Molecular Cloning and Analysis of Abundant and Stage-Specific mRNAs from *Puccinia graminis*

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To characterize highly expressed mRNAs from germinated urediniospores of *Puccinia graminis* f. sp. *tritici*, we isolated 68 cDNA clones of abundant mRNA species belonging to at least six homology groups. The two most abundant homology groups, HG1 and HG2, contained 54 of the 68 cDNA clones and accounted for 2.4 and 0.6% of the poly(A)$^+$ RNA in germinated urediniospores, respectively. By sampling different developmental stages of the uredinial cycle, we showed that the *uam* transcript, corresponding to HG2, accumulated in all stages of hyphal and urediniospore development, whereas the accumulation of *usp* transcript, corresponding to HG1, was specific to the sporulation stage. Southern blot analysis indicated that *usp* is a small gene family consisting of three to four members. Sequence analysis of 10 cDNA clones indicated that two different members of the *usp* gene family were expressed in germinated urediniospores. This gene family encodes small hydrophobic polypeptides of 113 amino acids with an unusual amino acid composition, in that alanine, glycine, leucine, and proline represent 48% of the protein. These polypeptides are predicted to be localized extracellular because they contain a putative signal sequence and may be functionally related to hydrophobins, a family of small hydrophobic proteins abundantly expressed during sporulation in *Schizophyllum commune* and *Aspergillus nidulans*. The *uam* and *usp* genes deserve further investigation, including isolation of genomic clones. The regulatory regions of the *uam* gene, which is highly expressed in hyphae, may be useful in the construction of a transformation vector for rust fungi.

Additional keywords: germination; wheat stem rust.

Puccinia graminis Pers. f. sp. *tritici* Eriks. & Henn., the cause of wheat stem rust, is a heteroecious fungus with a complex life cycle that includes five spore stages. In the asexual uredinial stage, which is parasitic on wheat (*Triticum* spp.), the fungus enters the leaf or stem through stomata and infects mesophyll tissues (de Bary 1867; Allen 1923). White flecks, the first macroscopic symptom of disease, appear at infection sites 5–7 days after inoculation. These flecks result from proliferation of rust mycelium in the intercellular spaces surrounding the mesophyll cells. Fungal cells at the center of this hyphal mass differentiate to form the uredinium (Harder 1976; Littlefield and Heath 1979). Sporulation begins in the uredinium and ruptures the host epidermis 8–10 days after inoculation. The uredinium continues to expand and reaches its maximum rate of production of urediniospores by 12–14 days after inoculation. Urediniospores can reinfect wheat, causing repeated cycles of infection and sporulation during the growing season and leading to severe epidemics when plants are susceptible and environmental conditions are favorable (Roelfs 1985). In spite of its importance, very little is known about the molecular biology of uredinial development.

The wheat stem rust fungus has several other important characteristics that are poorly understood. The fungus is an obligate biotrophic parasite that has race specificity governed by gene-for-gene interaction with its host (Loegering and Powers 1962; Loegering 1984) and requires an alternate host to complete its sexual life cycle. However, the difficulties inherent in studying obligate biotrophic parasites have discouraged attempts to apply molecular genetic technologies to *P. graminis* or other rust fungi. Some isolates of *P. graminis* have been grown in axenic culture, but these cultures tend to grow slowly, are often unable to sporulate, and lose their pathogenicity to wheat (Maclean 1982). Nevertheless, certain axenic cultures are useful for experiments requiring large quantities of vegetative rust mycelium, including metabolic studies (Maclean and Scott 1976; Manners *et al.* 1984), production of protoplasts (Huang *et al.* 1990) and the isolation of DNA and RNA.

We are developing tools for molecular genetic investigation of this fungus in preparation for the long-term objective of isolating race-specific genes for avirulence from *P. graminis*. The goal of the present investigation was to isolate and characterize highly expressed genes suitable for use in developing vectors for transformation of the fungus. In this research, we identified two highly abundant mRNA clones and characterized their patterns of expression during the uredinial stage of the life cycle. We also sequenced the cDNA corresponding to the most abundant message to elucidate the amino acid sequence and possible function of the encoded protein.

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RESULTS

Identification of highly abundant mRNAs.

To identify highly abundant mRNAs, clones from a

Table 1. Characteristics of abundant cDNAs in germinated urediniospores of *Puccinia graminis*

Homology group	Number of clones per group[a]	Representative clone[b] Designation	Insert size (bp)	mRNA size (nt)	Percentage of poly(A)$^+$RNA[b]
HG1	40	pCRL11	500	500	2.4
HG2	14	pCRL45	750	1,500	0.6

[a] Clones were classified into homology groups on the basis of cross-hybridization analysis.
[b] Determined from poly(A)$^+$ RNA from germinated urediniospores by RNA slot blot analysis.

cDNA library constructed from germinated urediniospore poly(A)$^+$ RNA were hybridized with ^{32}P-labeled first-strand cDNA from the same RNA. Approximately 4% of the cDNA clones showed a substantially higher level of hybridization than that of the majority of the clones. Sixty-eight cDNA clones that displayed high levels of hybridization were selected and analyzed by cross-hybridization to determine the number of homology groups. Among the 68 clones tested, 40 belonged to one homology group (HG1), 14 to a second homology group (HG2), and seven to six additional homology groups (HG2-8). The remaining seven could not be classified. The first two groups (HG1 and HG2), which apparently represented highly abundant messages in the germinated urediniospore, were selected for further analysis. Their characteristics are summarized in Table 1. The cDNA clones pCRL11 and pCRL45 were selected to represent homology groups HG1 and HG2, respectively.

