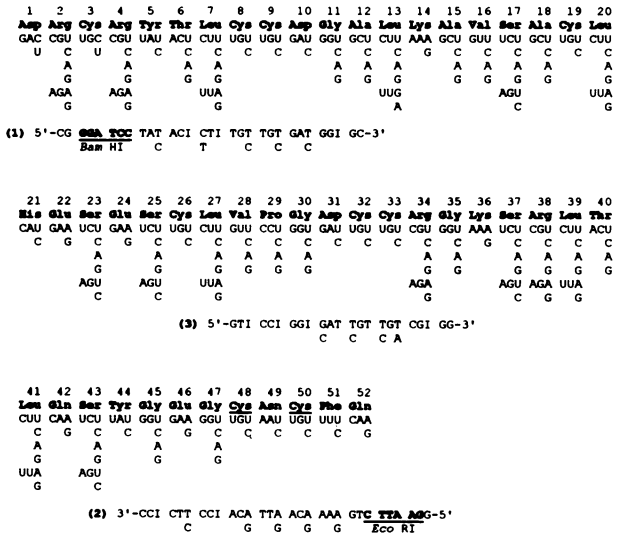

Fig. 2. N-terminal sequence of the NIP1 polypeptide. Degenerate oligonucleotides 1 and 2 were used as PCR primers, oligonucleotide 3 as hybridization probe. Sequencing of *nip1* genomic and cDNA clones revealed that cysteines at position 48 and 50 (underlined) at the 3′ primer site as well as the glutamine at position 42 did not agree with the deduced amino acid sequence.

Southern analysis of genomic DNA from fungal race US238.1 using pSR-NIP1 as probe indicated that the *nip1* gene was present in a single copy. After the isolation of a *nip1* clone, pMR-NIP1, from a size-enriched genomic library, sequence analysis revealed an open reading frame with the translation start codon located 153 bp downstream of the 5′ end of the clone. The deduced amino acid sequence included the known protein sequence C-terminal to a 22 amino acid secretory signal peptide (Figure 3B). A different size-enriched genomic library was then used to isolate another clone, pAG-NIP1^P, encompassing the putative promoter of the *nip1* gene. Finally, the insert of this clone was ligated via an internal *Sph*I restriction site to the *Sph*I–*Pst*I fragment from pMR-NIP1 to yield clone pAG-NIP1 (Figure 3A).

To characterize the exon–intron structure of the *nip1* gene, a cDNA library was constructed using RNA isolated from fungal race US238.1. Colony replica filters were hybridized using the genomic clone, pMR-NIP1, as a probe. The inserts of 24 positive clones fell into two size classes of ~400 (pHH-NIP1S) and 600 bp (pHH-NIP1L), each containing a poly(A) tail. Sequencing of a member of each class revealed that they both comprise the translated region. Primer extension studies (primer 4) revealed two major and two minor transcription start sites, demonstrating that none of the *nip1* cDNAs were of full size (Figure 3B). The size difference between the *nip1* cDNAs was mainly due to a 140 bp nucleotide stretch in the 3′-untranslated region. Presumably, two different polyadenylation sites were used because this sequence directly preceded the poly(A) tail in pHH-NIP1L, but was absent in pHH-NIP1S (Figure 3B). Comparison with the genomic sequence revealed that the gene consisted of two exons separated by a 65 bp intron. The entire deduced protein sequence contained 82 amino acids including a 22 amino acid secretory signal peptide. The three C-terminal amino acids are encoded by the second exon. Comparison with the N-terminal amino acid sequence (Figure 2) revealed

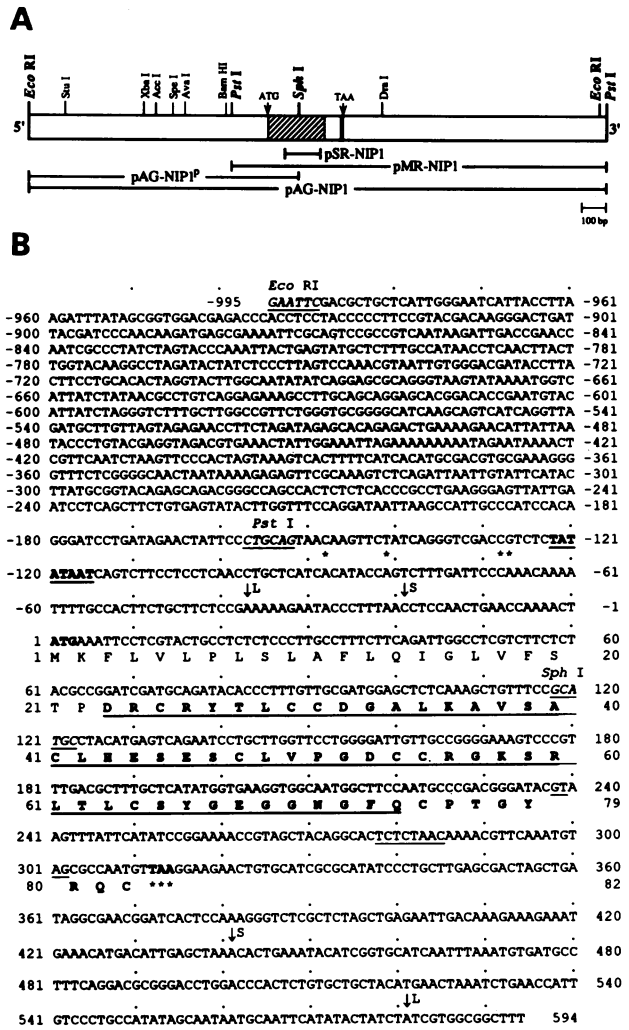

Fig. 3. Structure of the *nip1* gene from *R.secalis* race US238.1. (**A**) Restriction map of genomic *nip1* clones. Locations of the inserts of the various clones described in the text are indicated. Hatched boxes represent the two exons of the gene. (**B**) *nip1* nucleotide sequence and deduced amino acid sequence of the gene product, NIP1. Approximately 1.6 kb of the insert from pAG-NIP1 were sequenced. The putative TATA box is underlined and in bold. Two major (positions –89 and –82) and two minor transcription start sites are marked by asterisks. Arrows indicate the 5′ and 3′ ends of cDNA clones pHH-NIP1L and pHH-NIP1S. The amino acid sequence of the mature protein is indicated in bold; the N-terminal sequence obtained by protein sequencing of NIP1 is underlined. The *nip1* sequence has been deposited in the GenBank database and is available under accession number U20363.

that three positions (42, 48 and 50; corresponding to positions 64, 70 and 72 in Figure 3B) had originally been interpreted incorrectly. The mature protein consists of 60 amino acids, 10 of which are cysteines (Figures 3B and 8).

