Mutualistic Fungal Endophytes Express a Proteinase that Is Homologous to Proteases Suspected to Be Important in Fungal Pathogenicity

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Many cultivated and wild grass species are hosts to mutualistic fungal endophytes. These associations are ecologically and agronomically significant, yet little is known regarding the physiological aspects of the interaction. In the Poa annua/Acremonium typhinum interaction, a fungal serine proteinase, At1, is surprisingly abundant and may constitute 1 to 2% of the total leaf-sheath protein. Sequence analysis of cDNA and genomic clones indicates that proteinase At1 is a member of the eukaryotic subtilisin-like protease family. It is homologous to proteases suspected to be virulence factors in fungal pathogens of insects, nematodes, and other fungi. Gel blot analysis of RNA extracted from infected leaf-sheath tissue indicates that the proteinase At1 transcript level is extremely high. RNA gel blots and immunoblots of purified enzymes indicate that similar proteinases are produced by Epichloë festucae and Acremonium lolii, the fungal endophytes infecting Festuca rubra subsp. rubra and Lolium perenne, respectively. Fungal expression of proteinase At1-like enzymes may be a general feature of endophyte infection.

Endophytic infection of grasses by fungi of the tribe Balansieae is widespread in nature (for reviews, see Siegel and Schardl, 1991; Siegel, 1993). Many commercially important turf and forage grasses are naturally infected with endophytes of the genus Acremonium (Morgan-Jones and Gams, 1982), which is related to the teleomorphic genus Epichloë. These associations are considered to be mutualistic (Clay, 1988a), with the plants providing nutrients for the fungi and the fungi synthesizing alkaloids that protect the plants from herbivory. The presence of the toxic alkaloids in endophyte-infected forage grasses, however, can result in the poisoning of grazing livestock (Bacon et al., 1975; Fletcher and Harvey, 1981). Because of the importance of livestock toxicoses to agriculture, considerable research has been conducted on this aspect of endophyte infection of grasses.

In contrast to the information on alkaloids and animal toxicosis, the physiological aspects of the endophyte-grass interactions have not been well characterized in any system. The endophytic fungal hyphae ramify within the intercellular spaces of the aerial plant parts, in particular the leaf sheaths (Hinton and Bacon, 1985). They do not invade the plant cells and must therefore obtain all of their carbon and nitrogen compounds from the nutrient-poor apoplast space. Very little is known about the factors important in host colonization or nutrient exchange between the plants and the fungi.

We are using the Poa annua Merr. cv Service (big bluegrass)/Acremonium typhinum Morgan-Jones & Gams interaction as a model system for the grass/fungus interaction (Lindstrom et al., 1993). Previously, we reported the characterization of a surprisingly abundant fungal protease, designated proteinase At1, which is expressed in the infected plants (Lindstrom and Belanger, 1994). It was estimated that proteinase At1, a single fungal protein, may represent 1 to 2% of the total leaf-sheath protein. The enzyme was localized both within membrane vesicles and in the fungal and/or plant cell walls. The abundance and cell-wall location suggest that the enzyme may be an important feature in the interaction between the grass and the fungus.

Because of the potential biological significance of proteinase At1, we have undertaken cloning and further characterization of the enzyme. Sequence analysis of the cDNA clone revealed that proteinase At1 is homologous to the subtilisin-like Ser proteases produced by fungal pathogens of insects, nematodes, and other fungi. In these systems, expression of the homologous proteases is believed to be an important factor in fungal pathogenicity. RNA gel blot and immunoblot analysis revealed that proteinase At1-like enzymes are expressed in other Acremonium and Epichloë endophyte/grass combinations. Fungal expression of such proteases appears to be a general feature of endophyte infection.