The relative abundance of mRNA of the two homology groups, HG1 and HG2, in germinated urediniospores was determined by slot blot hybridization. Probes were prepared from representative cDNA clones and hybridized to known amounts of poly(A)$^+$ RNA and to *in vitro* transcription products from these two cDNAs at varying concentrations comprising a standard curve. Values were then calculated based on the relative radioactivity of the samples compared to the standards and were corrected for the amount of rRNA contamination in the poly(A)$^+$ RNA fraction, which was determined to be 5% by slot blot hybridization. These two homology groups (HG1 and HG2) accounted for 2.4 and 0.6% of the total poly(A)$^+$ RNA, respectively (Table 1).

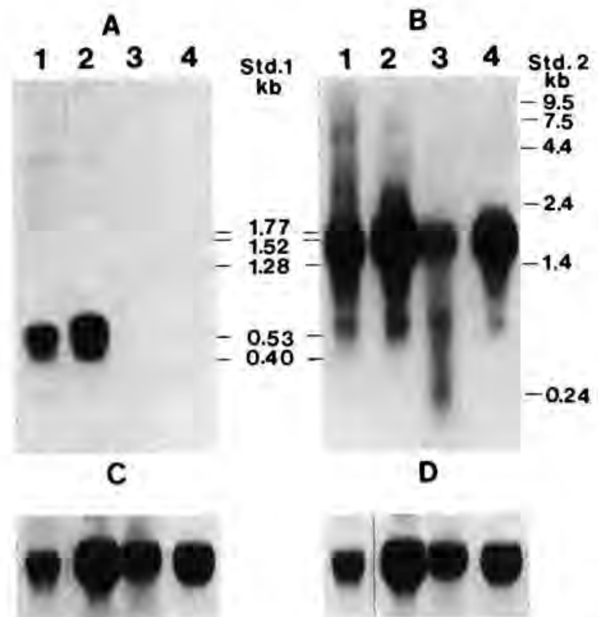

Fig. 1. Hybridization of total RNA isolated from different stages of the uredinial cycle, with probes from HG1 and HG2. Each lane contains 16 μg of RNA from: 1, urediniospores; 2, germinated urediniospores; 3, axenic culture V1B; 4, infected wheat leaves 12 days after inoculation containing uredinia. **A,** Probed with insert DNA from HG1 clone pCRL11; **B,** probed with insert DNA from HG2 clone pCRL45. **C** and **D,** represent the blots shown in A and B, respectively, which have been stripped and reprobed with the oligonucleotide RUST1. Standards 1 and 2 were low (0.16–1.77 kb) and high (0.24–9.5 Kb) RNA ladders (Gibco BRL). The 0.78, 0.28, and 0.16 kb markers of the low RNA ladder were unclear and therefore omitted.

RNA analysis.

The patterns of HG1 and HG2 mRNA accumulation in different stages of the uredinial cycle were examined by RNA blot analysis (Fig. 1). Total RNA was prepared from urediniospores, germinated urediniospores, and infected wheat seedlings (12 days after inoculation) with sporulating uredinia. In addition, RNA was isolated from V1B, a nonsporulating axenic culture of *P. graminis*, which has been maintained in the laboratory for several years. The rust-specific oligonucleotide (RUST1) enabled us to determine the amount of fungal RNA in each of the samples and therefore to directly compare RNA from infected wheat with RNA from isolated rust.

Hybridization data obtained with the two cDNAs indicate that they have different patterns of transcript accumulation. The cDNA clone pCRL11 (representing HG1) hybridized strongly to RNA from spores and germinated spores, but not from axenic hyphae (Fig. 1A and Table 2), indicating that accumulation of this transcript is associated with sporulation. In contrast, the clone pCRL45 (representing HG2) hybridized strongly to RNA extracted from spores, germinated spores, and axenic hyphae (Fig. 1B and Table 2), indicating that accumulation of this transcript is constitutive. This difference in the pattern of transcript accumulation was also clearly shown in the RNA sample isolated from infected wheat plants to which pCRL45 hybridized and pCRL11 did not (Fig. 1A,B and Table 2). Although the infected leaves contained pustules filled with urediniospores, the procedure used for grinding the leaf tissue was ineffective in rupturing the urediniospores. Therefore, the major fraction of fungal RNA in this sample was from hyphae produced within the leaf and not from urediniospores. The difference in the hybridization levels indicates pCRL11 mRNA is predominantly localized within the spore, while pCRL45 mRNA is found in both spores and hyphae. No hybridization was

