Characterization of SNP1, a Cell Wall-Degrading Trypsin, Produced During Infection by *Stagonospora nodorum*

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Accepted 25 January 2000.

Stagonospora (= *Septoria*) *nodorum* when grown in liquid culture with wheat cell walls as the sole carbon and nitrogen source secretes numerous extracellular depolymerases, including a rapidly produced, alkaline, trypsin-like protease (SNP1). The enzyme was purified 417-fold by cation exchange chromatography and has a molecular mass of 25 kDa on sodium dodecyl sulfate gels, pI 8.7, and pH optimum of 8.5. It cleaved peptide bonds on the carboxyl side of lysine or arginine, was strongly inhibited by the trypsin inhibitors aprotinin and leupeptin and weakly by phenylmethylsulfonyl fluoride, and its activity was stimulated by calcium. SNP1 has the characteristic, conserved, fungal, trypsin N terminus. Polymerase chain reaction (PCR) primers based on this sequence and the conserved trypsin active site were used to amplify a DNA fragment that facilitated isolation of the corresponding genomic clone from a lambda library of *S. nodorum*. The full-length sequence confirmed its identity as a trypsin-like protease containing the N-terminal sequence of the previously purified enzyme. Infected leaf tissue contained a protease, not present in controls, that coeluted with the fungal trypsin from cation exchange, and had properties (pI and inhibitor characteristics) similar to those of the fungal trypsin. SNP1 expression in planta was detected by Northern (RNA) blotting, reverse transcription PCR, and green fluorescent protein confocal microscopy. SNP1 released hydroxyproline from wheat cell walls. The release of hydroxyproline, together with its early expression in planta, suggests that SNP1 participates in the degradation of host cell walls during infection.

Additional keywords: cell wall proteins, plant pathogen, virulence.

Stagonospora nodorum causes a leaf and glume blotch of wheat (Weber 1922). Despite the widespread and economic impact of this necrotrophic pathogen (King et al. 1983), very little is known about how it causes disease. Extensive epi-

phytic growth precedes penetration and then subcuticular, lateral growth occurs within the epidermal wall. Subsequent invasion of the mesophyll involves sparse intercellular growth with occasional cell wall penetration and collapse of surrounding cells (Karjalainen and Lounatmaa 1986; Bird and Ride 1981; Zinkernagel et al. 1988). Regions beneath penetration sites are more diffuse than the normal wall (Karjalainen and Lounatmaa 1986). Overall, this evidence suggests a possible role for cell wall-degrading enzymes in penetration and colonization.

A direct involvement for cell wall-degrading enzymes in infection by *S. nodorum* has not been established but their production both in vitro (Lehtinen 1993; Magro 1984) and in vivo (Magro 1984) has been detected. Both of these studies focused largely on polysaccharide-degrading enzymes, the major activities of which reflected the composition of the wheat cell wall, in which arabinoxylan is a key matrix component (Carpita and Gibeault 1993). Given that the cell wall-degrading enzymes produced by plant pathogens are adapted to their host cell walls (Cooper et al. 1988), it is surprising that one class of enzymes often overlooked are proteases. In some plant species proteins can account for up to 15% of the cell wall, where they serve both structural and enzymatic functions (Showalter 1993). There are five major classes of cell wall proteins mostly characterized from advanced dicots. These are extensins (hydroxyproline-rich glycoproteins, HRGPs), glycine-rich proteins (GRPs), proline-rich proteins (PRPs), arabino-galactan proteins (AGPs), and solanaceous lectins. Some have been found in different forms in graminaceous monocots such as threonine-hydroxyproline-rich glycoproteins (THRGPs) and a histidine-rich form (HHRGPs) (Kieliszewski et al. 1989; Showalter 1993). HRGPs can be produced at elevated levels during pathogenesis (Clarke et al. 1981; Mazau and Esquerré-Tugayé 1986). If invading fungi can degrade these proteins, proteases may play an important role as virulence factors. Proteases may also counter host defense-related proteins, such as the antifungal enzymes chitinases and glucanases (Mauch et al. 1988). Proteases can be involved in processing fungal toxins (Howard and Buckley 1985), elicitors (van den Ackerveken et al. 1993), and zymogens (Rypniewski et al. 1994). Alternatively they may be toxic per se by their action on host cell walls (Movahedi and Heale 1990b) or on the host plasma membrane (Tseng and

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Nucleotide and/or amino acid sequence data for *Snpl* can be found at the GenBank data base as accession no. AF092435.

Mount 1974), or they may disrupt cortical microtubules by degrading a linked transmembrane-extensin system (Akashi and Shibaoka 1991). Proteases may also serve a nutritional role, e.g., in the interaction between the endophyte *Acremonium typhinum* and its host grass (Lindstrom and Belanger 1994). The production of protease inhibitors by many plants, including monocots and the demonstration that they can affect fungal pathogens (Joshi et al. 1998; Koiwa et al. 1997; Lorito et al. 1994) suggest that there is a need for plants to defend themselves against pathogen proteases. Moreover, the induction of a protease inhibitor during the incompatible interaction between *S. nodorum* and barley (Stevens et al. 1996) strengthens this hypothesis.

Proteases are produced by many plant-pathogenic fungi and bacteria, including the biotrophs *Uromyces viciae-fabae* (Raucher et al. 1995) and *Ustilago maydis* (Hellmich and Schauz 1988), the hemibiotrophic *Colletotrichum lindemuthianum* (Ries and Albersheim 1973), and necrotrophs such as *Botrytis cinerea* (Movahedi and Heale 1990a). Attempts to establish involvement of proteases in virulence have given conflicting results, such as positive associations for *Magnaporthe poae* infecting roots of Kentucky bluegrass, *Xanthomonas campestris* and turnip leaves, and *Pyrenopeziza brassicae* and oilseed rape (Sreedhar et al. 1999; Dow et al. 1990; Ball et al. 1991); in no case was the cellular function of the protease established. In contrast, protease-defective mutants of *Erwinia chrysanthemi*, *Cochliobolus heterostrophus*, *C. carbonum*, and *Cladosporium cucumerinum* were unaffected in pathogenicity (Dahler et al. 1990; Walton 1994; Murphy and Walton 1996; Robertson 1984).

Here we report the purification, characterization, and sequence of an alkaline, trypsin-like, extracellular protease produced by *S. nodorum* when grown on wheat cell walls, its detection in planta during infection, and its ability to degrade wheat cell walls. The putative roles of a trypsin-like protease, SNP1 (*S. nodorum* protease 1), as a cell wall-degrading enzyme and as a virulence factor are discussed.

RESULTS

When *S. nodorum* was grown in liquid culture on wheat cell walls a number of different extracellular depolymerases were produced. Major activities were xylanase and β 1-3 glucanase, both detectable 12 h after transfer onto cell walls and peaking at 24 and 72 h, respectively. High protease activity was detected with the general protease substrate azocasein at 12 h and maximum production was at 24 h. Preliminary studies in vivo also showed elevated levels of protease activity in infected plants, compared with controls, throughout (2 to 14 days) infection. The only other putative fungal enzyme activities clearly detected in planta were xylanase (confirmed as *S. nodorum* xylanase by Western blotting [immunoblotting]) and arabinosidase, but these activities were associated with later stages (>3 to 4 days) of lesion development (data not shown). Therefore, our initial research centered on *S. nodorum* protease(s).

Purification of SNP1.

