

Highly Abundant and Stage-Specific mRNAs in the Obligate Pathogen *Bremia lactucae*

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Germinating spores of the obligate pathogen *Bremia lactucae* (lettuce downy mildew) contain several unusually abundant species of mRNA. Thirty-nine cDNA clones corresponding to prevalent transcripts were isolated from a library synthesized using poly(A)⁺ RNA from germinating spores; these clones represented only five distinct classes. Each corresponding mRNA accounted for from 0.4 to 9 percent by mass of poly(A)⁺ RNA from germinating spores and together represented greater than 20 percent of the mRNA. The expression of the corresponding genes, and a gene encoding Hsp70, was analyzed in spores during germination and during growth *in planta*. The *Hsp70* mRNA and mRNA

from one abundant cDNA clone (ham34) were expressed constitutively. Two clones (ham9 and ham12) hybridized only to mRNA from spores and germinating spores. Two clones (ham37 and ham27) showed hybridization specific to germinating spores. Quantification of the number of genes homologous to each cDNA clone indicated that four clones corresponded to one or two copies per haploid genome, and one hybridized to an approximately 11-member family of genes. A sequence of the gene corresponding to ham34 was obtained to investigate its function and to identify sequences conferring high levels of gene expression for use in constructing vectors for the transformation of *B. lactucae*.

Additional keywords: differentiation, *Lactuca sativa*.

The fungus *Bremia lactucae* Regel (Oomycotina) is an obligate biotroph that causes the downy mildew disease of lettuce. The specificity of the interaction between *B. lactucae* and lettuce is governed by a gene-for-gene interaction (Flor 1956). Thirteen pairs of resistance and avirulence genes have been identified by classical genetic analyses, but they remain uncharacterized at the molecular level. Lettuce downy mildew is now one of the genetically best understood interactions (Crute and Johnson 1976; Farrara *et al.* 1987; Ilott *et al.* 1989).

B. lactucae passes through a series of developmental stages during infection and colonization of its host that have been well studied cytologically and physiologically (Sargent 1981; Tommerup 1981; Woods *et al.* 1988). However, little is known at the molecular level concerning changes in gene expression during the life cycle of the fungus and of the gene products required during these stages. Some gene products are probably similar to those present in saprophytic relatives; others are likely to be important for the rapid establishment of a parasitic relationship.

Germination of asexual spores of *B. lactucae* commences after the spores are washed from the sporangiophores, if the proper environmental conditions are present. The germ tube directly penetrates the lettuce epidermis and forms primary and secondary vesicles in an epidermal cell, usually within 12 hr after inoculation. Until the secondary vesicles

are formed, little increase in net cytoplasmic volume is observed and nuclear division does not occur (Sargent 1981; Tommerup 1981). In compatible interactions, after several more hours the fungus grows from the cell that was initially invaded and proliferates intercellularly for several days, forming haustoria which invaginate the host plasmalemma, until sporulation occurs. The specific signals that induce sporulation are unknown, but the process is influenced by environmental conditions such as humidity and light (Raffray and Sequeira 1971). In contrast to compatible interactions, an incompatible reaction occurs when the fungus expresses an avirulence gene that matches a resistance gene in the host. The incompatible response involves rapid hypersensitive death of the penetrated host cell and cessation of fungal growth. Incompatible and compatible interactions are indistinguishable prior to penetration of the host (Maclean and Tommerup 1979; Woods *et al.* 1988).

In preliminary molecular studies of germination in *B. lactucae*, we had observed that when total RNA from germinating spores was visualized after separation by gel electrophoresis, prominent bands were apparent in addition to the bands expected for the ribosomal RNAs (unpublished observations). Many of these bands were not seen in profiles of RNA from spores. This suggested that gene expression during germination involved the differential synthesis of highly abundant mRNAs which could play important roles in early infection events including germination, penetration, or host recognition. In this study, we have identified several classes of highly expressed genes that correspond to these abundant mRNAs and characterized their pattern of expression during the life cycle of *B. lactucae*. The structure of one gene was analyzed in detail to determine the possible function of the gene product and to characterize strong promoter sequences from *B. lactucae* that might be useful in developing a transformation system.

