Solution Structure of the Plant Disease Resistance-triggering Protein NIP1 from the Fungus *Rhynchosporium secalis* Shows a Novel β-Sheet Fold*

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Activation of the disease resistance response in a host plant frequently requires the interaction of a plant resistance gene product with a corresponding, pathogen-derived signal encoded by an avirulence gene. The products of resistance genes from diverse plant species show remarkable structural similarity. However, due to the general paucity of information on pathogen avirulence genes the recognition process remains in most cases poorly understood. NIP1, a small protein secreted by the fungal barley pathogen *Rhynchosporium secalis*, is one of only a few fungal avirulence proteins identified and characterized to date. The defense-activating activity of NIP1 is mediated by barley resistance gene *Rrs1*. In addition, a role of the protein in fungal virulence is suggested by its nonspecific toxicity in leaf tissues of host and non-host cereals as well as its resistance gene-independent stimulatory effect on the plant plasma membrane H⁺-ATPase. Four naturally occurring NIP1 isoforms are characterized by single amino acid alterations that affect the different activities in a similar way. As a step toward unraveling the signal perception/transduction mechanism, the solution structure of NIP1 was determined. The protein structure is characterized by a novel fold. It consists of two parts containing β-sheets of two and three anti-parallel strands, respectively. Five intramolecular disulfide bonds, comprising a novel disulfide bond pattern, stabilize these parts and their position with respect to each other. A comparative analysis of the protein structure with the properties of the NIP1 isoforms suggests two loop regions to be crucial for the resistance-triggering activity of NIP1.

Plant disease resistance frequently results from the specific interaction of disease resistance (*R*) genes with corresponding pathogen avirulence (*Avr*) genes. When an *R* gene product recognizes the matching microbial *Avr* gene, plant defense reactions are activated to arrest further pathogen development. In recent years, many *R* genes have been characterized from various plant species, their products forming five classes of proteins with common structural motifs such as leucine-rich repeat domains, nucleotide-binding sites, leucine-zipper domains or domains similar to the cytoplasmic Toll/interleukin-1 receptor (1). Furthermore, studies on the signal transduction components that play a role in plant disease resistance have uncovered remarkable similarities with innate immunity pathways in insects and mammals (2).

Originating from very diverse pathogens the products of *Avr* genes, with the exception of the *Xanthomonas AvrBs3* gene family, lack such structural similarities. However, the increasing number of *Avr* proteins for which an impact on virulence has been demonstrated (3) suggests a common genuine function. In the broader sense, these proteins are produced by pathogens to allow or optimize development on the host. Their secondary role as specific signals in plant resistance triggering thus appears to be a function of the recognition capability of the plant. This is best described by the “guard hypothesis,” which assumes that *Avr* proteins target host proteins to modulate critical plant activities. These host targets are “guarded” by *R* proteins, which initiate defense reactions when their “guardees” are disturbed by *Avr* proteins (1, 4). Currently, the tomato Pto kinase (4) and the *RIN4* protein of *Arabidopsis thaliana* (5) best exemplify this scenario. According to the model, Pto is part of a general host defense-signaling pathway. The *R* protein *Prf* recognizes the interaction of Pto with bacterial *AvrPto*, which suppresses this pathway. *RIN4* is a negative regulator of basal defense responses, an activity that is enhanced upon interaction with bacterial *AvrR* or *AvrRpm1*. *RIN4* integrity is guarded by the *A. thaliana* *RPM1* protein, which hence “recognizes” two different *Avr* proteins targeting the same plant factor.

More than 40 *Avr* genes from bacterial, but only very few from fungal, plant pathogens have been characterized. Most bacterial *Avr* proteins that are transferred into host cells via the bacterial type III secretion system (6) and the product of the *Avr-Pita* from the rice pathogen *Magnaporthe grisea* (7) have intracellular targets. In contrast, the *Avr* proteins from the tomato pathogen *Cladosporium fulvum* and from the barley pathogen *Rhynchosporium secalis* function extracellularly as...
elicitors of plant defense reactions (8). High affinity binding sites for two of the AVR proteins, AVR9 from C. fulvum (9) and NIP1 from R. secalis, have been identified, although on membranes of both resistant and susceptible host plants as well as of related non-host species. In addition, the tomato Cf-9 protein that recognizes AVR9 without direct physical interaction (10) was recently found to be part of a membrane-associated protein complex (11). Nevertheless, the mechanism of AVR protein perception and signal transduction into the host cell is still not fully understood.