Table 2. Amount of hybridization between RNA from different stages of the uredinial cycle of *Puccinia* and ^{32}P-labeled probes of pCRL11 and pCRL45 by RNA blot analysis

RNA source	pCRL11			pCRL45		
	pCRL11 (cpm)	Ribosomal[a] (cpm)	Corrected value[b] (cpm)	pCRL45 (cpm)	Ribosomal[a] (cpm)	Corrected value[b] (cpm)
Urediniospore	956	8,430	5,865	3,470	11,166	15,889
Germinated urediniospore	3,156	51,720	3,156	10,219	51,124	10,219
Axenic hyphae[c]	23	21,907	54	1,087	17,210	3,229
Infected wheat leaves[d]	37	20,791	92	5,182	18,778	14,111

[a] The relative amount of fungal 25S ribosomal RNA was determined using a rust-specific oligonucleotide (Rust-1) as a probe.
[b] Corrected values were determined by adjusting for differences in the amount of fungal 25S ribosomal RNA in each lane.
[c] Culture V1B, a non sporulating axenic culture of *P. graminis*.
[d] Wheat seedlings, 12 days after inoculation, containing sporulating pustules of *P. graminis*.

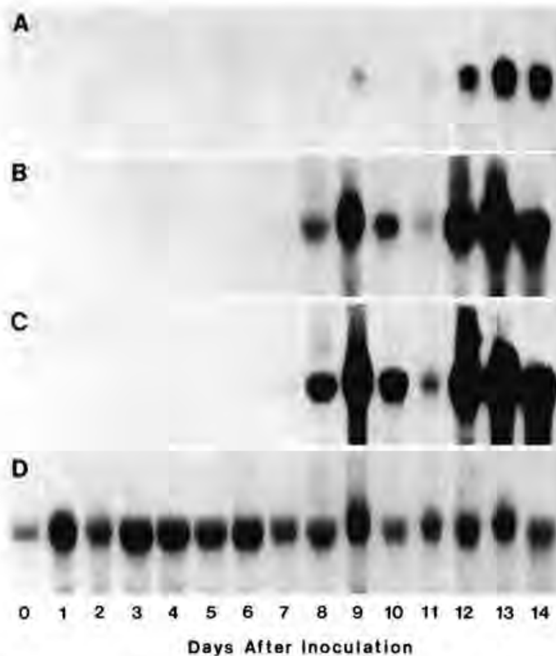

Fig. 2. Detection of HG1 and HG2 mRNAs during uredinial cycle of *Puccinia graminis*. Each lane contained 20 μg of total RNA isolated from wheat leaves 0–14 days after inoculation (lanes 0–14). **A,** Probed with insert DNA from pCRL11 (HG1); **B,** probed with insert DNA from pCRL45 (HG2); **C,** probed with oligonucleotide RUST1 (specific for rust 25S RNA); **D,** probed with oligonucleotide R635 (25S RNA). A–D represent autographs of single RNA blot that was stripped and then reprobed.

observed with either of the probes to RNA isolated from uninfected wheat leaves (data not shown).

To determine more precisely the temporal pattern of transcript accumulation with respect to fungal development, RNA was extracted from infected wheat leaves every day for 15 days starting with the day of inoculation. Equal amounts of total RNA (determined by absorbance at 260 nm) from each time point were separated by gel electrophoresis and analyzed by blot hybridization using pCRL11 and pCRL45 as probes (Fig. 2). The filters were also hybridized with a conserved 25S ribosomal RNA oligonucleotide probe (R635) to determine actual amounts of total RNA in each lane (Fig. 2D) and a rust-specific oligonucleotide (RUST1) to determine the amount of fungal RNA in each of the samples (Fig. 2C).

The first significant amount of fungal 25S ribosomal RNA was detected 7 days after inoculation when white flecks first appeared on the leaf surface. The radioactivity for the 7-day sample was visible only faintly in the autoradiograph (Fig. 2C), but a significant amount of radioactivity above background was detected by radiometric analysis (data not shown). Radioactivity increased then through the 13th day, with the exception of day 11 (Fig. 2C). (The low level of fungal RNA on the 11th day probably represents a sampling error in which the leaves collected on this day had a lower level of infection than did the other samples.) The first significant amount of hybridization to pCRL45 occurred also on the seventh day (when radiometric analysis again revealed significant radioactivity) and increased thereafter (Fig. 2B), following the pattern of 25S ribosomal RNA. On the other hand, the first detectable level of hybridization with pCRL11 was observed on the ninth day and the level of this mRNA increased through the 14th day (Fig. 2A). This pattern of transcript accumulation correlated with the timing of spore development. Sporulation was first observed in a few of the pustules late on the eighth day and the level of sporulation progressed so that the leaves were heavily covered with uredinia by day 13. The results from these two experiments indicate that the expression of the gene(s) represented by pCRL11 is associated with sporulation and therefore this gene has been designated *usp* (*u*redinial *sp*ore). Transcripts represented by pCRL45 accumulate both in vegetative and sporulating hyphae and therefore the gene(s) corresponding to these transcripts has been designated *uam* (*u*redinial *a*bundant *m*essage).