Isoelectric-focusing gelatin overlays of crude culture fluids showed a single major protease activity with a pI of approximately 8.7. Purification was achieved by two steps of cation exchange chromatography. The first round of cation exchange

chromatography (SP Sepharose) was performed on fast protein liquid chromatography (FPLC). Figure 1A shows a typical profile; peak protease activity was subjected to high resolution cation exchange separation by perfusion chromatography. Peak activity in four fractions appeared as a single peak during both steps. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE; Fig. 2) monitored each stage and revealed that peak fractions from the second cation exchange chromatography step contained a major band of 25 kDa. The protein content and the activities of the protease at the various stages of the purification procedure are given in Table 1. A 417-fold purification was achieved.

Fig 1. A, Elution profile of SNP1 from an S-Sepharose cation exchange column in 25 mM 2-(N-morpholino)ethane sulfonic acid (MES) pH 6.0 eluted over a linear 0–200 mM NaCl gradient. Elution profiles of (B) infected and (C) control plant protein extracts eluted under the same conditions. Protein mg ml^{-1} (----); protease activity (—) is expressed either (A) as $\mu\text{kats ml}^{-1}$ after the standard 15-min assay or (B,C) as absorbance units following a 12-h assay. All assays were performed with the trypsin substrate Benz-Phe-Val-Arg-NA.

N-terminal sequence.

Peak fractions from the perfusion chromatography step were run on SDS-PAGE, blotted onto polyvinylidene difluoride (PVDF) membrane and stained as described by Packman (1993). N-terminal sequencing of the 25-kDa band (underlined sequence in Figure 3) revealed it to be a trypsin-like protease showing 89.5% identity with a trypsin protease from *Fusarium oxysporum* (Rypniewski et al. 1993) and 84.2% identity with ALP1 of *Cochliobolus carbonum* (Murphy and Walton 1996) over the 19 amino acids determined.

Temperature and pH optima.

SNP1 has a temperature optimum of 35°C but was most stable at 4°C after a 1-h incubation period. It has a broad pH optimum over the range 7 to 9 but activity rapidly declines above and below this. Consistent with this was maximum stability at pH 8 (data not shown).

Metal ions.

Protease activity was unaffected by the metal ions $MnCl_2$, $MgCl_2$, $NaCl$, $ZnCl_2$, over the range 1.25 to 10 mM or by $PbAc_2$ and $FeCl_3$ over the range 1.25 to 5 mM, but was inhibited by 85 and 60%, respectively, at 10 mM. Protease activity was stimulated approximately 22% by calcium ions, optimally at 2.5 mM (data not shown). This calcium re-

quirement is consistent with the enzyme being a trypsin-like protease.

Inhibitors.

The pure protease (assayed with Benz-Phe-Val-Arg-NA) was classified with the use of standard inhibitors (Fig. 4). The purified protease was strongly inhibited by aprotinin (99%) and leupeptin (99%) and weakly inhibited by phenylmethylsulfonyl fluoride (PMSF; 30%), characteristic of trypsin-like proteases (Gebhard et al. 1986; Powers and Harper 1986). Soybean trypsin inhibitor and turkey egg white inhibitor also inhibited the protease, with 98 and 90% inhibition, respectively. EDTA inhibited activity by 20%, consistent with the metal ion requirement of calcium; this also coincided with some loss of activity of protease after extensive dialysis. The cysteine and aspartic inhibitors iodoacetic acid and pepstatin were ineffective against the protease.

Substrate range and kinetics.

Pure SNP1 was assayed against a range of nitroanilide substrates to assess substrate specificity. It showed no activity against the chymotrypsin substrate Suc-Ala-Ala-Pro-Phe-NA or the subtilisin substrate CBZ-Gly-Gly-Leu-NA.

A kinetic analysis was performed with the pure protease against a range of trypsin nitroanilide substrates and the K_m and V_{max} calculated for each with the direct linear plot (Cornish-Bowden and Eisenthal 1974). The substrate range used was 0.125 to 2 mM with a fixed enzyme amount of 0.02 μg (Table 2).

SNP1 showed a preference for the substrates Benz-Phe-Val-Arg-NA and D-Val-Leu-Arg-NA. It was least active against Benz-Pro-Phe-Arg-NA but higher activity occurred when Arg was replaced by Lys in the substrate D-Val-Phe-Lys-NA. The ability to cleave at the carboxyl side of both Arg and Lys is indicative of a trypsin-like protease.

Effect of SNP1 on wheat cell walls.

SNP1 could degrade collagen (based on degradation of azocoll), a hydroxyproline-rich protein, suggesting that this protease may be able to degrade some plant cell wall structural proteins. Two different concentrations of pure SNP1 were used to digest 5 mg of wheat cell walls. With 0.4 μg of protein (corresponding to 1.6 μk ats) there was no significant degradation of wheat cell walls, compared with controls (Fig. 5). However, 2 μg of protein (8 μk ats) resulted in an approximately 3.5-fold increase in release of hydroxyproline-containing proteins from cell walls. This was equivalent to a total of 1.6 μg of hydroxyproline, which is approximately 40% of that present in the wall. The estimate of 0.8 μg of hydroxyproline per mg of cell wall agrees closely with that found in wheat cell walls by Clarke et al. (1981).

Fig. 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (12%) showing purification of the trypsin-like protease secreted by *Stagonospora nodorum*. M, molecular mass standards expressed in kDa; lane 1, culture filtrate after 48 h of growth on wheat cell walls; lane 2, proteins from fast protein liquid chromatography (FPLC) cation exchange chromatography fractions showing peak protease activity; lanes 3 and 4, 25-kDa protein from BioCad HS20 cation exchange chromatography showing peak protease activity.

Table 1. Purification summary for the isolation of an alkaline protease from the culture filtrate of *Stagonospora nodorum*^a

Purification step	Protein mg/ml	Volume ml	Total protein (mg)	Activity per μl (nkats)	Total activity (μk ats)	Specific activity per mg (μk ats)	Purification (n-fold)
Culture filtrate*	0.3	100	30	3	300	10	
Ammonium sulfate*	2.5	10	25	30	300	12	1.2
Cation exchange 1†	0.4	2.5	1	46.7	116.7	117	11.7
Cation exchange 2†	0.004	1.5	6 μg	16.67	25	4167	417

^a Protease activity was initially determined on azocasein* then on Benz-Phe-Val-Arg-NA. †

Addition of the trypsin inhibitor aprotinin at 1 μ m reduced levels of hydroxyproline release by pure SNP1 to that of controls. This clearly demonstrates that pure SNP1 alone can degrade the wheat cell wall.

Effect of SNP1 on tritordeum suspension cells.

SNP1 activities ranging from 35 nkats to 20 μ kats were assayed for their ability to kill tritordeum (fertile amphidiploid between *Hordeum chilense* and *Triticum durum*) suspension