The NIP1 gene of R. secalis encodes an 82-amino acid protein, which upon cleavage of a signal peptide yields a 60-amino acid mature protein that contains 10 cysteine residues (12) in five intramolecular disulfide bonds (13). Application of NIP1 to leaves of barley lines carrying the R gene, Rts 1, but not of lines lacking this gene, results in the induction of defense reactions (14). In addition, NIP1 has a virulence-associated function; leaves of barley lines carrying the R gene of R. secalis have been characterized that either lack the gene, but not of lines carrying the gene. In contrast, virulent races either lack the gene or carry alleles containing point mutations that translate into resistant host plants as well as of necrotic lesions, a process that appears to be independent of the plant genotype it stimulates the formation of (14).

Following the protein isolation protocol described previously (13), 7.2 mg of native NIP1, type I (80 kDa), was dissolved in 10 mM 2-[morpholinoo]ethane sulfonic acid (pH 7.5; 100 mM NaCl, 5 mM TCEP, 0.5 mM PFP) and a second fraction with a slightly lower molecular weight (5734.6 Da) and bovine cytochrome c (12290.9 Da) were collected. NMR Experiments—The NMR experiments were performed on Bruker Avance DMX 500 and Varian 600 spectrometers equipped with a pulsed-field gradient unit and triple resonance probe. The sample concentration was about 0.1 M and the sample preparation was performed at 25 °C and pH 6.0 (pH meter reading). Three-dimensional 15N-edited NOESY spectra were recorded with mixing times of 80 ms and 150 ms, 15N-edited TOCSY (30 ms mixing time), 1H HMBC-NOESY-HSQC (150 ms mixing time), two-dimensional 1H-1H HSQC, 1H COSY (H2O and D2O), 1H NOESY (H2O and D2O, 80 and 150 ms mixing time), and 1H TOCSY (H2O and D2O, 25 ms mixing time) spectra were used for the assignments. The spectra were prepared using the NMRPipe program (22) on Silicon Graphics workstations. The data were interpreted using the program XEASY (23). Resonances were assigned following the standard strategy for the assignment of NMR spectra to tetratricopeptide repeat proteins. Coupling constants were determined in three-dimensional HNHA (26), two-dimensional HMBC-J (27), and three two-dimensional MJ-HMQC (28) spectra. Two-dimensional NOE and TOCSY spectra were analyzed as in the HMBC-J and the MJ-HMQC spectra the coupling constants were determined from the in-phase doublets in the 1H dimension using the fitting procedure INFIT (29).

Spectra were acquired on a 500 MHz Varian Inova spectrometer. The combination of NMR experiments using NOE and TOCSY experiments with high spectral resolution were recorded with (NOE) and without (NOE) chemical exchange. The relaxation times were determined using gradient enhanced sensitivity pulse sequences (30). Spectra were recorded on a 500 MHz Varian Inova spectrometer. Two pairs of NOE experiments were recorded with (NOE) and without (NOE) the use of 1H saturation applied before the start of the experiment, respectively. Series of R1, with time delays of 15, 45, 105, 200, 400, 600, 800, 1000, 1300, 2000 ms, and R2, with time delays of 8.2, 42.6, 4, 15, 57.5, 73.9, 90.3, 121.3, 172.4, 238, 320 ms, experiments were collected. NOE spectra were acquired using 2048 × 170 complex points. The R1 and R2 spectra were obtained using 2048 × 500 complex points. The relaxation parameters were determined from the peak heights. Data were analyzed using the Modelfree software (31) and reduced spectral density mapping (30).

Slowly exchanging amide protons were determined from the NH resonances from the fingerprint region cross peaks present in a TOCSY, COSY, and NOESY, recorded at 298 K of a NIP1 sample that was fully protonated and lyophilized. The spectra were recorded after dissolving the sample in D2O within the first 12 h of exchange. These slow exchanging amides are thought to arise from strong hydrogen bonds within the structure.