Transformation of R.secalis

In order to demonstrate that the *nip1* gene is causal for the expression of avirulence on plants carrying resistance gene *Rrs1*, a transformation system was established for *R.secalis* (M.Rohe and W.Knogge, unpublished). For this purpose, the *nip1* gene from race US238.1 was cloned into a modified pAN7-1 vector (Punt *et al.*, 1987; Bowyer *et al.*, 1995) and transferred into race AU2 which is virulent on *Rrs1* plants. Four hygromycin-resistant trans-

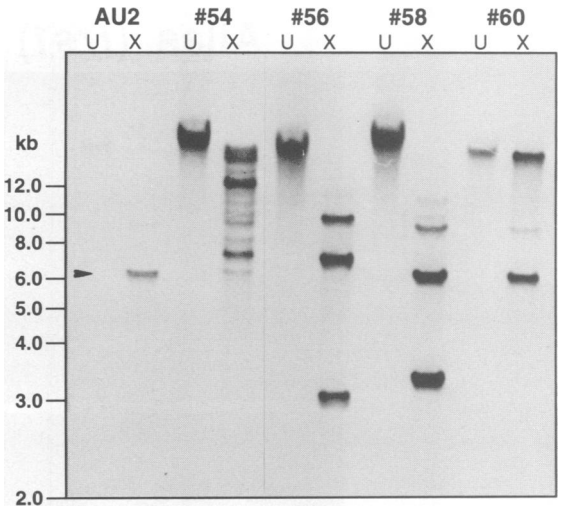

Fig. 4. Transformation of race AU2 with the *nip1* gene from race US238.1. Undigested (5 µg; U) and *Xho*I-digested DNA (10 µg; X) from race AU2 and four transformants were separated by agarose gel electrophoresis, transferred to a nylon filter and probed with the *Eco*RI insert of pAG-NIP1. The arrow tip marks the endogenous *nip1* gene fragment from race AU2.

formants were analyzed for integration of the foreign *nip1* gene and for their phenotype on Atlas 46 (*Rrs1*) and Atlas (*rrs1*). Southern blots revealed that all transformants carried one or more copies of the *nip1* transgene in addition to the endogenous gene (Figure 4). Upon inoculation of plants, all transformants retained virulence on the *rrs1* cultivar. On *Rrs1* plants, however, three transformants proved to be avirulent and induced PR*Hv-1* mRNA accumulation (Figure 5), while one (#54) showed a delay in the expression of virulence (data not shown).

Presence and expression of the nip1 gene in different R.secalis races

Different fungal races were analyzed for the presence of the *nip1* gene to enable a correlation with their virulence phenotype on *Rrs1* plants. Therefore, DNA was isolated from 12 races of *R.secalis* and analyzed by Southern hybridization using the insert of pHH-NIP1L as a probe (Figure 6). The *nip1* gene or a cross-hybridzing *nip1* homolog was present in a single copy in seven races in addition to race US238.1. In four races, US262, CV1, UK5 and UK8, the gene was undetectable. The restriction fragments hybridizing with the *nip1* probe were of similar size (*Eco*RI: 2.4 kb; *Pst*I: 1.6 kb), with the exception of those of race AU1 (4.4 kb and 3.6 kb, respectively). When PCR was performed using primers directly flanking the coding sequence (primers 5 and 6) and DNA from the 12 fungal races as templates, amplification products of the expected size of 353 bp were obtained for the eight races possessing a *nip1* homolog, indicating that the gene was of the same size in all races (data not shown).

Expression of the *nip1* gene in different fungal races was analyzed at the mRNA and protein levels. For this purpose, reverse transcription PCR was carried out using primers 5 and 6. Again, all eight races carrying a *nip1* homolog showed amplification products of the same size, demonstrating that the gene is transcribed in these races (data not shown). This result was also confirmed by Northern blot analysis using the PCR-amplified 353 bp

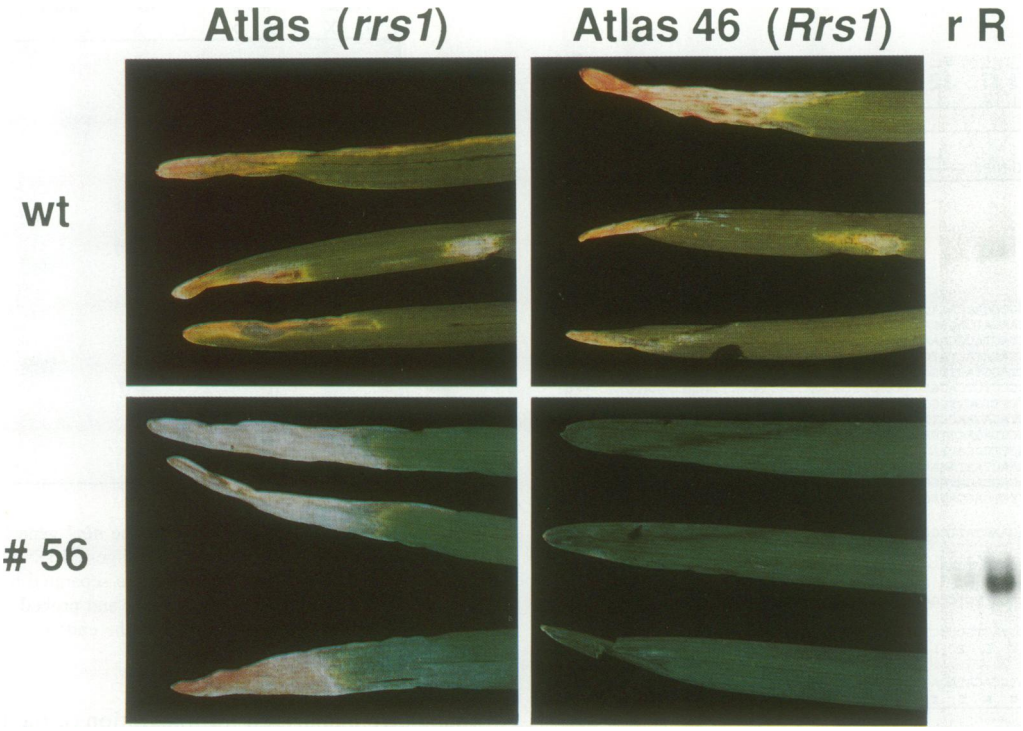

Fig. 5. Alteration of the fungal virulence phenotype by transformation with the *nip1* gene. The near isogenic cultivars Atlas and Atlas 46 were inoculated with race AU2 wild-type (wt) or *nip1* transformant #56. The presence of the *nip1* gene from race US238.1 converted the compatible wild-type interaction to incompatibility on *Rrs1* plants. In addition, PR*Hv-1* mRNA accumulation was induced by the transformant in Atlas 46 (R), but not in Atlas (r) 24 h post-inoculation.