Structure of the *usp* gene family.

Given that the cDNA clone pCRL11 appeared to be full-length (Table 1), this clone was selected for further analysis. Southern blot hybridization of total DNA isolated from germinated urediniospores revealed that *usp* was a small gene family with three to four members (Fig. 3). The cDNA clone pCRL11 was sequenced, revealing that it contained an insert of 491 bp that included a poly(A) tail of 23 bp. To determine if this cDNA clone represented the complete message, primer extension analysis was performed using a 23-nucleotide primer complementary to a region near the 5′ end of the sense strand of the cDNA. A prominent primer extension product of 100 nucleotides and a number of minor bands extending to 105 nucleotides were observed, indicating that the cDNA clone pCRL11 was approximately 50 bp short of being full-length (data

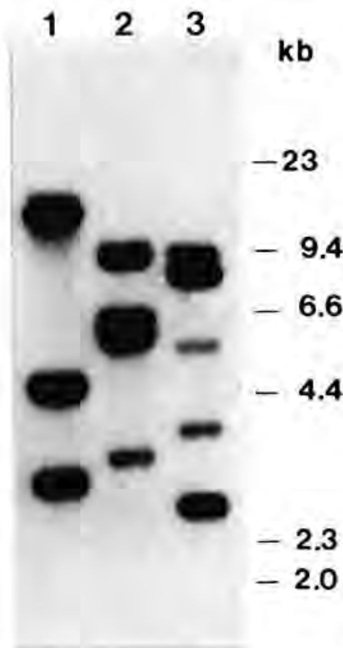

Fig. 3. Hybridization of genomic *Puccinia graminis* DNA with *usp* cDNA probe. Genomic DNA was digested with *Eco*RI (lane 1), *Hin*dIII (lane 2), or *Sst*I (lane 3) separated on 0.8% agarose gel and blotted onto nitrocellulose membrane. The blot was hybridized with a random primer, labeled insert DNA from pCRL11. Sizes of bacteriophage λ *Hin*dIII/*Eco*RI fragments are given in kilobases.

not shown). Additional clones from HG1 were analyzed for insert size, and 13 clones containing inserts equal to or larger than pCRL11 were sequenced. Ten of the cDNA clones were of identical length and corresponded to the longest of the primer extension product (105 nucleotides), indicating that these clones were full length. This group of clones could be subdivided into two equal groups (HG1-A and HG1-B) based on differences in their DNA sequences. The three remaining cDNA clones were essentially identical to HG1A, except one was longer by 23 bp (HG1-C) and two were longer by 51 bp (HG1-D) at the 5′ end. These three cDNA clones may represent transcripts that were not detected by primer extension due to premature termination of reverse transcriptase or artifacts due to cloning. To test this, Southern blot analysis of digested total DNA, using oligonucleotide probes specific to each of the four groups of cDNAs was carried out. Only oligonucleotide probes for HG1-A and HG1-B hybridized specifically to fragments that also hybridized to pCRL11, indicating the cDNA clones in groups HG1-C and HG1-D represent artifacts of cloning (data not shown). These results further support the conclusion that the 10 cDNA clones from HG1-A and HG1-B are full length.

The DNA sequences of two cDNA clones (pCRL8 and pCRL9), representing the cDNA classes HG1-A and HG1-B, respectively, are shown in Figure 4. Analysis of pCRL8 DNA sequence indicates a single large open reading frame encoding a small polypeptide (USP1) of 113 amino acids (Fig. 4). This putative polypeptide has an unusual amino

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pCRL8                                     CTTTAGAGCCCACACAACAATTACTATTTTCAACCCAACCAATTTTAA   48
pCRL9                                     *************C**********************************

pCRL8  TCATCACCCTTCAACAACTCCGAGCTCAGTCTTTCAACAACGGTAACTTATCCCAACTTTTAAACTTTCGAAACCAATCGAAG  131
pCRL9  *******************T******************************************************T********

        M   K   I   T   M   L   F   A   A   L   S   A   A   S   G   A   F   A   A   P   A    21
pCRL8  ATG AAG ATC ACC ATG CTT TTT GCT GCC CTT TCT GCT GCC AGC GGT GCC TTC GCG GCC CCT GCT  184
pCRL9  *** *** *** *** *** T** *** **C *** *** *** *** *** *** *** *T* *** *** *** *** ***
                            F                                       V

        Q   A   V   A   A   A   K   D   L   S   I   G   A   G   V   G   I   G   I   G   A    42
pCRL8  CAA GCC GTC GCC GCC GCT AAG GAT CTC TCT ATC GGC GCC GGT GTT GGT ATC GGT ATT GGA GCT  247
pCRL9  *** *** *C* *** *** *** *** *** *** *** *** *** **A *** *** *** *** *** *** *** ***
                A