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Nucleotide and/or amino acid sequence data is to be submitted to EMBL/GenBank/DDBJ as accession number X16984.

MATERIALS AND METHODS

Manipulation of *B. lactucae* and isolation of nucleic acids. Isolate CS9 was propagated on lettuce cultivar Cobham Green (Dark Green Boston), at 15° C (Farrara *et al.* 1987). Spores were harvested from sporulating cotyledons (1 day after the observation of initial sporulation) by shaking in deionized water at 15° C. Spores were germinated by washing the spores once in water and then placing 10⁷ spores in 10 ml of water in a 100-mm glass petri plate in the dark at 15° C. RNA was prepared from germinating spores (>80% of spores having germ tubes of 20 µm or greater), directly from spores, or from infected or uninfected lettuce tissue as described by Judelson and Michelmore (1989). DNA was isolated as described by Hulbert and Michelmore (1988).

Construction of a cDNA library. First strand cDNA was synthesized from 2 µg of oligo(dT)-purified RNA from germinating spores. The RNA was heated to 60° C for 5 min, cooled on ice, and added to a 20-µl reaction mixture that contained 1.7 µg oligo(dT)₁₂₋₁₈, 10 mM dithiothreitol, 50 mM Tris-Cl, pH 8.5, 75 mM KCl, 3 mM MgCl₂, 50 µg/ml actinomycin D, 1 µl of RNAGuard (Pharmacia LKB, Piscataway, NJ), 1 mM of each deoxynucleotide triphosphate, and 24 units of MMLV reverse transcriptase. After incubation for 1 hr at 37° C, second strand synthesis was performed by adding 80 µl of a solution containing 25 mM Tris-Cl, pH 7.5, 6.2 mM MgCl₂, 125 mM KCl, 25 units of DNA polymerase I holoenzyme, plus 2 units of RNase H and incubating the mixture for 1 hr each at 12° C and 22° C. Using the methods of Gubler and Hoffman (1983), the resulting cDNA was tailed with dCTP using terminal transferase, annealed to *Bam*HI-digested pBS- (Stratagene, La Jolla, CA) that had been blunt-ended with the Klenow fragment of DNA polymerase I and tailed with dGTP, and transformed into *Escherichia coli* DH5α. Analysis of the unamplified library indicated that >80% of the clones had inserts longer than 200 base pairs (bp), with the average insert being 490 bp.

RNA and DNA hybridization. The cDNA library was screened with a total cDNA probe that was prepared from 0.5 µg of poly(A)⁺ RNA from germinating spores using MMLV reverse transcriptase, an oligo(dT) primer, 50 µg/ml of actinomycin D, and [³²P]dCTP (Maniatis *et al.* 1982). The probe (10⁷ cpm) was hybridized to colonies replicated on nitrocellulose filters for 24 hr (Maniatis *et al.* 1982), with the addition of 0.1 mg/ml each of yeast tRNA, poly(C), and poly(A) to the hybridization buffer. After hybridization, the filters were washed three times in wash buffer (1× SSC, 1% sodium dodecyl sulfate, 0.1% sodium pyrophosphate) at 65° C before autoradiography.

Cross-hybridizations between individual cDNA clones were performed by colony hybridizations as described above or by Southern blotting of DNA isolated from each clone by the alkaline lysis technique (Maniatis *et al.* 1982). Probes were prepared by digesting clones from the library with *Eco*RI and *Hind*III and labeling the liberated inserts with [³²P]dCTP using random primer labeling (Amersham, Arlington Heights, IL).