DNA fragment from race US238.1 as a probe (Table II). Transcript sizes were found to be ~0.5 kb. When culture filtrates from the 12 fungal races were subjected to Western analysis using antisera raised against NIP1 (Wevelsiep *et al.*, 1991), all races which carry the *nip1* gene and produce the *nip1* transcript were also found to secrete NIP1 protein (Table II). In contrast, elicitor assays using the purified protein from all eight NIP1-producing races revealed that the *nip1* gene products from fungal races AU2 and AU3 were not biologically active (Figure 7; Table II)

Comparison of the nip1 gene from different fungal races

Fungal race AU2 is virulent on *Rrs1* barley plants and carries a *nip1*-homologous gene (Figure 6) encoding an elicitor-inactive NIP1 protein (Figure 7). To study the molecular basis of this inactivity, the gene was isolated from a genomic library. Sequence analysis of ~1.6 kb of a 2.4 kb *Eco*RI fragment revealed a difference in eight nucleotide positions as compared with the *nip1* gene from race US238.1 (Figure 3B). Three of these mutations were located 5′ to the coding sequence in the putative promotor region (position –953: T→C, –595: C→T, –85: T→A), four within the coding sequence (position 119: C→A, 129: T→A, 199: G→C, 228: C→A) and one in the 3′-untranslated sequence (445: C→T; cf. Figure 3B). The exchange at position 119 destroys the *Sph*I restriction site found in the gene from race US238.1. Direct sequencing of PCR-amplified DNA from the other six *nip1*-carrying races revealed that three of the sequence alterations within the coding region, all of which led to amino acid exchanges (position 40: Ala→Glu, 43: His→Gln, 77: Thr→Lys), are

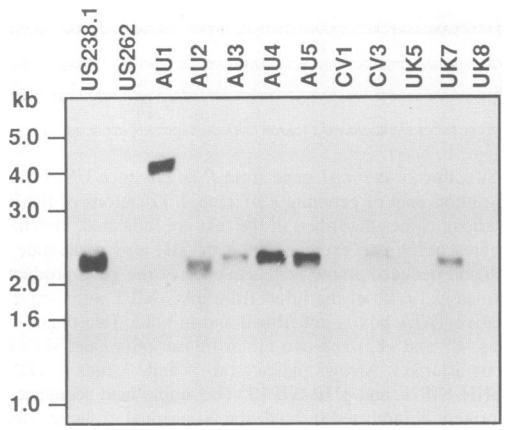

Fig. 6. Occurrence of the *nip1* gene in different races of *R.secalis*. DNA was isolated from fungal races, digested with *Eco*RI and separated by electrophoresis. After transfer to a nylon filter, the insert of cDNA clone pHH-NIP1L was used as a probe.

also present in the *nip1* homologs of races AU1, AU4, AU5 and CV3 (Figure 8). These gene products were elicitor-active, although apparently to a lesser extent than those of races US238.1 and UK7 (Figure 7; Table II). Furthermore, the coding regions of the *nip1* genes of races US238.1 and UK7 were found to be identical, and the levels of PR*Hv-1* mRNA accumulating upon treatment with either NIP1 protein were similar. In contrast, the *nip1* genes from the virulent fungal race, AU2, and from race AU3, whose interaction with barley is not controlled by the *Rrs1* gene, both contained additional single nucleotide exchanges, leading to a replacement of Gly67 by Arg in the NIP1 protein from race AU2 and of Ser45 by Pro

Table II. Comparison of avirulence phenotype, *nip1* expression and NIP1 elicitor activity in different races of *R.secalis*

Race	US		AU					CV		UK		
Cultivar	238.1	262	1	2	3	4	5	1	3	5	7	8
AvrRrs1 phenotype	+[a]	–	+	–	?[b]	+	+	–	+	–	+	–
nip1 genotype	+	–	+	+	+	+	+	–	+	–	+	–
nip1 transcript	+	–	+	+	+	+	+	–	+	–	+	–
NIP1 protein	+	–	+	+	+	+	+	–	+	–	+	–
Elicitor activity	+	–	+	–	–	+	+	–	+	–	+	–

[a]The avirulence phenotype was taken from Table I with + indicating that a race was avirulent on *Rrs1* plants but virulent on *rrs1* plants.
[b]Race AU3 did not express an avirulence phenotype that is controlled by barley resistance gene *Rrs1*. The involvement of a different avirulence/resistance gene pair could not be determined with the four barley cultivars used in this study.

in the protein from race AU3 (Figure 8). These amino acid deviations from the NIP1 protein of races US238.1 and UK7 apparently render the gene products inactive as elicitors of defense responses on plants carrying resistance gene *Rrs1* (Figure 7; Table II).

Discussion

The race-specific elicitor NIP1 from *R.secalis* induces a resistance response, the accumulation of PR*Hv-1* mRNA, in barley cultivars carrying resistance gene *Rrs1*, but not in plants of the *rrs1* genotype (Hahn *et al.*, 1993). Therefore, this protein was a likely candidate for the product of an avirulence gene. The hallmark of such a gene is that it is necessary and sufficient for the expression of the pathogen avirulence phenotype in combination with the complementary host resistance gene. Hence, the product of an avirulence gene, when present during the infection by a virulent race of the pathogen, should prevent pathogen development in a host cultivar carrying the complementary resistance gene. In such physiological complementation experiments, it was demonstrated that the *nip1* gene product from the avirulent race US238.1 converted the normally compatible interaction of fungal race AU2 to incompatibility only in plants of the *Rrs1* resistance genotype, but not in those of the *rrs1* genotype. After development of a transformation system for *R.secalis*, the avirulence-determining function of the *nip1* gene was confirmed by genetic complementation. Transfer of the *nip1* gene from race US238.1 into the virulent fungal race, AU2, yielded transformants that are avirulent on *Rrs1* plants, but remain virulent on *rrs1* plants. In addition, a transformant was identified that showed delayed virulence. Quantitative analysis of the expression of the foreign *nip1* gene in comparison with the endogenous gene encoding an elicitor-inactive product is needed to interpret the observed virulence phenotype of this transformant.