MATERIALS AND METHODS

Plant and Fungal Culture

Endophyte-infected and endophyte-free grasses were identified and maintained as previously described (Lindstrom et al., 1993). The Acremonium and Epichloë spp. endophytes were isolated from endophyte-infected leaf sheaths and cultured as previously described (Lindstrom et al., 1993).
Protein Sequencing

For protein sequencing, proteinase At1 was purified from endophyte-infected Poa ampla leaf-sheath tissue as previously described (Lindstrom and Belanger, 1994). The purified protein was subjected to SDS-PAGE (Laemmli, 1970) and electroblotted to a PVDF membrane (ProBlott, Applied Biosystems). The protein was visualized by staining with 0.2% Ponceau S (Sigma) in 1% acetic acid. The membrane was destained in 1% acetic acid, and the protein band was excised. N-terminal amino acid sequencing of the intact protein and two peptides generated by trypsin digestion was performed by the Harvard Microchemistry Facility (Harvard University, Cambridge, MA).

Nucleic Acid Isolation and Gel Blot Analysis

For extraction of fungal DNA, fungal mycelium was ground to a fine powder in liquid nitrogen. The powdered tissue was extracted in 500 mM NaCl, 100 mM Tris, pH 8.0, 50 mM EDTA, 1% SDS, 10 mM 1,10-phenanthroline, and 0.07% β-mercaptoethanol (Dellaporta et al., 1983) in a ratio of 5 mL of buffer/1 g of tissue. The sample was extracted twice with phenol:chloroform:isoamyl alcohol (25:24:1, v/v/v) and once with chloroform:isoamyl alcohol (24:1, v/v). DNA in the final aqueous layer was precipitated by adding an equal volume of isopropanol. The sample was incubated overnight at −20°C, and the DNA was collected by centrifugation at 5000 g for 10 min. The DNA pellet was redissolved in 10 mM Tris, pH 8.0, and 1 mM EDTA, and DNA solution was extracted with phenol:chloroform:isoamyl alcohol as above and ethanol precipitated.

Facility (Harvard University, Cambridge, MA).

Library Construction, Screening, and Clone Characterization

A cDNA library was constructed using poly(A)+ RNA prepared from endophyte-infected Poa ampla leaf-sheath tissue. cDNA synthesis and phage packaging was carried out using a commercial kit (ZAP Express, Stratagene). The primary cDNA library contained 2 × 10⁶ plaque-forming units. The library was screened with the proteinase At1 PCR clone. The hybridization conditions were the same as those for the DNA gel blots. The cDNA inserts from positive plaques were excised from the lambda vector as recombinant pBK-CMV phagemids (Short et al., 1988).

To obtain genomic clones, two size-selected libraries of fungal genomic DNA were prepared. DNA gel blot analysis, using the proteinase At1 cDNA clone as a probe, revealed two BamHI fragments at about 4.8 and 2.6 kb. Since there was a single BamHI site in the cDNA, the two fragments were postulated to constitute the proteinase At1 gene. BamHI-digested DNA from the appropriate regions of an agarose gel was extracted as for the PCR product and ligated to a BamHI-digested phage vector (ZAP Express). Library screening and processing of positive clones was the same as for the cDNA clones.

Dideoxynucleotide sequencing (Sanger et al., 1977) of double-stranded plasmids with T7 DNA polymerase (Toneguzzo et al., 1988) was performed using a commercial sequencing kit (United States Biochemical) with synthetic oligonucleotides as primers.

Enzyme Assays

Proteinase At1 was purified from endophyte-infected Poa ampla leaf sheaths as described previously (Lindstrom and Belanger, 1994) and dissolved in water at 0.1 μg μL⁻¹. Reaction mixtures contained 100 mM Tris, pH 8.0, 20 mM DTT, and 2.5 mM peptide substrate dissolved in 100 μL of DMSO. The final volume was 1 mL. The assay was started by adding 10 μL (1 μg) of enzyme to the mixture. The reaction was followed spectrophotometrically at 400 nm. N-Succinyl-Ala-Ala-Pro-Met-p-nitroanilide was from Bachem (Torrance, CA), and N-succinyl-Ala-Ala-Phe-p-nitroanilide
was from Calbiochem-Novobiochem. All other peptide substrates were from Sigma.