Northern blotting was performed by separating RNA by electrophoresis on 1% agarose gels containing 2.2 M

formaldehyde (Maniatis *et al.* 1982), followed by capillary blotting in 20× SSC to Zetaprobe membranes (Bio-Rad, Richmond, CA). Parallel lanes were stained with 0.5 µg/ml of ethidium bromide. Synthetic RNAs of known size and rRNAs from lettuce and *E. coli* were used for molecular weight markers. The filters were hybridized for 24 hr with DNA probes that were labeled by random primer synthesis using [³²P]dCTP, and washed three times at 50° C in wash buffer before autoradiography. The *Hsp70* probe, pBH3-1, has been described previously (Judelson and Michelmore 1989).

Quantitation of RNA levels and gene copy number. The levels of mRNA were determined by RNA dot blotting. For each cDNA clone, a filter was prepared that contained poly(A)⁺ RNA from germinating spores (usually 10 and 20 ng) and an RNA standard curve composed of fivefold serial dilutions of RNA standards (see below) which ranged from 15.6 ng to 5 µg. The RNA was prepared by two rounds of oligo(dT)-cellulose chromatography and was judged to be of adequate purity since rRNA bands could not be detected by ethidium bromide staining following agarose gel electrophoresis. RNA standards were prepared from the cDNA inserts by *in vitro* transcription. These reactions contained, in 25 µl, 1 µg plasmid DNA (cut opposite the appropriate promoter with *Eco*RI or *Hind*III), 40 mM Tris-Cl, pH 8.0, 25 mM NaCl, 8 mM MgCl₂, 2 mM spermidine-HCl, 0.4 mM nucleotide triphosphates, 6 units of RNAGuard, and 10 units of T3 or T7 RNA polymerase. The enzymes were chosen to synthesize a sense copy of the RNA, as determined by prior localization of poly(A) tails by DNA sequencing. After 30 min at 37° C, 225 µl of a solution containing 40 mM Tris-Cl, pH 7.5, 6 mM MgCl₂, 10 mM NaCl, and 1 unit RNase-free DNase was added. The mixture was incubated at 37° C for 15 min, extracted twice with phenol:chloroform (1:1), and RNA precipitated using one-third volume of 3 M NaOAc, pH 5.2, and 2.5 volumes of ethanol. To ensure removal of unincorporated nucleotides, the pellet was resuspended in 0.3 M NaOAc, pH 5.2, and reprecipitated, with the final pellet being washed three times with warm 70% ethanol. RNA concentrations were determined spectrophotometrically, assuming 40 µg/ml for an OD₂₆₀ of 1; the concentrations calculated were verified by an independent assay that involved spotting samples of each product and known concentrations of yeast tRNA on 1% agarose plates containing 0.5 µg/ml of ethidium bromide. The fidelity of the reactions was confirmed by sizing the RNAs by gel electrophoresis. The experimental or standard RNAs were mixed with yeast tRNA to a total of 100 µg and transferred using a vacuum manifold to Zetaprobe membranes (Bio-Rad) according to the manufacturer's instructions.

Gene copy numbers were determined by DNA dot blotting. A standard curve was prepared for each cDNA clone from CsCl-purified plasmid DNA that was linearized with *Eco*RI. Amounts used corresponded to 0.5, 1.0, 2.0, 10, 50, and 100 copies per genome relative to 250 ng of genomic DNA (calculated from the length of the insert and assuming a haploid genome of 5 × 10⁷ bp; D. Francis, S. Hulbert, and R. Michelmore, unpublished). Experimental samples contained 50, 250, or 1,000 ng of genomic DNA.

Salmon sperm DNA was added to each sample to bring the total to 1 μg , and then the experimental samples and the standard curves were transferred in duplicate to Zeta-probe membranes, as recommended by the manufacturer, using a vacuum manifold.

The filters were hybridized with an excess amount of the appropriate cDNA probe as detailed above and washed three times in wash solution at 50° C; the low stringency was selected to facilitate detection of related sequences. The spots were localized by autoradiography and excised, and radioactivity was determined by Cerenkov counting to 2% accuracy. RNA abundance and gene copy number were determined by comparing the hybridization intensity of the experimental samples with the standard curves. A mass percent value for the RNA was obtained by multiplying the relative abundance by the ratio of mRNA size to cDNA insert size (less the size of the homopolymeric tails). Each hybridization was performed at least twice using duplicate filters. Calculations were made using data from two concentrations of the experimental samples on each filter.