Genetic complementation has previously been used to confirm the avirulence genes *Avr4* and *Avr9* of the tomato pathogen, *Cladosporium fulvum*, on host cultivars possessing resistance genes *Cf4* and *Cf9*, respectively (van den Ackerveken *et al.*, 1992; Joosten *et al.*, 1994). In addition, disruption of the *Avr9* gene resulted in transformants that were virulent on plants of the *Cf9* genotype (Marmeisse *et al.*, 1993). In the case of *Avr4*, a naturally occurring partial disruptant caused by a frameshift mutation was found to be virulent on *Cf4* plants (Joosten *et al.*, 1994). This indicated that the products of

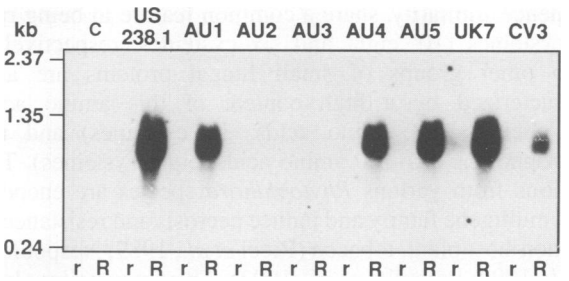

Fig. 7. Elicitor activity of NIP1 proteins from different *R.secalis* races. Leaves of barley cultivars Atlas (r) and Atlas 46 (R) were treated with the NIP1 polypeptides from different fungal races. RNA was isolated after 24 h and separated by electrophoresis. Northern blots were probed with PR*Hv-1* cDNA, encoding a PR5-type protein. C; control: mock-inoculation with 0.05% Tween 20.

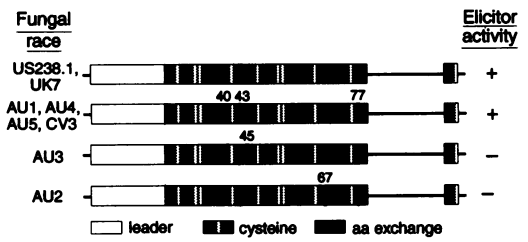

Fig. 8. Structure of NIP1 polypeptides from different *R.secalis* races and correlation with their elicitor activity on *Rrs1* barley plants. The deduced amino acid sequences of the different NIP1 polypeptides fell into two groups, one comprising the protein from races US238.1 and UK7. In the gene products from all other races, amino acid positions 40 (Ala→Glu), 43 (His→Gln) and 77 (Thr→Lys) were altered. In comparison, the sequences of the elicitor-inactive *nip1* gene products from races AU3 and AU2 each contain additional single amino acid exchanges at positions 45 (Ser→Pro) and 67 (Gly→Arg), respectively.

these genes are the only avirulence-determining fungal factors in these interactions. Analogous experiments are under way in which the *nip1* gene from *R.secalis* will be replaced by a disrupted gene via homologous recombination. If transformants can be isolated that are virulent on *Rrs1* plants, this will demonstrate unequivocally that NIP1 is not only sufficient but also necessary for avirulence expression in combination with resistance gene *Rrs1* and, thus, that the *nip1* gene is identical with avirulence gene, *AvrRrs1*.

The deduced amino acid sequence of the *nip1* gene product revealed a cysteine-rich protein. The structures of the *nip1* alleles from different fungal races fell into two groups: the first comprising the alleles of races US238.1 and UK7; the second, those of all other *nip1*-carrying

races. The sequences in the second group share three nucleotide exchanges relative to the sequences of the first group, all of which result in amino acid exchanges in the gene products. However, *Rrs1*-specific elicitor activity of the altered NIP1 polypeptides was retained. More detailed future analyses will examine whether the lower PR protein transcript levels observed upon treatment of plants with NIP1 from races AU1, AU4, AU5 or CV3 were due to the alterations in the primary structure of these proteins. In contrast, single additional amino acid exchanges in the *nip1* gene products from races AU2 and AU3 led to a loss of elicitor activity.

NIP1 from *R.secalis* and the avirulence gene products AVR4 and AVR9 from *C.fulvum*, although lacking any sequence similarity, share a common feature in being rich in cysteines (10, eight and six cysteines, respectively). Two other groups of small fungal proteins are also characterized by a high content of this amino acid, the elicitins (98 amino acids, 10 cysteines) and the hydrophobins (90–150 amino acids, eight cysteines). The elicitins from various *Phytophthora* species are encoded by a multigene family and induce necrosis and resistance in the non-host plant, tobacco (Ricci *et al.*, 1989; Nespoulous *et al.*, 1992; Pernollet *et al.*, 1993). They may be involved in limiting the host range at the species level (Kamoun *et al.*, 1993). Hydrophobins have been characterized from a number of fungi including pathogens (Wessels *et al.*, 1991; Carpenter *et al.*, 1992; St. Leger *et al.*, 1992; Talbot *et al.*, 1993). Due to their strong hydrophobicity they assemble into insoluble rodlet arrays on spores and aerial hyphae to confer hydrophobic properties to the surface of these fungal structures (Beever and Dempsey, 1978; Bell-Pedersen *et al.*, 1992). Some of them are thought to be involved in attaching hyphae to each other (Wessels, 1992) and to hydrophobic surfaces (St. Leger *et al.*, 1992), as well as in appressorium formation (Talbot *et al.*, 1993). In addition, a fungal virulence factor, the phytotoxin cerato-ulmin from *Ceratocystis ulmi*, the pathogen causing the Dutch elm disease, was found to be a hydrophobin (Stringer and Timberlake, 1993). The hydrophobins from different species are characterized by a high conservation of the cysteine pattern (C-CC-C-C-CC-C) and of hydrophobic stretches despite a low overall sequence resemblance. Another common feature of the hydrophobins is that the second and third cysteine form a doublet which is usually followed by an asparagine (Stringer and Timberlake, 1993). The hydrophobin cysteine pattern, not however the conservation of hydrophobic amino acid stretches, is also found in NIP1 from *R.secalis*. The amino acid following the first cysteine doublet is an aspartic acid, not an asparagine. For an Asn→Asp replacement, only a single nucleotide exchange at the first codon position (AAT→GAT) is required.