Structure Calculation—Quantitative distance constraints were obtained from the three-dimensional 15N NOESY HSQC (80 ms mixing time), 15N NOESY-HSQC (80 ms mixing time), and 1H NOESY-HSQC (80 ms mixing time) spectra. Upper limits were calibrated using the program DYANA (32). Since DYANA only calibrates upper limits, lower limits were introduced at a later stage. Interstrand dHN and sequential dNOE and ddeo distances (taken from the three-dimensional structures) were used for the calibration of the NOESY-HSQC. The two-dimensional NOE (D2O, 80 ms mixing time) spectra were used for the calibration of the 15N NOESY-HSQC, 1H NOESY-HSQC, and NOESY spectrum, respectively. Lower limits were set by shortening the distances r by 20%. Stereo-specific assignments and angle restraints for NIP1 were obtained from a quantitative analysis of the various J-coupling spectra. Structures were calculated using 34 φ and 11 ψ angle
The energetic and geometric statistics of the best 25 structures are presented in Table II. All structures are in good agreement with the experimental restraints. No structures showed consistent (>60% of all structures) NOE structure violations larger than 0.2 Å or dihedral angle restraints larger than 3°. Ramachandran analysis of the ensemble showed that 97% of the residues lie in the allowed regions. From the relaxation measurements, R1 and R2 rates and NOE values were determined and subsequently used to calculate spectral densities, employing the reduced spectral density mapping method (30). Fig. 5 presents the spectral densities, which were calculated at zero, 0.02, and 0.04 (50.7 and 500 MHz, respectively). From a comparison of the χ² values of the “isotopic” and “anisotropic” Modelfree analyses followed that the dynamics of NIP1 can be described with an isotropic model. The overall rotational correlation time, derived from the mean value of the R2/R1 ratio was determined to be 4.8 ns. The reduced spectral density mapping indicates that the dynamic properties are quite similar for most residues in the protein (Fig. 5). The behavior of residues with spectral density values strongly different from the average can be explained by increased internal flexibility or conformational exchange (see below).

**DISCUSSION**

The NIP1 Structure—NIP1 has a well defined structure predominantly formed by β-strands (Figs. 4, A and B). It consists of two parts that have a well defined mutual orientation. The five intramolecular disulfide bonds play a major role in the NIP1 fold, providing a high level of stability. This was illustrated by circular dichroism measurements, which showed that most of the secondary structure elements are preserved even at 100 °C. The disulfide bond pattern determined with the “partial reduction procedure” was confirmed by the NMR measurements.

A closer look at the NIP1 structure reveals that if the N-terminal two residues and β2 were discarded, the structure can be considered as a tandem repeat of similar structural parts. Each part contains four cysteines in structurally equivalent positions (Cys⁶, Cys⁸, Cys¹⁹, and Cys²⁶ in the first, and Cys³², Cys³³, Cys⁴², and Cys⁵³ in the second part). The first and fourth cysteines of each domain form a disulfide bond, whereas the second cysteine of the first (Cys⁷) and the third cysteine of the second part (Cys⁴²) form a disulfide bond between the parts. The remaining two cysteines (Cys¹⁸ and Cys³⁸) form disulfides with the cysteines at the N and C termini (Cys⁵ and Cys⁶⁰, respectively), and it can readily be imagined how longer structures can be formed as tandem repeats of these structural motifs. However, no higher repeat number proteins were found in the database.

With the exception of the first two amino acids at the N terminus, loop I and in particular loop III, the structure of NIP1 is rather rigid (Fig. 4, A and B). This is mainly a consequence of the presence of the five disulfide bonds. Starting at the N terminus, the first β-sheet is formed by residues 4–8 (β1) and 16–20 (β2). By forming a covalent linkage between the flexible N-terminal part of the protein and β2, thereby crossing the first β-sheet, the disulfide bond between Cys⁶ and Cys¹⁹ stabilizes this β-sheet. However, the amide protons in this β-sheet exchange faster than those in other parts of the protein (data not shown) indicating that conformational exchange oc-

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3 V. Li and W. Knogge, unpublished results.
4 A. Murzin, personal communication.
curs. This is supported by the observation that residues 5 and 6 in the center of β1, and residue 16 in β2, have much higher \( J(0) \) values than their neighboring residues, while their \( J(\omega_{\alpha}) \) and \( <J(\omega_{\alpha})> \) values are comparable (Fig. 5). The flexible loop between β1 and β2 (loop I, residues 9–15) is less well defined (Fig. 4, A and B). In addition, residue 13 experiences conformational exchange (Fig. 5).