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1  GTCGACGGGTGAATTTGCGGGACGGTGTTCGGTCTCCTGCACGCCTGATCGCGCGATGC
61  GATGCAAAGTGGCACCTGCAAAGAAGCAAAGGACCGTCTGAGGAGGCATCCGGACAAC
121 CCGACCATCTCGTTTGAATGAAACGCCAGCACGTGATAGACCACAGGACTTGCCTCCTCC
181 CTATTCCTGCCTCATTCAGTAGCTGTCCAATGCGAGCGGCTGCACTTGAAGTGTCCAAG
241 CCAGCCCGCCTGTGGTGTTCGAAAGTCCCGCCTCCACTGGGCATGATCGCCACCATGGC
301 CCCGGGGCTTGATTGACTACGTACGTGATGCCAAGCACCAATGTCTACGTGCAACTGC
361 CTTTCTGGGCGCTGACCCGCTCGCTAAGTCGCATCACGTACGTCTCCTACACCAACCAC
421 GCGCCGAGGCGTATCTTGTCTCCTGTTACTGCAATATAAGAAAAGCTTGGTATTCTCTTG
481 CCAAGAAGGCAGCGTGAAGATCATTGACCCACGTCCGATCGCCTACTCCGAGTATGGCA
541 GGTCTGCCTGTTTCATGATTTGTACCACGGTTTGCTCATGGACGTCAACCATGTTATCGC
601 TCTTGATCGACAGTGTTCTAGCATGATTATGCAGTCTACTATACGGCGTGGCCGCTTTCG
661 GCTGCTTTGCCCGCGAAACTTTGCAGATATGTCGGGAATGGCCGCGCTACATAGATCT
721 ACACAGGCCTTCTACACTGCACGGCGCCCGAAAGCAGATCTAACACGTCTGATTACACTC
781 GCGAGACTTCGCCACCAGGCCTCACAAGGCAGAAGACGCGGCGCGGAATTACTCGTGCG
841 TGCGTGAAAGAGGTCGGCAACGAAACAGTTCTGCACTACGCTTAGCATAGCGCCGAAAG
901 TCTCCTAATATCGGCCGTTGGCATTAAAGACTACACGGGAAGTAGACGATCCACTTCGCCA
961 ACTTCTCTTTGGCACTGTACAGGCTACCCTAGAGCGCGCTTAAGCGACGGCAGATAAG
1021 TACCACTTCATCGGAGGCAAGGCACAACATGGACCTACATGTCCCACTAAGCCAAAGAA
1081 GGTACTTGTAACCTTAGCAGCTCCAATCACTTCTCTGCCAGACGTGATTAGACCCTATC
1141 TGGAAGGACCTGATCGTCCCCTGGCCTAATGACCTTCGAGCATCTACGACGACAGGACAG
1201 GAAGGTTGATGGACATCAGATGAAGCCATACACCATGTAGCACGCAACTGAAGTACAAAT
1261 ATCAGGGCCTCAACCCTCTCTTTGCAAACAGTCCCTCACTACAACCTCTCGCTACAGACA
1321 CCTTCCAGCCAGCCAGCTTCAGACCTACCCTCAGCCTCATTCAGATGCACCTTCACAGAC
                                     M H F T D      5
1381 GTCCTCATTGCCCTCGCGGCTCCCGCTCTCATCAGCGCTGCGGCTATCCCCAGGACCCT
V L I A L A A P A L I S A A A* I P Q D P      25
1441 GAGTTCCCTGAGGGCGAGTTCCCGGAGGACCCCATCGTTGGCGGAACCACCGCCAGCGCT
E F P E G E F P E D P I V G G T T A S A      45
1501 GCGGACTTCCCCTTCATCGTCTCCATCCAGCAGGGCGGAAGCCACTTCTGCGGTGGTTTC
G D F P F I V S I Q Q G G S H F C G G S      65
1561 CTGCTGAACGCCAACACCGTCTGACCGCGCTCACTGCGCTGTGCGACAGACTGCTTCC
L L N A N T V L T A A H C A V G Q T A S      85
1621 AGCCTTCAGATCCGCGCTGGCTCTCTCgtaagacatgccatatttcaccatctcatccaa
S L Q I R A G S L      94
1681 cttcccactaacaccccttcagAGCCGTACCTCCGGCGGAACCGTCGTCAAGGTGTCCT
S R T S G G T V V K V S S      107
1741 CCATCAAAGTCAACCCCTCGTACGCCGCCGGCACCTACAACAACGACGTTGCCATCATGA
I K V N P S Y A A G T Y N N D V A I M K      127
1801 AGCTCGCCACCTCCATCCCACCAGCTCCACCATCAGCTACGCCAAGCTCGCCTCTTCTG
L A T S I P T S S T I S Y A K L A S S G      147
1861 GCTCCGACCCTGCCGCTGGCACCGGCCTCACCGTCGCCGCTGGGGCACCAACAGCTCCG
S D P A A G T G L T V A G W G T T S S G      167
1921 GCGGCCAGTCCCTGCCGCTCAACCTCCTCAAGTCTGACGTGCGCGTCATCAGCCGCTCCA
G Q S L P V N L L K V D V P V I S R S T      187
1981 CTTGCAACTCCAACACGCGAGCGACGCATCACGACGGCCATGTTCTGCGCTGGCCTGT
C N S N Y G S G R I T T A M F C A G L S      207
2041 CCCAGGGTGGAAGGACTCGTGCCAGGTGACTCTGGCGGCCCATCATCAACACCTCTT
Q G G K D S C Q G D S G G P I I N T S S      227
2101 CGCGCGTGCAGGTTGGTGTGGTTTCTTGGGGTGAGGGTTGCGCGTCTGCGGGTGCGCCTG
R V Q V G V V S W G E G C A S A G A P G      247
2161 GTGTCTACTCCAACCTTGGGCCAGAGCAGCCTTGCGAGCTTTGTTGCTGCCAAGTGTAGA
V Y S N L G Q S S L A S F V A A N L      265

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Fig. 3. Sequence of *SNP1*. The N terminus amino acid sequence (IVGGTTASAGDFPFIVSIQ) obtained directly from the mature protein SNP1 is indicated by underlining. Active site sequence, conserved among other trypsin-like proteases, CQGDSGGP, used to design the second oligonucleotide primer for polymerase chain reaction (PCR) amplification is also indicated by underlining. Intron is indicated in lowercase letters; putative signal peptide cleavage site is indicated by *.

cells. None of the concentrations tested affected viability, compared with controls, after 48 h (data not shown). Therefore, no direct, phytotoxic effect could be assigned to *SNP1*.

Isolation and characterization of *SNP1*.

Degenerate primers based on the N-terminal amino acid sequence IVGGTTA and the active site conserved sequence CQGDSGGP were used to amplify a single 610-bp polymerase chain reaction (PCR) product from genomic *S. nodorum* DNA. The sequence of this fragment, which encodes the *SNP1* N terminus, was highly similar to that of trypsin-like proteases (data not shown). The PCR product contained an open reading frame interrupted by one putative intron of 56 bp. Southern blot analysis suggested that this was a single-copy gene (Fig. 6). ³²P-labeled PCR product was used to screen a lambda library of the pathogenic wheat isolate of *S. nodorum*, LAW95. Positive plaques were identified and purified, and DNA isolated from a single, strongly hybridizing clone. After restriction analysis and Southern hybridization with the labeled PCR product, the appropriate restriction fragments were subcloned into pUC18 and both strands se-

quenced. The full-length sequence is shown in Figure 3. *SNP1* encodes an open reading frame of 855 bp interrupted by a single, putative intron of 56 bp, indicated by lowercase letters. The intron was confirmed by sequencing the corresponding cDNA. The DNA sequence obtained from the genomic library of *S. nodorum* isolate LAW95 was identical to the sequence of the PCR product obtained from isolate BS171.

SNP1 is predicted to encode a protein of 265 amino acids (29.4 kDa) that appears to be a preprotrypsin with a leader sequence of 36 amino acids, subject to post-translational modification. A putative signal-peptide cleavage site was identified after Ala at amino acid 20, based on the (−3−1) rule according to von Heijne (1986). This indicates a signal peptide of 20 amino acids, which would leave a possible propeptide of 16 amino acids between this signal peptide cleavage site and the consensus trypsin N-terminal sequence. Further proteolytic cleavage of this propeptide would result in a ma-

Fig. 4. Effect of protease inhibitors on the *Stagonospora nodorum* trypsin-like protease. Enzyme was pre-incubated with each inhibitor for 30 min before assay with Benz-Phe-Val-Arg-NA. Protease activities are expressed as a percentage of activity obtained in the control. Final concentrations of inhibitors: aprotinin, 1 μ M; leupeptin, 1 mM; pepstatin, 10 μ M; iodoacetic acid, 100 μ M; phenylmethylsulfonyl fluoride (PMSF), 1 mM; ethylenediaminetetracetic acid (EDTA), 10 mM; soybean trypsin inhibitor (SBT), 100 μ g/ml; and turkey egg white inhibitor (TEW), 100 μ g ml^{−1}. Error bars represent the standard deviation about the mean of three replicates.