**Immunoblot Analysis**

Affinity-purified polyclonal antibodies to proteinase At1 were prepared, and immunoblots were processed as previously described (Lindstrom and Belanger, 1994). Detection was either by alkaline phosphatase (Sambrook et al., 1989) or by chemiluminescence using a commercial kit (ECL Western Blotting Detection System, Amersham).

**RESULTS**

**Proteinase At1 cDNA and Genomic Clones**

Our strategy for obtaining a cDNA clone for proteinase At1 was to first obtain a partial amino acid sequence of the protein. N-terminal and two internal peptide sequences were obtained: ARVVQKNAPWGL, AHQIQSYVAG, SNVVDDNHGHTHVAGTI, and YFVAVASGNNNR, respectively. Both of the internal peptides were homologous to other fungal Ser proteases such as proteinase K (Gunkel and Gassen, 1989), so their relative orientations could be surmised. Because proteinase At1 and proteinase K have several features in common (Lindstrom and Belanger, 1994), we suspected that they may have some sequence homology.

Degenerate oligonucleotides were designed based on the amino acid sequences of the internal peptides. PCR was used to amplify the corresponding region from A. typhinum genomic DNA. A PCR clone was used to screen a cDNA library prepared from endophyte-infected P. ampla leaf-sheath poly(A)+ RNA. Since proteinase At1 is abundant in infected leaf sheaths and its expression level in culture is variable (Lindstrom and Belanger, 1994), we expected that the leaf-sheath library to be a superior source of proteinase At1 clones.

A full-length cDNA clone was used to screen size-selected genomic libraries to obtain two genomic clones that spanned the entire proteinase At1 gene. A diagram of the gene structure is shown in Figure 1. The nucleotide and deduced amino acid sequence of proteinase At1 are shown in Figure 2.

Comparison of the cDNA and genomic sequences indicated that the coding sequence is interrupted by three small introns. The consensus 5' GT and 3' AG sequences at the intron-exon junctions (Breathnach and Chambon, 1981) were absolutely conserved. There are two adjacent Mets, one of which is the presumed initiator. The sequence surrounding the first Met is nearly identical to the consensus sequence of CCA/GCCAUG (Kozak, 1984). In general, the first AUG in a transcript is the site of initiation of translation (Kozak, 1995). If the first Met is the initiation codon, then the proteinase At1 gene encodes a 387-amino-acid peptide with a calculated molecular mass of 40,860 D. The N terminus of the protein is hydrophobic, suggesting a signal sequence (Watson, 1984), which would be expected. The cell-wall localization of proteinase At1 indicates that it is indeed a secreted protein (Lindstrom and Belanger, 1994). Based on the weighted matrix method of von Heijne (1986), the predicted signal-sequence cleavage site is between amino acids 16 and 17. The position of the N terminus of the mature protein, as determined from the protein sequence data, indicates that there is also a propeptide sequence of 90 amino acids.

There are three potential N-glycosylation sites of Asn-X-Thr/Ser at amino acids 33, 239, and 277. One is found in the Pro region of the protein and two are found in the mature region. There are also numerous potentially O-linked glycosylation sites of Ser and Thr. Lectin binding to purified mature proteinase At1 indicated that it is indeed a glycoprotein (Lindstrom and Belanger, 1994). The calculated molecular mass of the mature protein is 29,527 D. The higher observed molecular mass of 34 kD estimated from SDS-PAGE gel analysis likely reflects the glycoprotein nature of proteinase At1 (Lindstrom and Belanger, 1994). The mature protein has a high theoretical pl of 10.83.