Assuming that all cysteines are engaged in disulfide bridges, the predicted structural features of hydrophobins resemble the 'toxin–agglutinin fold' topology, described for functionally very diverse proteins from different sources including wheat germ agglutinin and neurotoxins from snake venom (Drenth *et al.*, 1980; Templeton *et al.*, 1994). These proteins are folded into a series of loops of variable length and amino acid composition organized around a core formed by four disulfide bonds. They exert their activity by specifically binding to membrane-bound receptors. This is also anticipated for the products of avirulence genes. Analysis of the three-dimensional folding pattern of NIP1 must reveal whether this protein is structurally related to other small cysteine-rich proteins such as the hydrophobins or the elicitins.

The specific elicitor/receptor model (Keen, 1982, 1990, 1992; Gabriel and Rolfe, 1990; Knogge, 1991; De Wit, 1992) implies that products of avirulence genes function as elicitors of plant defense responses in host cultivars possessing the complementary receptors, the putative products of resistance genes. More than 30 avirulence genes have been isolated from phytopathogenic bacteria (for review, see Dangl, 1994). However, none of the gene products has been demonstrated to possess elicitor activity. In contrast, three of the four cultivar-specific fungal avirulence genes cloned to date encode elicitors of plant defense reactions. NIP1 from *R.secalis* was identified by its ability to induce PR protein biosynthesis specifically in *Rrs1* barley plants (Hahn *et al.*, 1993). The products of *Avr4* and *Avr9* from *C.fulvum* trigger a hypersensitive response in tomato cultivars of the *Cf4* and *Cf9* resistance genotype, respectively (van den Ackerveken *et al.*, 1992; Joosten *et al.*, 1994). In contrast, the *AVR2-YAMO* gene from *Magnaporthe grisea*, which prevents infection of rice cv. Yashiro-mochi (Valent and Chumley, 1991) carrying resistance gene Pi-62 (B.Valent, personal communication), might function in a different way. A stretch of the deduced amino acid sequence of this gene shares homology with the active center of neutral Zn^{2+} proteases. The biochemical activity of the *AVR2-YAMO* gene product remains to be determined. However, the occurrence in some virulent fungal races of point mutations in the protease motif as well as the introduction of additional mutations into this region suggest that the intact active center is needed for avirulence expression, possibly by releasing the actual elicitor from an as yet unknown protein (De Wit, 1995; B.Valent, personal communication).

Virulence of *C.fulvum* races on *Cf9* tomato cultivars is the result of complete deletion of the *Avr9* gene (van Kan *et al.*, 1991). *Avr4*, on the other hand, is present in all fungal races but virulent races carry alleles with single nucleotide exchanges (Joosten *et al.*, 1994). In the case of the *nip1* gene from *R.secalis*, both situations were found. Several virulent races lack the gene and one, AU2, carries an altered allele encoding an elicitor-inactive protein. In addition, avirulent race AU3, whose interaction with *Rrs1* plants is not determined by the *nip1* gene, secreted an altered protein lacking elicitor activity. In contrast to the product of *Avr4* from *C.fulvum* (Joosten *et al.*, 1994) however, no cysteine residues were affected by amino acid exchanges. Therefore, virulence of *R.secalis* on *Rrs1* barley plants is achieved either by deletion of the *nip1* gene in virulent fungal races or by incorporation of other amino acids at distinct positions in the NIP1 polypeptides that impaired elicitor activity, thus enabling the fungus to circumvent recognition by the host plant.

The product of avirulence gene *nip1* from *R.secalis*, like the AVR9 and the AVR4 proteins from *C.fulvum* (van Kan *et al.*, 1991; Joosten *et al.*, 1994), were found in significant amounts during development of the fungus in a susceptible cultivar, while the gene product was undetectable in resistant plants (Wevelsiep *et al.*, 1991). This indicates that these gene products may play essential

roles in the virulence of the pathogens. The intrinsic functions of AVR4 and AVR9 are not known. However, races of *C.fulvum* which lack the *Avr9* gene, or in which this gene was disrupted, as well as a race carrying an *Avr4* gene with a frame-shift mutation, leaving only the 13 N-terminal amino acids of the 135 amino acid wild-type AVR4 protein, did not show impaired development on the host plant, indicating that these genes are dispensible for growth and pathogenicity (Joosten *et al.*, 1994). The same holds true for the *AVR2-YAMO* gene from *M.grisea* which is absent in several virulent races of the pathogen (Valent and Chumley, 1991; De Wit, 1995; B.Valent, personal communication).

The NIP1 protein from *R.secalis* was detected originally as a member of a small family of non-specific phytotoxins that induced necrosis in all barley cultivars tested, as well as other cereals and a dicotyledonous species, bean (Wevelsiep *et al.*, 1991). The toxicity of NIP1 was manifested by its stimulatory effect on the activity of the plant plasmalemma H^+-ATPase from barley (Wevelsiep *et al.*, 1993) and bean (W.Knogge, unpublished data). The occurrence of the protein *in planta* correlated with lesion development. Therefore, the protein appears to be a virulence factor evolved by the fungus to kill host cells in order to gain access to plant nutrients. Races lacking the *nip1* gene appear to be less virulent than races carrying this gene, as race AU2 displayed the greatest virulence. Together with the high conservation of both the structural sequences and the promoter region, this implies a significant role of NIP1 in fungal virulence and suggests that the toxic activity of the mutated NIP1 in race AU2 is retained. Current work is addressing the question of whether the ATPase-stimulating activity of NIP1 from the different races is affected by the observed amino acid exchanges or whether this virulence function indeed remains unchanged. Conclusive evidence of a quantitative role for the protein in overall virulence expression should be provided by gene disruption experiments. Nevertheless, irrespective of its potency, NIP1 is a virulence factor of *R.secalis*, and barley plants carrying resistance gene *Rrs1* appear to have utilized this offensive weapon of the fungus in the recognition process that results in the onset of the defense response. Cloning and characterization of the NIP1 receptor is expected to provide further insight into the molecular mechanisms underlying signal perception and transduction in the host plant.