Table 2. Kinetic constants of substrates cleaved by *SNP1*

Substrate	K _m (mM)	V _{max} (mkat/mg)
Benz-Phe-Val-Arg-NA	0.25	7.3
D-Val-Leu-Arg-NA	0.3	7.5
Benz-Pro-Phe-Arg-NA	0.63	1.5
D-Val-Phe-Lys-NA	0.39	2.8

Fig. 5. Release of hydroxyproline-containing proteins from wheat cell walls by *SNP1*. Pure *SNP1* was incubated with 5 mg of cell walls for 8 h. Soluble digestion products were hydrolyzed before assay for hydroxyproline content.

Fig. 6. Southern blot demonstrating the 610-bp polymerase chain reaction product from *SNP1* hybridizes to a single band. Genomic DNA (10 μ g) was cut with *Bam*HI (B), *Eco*RI (E) and *Hind*III (H).

ture protein of 229 amino acids (25.4 kDa), which is in agreement with the size of *SNP1* estimated by SDS-PAGE. The sequence alignment of *SNP1* with other trypsins from filamentous fungi indicates that there is significant identity with *F. oxysporum* trypsin (64%), *C. carbonum* ALP1 (56%), and *Metarhizium anisopliae* TRY1 (55%) (Fig. 7). *SNP1* contains the catalytic triad common to all serine proteases, His-77, Asp-122, and Ser-218 (Neurath 1984).

Detection of protease in planta.

Protease activity was detected in both control and infected plant material throughout (2 to 14 days) infection (with Benz-Phe-Val-Arg-NA as substrate). Levels in extracts from infected plants were approximately twice that observed in controls. When extracts from day 14 were eluted from a S-cation exchange column, the plant trypsin-like activity eluted at around fraction 19 and was present in both control and infected plants (Fig. 1B,C). Another peak at around fraction 13, which was absent or minimal in control plants, coeluted with the fungal protease (Fig. 1A). A similar peak was also apparent at days 2 and 7 (data not shown). Isoelectric focusing and

subsequent gelatin overlay showed a single protease in these coeluting fractions with a pI of approximately 8.5, again consistent with the fungal trypsin with a pI of approximately 8.7 (data not shown). Activity in these fractions was also completely inhibited by the trypsin inhibitors aprotinin and leupeptin.

Expression of *SNP1* in planta.

Expression of *SNP1* could be detected by Northern (RNA) blot analysis in infected but not in healthy leaves 7 days after inoculation with the aggressive isolate LN97, but was not detectable with the less aggressive isolate BS171 (Fig. 8A). However, *SNP1* transcripts were detected from infected leaves 7 days after inoculation with either LN97 or BS171 by reverse transcription (RT)-PCR (Fig. 8B,C). As a positive control, expression of *S. nodorum* chitin synthase was also detected in infected leaves (Fig. 8D) whereas no amplification was detected in noninfected leaves or with wheat genomic DNA.

To investigate further *SNP1* expression in planta, isolate LAW95 was transformed with a vector carrying the *SNP1* promoter fused to a green fluorescent protein (GFP) reporter

			*	20	*	40	*	60		
snp1	:	MHFTDVLIALAA-PALISAAAI	QDPEFPEGEFPED	PIVGGTTASAGDEFFIVS	IQQGG	:	59			
foxy	:	-----MVKFASVVALVAPLAAA	PQEIPN-----	IVGGTSASAGDEFFIVS	ISRNGG	:	47			
hc	:	MRFQSMIT--AALPALVLSAPTP	QWDDVPE-----	DSIVGGTTAAAGEYPPFIVS	IQLGGR	:	53			
pr2	:	MELKSL LAVYLPLCAAPLAA	ARPASNAV-----	FIVGGSPAAAGEFFIVS	TLNGR	:	52			
			*	80	*	100	*	120		
snp1	:	HFCCGSSLINANTVLTAAHC	AVGQTASS-----	LQIRAGSLSRTSGCTVVKV	SSIKVNPSYA	:	115			
foxy	:	PWCCGSSLINANTVLTAAHC	VSGYAQSG-----	FQIRAGSLSRTSGGITSSL	SSVRVHPSYS	:	103			
hc	:	HNCGGTLINGNTVVTAAHC	SVSSAIGGSINNVAVRV	GSLSANS	GGQVIKVS	SKIIHPSYQ	:	113		
pr2	:	HWCCGVLLINANTVLTAAHC	VESTPAIS-----	QVRAGSLAHASGGV	VANISSITPHPKYE	:	107			
			*	140	*	160	*	180		
snp1	:	AGTYNNDVAIMKLATSIP	TSSTISYAKLASSGSD	PAAGTGLTVAGWGT	TSSGGQSLP	VNLI	:	175		
foxy	:	GN--NNDLAILKLSTSI	PSGGNIGYARLAASGSD	PVAGSSATVAGWG	ATSEGGSS	STP	VNLI	:	161	
hc	:	ASTSNNDIAIWKLSST	VTAGGNIGFASLAASGSD	PASGSTTSVAGWG	ATREGGG	ANN-AL	:	172		
pr2	:	G--LGYDMAILKLST	PIEANGTI--VRHIARGSD	PVGGADATVAGWG	DLEYACQA-PEEL	:	162			
			*	200	*	220	*	240		
snp1	:	LKVDVPVISRSTCNS	SNYGSGR-----	ITTAMFCAGLSQGGK	DS	COGDSGGPIINTSS	SRVQV	:	231	
foxy	:	LKVTVPIVSRATCRAQ	YGTSA-----	ITNQMFCA	GVSSGGKDS	COGDSGGPIVD-SS	N	TLI	:	216
hc	:	LKVSVPVARSTCVS	NYNAV--LTVITNMVC	AGVTAGGRD	SCOGDSGG	ELVD-ANK	TLI	:	229	
pr2	:	QKVTVPVVDRATCSA	AYQAI PNMPNITDAM	FCAGLKEGGQDAC	NGDSGGPIID	TETRVLI	:	222		
			*	260	*					
snp1	:	GVVSWGEGCASAGAP	GVYSNLGQSS	LASEVAANL	:	265				
foxy	:	GAVSWGNGCARPNY	SGVYASV--ALR	SFIDTYA	:	248				
hc	:	GVVSWGTCARP	NLPGVYSRVG--TLR	SFIDQNA	:	261				
pr2	:	GVVSWGKCAAPNAY	GVYTRLGADI--EF	IKSHL	:	254				

Fig. 7. Comparison of predicted amino acid sequences of *SNP1* and three related trypsin-like proteases with PILEUP (Devereux et al. 1984). Sequence references: *Fusarium oxysporum* (foxy), SwissProt P35049, Rypniewski et al. (1993); *Cochliobolus carbonum* (hc), ALP1, Murphy and Walton (1996); *Metarhizium anisopliae* (pr2) *try1* Smithson et al. (1995). Amino acid identity indicated by black background shading.

gene (Spelling et al. 1996). As a positive control, the same isolate was also transformed with a vector carrying GFP fused to the constitutive β -tubulin gene promoter (Cooley and Caten 1993). To confirm inducibility of the *SNP1* promoter, transformants were grown in complete medium, starved, and then transferred to basal salts plus 1% gelatin. pSNP1-GFP transformants expressed GFP when grown on gelatin, but not on complete medium. Transformants in which GFP was under the control of the β -tubulin promoter expressed GFP in both media (data not shown).