**Proteinase At1 Is Homologous to Other Fungal Subtilisin-Like Enzymes**

A comparison of the deduced amino acid sequences of the A. typhinum proteinase At1 with three closely related sequences from other organisms is shown in Figure 3. Proteinase At1 is clearly homologous to other fungal subtilisin-like Ser proteases. The positions of the Asp, His, and Ser residues of the catalytic site are completely conserved in proteinase Atl. Reduced by pathogenic fungi. Paecilomyces lilacinus (Thom.) Samson and Metarhizium anisopliae (Metschn.) Sorokin are fungal pathogens of nematode eggs and insects, respectively (St. Leger et al., 1992; Bonants et al., 1995). In these two organisms expression of the homologous protease is believed to be important in the pathogenicity of the fungus. The homologous protease produced by Tritirachium album Limber is the well-studied protease proteinase K (Gunkel and Gassen, 1989). T. album is a saprophyte, and expression of proteinase K is believed to serve a nutritional function. Lower levels of homology exist between proteinase At1 and other fungal subtilisin-like proteases.

The similar fungal proteases have the same general structure: a presequence that targets the enzyme to the ER and a long prosequence that is ultimately cleaved to generate the mature form of the protein. The prepeptides of
such proteases are considered to be important in proper folding of the enzymes and have been referred to as intramolecular chaperones (Shinde and Inouye, 1993).

Pair-wise comparisons of the mature proteinase A11 sequence with homologous fungal proteases are shown in Table I. As would be expected, there is more sequence variation in the pre- and prosequence regions of the protease than in the two regions (Jarai et al., 1992; Frederick et al., 1993; Geremia et al., 1993; Jarai et al., 1994), indicating the existence or location of additional introns, which has no in-...
Although the three proteases are likely to be evolutionarily related, they are clearly distinct from each other in their preferred cleavage sites.

### Substrate Specificity of Proteinase At1

The substrate specificity of proteinase At1 was investigated using synthetic tripeptide substrate esters (Table II). The best substrates for proteinase At1 were Suc-Ala-Ala-Pro-Met-Na and Suc-Ala-Ala-Pro-Phe-Na. Proteinase At1 also cleaved substrates with C-terminal Arg, Leu, and Ala, but at slower rates. The amino acids at the P2 and P3 sites of the substrate were also factors in the rate of cleavage.

Relative substrate specificities of the highly homologous protease Pr1 from *M. anisopliae* have been reported (Samuels and Paterson, 1995) and are included in Table II. The nematode-trapping fungus *Arthrobtos oligospora* Fries. produces a subtilisin-like protease (PPII) that may be a virulence factor (Tunlid et al., 1994). The properties of the enzyme and partial amino acid sequence indicate that this protease likely is also related to proteinase At1, so its relative substrate specificities are also included in Table II. Although the three proteases are likely to be evolutionarily related, they are clearly distinct from each other in their preferred cleavage sites.

### Proteinase At1 Message in Infected Plant Tissue and in Cultured Fungus

We previously reported that proteinase At1 is an abundant protein in infected *P. ampla* leaf-sheath tissue (Lindstrom and Belanger, 1994). RNA gel blot analysis indicated that the proteinase At1 transcript level was also high (Fig. 4). An intense hybridizing band was detectable from both endophyte-infected leaf-sheath poly(A)⁺ RNA and total RNA with only an overnight exposure. Since most of the RNA probably originated from the plant, the fact that the transcript for a single fungal protein was so readily detectable indicates that the message must be an extremely large proportion of the fungal message pool. No hybridization

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**Table I.** Pair-wise comparison of *A. typhinum* proteinase Atl to the homologous secreted proteinases from other fungi

<table>
<thead>
<tr>
<th>Accession No.</th>
<th>Organism</th>
<th>Identity to Proteinase Atl % amino acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>L29262</td>
<td><em>Pacilomyces lilacinus</em></td>
<td>56</td>
</tr>
<tr>
<td>M73795</td>
<td><em>Metarhizium anisopliae</em></td>
<td>55</td>
</tr>
<tr>
<td>S71812</td>
<td><em>Fusarium sp.¹</em></td>
<td>55</td>
</tr>
<tr>
<td>X14688</td>
<td><em>Tricharium album</em></td>
<td>52</td>
</tr>
<tr>
<td>U16305</td>
<td><em>Beauveria bassiana</em></td>
<td>46</td>
</tr>
<tr>
<td>Z11580</td>
<td><em>Aspergillus fumigatus</em></td>
<td>43</td>
</tr>
<tr>
<td>X17561</td>
<td><em>Aspergillus oryzae</em></td>
<td>41</td>
</tr>
<tr>
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<td><em>Aspergillus flavus</em></td>
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</tr>
<tr>
<td>L31778</td>
<td><em>Aspergillus nidulans</em></td>
<td>41</td>
</tr>
<tr>
<td>L19059</td>
<td><em>Apergilus niger (Pep D)</em></td>
<td>39</td>
</tr>
<tr>
<td>D00923</td>
<td><em>Acremonium chrysogenum</em></td>
<td>37</td>
</tr>
<tr>
<td>M87516</td>
<td><em>Trichoderma harzianum</em></td>
<td>32</td>
</tr>
</tbody>
</table>