Materials and methods

Fungal culture

The 12 fungal races of *R.secalis* used in this study were of different geographical origin (US, California; AU, Australia; CV, Cologne; UK, Great Britain). Fungal mycelia were grown in liquid culture as previously described (Wevelsiep *et al.*, 1991), harvested after 2 weeks and separated from culture fluids by filtration.

Isolation of NIP1 and amino acid sequence analysis

For the purification of NIP1 from different races of *R.secalis*, a modification of the method described previously (Wevelsiep *et al.*, 1991) was used. After ultrafiltration of culture filtrates through an Ultrasette Ω3 membrane (FILTRON GmbH, Karlstein,Germany) and chromatography over a DEAE–cellulose column, the non-bound protein was subjected to cation-exchange chromatography (FPLC, Resource S; Pharmacia, Freiburg, Germany) using the following elution system: buffer A, 50 mM MES/KOH (pH 6.2); buffer B, 50 mM MES/KOH, 0.5 M KCl (pH 6.2); with a linear 0–30% gradient of buffer B in 10 min. NIP1-containing protein fractions were pooled and subjected to reverse-phase HPLC (Hewlett-Packard HP1090, Waldbronn, Germany) using a Vydac C_4 column (300 Å, 5 µm, 4.6×250 mm; Separations Group, Hesperia, California) at a flow rate of 0.5 ml/min and the following elution system: solvent A, 0.1% trifluoroacetic acid (TFA); solvent B, 0.1% TFA, 70% isopropanol; with a linear 0–74% gradient of solvent B in 122 min. NIP1 from race US238.1 was sequenced from the N-terminus after reductive carboxymethylation of the sulfhydryl groups with iodoacetic acid (Hollecker, 1989) by automated Edman degradation using a 477A pulsed liquid phase protein/peptide sequencer and a 120A on-line PTH amino acid analyzer (Applied Biosystems, Weiterstadt, Germany) according to the manufacturer's instructions.

Elicitor activity of NIP1

Elicitor activity of NIP1 was analyzed as previously described (Hahn *et al.*, 1993). To demonstrate the avirulence-determining function of NIP1, the purified protein from race US238.1 was mixed with spore suspensions (5×10^6 spores/ml) from races US238.1 or AU2 to yield final concentrations of 40 µg/ml. Fifteen µl of these mixtures were used to inoculate barley cultivars carrying or lacking resistance gene *Rrs1*.

Nucleic acid isolation

Fungal mycelia were washed several times with water, dried with Whatman paper, frozen in liquid nitrogen and ground to a fine powder. Nucleic acids were extracted in a mixture of 1 vol. of 150 mM Na acetate, 1 mM EDTA, 4% (w/v) SDS (pH 5.0) and 1 vol. of phenol/$CHCl_3$ (1:1, v/v) by shaking vigorously for 30 min at room temperature. Purification of nucleic acids was then essentially as described by Sambrook *et al.* (1989). DNA was precipitated with EtOH for 1 h on ice from the first LiCl supernatant and the resulting pellet was washed with 70% (v/v) EtOH. After redissolving the pellet in water, DNA was precipitated with Na acetate/EtOH. Total RNA from plants was extracted according to previously published methods (Logemann *et al.*, 1987; Hahn *et al.*, 1993). DNA was extracted using either the cetyltrimethylammonium bromide (CTAB) procedure essentially as described by Murray and Thompson (1980) or the method described by Bedbrook (1981).

Oligonucleotides

1–3: see Figure 2
4: 5′-C_{47}ATCTGAAGAAAGGCAAGGGAGAGAGGCA$_{18}$-3′
5: 5′-C_{-20}CTCCAACTGAACCAAAAC$_{-2}$-3′
6: 5′-C_{334}GCGATGCACAGTTCTTCC$_{316}$-3′
7: 5′-C_{176}CCGTTTGACGCTTTGCTCATA$_{199}$-3′
8: 5′-G_{124}GCATGCGGAAACAGCTTTGAGA$_{103}$-3′
9: 5′-C_{316}TTAACATTGGCGCTAC$_{299}$-3′.

Isolation of nip1 genomic clones from R.secalis, races US238.1 and AU2

Based on the N- and C-terminal regions of the partial protein sequence of 52 amino acids, degenerate PCR primers consisting of mixtures of 32 different 23 nt long oligonucleotides (Figure 2, primers 1 and 2) were synthesized with inosine as neutral base at positions of 4-fold ambiguity. The 5′ primers contained an additional 8 nt long sequence with a *Bam*HI site, the 3′ primers a 7 nt long sequence with an *Eco*RI site. Total RNA was isolated from 2-week-old mycelia of *R.secalis*. First strand cDNA synthesis was carried out with 10 µg RNA and 100 pmol of primer 2. After reverse transcription, an aliquot of the cDNA mixture was used in an amplification reaction with 100 pmol of primers 1 and 2 (Figure 2) in a thermocycler (Pharmacia, Freiburg, Germany) with 30 cycles of 1 min at 93°C, 2 min at 60°C and 2 min at 72°C. Aliquots of the reaction mixture were subjected to a second round of amplification using the same conditions. The specific amplification product of ~150 bp was gel purified and recovered using the QIAEX Gel Extraction Kit (QIAGEN, Hilden, Germany), followed by fill-in and ligation into the *Sma*I site of pUC18. After transformation of *Escherichia coli* strain DH5α, plasmid DNA prepared from white colonies was screened by Southern blot analysis using oligonucleotide 3 (Figure 2) as probe. This procedure yielded one positive clone, pSR-NIP1, which was confirmed by sequence analysis.

Genomic DNA was isolated from *R.secalis*, race US238.1. A size-enriched genomic library was prepared by cloning *Pst*I fragments of a size range of 1.6 kb into pBluescript II KS- (Stratagene, La Jolla, CA). Screening of replica filters carrying 2400 colonies with the pSR-NIP1 insert as a probe led to the identification of two clones that yielded restriction fragments of the same size. One of them, pMR-NIP1, was

analyzed further by sequencing. To isolate a genomic clone containing the promoter of the *nip1* gene, *Eco*RI–*Sph*I fragments of a size range of 1.1 kb were cloned into pBluescript II KS–. Screening of 7200 colonies with the *Pst*I–*Sph*I fragment from the insert of pMR-NIP1 as a probe led to the identification of clone pAG-NIP1^P. Finally, clone pAG-NIP1 was obtained by ligating the insert from pAG-NIP1^P and the *Sph*I–*Pst*I fragment of pMR-NIP1.