On wheat leaves inoculated with 5 μ l of spores transformed with the pSNP1-GFP construct, GFP fluorescence was apparent in hyphae after 24 h and was still detected 4 days post inoculation. Moreover, fluorescence was stronger in hyphae above or penetrating stomates (Fig. 9A,B). Fluorescence was uniform in hyphae of the pTUBA-GFP transformant expressing GFP constitutively (Fig. 9C).

DISCUSSION

SNP1 has been isolated and characterized from *S. nodorum* as a typical fungal trypsin-like protease. We have shown that

SNP1 is able to degrade native protein(s) in wheat cell walls, and that the mRNA is expressed during infection as shown by Northern blot analysis, RT-PCR, or GFP expression under control of the SNP1 promoter. This coincided with the production of a trypsin by *S. nodorum* in planta.

The substrate specificity of this enzyme is typically trypsin-like, specifically cleaving the peptide bond on the carboxyl side of lysine or arginine residues (Kraut 1977; Steitz and Shulman 1982). The exact Arg or Lys preference for this enzyme requires further study. Bovine trypsin shows a preference for lysine over arginine residues, while in *F. oxysporum* this specificity is reversed (Rypniewski et al. 1993). Alterations in the p2 and p3 subsites also alter activity; there was an 80% reduction in $V_{\max}$ between the Benz-Phe-Val-Arg-NA substrate and Benz-Pro-Phe-Arg-NA. A trypsin-like protease of the entomopathogenic fungus *M. anisopliae* showed a 92% reduction in $V_{\max}$ against the same substrates (St. Leger et al. 1996). When Phe is at the p2, Lys was preferentially cleaved over Arg by SNP1. The ability of SNP1 to cleave Lys residues may be of relevance in view of their abundance in the plant cell wall extensins (Showalter 1993).

Although frequently implicated in pathogenicity, the exact role of proteases in infection by plant pathogens has yet to be determined. If they are cell wall-degrading enzymes, then they would be expected to degrade structural wall proteins, which mainly consist of HRGPs. It has been suggested previously that protease(s) produced by *S. nodorum* may play a role in degradation of protein in the aleurone layer of seeds when causing glume blotch (Sieber 1989). SNP1 is highly active against the substrate azocoll and is produced in large amounts when grown on hydroxyproline-rich collagen in liquid culture. Also, pure SNP1 with no other associated enzyme activities released hydroxyproline, presumably from structural proteins in the wheat cell wall. There are few reported cases of proteases from plant pathogens able to degrade host cell wall proteins other than metalloprotease(s) from *Erwinia carotovora* subsp. *carotovora* against HRGPs from potato and tomato (Heilbronn et al. 1995; Willis et al. 1987) and a metalloprotease from *X. campestris* that degraded a PRP from *Brassica campestris* but had no activity on model protein substrates (Dow et al. 1998). Thus, cell wall degradation is a new function that might be assigned to proteases. However, Alp1a and Alp1b, trypsin-like proteases of *C. carbonum*, although produced on collagen, had no activity against maize extensins (Murphy and Walton 1996). Trypsins can cleave extensins of dicots and monocots but usually only after removal of the extensive glycosyl groups; thus, the one to four arabinosyl residues found on most hydroxyproline residues in dicots must first be removed with anhydrous hydrogen fluoride (Kieliszewski et al. 1989; Smith et al. 1986). Typically in monocots only less than half the hydroxyproline residues are glycosylated (Lampert and Miller 1971; Showalter 1993). Linkages in cereal cell walls vulnerable to SNP1 will presumably involve arginyl and lysyl bonds. Extensins and PRPs such as maize HRGP, which consists of 13 mol% Lys, are lysine rich. There are repeats containing Arg in GRPs and in maize PRP (Showalter 1993). Lys-Pro links are usually trypsin resistant but there is one that is trypsin vulnerable in a palindromic Lys-Pro-Lys repeat of a maize THRGP (Kieliszewski et al. 1989). Therefore, it will be our aim to determine the putative protein target(s) of SNP1 in the wheat cell wall.

Fig. 8. Detection of SNP1 RNAs from in vitro cultures and infected wheat leaves. Myc: 2-day-old *Stagonospora nodorum* isolate BS171 cultured in Czapek-Dox V8 liquid medium was starved for 8 h in basal salts prior to transfer to basal salts containing 1% (wt/vol) wheat cell walls for 16 h of growth; wheat leaves with no spores as a negative control (Con), or infected with 10^6 spores per ml of *S. nodorum* strain LN (LN) or BS171 strain (171). Leaves were harvested 7 days post inoculation. **A**, Northern (RNA) blot hybridized with SNP1. **B–D**, Agarose gel of reverse transcription-polymerase chain reaction products amplified with **(B)** SNP1 primers F1b-R11, **(C)** SNP1 primers F12-R15, or **(D)** chitin synthase primers CHSF-CHSR.

Unfortunately, there is little information on wheat cell wall proteins, although wheat, barley, and rice of the Festuciodeae subfamily share nucleotide sequences related to that of maize HRGP (Raz et al. 1991). Thus, there are potentially vulnerable peptide bonds in wall proteins from cereals, which becomes more relevant in light of their low glycosylation, compared with HRGPs of dicots. Pathogens of dicots could require arabinosidases to act synergistically with proteases to achieve extensin degradation but proteases may be able to degrade monocot extensins directly, as suggested by this work. Nevertheless, high levels of arabinosidases of *S. nodorum* are also rapidly produced in vitro and are present in vivo (data not shown). It is also interesting to note that a high pI xylanase initially co-purified with SNP1, and this is one of the most abundant enzymes produced by *S. nodorum* when grown on wheat cell walls. The importance of xylan in the wheat cell wall (>40% wt/wt) (Carpita and Gibeau 1993) and the demonstration that this pure xylanase can also degrade the wheat cell wall (data not shown) suggest that these two enzymes, with similar charge and regulation by host cell walls, may have a very destructive effect on the wall when secreted together in vivo.

Detection of enhanced putative pathogen depolymerases in infected host plants is complicated by the presence of related plant enzymes such as proteases, which may also be induced during plant-pathogen interactions (Lusso and Kuc 1995; D'Silva et al. 1998). However, primers used for the RT-PCR were specific to SNP1. Moreover, GFP transformants showed that the SNP1 promoter is activated in growing hyphae by 24 h after inoculation with *S. nodorum* spores. Therefore, the increased trypsin activity detected in infected plants is likely to

be of fungal origin since it shares properties with SNP1 (inhibitor sensitivity, M_r , pI, elution from S-column). Similarly, Movahedi and Heale (1990a) showed the coelution of an aspartic protease from infected carrot tissue with an aspartic protease from *Botrytis cinerea*. Also, a subtilisin-like protease was produced by *Magnaporthe poae* in infected roots of Kentucky bluegrass (Sreedhar et al. 1999). Nevertheless, the importance of proteases as virulence factors remains unclear despite reports on protease-deficient mutants of *X. campestris* and *P. brassicae* (Dow et al. 1990; Ball et al. 1991) that are less pathogenic than the wild-type strain. In many cases, gene mutation, disruption, or deletion of proteases did not affect pathogenicity, as reported for *Erwinia chrysanthemi*, *C. heterostrophus*, *C. cucumerinum*, and *C. carbonum* (Dahler et al. 1990; Walton 1994; Robertson 1984; Murphy and Walton 1996). In some of these cases, the lack of phenotype modification might be due to residual protease activity because of the presence of other isoforms, or result from the expression of proteases from other families. Moreover, virulence may be conferred by a set of cell wall-degrading enzymes acting in concert, such as xylanases, arabinosidases, and SNP1 of *S. nodorum*, rather than each component functioning independently.