¹ In cases where the N terminus has not been experimentally determined, the propeptide cleavage site was assigned by analogy to the other enzymes.

**Table II.** Substrate specificities of *A. typhinum* proteinase At1, protease Pr1 from *M. anisopliae*, and protease PPI from *Arthrobtos oligospora*

<table>
<thead>
<tr>
<th>Substrate</th>
<th>At1</th>
<th>PPI³</th>
<th>Pr1³</th>
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<tbody>
<tr>
<td>Suc-Ala-Ala-Pro-Met-Na</td>
<td>100%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Suc-Ala-Ala-Pro-Phe-Na</td>
<td>70%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bz-Val-Gly-Arg-pNa</td>
<td>59%</td>
<td>100%</td>
<td></td>
</tr>
<tr>
<td>Bz-Ile-Gly-Gly-Arg-pNa</td>
<td>19%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Suc-Ala-Ala-Pro-Leu-pNa</td>
<td>13%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Suc-Ala-Ala-Pro-Val-pNa</td>
<td>6%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Suc-Ala-Val-Gly-Arg-pNa</td>
<td>4%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Suc-Ala-Ala-Val-Ala-pNa</td>
<td>4%</td>
<td>4.4%</td>
<td></td>
</tr>
<tr>
<td>Suc-Phe-pNa</td>
<td>0%</td>
<td></td>
<td>1.6%</td>
</tr>
<tr>
<td>Suc-Gly-Gly-Phe-pNa</td>
<td>0%</td>
<td></td>
<td>2.4%</td>
</tr>
<tr>
<td>Suc-Ala-Ala-Phe-pNa</td>
<td>0%</td>
<td></td>
<td>45.6%</td>
</tr>
</tbody>
</table>

³ Data from Tulind et al. (1994). ² Data from Paterson (1995).
was detectable in RNA from endophyte-free plants, indicating that the host, *P. ampla*, does not express a highly similar proteinase.

When *A. typhinum* from *P. ampla* was isolated and grown in culture, proteinase At1 was not detected constitutively, but was detected after 1 to 2 weeks in culture, indicating that it may be induced by nutrient depletion (Lindstrom and Belanger, 1994). As would be expected, proteinase At1 message was detectable in the RNA from a 16-d cultured fungus, which was extracted at a time when it was producing proteinase At1 (Fig. 4).

**Proteinase At1 Is Encoded by a Single Gene**

Gel blot analysis of *A. typhinum* DNA hybridized with the proteinase At1 cDNA clone indicated that proteinase At1 is encoded by a single gene. When *A. typhinum* DNA was digested with the restriction endonuclease BgIII, an enzyme for which there are no sites in the proteinase At1 gene, only a single hybridizing band was detected (Fig. 5). When the DNA was digested with restriction endonucleases for which there are sites within the proteinase At1 gene, the predicted number of hybridizing bands were detected (Fig. 5). The lack of unpredicted hybridizing bands indicates that there are no additional closely related *A. typhinum* proteinase At1 genes.