Using DNA from fungal race AU2, a genomic library was constructed in λEMBL3 (Stratagene) according to the manufacturer's protocol using the *Bam*HI and *Eco*RI restriction sites. The library contained 2.17×10^5 recombinant phages and was amplified in *E.coli* strain XL1-Blue MRA, yielding 2.8×10^{10} p.f.u./ml. Of the positive clones identified by screening the library with the *Sph*I–*Pst*I fragment from pMR-NIP1, five clones were found to yield identical restriction patterns. One of them was subcloned into pBluescript II SK– and analyzed by sequencing.

Primer extension analysis

The transcription start site of the *nip1* gene from fungal race US238.1 was determined essentially as described by Sambrook *et al.* (1989) by extending primer 4.

Construction and screening of an R.secalis cDNA library

Total RNA was extracted from 10-day-old mycelia of *R.secalis*, race US238.1. Poly(A)$^+$ RNA was isolated by chromatography on oligo(dT)-cellulose (Pharmacia). cDNA was synthesized from 7 μg poly(A)$^+$ RNA using the λZAP cDNA synthesis kit (Stratagene). The library contained 2.5×10^5 p.f.u./μg cDNA. 10^6 plaques were amplified in *E.coli* strain 'Sure' following the manufacturer's instructions (Stratagene). Screening of the amplified cDNA library with the pMR-NIP1 insert as a probe was performed with 120 000 p.f.u. and 30 positive clones were identified. After excision using the ExAssist/SOLR system (Stratagene), pBluescript II SK– phagemids were amplified in *E.coli* strain SOLR. Plasmid DNA was isolated from 24 positive clones. Inserts fell into two size classes of 400 bp (18 clones) and 600 bp (six clones). Inserts from two clones of each size class were sequenced.

Polymerase chain reaction

PCR was carried out using 100 ng of genomic DNA or 0.1–2 ng of first-strand cDNA after reverse transcription of RNA from different fungal races. Fifty ng of each primer (primers 5 and 6) were used. After denaturation (4 min, 95°C) 31 cycles of 1 min 95°C, 1 min 60°C, 1 min 74°C (last cycle: 10 min 74°C) were performed in a thermocycler (Pharmacia).

RNA hybridization analysis

Fungal RNA (15 μg) was separated by electrophoresis on 1.2% agarose gels (5.1% formaldehyde), transferred and fixed to Hybond-N membranes (Amersham-Buchler, Braunschweig, Germany). As probes, PCR-amplified cDNA (288 bp fragment covering the coding region) or cDNA (pHH-NIP1L) were labeled using the digoxigenin oligonucleotide labeling method according to the manufacturer's protocol (Boehringer, Mannheim, Germany) and purified by chromatography on Sephadex G-50. Pre-hybridization and hybridization were carried out at 42°C using 50% (v/v) formamide, 5× SSC, 2% (w/v) blocking reagent, 0.1% (w/v) *N*-lauryl sarcosine, 0.02% (w/v) SDS. Hybridizing bands were visualized using the DIG luminescent detection kit (Boehringer).

DNA hybridization analysis

Genomic fungal DNA (10 μg) was digested with *Eco*RI, electrophoresed in 1.0% agarose gels, transferred and fixed onto nylon membranes (Hybond-N). DNA probes were labeled using the digoxigenin oligo-labeling procedure essentially according to the manufacturer's protocol except that the labeled probe was purified by chromatography on Sephadex G-50. Pre-hybridization for a minimum of 5 h and hybridization were performed at 42°C in 50% formamide, 5× SSPE, pH 7, 0.2% SDS, 0.1 mg/ml herring sperm DNA, 0.2% PVP, 0.2% Ficoll, 0.2% BSA. Filters were washed for 1 h at 68°C in 2× SSC, 0.1% SDS and 1 h in 0.2× SSC, 0.1% SDS. Hybridizing bands were visualized using the DIG luminescent detection kit.

Western blot analysis

Proteins were separated under denaturing conditions by electrophoresis on 12–20% polyacrylamide gradient gels and transferred onto Hybond-ECL membranes (Amersham-Buchler). After incubation, immunological detection of NIP1 was achieved using the ECL Western Blotting Detection Kit (Amersham-Buchler).

Sequence analysis and data processing

For DNA sequencing, the dideoxy chain termination method (Sanger *et al.*, 1977; Chen and Seeburg 1985) was used with T7 DNA polymerase (Pharmacia, Freiburg, Germany) or sequenase (USB, Cleveland, OH). Direct sequencing of PCR-amplified DNA was performed according to Rao (1994) using the Cyclist Exo$^-$ *Pfu* DNA sequencing kit (Stratagene) and primers 5 and 6 as well as primers 7, 8 and 9. Processing of sequence data was performed using the University of Wisconsin Genetics Computer Group sequence analysis software with GenBank (11/94) and EMBL (11/94) databases.

Fungal transformation

Isolation of fungal protoplasts and transformation were carried out essentially as described for *Septoria nodorum* (Cooley *et al.*, 1988). After cloning of the *Eco*RI *nip1* fragment from pAG-NIP1 into pBluescript II KS–, the *Hin*dIII–*Not*I fragment from this plasmid was isolated and cloned into a modified pAN7-1 vector (Punt *et al.*, 1987; Bowyer *et al.*, 1995). As selection marker this vector carries the *hph* from *E.coli* under the control of the *gpd* promotor and the *trp*C termination signal from *Aspergillus nidulans*. Selection of transformants was performed in the presence of 100 μg/ml hygromycin B (Rohe and Knogge, unpublished).