        G   V   G   P   Y   G   Y   P   Y   G   A   Y   P   G   W   R   L   Q   L   L   P    63
pCRL8  GGC GTA GGT CCT TAC GGT TAC CCG TAC GGT GCG TAC CCC GGA TGG AGG TTA CAA CTA CTA CCC  310
pCRL9  *** *** *** T** *** **C *** *** *** **C **C *** *** **G *** *** *** *** *** *** ***
                    S

        L   R   W   L   S   L   Q   W   I   P   L   R   I   P   I   L   P   M   V   N   T    84
pCRL8  TTA CGG TGG CTA TCC TTA CAG TGG ATA CCC TTA CGG ATA CCG ATA CTA CCA ATG GTA AAC ACT  373
pCRL9  *** *** *** *** C** *** *G* C** C** *** *** *** *** **A *** *** *** *** *** *** ***
                        P       R   R   L

        W   C   S   V   A   C   A   Q   N   P   S   P   A   H   L   H   T   F   K   P   F   105
pCRL8  TGG TGT TCT GTG GCC TGC GCG CAG AAT CCT AGC CCG GCT CAT CTG CAT ACT TTC AAA CCC TTC  446
pCRL9  *T* *** *** *** *** *** *** *** *** *** C** *** *** *** **T *T* *T* A** *** *** **A
        L                                       R                   L   I   I           L

        T   S   E   Q   F   L   F   L   -                                                 113
pCRL8  ACT TCC GAA CAA TTT TTA TTT CTT TAA ACATTAGCTTGCTCGCGCAATTTAAGTGATGATTTTTATTCTCT(A)  518
pCRL9  *** C** *** *** *** *** *** *** *** ***************************************C****(A)
            P
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Fig. 4. DNA sequences of two *usp* cDNA clones and their predicted amino acid sequences. Asterisks indicate for pCRL9 a nucleotide that is identical to one in pCRL8. A single letter code for amino acids is indicated above the pCRL8 DNA sequence. In cases in which base pair substitution in pCRL9 resulted in an amino acid change, this amino acid is indicated below the pCRL9 DNA sequence. The site of polyadenylation is indicated by (A).

acid composition in which alanine, glycine, leucine, and proline compose 48% of the protein. In addition, there are three discrete repeats contained within the polypeptide. The first is a glycine repeat (aa 32–45) where every other amino acid is a glycine. Internally, this is organized into a repeat of IGAGVG. This glycine repeat has similarities to glycine-rich proteins found in plant cell walls (Condit and Meagher 1986; Keller *et al.* 1988) which have G - X repeats, as does the GRP 1.8 protein from *Phaseolus vulgaris* (Keller *et al.* 1988) and fibroin, the structural protein in silk in which glycine alternates with alanine (Mita *et al.* 1988). This G - X motif is proposed to form a β-pleated sheet configuration in the native protein and is a common feature of many structural proteins. However, in contrast to the putative USP1 protein, these glycine-rich proteins contain more than 60% glycine and the protein is almost entirely composed of this repeat. The other two repeats in USP1 are imperfect and are characterized by motifs of PYGY (aa 46–55) and LQXXPLR (aa 59–75). The putative USP1 polypeptide is hydrophobic in nature and contains three hydrophobic domains (Fig. 5) with average hydrophobicity values of 0.56, 0.61, and 0.44, respectively (Eisenberg *et al.* 1984). These values are based on an average more than 21 amino acid residues. The first hydrophobic domain, located at the amino terminus, is flanked by positive charges and resembles a signal sequence (Boyd and Beckwith 1990) indicating that USP1 may be secreted.

The clones pCRL8 and pCRL9 differ by 27 base pair substitutions, 14 of which resulted in amino acid changes (Fig. 4). Four of these amino acid changes are within the PYGY and LQXXPLR repeats, two of which are in the variable sites in the LQXXPLR repeat. Each of the four amino acid changes are conservative substitutions. Because pCRL8 and pCRL9 represent different classes of cDNA clones and are assumed to be transcribed from two different

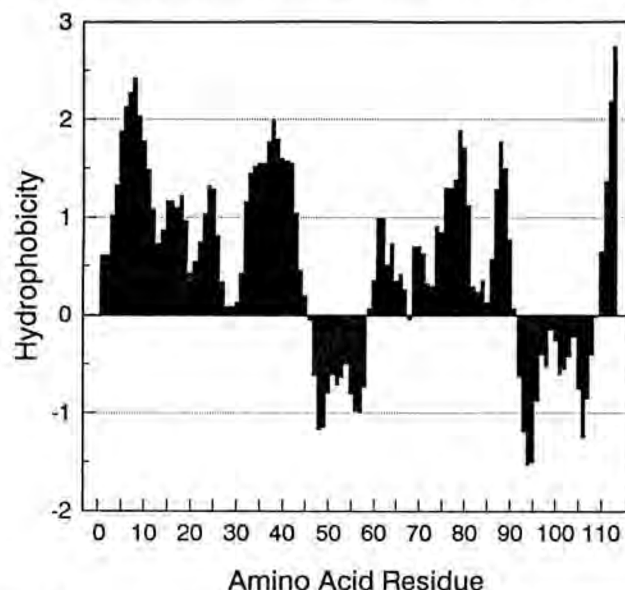

Fig. 5. Hydrophobicity plot of the predicted USP1 protein. The Kyte-Doolittle algorithm was used with a window size of 7 (Kyte and Doolittle 1982). Points above the *x*-axis represent hydrophobic regions.