**Proteinase At1 Expression in Other Acremonium Endophyte Isolates**

DNA gel blot analysis indicated that sequences homologous to proteinase At1 were present in *Acremonium* endophytes isolated from other grass species (Fig. 6). The species of the *Acremonium* endophytes infecting the other *Poa* species have not been determined. The species of endophytes infecting *Lolium perenne* L. (perennial ryegrass) and

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**Figure 4.** RNA gel blot analysis of proteinase At1 transcripts. Total and poly(A)+ RNA was isolated from endophyte-infected (E+) and endophyte-free (E−) *P. ampla* leaf-sheath tissue and from a 16-d-old *A. typhinum* fungal culture grown on potato dextrose agar plates. Ten micrograms of total RNA was used for the leaf-sheath samples. For the leaf-sheath poly(A)+ RNA and for both total and poly(A)+ fungal RNA, 5-μg samples were used. The radiolabeled proteinase At1 cDNA insert was used as a probe.

**Figure 5.** DNA gel blot analysis of *A. typhinum* DNA. Twelve micrograms of total DNA per sample was digested with the indicated restriction enzymes, fractionated by electrophoresis on an agarose gel, transferred onto a nylon membrane, and hybridized with the radiolabeled proteinase At1 cDNA.

**Figure 6.** DNA gel blot analysis of fungal DNA from *Epichloë festucae* and *Acremonium* species isolated from various endophyte-infected grass species. Four micrograms of total DNA per sample was digested with either BglII or BamHI. The host plant from which the fungal endophyte was isolated is indicated. The *Festuca rubra* plant was *F. rubra* L. subsp. *litoralis* Meyer Auquier. The blot was prepared and hybridized as described for Figure 5. A, DNA digested with BglII. B, DNA digested with BamHI.
the *Festuca rubra* subspp. (fine fescues) are *Acremonium lolii* Latch, Christensen, and Samuels (Latch et al., 1984) and *Epichloë festucae* (Leuchtmann et al., 1994), respectively. The blot in Figure 6 was hybridized and washed under the same stringency conditions as the *A. tpyhinum* DNA gel blot in Figure 5. The fact that hybridizing sequences were so readily detectable from the other fungal isolates indicates that those sequences must be highly homologous to proteinase At1 from *A. tpyhinum*.

We previously reported immunological detection of similar proteases in other endophyte-infected *Poa* species (Lindstrom and Belanger, 1994), so detection of homologous genes in the fungal isolates from these plants was expected. We had not, however, been able to detect either proteinase At1-like activity or immunoreactive protein in crude extracts of endophyte-infected ryegrass (Lindstrom and Belanger, 1994). Detection of proteinase At1 hybridizing sequences in the DNA of the endophytes from ryegrass and fine fescue raised the question of whether they may be expressing proteinase At1-like enzymes in quantities undetectable by the methods previously used.

RNA gel blot analysis of total or poly(A)+ RNA extracted from leaf-sheath tissue of different endophyte-infected grass species indicated that transcripts homologous to proteinase At1 were indeed being produced by the other *Acremonium* species (Fig. 7). As was found for *P. ampla*, the transcript level in the other endophyte-infected *Poa* species was very high and easily detected in total RNA.

Proteinase At1-homologous transcripts were also detectable from poly(A)+ RNA extracted from endophyte-infected fine fescue and perennial ryegrass (Fig. 7) leaf sheaths. The need to use poly(A)+ RNA indicates that proteinase At1-like transcripts constituted a lower proportion of the RNA extracted from endophyte-infected fine fescue and perennial ryegrass leaf sheaths. Whether this is due to a lower level of proteinase expression by those fungal species or a lower fungal mass within the infected plant tissue is not known.

Detection of transcripts homologous to proteinase At1 suggested that similar proteinases were being expressed in endophyte-infected fine fescue and perennial ryegrass, although the protein levels were too low to be detectable in crude extracts (Lindstrom and Belanger, 1994). To confirm that such proteinases were present, leaf-sheath extracts of endophyte-infected fine fescue and perennial ryegrass were subjected to the proteinase At1 purification protocol (Lindstrom and Belanger, 1994), followed by immunoblot analysis. In samples concentrated by purification, proteins that were recognized by the proteinase At1 antisera were clearly detectable (Fig. 8).