Loop II, between β2 and β3, connects the two parts of the molecule. The first β-sheet ends in a type I β-turn (residues 21–24). Examination of the \( \phi \) and \( \psi \) angles shows that the orientation of residues 24–28 in loop II are well defined and can be recognized as β-strand. However, no hydrogen bond partners are found for these amino acids. The exception is residue 27, the oxygen of which is hydrogen-bonded to the NH of residue 9, thereby forming an isolated β-bridge, which restricts the number of possible conformations. In addition, the cystine formed by residues 8 and 26 makes this part of the loop even more stable. Residue 9 is also the cystine partner of residue 42, providing an additional connection between the two structural motifs. The stabilized mutual orientation of the two structural motifs is further indicated by the presence of multiple NOE between the motifs. Especially, the aromatic residue, Phe51, has many interactions with residues in both parts of the molecule.

The β-sheet in the second structural motif consists of three anti-parallel β-strands: β3(30–34), β4(37–41), and β5(58–59). The sheet is well defined (Fig. 4, A and B) and ends at the C terminus of the protein, where the cysteine bridge between residues 33 and 60 provide additional stability. β3 and β4 are connected by a β-turn (residues 34–37), while β4 and β5 are connected by loop III. The conformational freedom in this loop is restricted by two interactions, \( i.e. \) by the cystine formed by residues 32 and 53, and by the hydrogen bond between residues 44 and 51, forming another isolated β-bridge. The relaxation data indicate that the sequence comprising residues 45–51 is rather flexible. The relatively high \( <J(\omega_{\alpha})> \) values indicate high frequency motions for these residues. However, the \( J(0) \) values of these residues are also higher, indicating the occurrence of more complex dynamics. Loop III ends in a type II β-turn (residue 54–57).

The dynamic variation in different parts of the molecule is corroborated by the biochemical characterization of the disulfide bonds. The accessible surface, as determined by MOLMOL (35) (Table III), is large for the two sulfur atoms involved in the bridge between Cysx and Cysy. This bond could be reduced with significantly more ease than the other bonds. On the other hand, the sulfur atoms of Cysz and Cysz, which are completely buried in the interior of the protein, are inaccessible to

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**Table I**

| Reduction of disulfide pair | N terminus to 1st Cys | itz-1st Cys to 2nd Cys | itz-2nd Cys to C terminus | β-Elimination N terminus | β-Elimination C terminus |
|-----------------------------|-----------------------|------------------------|--------------------------|-------------------------|-------------------------|
| Cys\(^2\)-Cys\(^{59}\)      | 289.3                 | 1699.0                 | 4537.1                   | 1911.3                  | 6158.0                  |
| Cys\(^2\)-Cys\(^{36}\)      | 925.1                 | 1848.1                 | 3752.2                   | 2696.1                  | 5522.3                  |
| Cys\(^9\)-Cys\(^{42}\)      | 1027.2                | 3444.9                 | 2052.2                   | 4396.1                  | 5420.6                  |
| Cys\(^{32}\)-Cys\(^{53}\)   | 3310.8                | 2261.5                 | 957.0                    | 5495.3                  | 3136.6                  |
| Cys\(^{32}\)-Cys\(^{53}\)   | 3412.9                | 2964.3                 | 148.1                    | 6300.2                  | 3034.4                  |

**Fig. 2.** The 500 MHz \(^1\)H,\(^{15}\)N HSQC spectrum of 2 mM NIP1 at 25 °C, 500 MHz. All expected cross-peaks from 57 backbone amide protons and from 8 side chains, \( i.e. \) 2 Asn, 1 Gln, and 5 Arg residues, could be assigned using two-dimensional and \(^{15}\)N-edited three-dimensional NMR spectra. They are marked by the corresponding residue number. Folded cross-peaks are marked (*).

**Fig. 3.** Histogram showing the number of NOE-derived distance constraints per residue. The bars represent intraresidue (black), sequential (dark gray), medium range (light gray), and long range (white) NOEs restraints. From the 740 NOEs, 232 are intraresidual, 241 sequential, and 54 medium range (between 2 and 5 residues apart in the sequence). Furthermore, 213 long range NOEs (5 or more residues apart) were identified.
TCEP, and no four-disulfide polypeptide species could be detected in which the disulfide bond between Cys32 and Cys53 was reduced. Even the presence of 6 M GuCl does not facilitate the TCEP reduction of this disulfide bond. Apparently, the presence of the Cys32-Cys53 bridge, which is positioned between two strands of the anti-parallel \( /H9252\) sheet, strongly stabilizes the tertiary structure of this part of the protein. Also the Cys33-Cys60 is hardly reduced by TCEP, although Cys60 has quite an extensive solvent accessible surface. In conclusion, the derived tertiary structure of NIP1 provides a satisfactory explanation for the observed reduction of the individual disulfide bonds.