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References

Ayesu-Offei,E.N. and Clare,B.G. (1970) *Aust. J. Biol. Sci.*, **23**, 299–307.
Bedbrook,J. (1981) *Plant Mol. Biol. Newslett.*, **2**, 24.
Beever,R.E. and Dempsey,G. (1978) *Nature*, **272**, 608–610.
Bell-Pedersen,D., Dunlap,J.C. and Loros,J.J. (1992) *Genes Dev.*, 6, 2382–2394.
Bowyer,P., Clarke,B.R., Lunness,P., Daniels,M.J. and Osbourn,A.E. (1995) *Science*, **267**, 371–374.
Carpenter,C.E., Mueller,R.J., Kazmierczak,P., Zhang,L., Villalon,D.K. and van Alfen,N.K. (1992) *Mol. Plant-Microbe Interact.*, **5**, 55–61.
Chen,E.Y. and Seeburg,P.H. (1985) *DNA*, **4**, 165–170.
Cooley,R.N., Shaw,R.K., Franklin,F.C.H. and Caten,C.E. (1988) *Curr. Genet.*, **13**, 383–389.
Crute,I.R. (1985) In Fraser,R.S.S. (ed.), *Mechanisms of Resistance to Plant Diseases*. Nijhoff/Junk, Dordrecht, pp. 80–142.
Dangl,J.L. (1994) In Dangl,J.L. (ed.), *Bacterial Pathogenesis of Plants and Animals*. Springer-Verlag, Berlin, pp. 99–118.
De Wit,P.J.G.M. (1992) *Annu. Rev. Phytopathol.*, **30**, 391–418.
De Wit,P.J.G.M. (1995) *Adv. Plant Pathol.*, **12**, in press.
Drenth,J., Low,B.W., Richardson,J.S. and Wright,C.S. (1980) *J. Biol. Chem.*, **255**, 2652–2655.
Ebel,J. and Cosio,E. (1994) *Int. Rev. Cytol.*, **148**, 1–36.
Flor,H.H. (1955) *Phytopathology*, **45**, 680–685.
Flor,H.H. (1971) *Annu. Rev. Phytopathol.*, **9**, 275–296.
Gabriel,D.W. and Rolfe,B.G. (1990) *Annu. Rev. Phytopathol.*, **28**, 365–391.
Habgood,R.M. and Hayes,J.D. (1971) *Heredity*, **27**, 25–37.
Hahn,M., Jüngling,S. and Knogge,W. (1993) *Mol. Plant-Microbe Interact.*, **6**, 745–754.
Hollecker,M. (1989) In Creighton,T.E. (ed.), *Protein Structure—a Practical Approach*. IRL Press, Oxford, pp. 145–153.
Hosemans,D. and Branchard,M. (1985) *Phytopathol. Z.*, **112**, 127–142.
Joosten,M.H.A.J., Cozijnsen,T.J. and De Wit,P.J.G.M. (1994) *Nature*, **367**, 384–386.
Kamoun,S., Klucher,K.M., Coffey,M.D. and Tyler,B.M. (1993) *Mol. Plant-Microbe Interact.*, **6**, 573–581.
Keen,N.T. (1982) *Adv. Plant Pathol.*, **1**, 35–82.
Keen,N.T. (1990) *Annu. Rev. Genet.*, **24**, 447–463.
Keen,N.T. (1992) *Plant Mol. Biol.*, **19**, 109–122.
Knogge,W. (1991) *Z. Naturforsch.*, **46c**, 969–981.
Lamb,C.J. (1994) *Cell*, **76**, 419–422.
Lehnackers,H. and Knogge,W. (1989) *Can. J. Bot.*, **68**, 1953–1961.

Logemann,J., Schell,J. and Willmitzer,L. (1987) *Anal. Biochem.*, **163**, 16–20.
Lyngs Jørgensen,H.J., De Neergaard,E. and Smedegaard-Petersen,V. (1993) *Physiol. Mol. Plant Pathol.*, **42**, 345–358.
Marmeisse,R., van den Ackerveken,G.F.J.M., Goosen,T., de Wit,P.J.G.M. and van den Broek,H.W.J. (1993) *Mol. Plant-Microbe Interact.*, **6**, 412–417.
Murray,M.G. and Thompson,W.F. (1980) *Nucleic Acids Res.*, **8**, 4321–4325.
Nespoulous,C., Huet,J.C. and Pernollet,J.C. (1992) *Planta*, **186**, 551–557.
Pernollet,J.C., Sallantin,M., Sallé-Tourne,M. and Huet,J.C. (1993) *Physiol. Mol. Plant Pathol.*, **42**, 53–67.
Punt,P.J., Oliver,R.P., Dingemanse,M.A., Pouwels,P.H. and van den Hondel,C.A.M.J.J. (1987) *Gene*, **56**, 117–124.
Rao,V.B. (1994) *Anal. Biochem.*, **216**, 1–14.
Ricci,P., Bonnet,P., Huet,J.C., Sallantin,M., Beauvais-Cante,F., Bruneteau,M., Billard,V., Michel,G. and Pernollet,J.C. (1989) *Eur. J. Biochem.*, **183**, 555–563.
Riddle,O.C. and Briggs,F.N. (1950) *Hilgardia*, **20**, 19–27.
Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Sanger,F., Nicklen,S. and Coulson,A.R. (1977) *Proc. Natl Acad. Sci. USA*, **74**, 5463–5467
Scheel,D. (1990) *Z. Naturforsch.*, **45c**, 569–575.
Shipton,W.A., Boyd,W.J.R. and Ali,S.M. (1974) *Rev. Plant Pathol.*, **53**, 839–861.
St. Leger,R.J., Staples,R.C. and Roberts,D.W. (1992) *Gene*, **120**, 119–124.
Stringer,M.A. and Timberlake,W.E. (1993) *Plant Cell*, **5**, 145–146.
Talbot,N.J., Ebbole,D.J. and Hamer,J.E. (1993) *Plant Cell*, **5**, 1575–1590.
Templeton,M.D., Rikkerink,E.H.A. and Beever,R.E. (1994) *Mol. Plant-Microbe Interact.*, **7**, 320–325.
Valent,B. and Chumley,F.G. (1991) *Annu. Rev. Phytopathol.*, **29**, 443–467.
Van den Ackerveken,G.F.J.M., van Kan,J.A.L. and de Wit,P.J.G.M. (1992) *Plant J.*, **2**, 359–366.
Van Kan,J.A.L., van den Ackerveken,G.F.J.M. and de Wit,P.J.G.M. (1991) *Mol. Plant-Microbe Interact.*, **4**, 52–59.
Wessels,J.G.H. (1992) *Mycol. Res.*, **9**, 609–620.
Wessels,J.G.H., de Vries,O.M.H., Ásgeirsdóttir,S.A. and Schuren,F.H.J. (1991) *Plant Cell*, **3**, 793–799
Wevelsiep,L., Kogel,K.H. and Knogge,W. (1991) *Physiol. Mol. Plant Pathol.*, **39**, 471–482
Wevelsiep,L., Rüpping,E. and Knogge,W. (1993) *Plant Physiol.*, **101**, 297–301.

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