**DISCUSSION**

Infection of grasses by mutualistic endophytic fungi is both ecologically and agronomically significant, yet very little is known about how the two organisms interact. Knowledge of the factors necessary for the establishment of effective interactions could be useful in efforts to generate novel grass/fungus combinations with improved agronomic characteristics. We have found that a secreted fungal proteinase is abundantly produced in endophyte-infected *Poa* species (Lindstrom and Belanger, 1994). This proteinase is the first factor to be reported that may have a role in the symbiotic interaction between a grass and a fungus.

From RNA gel blot analysis and immunoblot analysis it is evident that proteinase At1-like enzymes are expressed in other grass/endophyte combinations, indicating that such expression is not unique to endophytes infecting *Poa* species. It may be that fungal expression of proteinase At1 is a general feature of endophyte infection. Clearly, how-

**Figure 7.** Detection of proteinase At1-like transcripts in RNA extracted from endophyte-infected *Poa* species, *Festuca rubra* L. subsp. *rubra*, and *Lolium perenne*. The radiolabeled proteinase At1 cDNA was used as a probe. A, Ten micrograms of total RNA from leaf sheaths of the indicated plants; B, 4 μg of poly(A)+ RNA from leaf sheaths of the indicated plants.

**Figure 8.** Purification of proteinase At1-like protein from endophyte-infected *Festuca rubra* subsp. *rubra* and *Lolium perenne*. Leaf-sheath extracts from the indicated plants were subjected to the proteinase At1 purification protocol, followed by immunoblot analysis using proteinase At1 antiserum. Purified enzyme from endophyte-infected *P. ampla* was also included. A, Chemiluminescent detection of antibody binding; B, alkaline phosphatase detection of antibody binding.
ever, there are quantitative differences in both enzyme activity levels and mRNA levels extracted from leaf sheaths of endophyte-infected *Poa* species when compared with the levels extracted from leaf sheath tissue from other endophyte-infected grasses. Whether this quantitative difference is relevant to the interactions between the fungal and grass species is unknown. It will ultimately be important to determine if the quantitative differences in proteinase Atl-like expression among endophyte-infected tissues are due to differences in expression levels among the *Acremonium* species or to differences in fungal mass within the different grass species.

Qualitatively, however, the enzymes from endophyte-infected fine fescue and perennial ryegrass must be very similar to proteinase Atl, since they are detectable using the antibody to proteinase Atl. At the nucleic acid level they must also be highly homologous, since their transcripts are detectable using the proteinase Atl cDNA hybridized under stringent conditions. Ultimately, sequence analysis and substrate specificity studies could be done to determine the degree of similarity among the other endophyte proteinase Atl-like enzymes.

Amino acid sequence analysis of proteinase Atl from *A. typhimurium* revealed that it is a member of the eukaryotic subtilisin-like protease family. Extracellular subtilisin-like proteases have been reported from a wide range of fungal species, including saprophytes, pathogens, and now mutualists. The two enzymes most similar to proteinase Atl were from fungal pathogens of nematode eggs (*P. lilacinus*) and insects (*M. anisopliae*) (St. Leger et al., 1992; Bonants et al., 1995). In both of these systems, expression of the homologous protease is hypothesized to be an important feature in pathogenicity. Application of purified protease from the nematode egg pathogen to immature eggs of *Meloidogyne hapla* resulted in an increase of nonviable eggs (Bonants et al., 1995). Antibodies to protease Pr1 from the entomopathogen *M. anisopliae* inhibited host colonization (St. Leger et al., 1988). The function of protease Pr1 in pathogenesis, therefore, may be to rapidly degrade insect cuticular proteins, thus allowing penetration of the fungus into the host (St. Leger et al., 1992).