members of the *usp* gene family, the genes corresponding to pCRL8 and pCRL9 are designated *usp1* and *usp2*, respectively. No significant similarities were found between the *usp* and DNA sequences in several data banks. However, amino acid sequence comparisons with proteins involved in fungal sporulation indicated that the putative USP1 polypeptide may be related to a class of hydrophobic proteins found in *Schizophyllum commune* (SC1, 3, and 4; Schruren and Wessels 1990) and *Aspergillus nidulans* (RodA; Stringer *et al.* 1991).

DISCUSSION

We have identified and characterized two classes of highly abundant messages from *P. g.* f. sp. *tritici*, which account for approximately 3% of the poly(A)$^+$ RNA in germinated urediniospores. The most abundant of these two classes, transcribed from a small gene family (*usp*), accumulated in a sporulation-specific manner. High levels of *usp* mRNA were found in urediniospores and represented 2.4% of the mRNA in germinated urediniospores. RNA extracted from nonsporulating hyphae of the axenic culture, V1B, and infected wheat leaves, showed very low levels of *usp* mRNA. This pattern of sporulation-specific transcript accumulation was also supported by a time-course study in which the accumulation of *usp* transcript corresponded with the appearance of pustules on infected wheat leaves. The second highly abundant message class, transcribed from the *uam* gene, showed a different pattern of accumulation. The *uam* transcript accumulated in both spores and nonsporulating hyphae. This pattern of transcript accumulation appeared to follow the levels of 25S RNA, indicating that the *uam* gene is constitutively expressed.

Very little is known about gene expression in plant pathogenic fungi during growth and sporulation. This is particularly true for obligate biotrophic fungi, such as *P. graminis*, where most studies must be done *in planta*. A major difficulty with these studies has been the quantitation of fungal RNA in samples that contain both fungal and plant material. In some systems, differences in the size of the ribosomal RNAs from the plant and the pathogen are sufficient to allow accurate quantitation of fungal RNA (Judelson and Michelmore 1990). Because this was not possible with *P. graminis*, we designed an oligonucleotide probe (RUST1), which is complementary to the 5′ end of the ribosomal large subunit. This rust-specific probe made it possible to directly compare levels of gene expression between samples from isolated rust and *in planta*. In addition, this probe should be useful in studying fungal growth within the leaf. Results from the time course study indicated fungal growth was very slow in the early stages of infection and that a rapid phase of growth started just prior to fleck stage. Similar results were obtained using a chemical assay for chitin in wheat leaves infected with *P. g.* f. sp. *tritici* (Mayama *et al.* 1975). It is unclear whether these results actually represent fungal growth in this early phase, or just the limits of detection for the assays.

A number of highly abundant messages from germinated spores of *Bremia lactucae*, an obligate biotrophic pathogen of lettuce, have been recently characterized (Judelson and

Michelmore 1990). *Ham*27 and *Ham*37 are expressed in a sporulation specific manner; *Ham*37 message is the most abundant mRNA in germinated spores, accounting for approximately 9% of the total poly$(A)^+$ RNA. Two genes expressed during the development of infection structures in another biotrophic pathogen, *Uromyces appendiculatus*, have been cloned and characterized (Bhairi *et al.* 1989). INF24, a single-copy gene that encodes a 16.4-kDa polypeptide, is expressed during the development of infection structures. INF56, however, is constitutively expressed, with increase in the level of expression during the development of infection structures (Xuei *et al.* 1992).

Recently, sporulation-specific genes have been characterized from the filamentous fungi *S. commune* (Sc1, Sc3, and Sc4; Schuren and Wessels 1990) and *Aspergillus nidulans* (*rodA*; Stringer *et al.* 1991). These genes encode small hydrophobic polypeptides (hydrophobins) and are thought to be involved in the development of the aerial mycelium and formation of fruiting bodies. The amino termini of each of the predicted proteins contain a hydrophobic core flanked by positive charges, which is characteristic of a signal sequence (Boyd and Beckwith 1990). The predicted extracellular location of the Sc3 and Sc4 polypeptides has been confirmed and accumulate in the walls of aerial cells (Wessels *et al.* 1991). The polypeptides encoded by the *usp* gene family share a number of similar features with hydrophobins, but further biochemical and genetic analysis will be necessary to determine if these polypeptides are functionally related.

P. graminis has a complex life cycle that includes production of five spore types: urediniospores, teliospores, basidiospores, pycniospores, and aeciospores. In this study, we have cloned and characterized a transcript that accumulates in a sporulation-specific manner during the uredinial stage of the life cycle. It will be interesting to determine if the expression of the *usp* gene family is associated with the other four spore types and if so, whether the members of this gene family are differentially expressed during these stages. Furthermore, the regulatory elements from the genes identified in this study, especially *uam*, which was highly expressed in hyphae, may be useful in the development of vectors for transformation of rust fungi.