Cloning and analysis of *Ham34*. A genomic clone for *Ham34* was isolated from a library of *B. lactucae* DNA (isolate SF5) in lambda EMBL4 (Frischauf *et al.* 1983) using standard techniques (Maniatis *et al.* 1982). Several bacteriophages with overlapping restriction maps were identified by hybridization with the insert from cDNA clone ham34; a 3.8-kilobase *Hind*III fragment from the bacteriophage reacted with the probe and was subcloned into pBS—

(pHAMG34-7-21). Deletion subclones were prepared by exonuclease III digestion (Henikoff 1984) and sequenced using a Sequenase kit (U.S. Biochemicals Corporation, Cleveland, OH). Full-length cDNAs related to ham34 were also sequenced to identify the transcriptional unit. Data management and hydropathy analyses were performed using the Pustell package of programs (International Biotechnologies, Inc., New Haven, CT). Similarity searches were performed in the NBRF/PIR and Swiss-Prot protein sequence data bases using the Fasta program (Pearson and Lipman 1988) available through Bionet.

The termini of the *Ham34* transcript were also identified by primer extension and nuclease S1 protection analyses by methods previously described (Judelson and Michelmore 1989) using 10 μg of RNA from germinating spores. The probe for primer extension analysis of the 5' end was a synthetic oligonucleotide (5'd(GGCAGCGAGTACGA-GAATC); bases 726–745) that was labeled with [³²P]ATP using T4 polynucleotide kinase. For nuclease S1 protection analysis of the 3' termini, the probe was prepared by digesting pHAMG34-7-21 with *Ban*I (located at base 1216), labeling with [³²P]dATP using Klenow polymerase, and digesting with *Hind*III (cleaving approximately 500 bp 3' of the *Ban*I site), with the probe then being gel-purified.

RESULTS

Identification of abundant mRNAs. To study further the unusually abundant mRNA species that we had detected

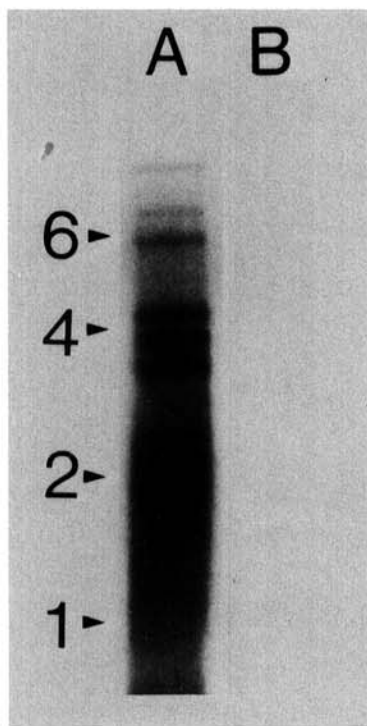


Fig. 1. Profile of ³²P-labeled cDNA. MMLV reverse transcriptase was used to synthesize cDNA from oligo(dT)-purified mRNA from germinated spores, with (A) or without (B) an oligo(dT)₁₂₋₁₈ primer. Reaction products were resolved by electrophoresis in a 1% agarose gel in 0.03 N NaOH and 1 mM EDTA. Size markers (in kilobases [kb]) are indicated in the left margin.