The NIP1 Elicitor Has a Novel Protein Fold

The disulfide pattern found in NIP1 has not been observed before in other proteins (motif.genome.ad.jp). In addition, to our knowledge the cysteine bonding pattern found in NIP1 has not been observed before in other proteins. The structure of NIP1 shows no homology to structures in the protein data bank, as was verified using the DALI server (www2.ebi.ac.uk/dali/) using a Z-score value of 2 as cut-off, nor with other elicitor proteins. For instance, the global fold of the AVR9 elicitor from the fungal tomato pathogen \( C. fulvum \) has been derived (36). Although the tertiary structure of the protein has not yet been completely elucidated, the presence of a cystine knot motif could be established. Furthermore, the preliminary three-dimensional structure of AVR9 shows homology to that of the carboxypeptidase inhibitor, whose structure was determined by x-ray diffraction (36, 37). The cystine knot motif was not found in NIP1. The only other structure currently available from a microbial elic-
itor protein, that of the elicitin cryptogein (38) from *Phytophthora cryptogae*, consists mainly of α-helical elements. Other small cysteine-rich proteins, which consist of two apparent domains, such as anti-stasin (Protein Data Bank accession code 1SKZ), display dissimilar folds. Therefore, the NIP1 structure can be regarded as a novel protein fold and it may be the first representative of an evolutionary superfamily.

**Structure-Function Relationship—**Association of NIP1 to a binding site on *Rrs1*-barley membranes is regarded as the initiating event in a signal transduction pathway that ultimately leads to resistance. The mechanism by which this and other elicitors trigger the plant defense response remains to be fully understood. One way to address the problem is by considering the elicitor activity of NIP1 isoforms. In fungal isolates, two types of NIP1 have been identified, in which the naturally occurring amino acid alterations S23P (type III) and G45R (type IV), respectively, yield proteins that show neither elicitor activity nor toxic activity (12, 17). The solution NMR studies of NIP1 reported here are an important step toward an understanding of the relationships between structure and the observed biological effects.

The amino acid alteration S23P occurs at the third residue in a type I β-turn. It is well known that this type of β-turn is compatible with any amino acid residue at its four positions with one exception; a Pro residue at the third position is incompatible with type I β-turns (39). Therefore, the introduction of a Pro at this position most probably results in the disruption of the protein structure causing the loss of its activity.

The mutation G45R is positioned in the part of loop III that exhibits complex internal dynamics (see above). Changing a single surface residue, in this case glycine into arginine, may have important consequences for the local stability of the protein. This has been elegantly demonstrated for the cold shock protein from the mesophile *Bacillus subtilis*, where the introduction of one arginine at the protein surface significantly increased its stability (40). Not only the size difference, but also the introduction of a positive charge in a region, which consists of two negatively charged glutamine residues, is likely to have dramatic consequences (Fig. 6). In particular, the presence of the arginine at position 45 may reduce the flexibility of loop III and thus affect the protein function.

Recently, a high affinity binding site for NIP1 was identified on barley plasma membranes. Interestingly, when the S29P or G45R alterations were introduced into the protein both resulting mutant isoforms (type III* and type IV*), although inactive, were efficient competitors for the binding site. This indicates that the regions where the mutations had been introduced are not involved in receptor binding. However, both mutations prevent signaling to occur. It is therefore tempting to speculate that NIP1 interacts with the primary receptor through a region in the structure opposite to residues 23 and 45. Binding may induce a conformational change that allows signaling to occur through the region around amino acid residues 23 and 45. Alternatively, the latter region may attract another plant component, possibly the *Rrs1* gene product, into the signaling complex. This would be in agreement with the guard hypothesis for *R* gene function (1), which proposes that *R* proteins recognize the intended manipulation of a plant target by a pathogen avirulence protein. In the present case, the NIP1 receptor would be the target of the virulence function of NIP1 and hence the guardee.

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