MATERIALS AND METHODS

Manipulation of *P. g.* f. sp. *tritici* and wheat seedlings.
Isolate SZA2C-1 (CRL 75-45-1781-3) was propagated by spraying urediniospores in light mineral oil onto leaves of 7-day-old wheat plants (cultivar Baart) that had previously been treated with maleic hydrazide to enhance spore production (Rowell 1984). Plants were incubated overnight in a dew chamber at 18° C then in a greenhouse at 18–28° C. Urediniospores were harvested with a cyclone collector 12–18 days after inoculation. For germination, 0.4 g of urediniospores was dusted onto 500 ml of germination buffer (50 μM nonyl alcohol, 0.005% w/v Tween 20, and 0.1% v/v ethanol) in a large glass baking dish covered with plastic wrap and incubated at 18–24° C. Germinated urediniospores were harvested 4–16 hr after dusting, blotted dry, and stored at −80° C after quick freezing in liquid nitrogen. In some cases, the frozen germinated spores were lyophilized. Inoculated and uninoculated wheat plants used for nucleic acid extractions were treated as described above, but they were not treated with maleic hydrazide. Axenic strain V1B of *P. graminis* (Maclean 1982), which has been maintained in the laboratory for several years, was cultured as previously described (Huang *et al.* 1990). This axenic culture is unable to infect wheat and does not sporulate.

Isolation of DNA and RNA.
DNA was isolated from frozen or lyophilized germinated urediniospores that were first ground to a fine powder with a mortar and pestle. One gram of powdered germinated spores was gently suspended in 7 ml of EB (200 mM Tris-HCl, pH 8.0, 250 mM NaCl, 25 mM EDTA, 0.5% w/v sodium dodecyl sulfate). The suspension was extracted with 6 ml of phenol, followed by 6 ml of chloroform/isoamyl alcohol (24:1, v/v). Nucleic acids were precipitated by isopropanol and resuspended in TEN (6 mM Tris-HCl, pH 7.5, 10 mM NaCl, 0.1 mM EDTA). Total DNA was further purified by CsCl-ethidium bromide gradient centrifugation (Sambrook *et al.* 1989). DNA was collected from the gradient, and ethidium bromide was removed by extraction with salt-saturated isopropanol (1:1, v/v, 5 M NaCl: isopropanol). The DNA was precipitated with ethanol after dilution with two volumes of water. The final DNA pellet was resuspended in TEN. DNA yield was typically 20–30 μg/g of spores. RNA was prepared from urediniospores, germinated urediniospores, axenic mycelial culture V1B, and wheat plants by the method of Chomczynski and Sacchi (1987). To rupture the urediniospores for RNA extraction, an MSK Braun homogenizer (B. Braun Biotech Inc., Allentown, PA) was used instead of a mortar and pestle.

Construction and screening of a cDNA library.
Poly$(A)^+$ RNA was isolated by oligo(dT)-cellulose column chromatography (Sambrook *et al.* 1989) from total RNA extracted from germinated urediniospores. The cDNA library was constructed by the procedure of Gubler and Hoffman (1983) using a Librarian kit from Invitrogen (San Diego, CA) according to manufacturer's instructions.

The cDNA library was screened with a total first-strand cDNA probe that was prepared from 1 μg of poly$(A)^+$ RNA from germinated urediniospores using M-MuLV reverse transcriptase (Boehringer Mannheim Biochemical, Indianapolis, IN), oligo (dT) primers, and $[^{32}P]$dCTP (Sambrook *et al.* 1989). The colonies were lifted onto nitrocellulose filters (Schleicher & Schuell, Keene, NH), prehybridized for 4 hr, and hybridized to the probe overnight in hybridization buffer (Sambrook *et al.* 1989) containing yeast tRNA and poly(A) at 100 μg/ml each. After hybridization, the filters were washed three times in wash buffer (0.2× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate] 0.5% sodium dodecyl sulfate) at 65° C and then subjected to autoradiography.

DNA and RNA blot hybridization.
Cross hybridization between cDNA clones was performed by Southern blotting (Southern 1975). DNA was isolated from each clone by an alkali lysis method (Sam-

brook *et al.* 1989). The inserts were released by restriction endonucleases, *Xho*I and *Hin*dIII. Probes were prepared by random primer labeling (Feinberg and Vogelstein 1983) of the isolated insert DNA fragments using a commercially available kit (Bethesda Research Laboratories, Gaithersburg, MD). Genomic Southern assays were performed by digestion of 2–4 μg of total DNA from germinated urediniospores with appropriate restriction enzymes and electrophoresed through an 0.8% agarose gel. The DNA was transferred by capillary blotting to nitrocellulose (Schleicher & Schuell) and hybridized to radiolabeled probes (Sambrook *et al.* 1989).