Similar proteases, although having lower levels of identity to proteinase Atl, are produced by other parasitic fungi and are also considered to be important components of pathogenicity. Protease-deficient mutants of *Beauveria bassiana*, an entomopathogen, had reduced virulence against the migratory grasshopper, *Melanoplus sanguinipes* (Bidochema and Khachatourians, 1990). The mycoparasite *Tritirachium albidum* produces a similar protease, designated Prb1, which is specifically expressed during the mycoparasitic process and is induced by fungal cell walls (Geremia et al., 1993). The nematode-trapping fungus *Arthroboitrys oligospora* produces an alkaline Ser proteinase, PII, which is capable of immobilizing free-living nematodes (Tunlid et al., 1994).

In none of the above systems, however, has the expression of the protease been shown by targeted gene inactivation to be absolutely required for pathogenicity. Even so, the available evidence suggests that these proteases are a component of overall pathogenesis. By analogy with the fungal pathogenic systems, and based on the abundance and cell-wall location, we speculate that proteinase Atl is an important factor in the symbiotic interaction of *A. typhimurium* with *P. ampla*. Proteinase Atl may facilitate colonization of the plant apoplast by degrading the plant cell-wall and/or apoplastic proteins; the degradation products may then serve as a nutritional source for the fungus.

In the case of the mammalian opportunistic pathogen *Aspergillus fumigatus* the homologous protease does not appear to be a virulence factor. Although correlations of protease expression with pathogenicity have been reported (Kothary et al., 1984), gene-disruption experiments have indicated no requirement for such expression (Tang et al., 1993; Jaton-Ogay et al., 1994). These results, however, do not invalidate the hypotheses that the homologous proteases described above may be factors in pathogenicity in those systems.

Expression of subtilisin-like proteases is not limited to pathogenic fungi and may in fact be a universal feature of Ascomycete and Deuteromycete fungi. A protease highly homologous to proteinase Atl produced by *Tritirachium albidum* is the well-known fungal enzyme proteinase K (Gunkel and Gassen, 1989). *T. albidum* is a saprophytic fungus, and proteinase K is believed to serve a nutritional function by degrading proteins in the environment to substrates that are usable by the fungus. Similar proteases produced by *Aspergillus* species are responsible for the postharvest decay of stored grains. In fact, *A. fumigatus* is actually a soil saprophyte and has only recently become known as a serious human pathogen due to medical treatments that result in immunosuppression. This suggests that it has not evolved specific virulence determinants (Holden et al., 1994). In addition to the homologous secreted proteases, vacuole-localized homologs have been reported: protease B from *S. cerevisiae* (Moehle et al., 1987) and PEP carboxylase from *Aspergillus niger* (Frederick et al., 1993; Jarai et al., 1994). Both of these proteins have C-terminal extensions relative to the secreted proteins, which may contain the vacuolar targeting information.

Proteinase Atl has a broad specificity with a preference for the hydrophobic amino acids Met or Phe in the P1 position. It also exhibits significant reactivity against substrates with the basic amino acid Arg in the P1 position. Comparison of relative substrate specificities for two other fungal subtilisin-like proteases indicated that, although the enzymes are clearly related, they have evolved a preference for cleavage at different amino acid sequences. This suggests that the considerable sequence variability outside of the highly conserved, tripartite active-site regions contributes to the substrate specificity.

St. Leger et al. (1992) speculated that the variability found in this family of proteases may reflect the evolutionary adaptations of various fungal lifestyles. Parasitic fungi are considered to have evolved from saprophytes (Evans, 1988), and mutualistic endophytes are considered to have evolved from parasites (Clay, 1988b). The proteases of the pathogenic fungi may have evolved substrate specificities and charge characteristics that
enhance their ability to gain access to their hosts. Data from the entomopathogen *M. anisopliae* support this hypothesis. Protease Pr1 from *M. anisopliae* is more effective at digesting insect cuticle than is proteinase K; it has a higher pI, which may enhance electrostatic binding to the cuticle (St. Leger, 1992). If the subtilisin-like proteases are important in the interactions between pathogenic and mutualistic fungi and their hosts, then it may be that the observed differences in substrate specificities have evolved to enhance these particular interactions.

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**LITERATURE CITED**


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