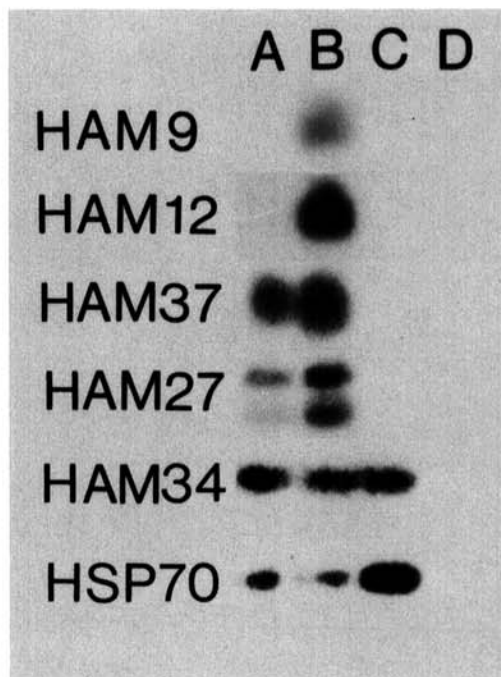


Fig. 2. Developmental expression of transcripts in *Bremia lactucae*. Total RNA from (A) spores (0.5 μg), (B) germinated spores (0.5 μg), (C) infected plants (5 μg), or (D) uninfected plants (5 μg) was resolved by electrophoresis, blotted, and hybridized with cDNA clones ham9, ham12, ham37, ham27, and ham34, and with a genomic clone of *Hsp70*. Uninfected lettuce RNA (4.5 μg) was added to spores and germinated spores to provide equal amounts of RNA per lane. Exposure times were varied to facilitate reproduction.

in germinating spores of *B. lactucae*, a cDNA library was prepared from poly(A)⁺ RNA isolated from germinating spores. An electrophoretic analysis of radiolabeled cDNA indicated the presence of distinct bands in addition to a background smear, consistent with our expectation of prevalent mRNA species (Fig. 1). These bands were not obtained in reactions performed without an oligo(dT) primer, and the relative abundance of these bands was higher in reactions using poly(A)⁺ RNA instead of total RNA, indicating that they were not the artifactual products of self-priming by structural RNAs.

Clones from the cDNA library that corresponded to abundant mRNAs were identified by preparing ³²P-labeled cDNA from the same RNA and hybridizing this probe to the library. Approximately 20% of the clones showed a level of hybridization significantly higher than that of the majority of colonies which showed only weak signals. Thirty-nine cDNA clones that initially displayed high to medium hybridization were selected; of these, 34 contained inserts long enough to be easily visualized by gel electrophoresis (>250 bp) and were analyzed further. The average length of inserts in the selected sample (570 bp) was a little longer than the average for the library (490 bp). The selected clones were cross-hybridized to each other and found to represent only five classes of mRNA species. One member of each class was selected for further analysis.

Stage-specific expression of genes. The clones were hybridized to total RNA prepared from spores, spores germinating *in vitro* (>85% germination), and infected plants

that were harvested 1 day before asexual sporulation (Fig. 2). Axenic culture conditions have not been developed for *B. lactucae*; therefore, infected plants are the only source for vegetative RNA. The rRNA bands of *B. lactucae* have lower electrophoretic mobilities than their counterparts in lettuce; a densitometric comparison of these bands from these mixed samples indicated that approximately 10% of the rRNA from the infected tissue after 5 days was of fungal origin. To compensate approximately for the lower prevalence of fungal RNA in these mixed samples, these blots were prepared using either 0.5 μg of fungal RNA or 5 μg of RNA from plant tissue.

The hybridization patterns obtained with the representative cDNA clones indicated that the mRNAs have different stage-specific patterns of expression. Two hybridized to RNA from germinating spores only (ham9 and ham12). Two detected mRNA from spores and the germinating sample (ham27 and ham37). One reacted with RNA from spores, germinating spores, and infected plants containing vegetatively growing fungus (ham34). A clone containing an *Hsp70* gene from *B. lactucae* was also hybridized to these blots as a control since previous studies suggested that this gene was expressed constitutively (Judelson and Michelmore 1989); as expected, reaction was observed in all fungal samples. None of the probes hybridized with samples from uninfected lettuce.