Total or poly(A)$^+$ RNA was electrophoresed through 1.5% agarose gels containing formaldehyde and transferred to Nytran (Schleicher & Schuell) as described by the manufacturer. The filters were hybridized, in the presence of 50% formamide, at 42° C overnight with DNA probes that were labeled by random primer synthesis using [^{32}P]dCTP. RNA blots were washed two times at room temperature (6× SSPE [1× SSPE is 0.18 M NaCl, 10 mM $NaPO_4$, pH7.7, and 1 mM EDTA], 0.1% SDS), two times at 37° C (1× SSPE, 0.1% SDS) and then washed for 1 hr at 60° C (1× SSPE, 0.1% SDS). Oligonucleotides R635 (5′d[GGTCCGTGTTTCAAGACGG]) and RUST1 (5′d[GCTTACTGCCTTCCTCAATC]) were used to determine the amount of 25S ribosomal RNA in each lane. R635 hybridizes to a conserved segment in the 5′ end of the ribosomal large subunit (Gueho *et al.* 1989), whereas RUST1 hybridizes to a variable region of the 5′ end and is rust specific. The nucleotide sequence of RUST1 is identical to the DNA sequence from several rust fungi but is different from the DNA sequence found in several other basidiomycetous fungi (P. Zambino, M. Berres, and L. Szabo unpublished data). Oligonucleotides were end labeled with terminal transferase (Boehringer Mannheim Corporation) using [^{32}P]dCTP and hybridized overnight to the RNA blots at 52° C in 6× SSPE, 1% SDS. The filters were washed two times in 6× SSPE, 0.1% SDS at room temperature, and once at 50° C in 6× SSPE, 0.1% SDS. Levels of mRNA from RNA blots were quantified using a radiometric analyzer (AMBIS Systems Inc., San Diego, CA) with a scan time from 3 to 13 hr. To correct for unequal amounts of total RNA, samples were standardized for the amount of 25S ribosomal RNA in each lane. A correction factor for each sample was calculated by dividing the value for the sample with the most radioactivity by the value for the sample. The corrected value for each sample was then calculated by multiplying the actual radioactivity for a particular sample by that sample's correction factor.

Quantitation of RNA levels.

The levels of mRNA were determined by RNA slot blot analysis. For each cDNA clone, 0.7, 1.4, and 2.8 μg of poly(A)$^+$ RNA from germinated urediniospores was applied to Nytran membranes (Schleicher & Schuell) with a slot blot apparatus (Hybri-slot, Bethesda Research Laboratories). Similarly, an RNA standard series for each clone, composed of serial dilutions ranging from 1.6 to 0.006 μg, was loaded adjacent to the RNA samples. The RNA standards were prepared from each cDNA clone by *in vitro* transcription according to manufacturer's instructions (Promega, Madison, WI). To ensure removal of free nucleotides from *in vitro* transcribed RNA, the RNA was precipitated three times by adding one-quarter volume of 5 M ammonium acetate and three volumes of ethanol, and the final pellet was washed three times with 75% ethanol. RNA concentrations were determined by spectrophotometric measurements at 260 nm. The quality of the RNA was checked by gel electrophoresis. The amount of contaminated rRNA in the poly(A)$^+$ RNA fraction was determined by slot blot analysis using a ribosomal DNA clone from *P. graminis* (L. Szabo, unpublished data) as a standard. The quantities of rRNA contamination were then subtracted from the total poly(A)$^+$ RNA, and these values were used in the calculations. To quantify the amount of hybridization, segments of the blot corresponding to each slot were excised and counted in a liquid scintillation counter.

Analysis of *usp* cDNA clones.

The nucleotide sequence of several *usp* cDNA clones was determined (Sanger *et al.* 1977) using double-stranded plasmids as substrate after denaturation and spun dialysis (Murphy and Kavanagh 1988). Ten micrograms of plasmid DNA, in 20 μl of TEN, were denatured by adding 5 μl of 1 M NaOH, 1 mM EDTA, and incubated at 18–24° C for 5 min. The denatured plasmids were loaded onto a column of Sepharose CL-6B-200 (Sigma, St. Louis, MO), equilibrated in TE (10 mM Tris-HCL, pH 7.5, 1 mM EDTA), and spun at 1,500 rpm for 3 min. The sample coming through the column was neutral in pH and was used immediately for sequencing (Sequenase, U.S. Biochemicals Corporation, Cleveland, OH). DNA sequence data management and analysis were performed using an IntelliGenetics software package (IntelliGenetics, Inc., Mountain View, CA). The DNA and the predicted protein sequences of *usp*1 were compared to sequences from GenBank (release 70), EMBL (release 29), PIR (release 31), and Swiss-Prot (release 20) using the program IFIND.

The 5′ end of the *usp* transcripts was determined by primer extension using poly(A)$^+$ RNA from germinated urediniospores as described by Ausubel *et al.* (1989) with the exception that M-MuLV reverse transcriptase (Boehringer Mannheim Biochemical) was used instead. An oligonucleotide (5′d[GTTGGGATAAGTTACCGTTGTTG]; Fig. 6, bases 83–105) complementary to the mRNA strand of the 5′ end of the *usp* cDNA clone pCLR11 was used as a primer.

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