SNP1 is a putative virulence factor in view of its activity on cell walls and its rapid induction in planta. Therefore, further studies are underway to determine the importance of SNP1 in disease by several approaches, which include degradation of wheat wall proteins (in situ and extracted) and targeted gene deletion. The unusual situation of a microbial depolymerase consisting of a single-copy gene and no other apparent isoforms should facilitate the latter strategy.

Fig. 9. Confocal microscopy of wheat leaves infected with *Stagonospora nodorum* transformed with a plasmid coding for green fluorescent protein (GFP) fused to (A,B) the SNP1 promoter (pSNP1GFP) or (C) with a plasmid coding for GFP fused to the constitutive promoter of *S. nodorum* tubulin (pTUBAGFP). Observations were made 1 or 4 days post inoculation. Excitation wavelength, 488 nm; detection, 510–520 nm (green channel) and 600–650 nm (red channel). Green fluorescence corresponds to GFP expression; red fluorescence comes from underlying chlorophyll. **A**, Three aligned stomata with more intense GFP green fluorescence in mycelium above stomata. **B**, A single stoma with associated hyphae showing stronger fluorescence than elsewhere on the surface. **C**, Two rows of four stomata but relatively uniform fluorescence in overlying hyphae. Position of stomata shown by arrows. Scale bars: **A**, 20 μ m; **B**, 100 μ m; **C**, 100 μ m. These images are representative of many observations from five replicate inoculations.

MATERIALS AND METHODS

Fungal isolate.

Septoria nodorum isolates BS171 (originally isolated from barley; Osbourn et al. 1986) and LN97 were obtained from AgrEvo (Saffron Walden, UK); a wheat isolate, LAW95, was from IACR, Long Ashton, Bristol (Hall et al. 1999). The fungus was grown on Czapek-Dox V8 agar (Newton and Caten 1988) under continuous UV at 20°C for 5 to 7 days to achieve sporulation.

Growth of *S. nodorum* in liquid culture.

For the production of protease the fungus was grown in shake culture. First, a fungal biomass was established in a complete liquid medium containing, per liter, 45.4 g of Czapek-Dox medium, 200 ml of filtered V8 juice, and 100 ml of Complete Supplement (Newton and Caten 1988). Spores (2×10^7) were inoculated into 250-ml Erlenmeyer flasks containing 100 ml of medium. The cultures were grown for 48 h at 24°C, 120 rpm on a rotary shaker (Sanyo Gallenkamp, Loughborough, UK), then were filtered through muslin, washed with 50 mM MES [2-(*N*-morpholino)ethanesulfonic acid] pH 6.0 and transferred for a 12-h period of starvation to a basal salts liquid medium that contained, per liter, 1 g of KH_2PO_4 , 0.5 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 9.76 g of MES (50 mM), and 10 ml of trace elements solution (containing, per liter, 1 g KH_2PO_4 , 0.5 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 20 mg of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 100 mg of ZnSO_4 , 2 mg of $\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$, 2 mg of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 2 mg of $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$). pH was adjusted to 6.0 with 1 M NaOH prior to autoclaving. After 12 h, cultures were strained through muslin and washed with 50 mM MES before transfer to inducing medium consisting of basal salts medium (as above) plus 1% (wt/vol) wheat cell walls.

Production of wheat cell walls.

Wheat cell walls were prepared from 14-day-old plants (cultivar Hornet) grown in trays at approximately 25°C under a 12-h photoperiod regime. Plants were chopped, then ground in liquid nitrogen. Ground plant material was then soaked in 2.5 volumes of ice-cold 0.1 M phosphate buffer, pH 6 before comminution in a Waring blender on high speed for 2 to 3 min. The resulting suspension was filtered through two layers of muslin and the insoluble plant material resuspended in 2 volumes of cold phosphate buffer and allowed to stand for 5 min. This was repeated to a total of four washes in buffer and finally one in cold, distilled water. Solvent extractions were performed with four washes of 1 volume chloroform:methanol (1:1, vol/vol) and three washes of 2 volumes of acetone. All extractions were made at 4°C. Cell wall material was left to dry overnight at room temperature then kept desiccated until required.

Enzyme assays.

Enzyme substrates were from Sigma (Poole, UK).

Trypsin-like protease activity was measured spectrophotometrically against the nitroanilide substrate, Benzoyl-Phe-Val-Arg-Nitroanilide. Reaction mixtures contained 50 µl of enzyme solution, 100 µl of 50 mM Tris-HCl pH 8, and 50 µl of 2 mM substrate made up in dimethyl sulfoxide (DMSO). Nitroanilide release was monitored at 405 nm over 15 min. General protease activity was measured against azocasein.

Reactions consisted of 100 µl of 3% azocasein in 10 mM Tris-HCl pH 8.0, 350 µl of 10 mM Tris-HCl, and 50 µl of enzyme sample or blank. Incubations at 37°C for 3 h were then stopped with 550 µl of 20% trichloroacetic acid (TCA), mixed, and clarified by centrifugation. Release of the red azo dye was monitored at A_{340} .

Substrate specificity.

Activity against other nitroanilide substrates was assayed under the same conditions with the substrates Suc-(Ala)₂-Pro-Phe-NA, CBZ-Gly-Gly-Leu-NA, Benz-Phe-Val-Arg-NA, Benz-Pro-Phe-Arg-NA, D-Val-Leu-Arg-NA, Benz-DL-Arg-NA, and D-Val-Phe-Lys-NA.

Protein assays.

Protein determinations were carried out by the dye binding method (Bradford 1976) in which Bio-Rad (Hemel Hempstead, UK) reagents were used with bovine serum albumin as standard.

Protease purification.

After 48 h of growth in inducing medium with 1% wheat cell walls, 500 ml of culture filtrate was harvested by filtration through two layers of muslin. Proteins were concentrated by precipitation with ammonium sulfate (80% saturation). The precipitate was resuspended in 50 ml of 25 mM MES pH 6.0 and dialyzed against several changes of MES buffer for 24 h. This retentate was then centrifuged at $14,000 \times g$ for 15 min and the supernatant filtered through a 0.2 µm nitrocellulose filter. Ten milliliters of this concentrated extract was applied to a 5-ml cation exchange column (High trap SP Sepharose; Amersham-Pharmacia Biotech, St. Albans, UK) run on a Pharmacia FPLC system and eluted over a 0–175 mM NaCl gradient in 25 mM MES pH 6.0 at 1 ml/min. Fractions with trypsin activity (versus Benz-Phe-Val-Arg-NA) were then buffer exchanged into 50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)-MES-Na Ac at pH 4.5 before injection onto a 0.8-ml-high resolution cation exchange column (HS20 Poros; Boehringer-Mannheim-Roche, Lewes, UK) run on a Biocad Sprint Perfusion Chromatography System (Perseptive Biosystems, Framington, MA) equilibrated in 50 mM HEPES-MES-NaAc at pH 4.5, and eluted in this same buffer over a 0–200 mM NaCl gradient. The molecular mass and purification of the protease were monitored with denaturing SDS-PAGE with 12% acrylamide (Laemmli 1970).

Isoelectric focusing.

Isoelectric focusing was performed with precast polyacrylamide gels and pI markers in the pH range 3 to 9.5 (Amersham-Pharmacia Biotech). Protease activity was detected in replica gels by overlaying gels with unprocessed, gelatin-coated X-ray film incubated previously for 10 min in water (Abraham and Breuil 1996). Protease activity was observed as a blue clearing zone on the film due to degradation of the gelatin layer after approximately 30 min at room temperature.

Temperature and pH optima.