The vegetative samples used in the preceding experiment were isolated on the day before sporulation; therefore, it was possible that *Ham34* (the gene corresponding to cDNA

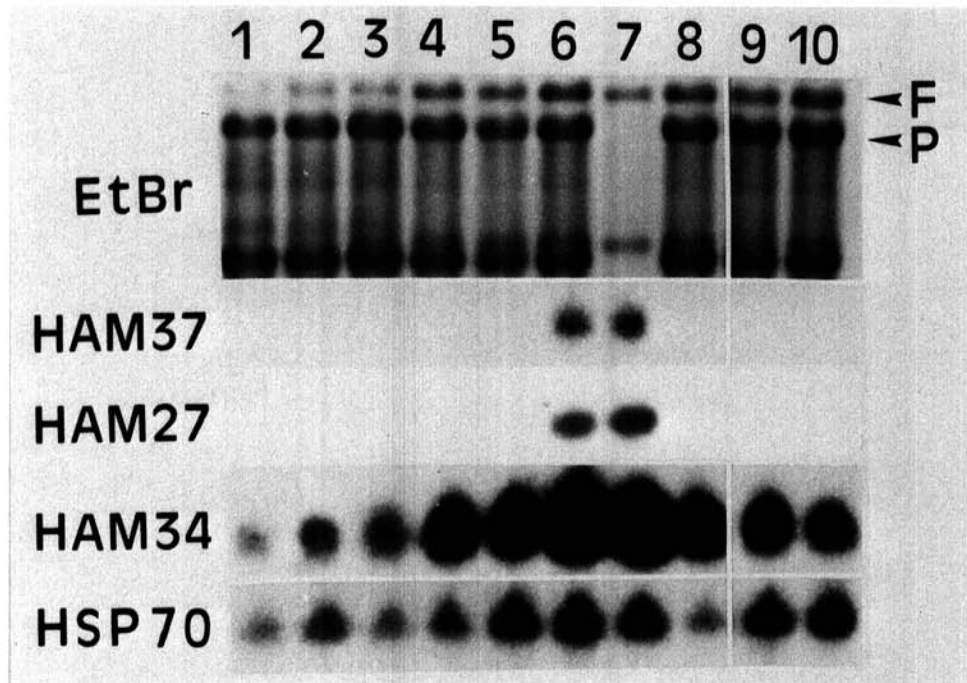


Fig. 3. Detection of mRNAs during growth of *Bremia lactucae* in lettuce. Seedlings were sprayed with spores at 0 hr and then incubated in alternating cycles of 16 hr of light (L) and 8 hr of dark (D). RNA was harvested from the entire seedling at (1) 60 hr, L; (2) 72 hr, D; (3) 84 hr, L; (4) 96 hr, D, and (5) 108 hr, L. Sporulation was observed at 120 hr; at this point, RNA was harvested from (6) total sporulating seedlings, (7) isolated spores, or (8) seedlings washed free of spores. At 108 hr some samples were moved to continuous light to prevent sporulation, and RNA was isolated at (9) 132 or (10) 156 hr. The RNA samples (5 μg of total RNA or 0.5 μg of spore RNA) were electrophoresed and stained with ethidium bromide to detect the plant and fungal rRNA bands (EtBr; negative image); indicated are the large bands of plant rRNA (P) and fungal rRNA (F). Other lanes were blotted and hybridized as in Figure 2 with cDNA clones ham27, ham37, and ham34, and with genomic clone *Hsp70*. Exposure times were varied to facilitate reproduction.

clone ham34) was not expressed constitutively but instead was induced at an early stage of sporulation. A similar possibility existed for *Hsp70* since studies in other fungi had shown that heat shock genes are induced in spores (Brambl *et al.* 1987; Kurtz *et al.* 1986). The expression of the genes was therefore examined throughout growth. Spores of *B. lactucae* were inoculated onto the surface of lettuce seedlings (0 hr). The seedlings were then incubated under standard conditions (16-hr light and 8-hr dark cycle). After the second day, infected plant samples were collected every 12 hr (after 8 hr of light or 4 hr of darkness) up to 120 hr, when sporulation was observed. Sporulation likely occurred between 118 and 120 hr since sporulation in *B. lactucae* is induced during the dark (Raffray and Sequeira 1971). Parallel sporulating cultures were harvested in their entirety, or the spores and sporangiophores were separated from the leaves (containing residual mycelium). In addition, at 108 hr some samples were shifted to continuous illumination to inhibit sporulation and then collected at 132 and 150 hr.