Pure protease activity was assayed over a range of pH values with five overlapping buffers to cover pH 5.5 to 11. All were used at 50 mM: MES pH 5.5 to 6.5; 3-(*N*-morpholino)

propanesulfonic acid (MOPS) pH 6.5 to 7.5; Tris-HCl pH 7.5 to 8.5; Glycine-NaOH pH 8.5 to 9.0; 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS) pH 9.5 to 11. Temperature optimum was estimated by assaying at a range of temperatures with a 15-min incubation time.

Temperature and pH stability.

Pure protease was incubated for 1 h at 4°C over the pH range 5.5 to 11 with five overlapping buffers before assay. Temperature stability was determined by assay following a 1-h incubation over the temperature range 4 to 60°C.

Metal ion requirement.

Pure protease was extensively dialyzed against several changes of 25 mM MES pH 6 and then incubated with a range of metal ions for 30 min before assay. Metal ions were used in the concentration range 1.25 to 10 mM.

Inhibitor studies.

Pure protease was incubated with the following inhibitors for 30 min before assay against Ben-Phe-Val-Arg-NA (concentrations are final concentrations in reaction mixtures): aprotinin (1 µM), leupeptin (1 mM), PMSF (1 mM), EDTA (10 mM), iodoacetate (100 µM), pepstatin (1 µM), soybean trypsin inhibitor (100 µg ml⁻¹), turkey egg white inhibitor (100 µg ml⁻¹).

Effect of SNP1 on wheat cell walls.

Five milligrams of wheat cell wall was washed for 1 h with agitation in 50 mM Tris-HCl, pH 8. Washed walls were then centrifuged at 11,000 × g for 15 min and the buffer removed. This was replaced with 950 µl of fresh buffer and 50 µl of test or control sample prepared in similar buffer. Samples were incubated with agitation at 25°C for 8 h before centrifugation at 11,000 × g for 15 min. Supernatants containing degradation products were removed and transferred to Pyrex tubes with Teflon-lined lids. Proteins in the sample were then hydrolyzed in 6 M HCl (final concentration) for 20 h at 110°C to release free hydroxyproline. Samples were then lyophilized before assay for hydroxyproline by the methods of Kivirikko and Liesmaa (1959). Solutions used for assay were as follows: A = 180 µl of bromine dissolved in 50 ml of ice-cold 1.25 M NaOH; B = 16% Na₂SO₃; C = 5% *p*-dimethylaminobenzaldehyde in propan-1-ol; D = 6 M HCl. Samples were resuspended in 300 µl of dH₂O and cooled on ice. Three hundred microliters of ice-cold A was then added, shaken vigorously, and incubated on ice for 10 min. Fifteen microliters of B was then added, followed by 300 µl of C, and finally 150 µl of D. Samples were shaken between each addition. Samples were then incubated at 95°C for 2.5 min followed by 10 min at 20°C. Absorbance was read at 560 nm and compared with a standard curve of hydroxyproline over the range 0.1 to 2.0 µg. Total hydroxyproline levels in cell walls were calculated following hydrolysis of walls with 6 M HCl as above.

Inoculation of plant material.

For extractions of protease, 7-day-old wheat plants of cv. Hornet, which is moderately susceptible to *S. nodorum*, were sprayed to just before run-off with a suspension of 5 × 10⁵ spores ml⁻¹ prepared in 0.02% Tween 20. Plants were covered in polythene for 48 h to maintain a high humidity and grown

at 20°C with a 12-h light and 12-h dark cycle. Control plants were sprayed with 0.02% Tween 20 alone. Infected material was harvested at 2, 7, and 14 days after inoculation, corresponding to water-soaking, chlorosis and some necrosis, and necrotic tissue, respectively.

For extraction of RNA, inoculation of excised leaves gave more reproducible and severe symptoms. First leaves of 15-day-old wheat cv. Brigadier were excised and rubbed with a wet paper pad. The apical 10 cm was laid on water agar plates containing 150 µg ml⁻¹ benzimidazole. The adaxial surface was inoculated by brush with 10⁶ spores ml⁻¹ of *S. nodorum* in 0.02% Tween 20. One-half to 1 g of control or infected leaves was harvested after 7 days for extraction with Trizol reagent (GibcoBRL Life Technologies, Paisley, UK) (see below).

Extraction of protease from wheat plants.

Forty grams of plant material was comminuted in liquid nitrogen and the powder suspended in 200 ml of cold extraction buffer, consisting of 50 mM sodium phosphate buffer pH 6 (5 ml g⁻¹ tissue) containing 5 mM dithiothreitol (DTT; to prevent oxidation), 0.2 M NaCl (to desorb proteins from cell walls), and 5% (wt/vol) polyvinyl pyrrolidone (to adsorb phenols). After being shaken on ice for 15 min, extracts were centrifuged at 4,000 × g for 15 min, then the supernatant was respun at 23,000 × g for 15 min. Proteins in the supernatant were precipitated with ammonium sulfate to 100% saturation and the precipitate resuspended in 25 mM MES pH 6 before extensive dialysis against 25 mM MES pH 6 at 4°C. Extracts were then concentrated to approximately 20 ml in a saturated solution of polyethylene glycol (PEG) 20,000 *M_r*.

Detection of fungal protease in infected plant tissue.

Ten milliliters of concentrated fungal culture filtrates prepared as previously described (see above) was applied to a S-Sepharose (fast flow; Sigma) cation exchange column (10 ml) equilibrated with 25 mM MES pH 6.0. The protease was eluted over a 0–250 mM NaCl gradient and fractions assayed for fungal trypsin activity. Ten milliliters of each dialyzed plant extract was loaded and eluted from this column under the same conditions. These fractions were subjected to gel isoelectric focusing and subsequent gelatin overlay to determine the pI of the protease activity.

Effects of SNP1 on viability of tritordeum cells.

The effect of SNP1 on the viability of tritordeum cells was studied with cells at 0.05 g/ml in assay medium containing 0.5 mM MES, 0.5 mM CaCl₂, 0.5 mM K₂SO₄, and 175 mM mannitol and adjusted to pH 5.7 (Baker and Mock 1994). Log concentrations of pure SNP1 from 0.04 to 40 µg, corresponding to 35 nkats to 20 µkats of activity/ml of reaction, respectively, were used in reactions performed in triplicate. SNP1 was incubated with suspension cells shaken at 120 rpm (Sanyo Gallenkamp, Loughborough, UK), 25°C, then 100 µl of suspension was removed at 2, 6, 24, and 48 h, and added to 1:1 (vol/vol) fluorescein diacetate (1 mg ml⁻¹); viability was determined by fluorescence microscopy.

Nucleic acid manipulations and sequencing.

Genomic DNA was prepared as described by Raeder and Broda (1985) from harvested mycelia grown for 48 h on complete medium (described above). Sequencing was performed

with an ABI-377 automated fluorescence sequencer with the Perkin-Elmer dye-terminator kit (PE Applied Biosystem, Warrington, UK). Sequence was compiled and analyzed by GCG programs (Devereux et al. 1984).

PCR isolation of *SNPI*.

PCR amplification of a 610-bp region of *SNPI* was performed in 25- μ l reaction with 0.2 mM concentrations of each dNTP, 1.5 mM $MgCl_2$, 1 U *Taq* polymerase, 50 pmol primer, and 50 ng of genomic DNA. Amplification cycle was as follows: 1 min 94°C; 35 cycles (1 min 94°C, 1 min 55°C, and 1 min 72°C); 5 min 72°C. PCR primers used were AT(H)GT(N)GG(N)GG(N)AC(N)AC(N)GC, corresponding to the N-terminal amino acid sequence IVGGTTA, and GG(N)CC(N)CC(N)SWRTC(N)CC(Y)TGRCA, corresponding to the active site amino acid sequence CQGDSGGP. The 610-bp product was eluted from the gel and sequenced directly with the two primers. Ten micrograms of genomic DNA was digested with *Bam*HI, *Eco*RI, and *Hind*III restriction endonucleases and after Southern transfer probed with the 610-bp *SNPI* PCR fragment. Hybridization conditions were 0.265 M $Na_2HPO_4 \cdot 2H_2O$ pH 7.2 with 7% SDS and 1% skimmed milk powder at 65°C; the most stringent wash was 15 min 0.5 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate) / 0.1% SDS at 65°C.

Isolation of full-length *SNPI*.

Duplicate Hybond-N filters (Amersham-Pharmacia Biotech) representing 10,000 plaques from a genomic library of *S. nodorum* isolate LAW 95, constructed in lambda GEM11 (Promega, Southampton, UK), were screened with the ³²P-labeled, 610-bp *SNPI* PCR fragment. Hybridization conditions were the same as above and final washes were 15 min each of 2 $\times$ SSC / 0.1% SDS and 0.5 $\times$ SSC / 0.1% SDS. Positive clones were identified and re-screened twice before lambda DNA was purified from the most strongly hybridizing clones. Following DNA isolation, restriction, and Southern blotting, positively hybridizing bands were subcloned into pUC18 and sequenced. Several different *Sal*I fragments were used as templates for sequencing with alternative restriction fragment templates used to confirm sequence across *Sal*I sites.

Northern blotting.

Two-day-old cultures of *S. nodorum* grown in Czapek-Dox V8 agar medium were starved for 8 h in basal salts. One gram fresh weight of starved mycelium was transferred to 15 ml of basal salts containing 1% wheat cell walls (wt/vol) and incubated for a further 16 h. Extraction of RNA and blotting were as described by Vallélian-Bindschedler et al. (1998). Probes were labeled with an oligolabeling kit (Amersham-Pharmacia Biotech). Prehybridization and hybridization were performed at 65°C in 0.5 M NaH_2PO_4 pH 7.4, 7% SDS, 1% skimmed milk. Conditions for the last wash was 0.2 $\times$ SSC, 0.1% SDS at 65°C.

Construction of a cDNA library and identification of *SNPI* cDNA clones.

Mycelium of *S. nodorum* isolate 171 was grown on cell wall inducing medium for 16, 27, or 40 h as described above. Six to eight grams of insoluble material (mycelium and plant cell walls) was frozen and comminuted in liquid nitrogen.

RNA was extracted with Trizol reagent (GibcoBRL Life Technologies) according to the manufacturer's instructions. Poly A⁺ RNA was purified with the PolyAttract mRNA Isolation System IV (Promega). Five micrograms of Poly A⁺ RNA (2.5 μ g from 16 h and 1.25 μ g each from 27 and 40 h) was used for the cDNA synthesis with the ZAP cDNA synthesis kit (Stratagene, Amsterdam). Fifty nanograms of cDNA was ligated in the Uni-ZAP XR Vector Kit (Stratagene). λ ZAPII recombinant DNA was packaged into a ZAP-cDNA Gigapack III Gold cloning kit (Stratagene). The primary library containing 4 $\times$ 10⁶ PFU was amplified.

RT-PCR.

RT-PCR was performed with RNA samples from 0.3 to 0.5 g of mycelium and from 0.5 to 1 g of leaf tissue with Trizol reagent (GibcoBRL Life Technologies) according to the manufacturer's protocol. After isopropanol precipitation, RNA was dissolved in 200 μ l of diethyl pyrocarbonate (DEPC)-treated water. DNA was removed by treating 20 μ g of RNA with 3 U of RNase-free DNase I (Promega) in the presence of 40 U RNasine (Promega) for 30 min at 37°C in a volume of 40 μ l. The reaction was stopped by adding 60 μ l of DEPC-treated water followed by a 1:1 (vol/vol) phenol:chloroform and a chloroform partition prior to precipitation with isopropanol. The RNAs were resuspended at 1 μ g ml⁻¹ in DEPC-treated water.

The following RT was performed by first incubating 0.5 μ g of oligo(dT)₁₅ and 2 μ g of RNA for 10 min at 70°C, then immediately chilling on ice. Enzyme buffer, 2 μ l of 0.1 M DTT, 1 μ l of 10 mM NTPs, and 40 U RNasine (Promega) was added to a final volume of 19 μ l then incubated 2 min at 42°C. Two hundred U Superscript II (Gibco BRL Life Technologies) was added and incubated for 50 min at 42°C. The reaction was stopped by heat inactivation for 10 min at 75°C.

PCRs were performed with 1 μ l of reverse transcribed RNA, 200 μ M NTPs, 1.5 mM $MgCl_2$, and 5 pmol of each primer in 25 μ l. The amplification cycle was 94°C 4 min, 40 $\times$ (94°C 50 s, 58°C 60 s, 72°C 60 s), 72°C 5 min. cDNAs were amplified with the *SNPI* specific primers F1b (GCACTTCA CAGACCTCCTCATTTGCCCTC)-R11 (CAGCAACAAAGC TCGCAAGGCTGCTCTG), F12 (GAGTTCCCTGAGGGCG AGTTCCCCGAG)-R15 (TAGCTGATGGTGGAGCTGGTG GGGATG). As a positive control, the C-terminal region of an *S. nodorum* chitin synthase was amplified with CHSF (CCTT CGCTACACCTGCACACTTCTCATC)-CHSR (GACGGCC CAGAGGATGAATGTCAAGTAG) primers.

GFP constructs and transformation.

pUCGFP was constructed by introducing the 1.1-kb *Eco*RI fragment coding for the GFP protein from the plasmid pCRGFP2 (Spellig et al. 1996) into pUC19. *SNPI* promoter was amplified by PCR with the primers SEPPY1 (ggcaGGATCCccacgatgc) and t11 (tgaagtCCATGGtgat gagctg). SEPPY1 was designed to introduce a *Bam*HI site in the promoter at 1,140 bp from the ATG site and the *Nco*I site was removed at this location. t11 was designed to introduce an *Nco*I site at the ATG. The resulting amplified product was cut with *Bam*HI and *Nco*I and ligated into the *Bam*HI-*Nco*I cut pUCGFP. This gave an in-frame fusion at the ATG. The GFP protein obtained from this pUCpSNPIGFP construct is cytoplasmic as the *SNPI* signal peptide was not included after the

SNP1 promoter. A vector pUCpTUBAGFP to express constitutively GFP was also constructed, introducing the -490-40 promoter region of the *S. nodorum* tubulin gene (Cooley and Caten 1993) into the *Bam*HI site of the pUCGFP vector. *S. nodorum* strain LAW 95 was co-transformed with one of the pUC GFP constructs and pAN7-1 (Punt et al. 1987) according to Cooley et al. (1988). Hygromycin-resistant transformants were screened for their ability to fluoresce when spores were germinated for 24 h in a basal salts medium containing 1% gelatin as sole carbon and nitrogen source. A strain expressing GFP constitutively was made by transforming the LAW 95 strain with pCRGFP2 and pAN7-1 plasmids.

Confocal microscopy.

Confocal microscopy was performed with a Leica TCS SP microscope. The light source was an argon laser. Excitation wavelength was 488 nm and detection was 510 to 520 nm (green channel) and 600 to 650 nm (red channel).

ACKNOWLEDGMENTS

We wish to acknowledge the invaluable help with enzyme purification from John Keon, IACR Long Ashton, supply of tritordeum cells and culture methods from P. A. Lazzeri, IACR, Rothamsted, and partial financial support from and interaction with Stephen Foster and Jane Dancer, AgrEvo UK Ltd. A. J. C. was supported by a BBSRC CASE studentship and L. B. by BBSRC grant no. 86/D09299.

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