RNA was harvested from seedlings and spores and separated by gel electrophoresis. The gels were stained with ethidium bromide to compare the relative intensities of the fungal and plant rRNAs and thus to assess the relative proliferation of the pathogen (Fig. 3). The staining pattern showed that the proportion of fungal RNA in these samples started at a low level, rose, and then plateaued before sporulation. The same RNAs were also transferred to nylon membranes after gel electrophoresis and hybridized to the cDNA or *Hsp70* probes (Fig. 3). The relative abundance of the *Ham34* and *Hsp70* mRNAs also rose throughout growth in parallel with the increase in fungal RNA, suggesting that these genes are expressed constitutively. Differences in regulation or stability may exist since the relative rate of increase was greater for *Ham34* than *Hsp70*. In contrast, mRNAs from spore-specific genes (*Ham37* and *Ham27*) did not appear before sporulation, and the same conditions of continuous light that inhibited sporangiophore development also prevented accumulation of these RNA species. These mRNAs were also not detected in the RNA isolated from leaves that had been stripped of spores, even though the ethidium bromide staining of the rRNAs showed that the majority of fungal mRNA remained within the leaf. The signal for induction of these genes, therefore, does not seem to be propagated through the coenocytic vegetative hyphae of *B. lactucae*, or induction may occur after delimitation of the sterigmata from the vegetative

mycelium. The germination-specific cDNA clones (ham9 and ham12) showed no hybridization to RNA isolated from tissues not bearing spores.

Quantification of mRNA abundance and gene copy number. The abundance of the mRNAs represented by the five cDNA clones in germinating spore mRNA was quantified using an RNA dot blot procedure (Table 1). Probes prepared from each cDNA clone were hybridized to known amounts of poly(A)⁺ RNA and to a standard curve constructed from the *in vitro* transcription products (sense copies) of each cDNA insert. Values were obtained by comparing the relative reactivity of the samples and correcting for the fractional size of each RNA represented by the cDNA clone. The abundance of these mRNAs was surprisingly high. Together, the five species accounted for more than 20% of the mRNA in germinating spores. During germination, other highly expressed mRNAs may have been present since some bands observed in the cDNA synthesis reaction (Fig. 1) were not accounted for by the mRNAs detected by the five cDNA clones. These results also indicated that the prevalence of the abundant species in the cDNA was not the result of the preferential synthesis of these molecules *in vitro* because the number of clones detected approximately reflected their abundance.

The prevalence of these species could result from either the activity of a single gene or more moderate expression of several members of a multigene family. Therefore, the number of genes hybridizing to each mRNA was quantified using a DNA dot blot method (Table 1). Four of the genes were of single or low copy number, while one (*Ham12*) had a moderate reiteration (approximately 11 copies). These values were consistent with the banding patterns and hybridization intensities observed in Southern blots of *B. lactucae* DNA probed with each cDNA clone (data not shown). Multiple bands were detected for *Ham12* suggesting that the gene family was not tightly clustered in the genome.

Structure of the *Ham34* gene. The structural gene for cDNA clone ham34 was cloned to investigate the putative product of the gene and to identify sequences that direct high levels of constitutive expression of genes in *B. lactucae*. A clone was obtained from a library of *B. lactucae* genomic DNA by using the ham34 cDNA as a hybridization probe. The primary structure of the gene and of the deduced protein product was determined by DNA sequence analysis (Fig. 4). Primer extension and nuclease S1 protection analyses identified a 674-nucleotide transcriptional unit that

Table 1. Abundant cDNAs detected in germinated spores of *Bremia lactucae*

Class	Representative member ^a	Number of clones in a class	mRNA size(s) ^b	Mass percent of poly(A) ⁺ RNA ^c	Genomic copies ^d
A	ham9 (410)	2	1,100	0.39 ± 0.09	1
B	ham12 (490)	15	1,250	2.4 ± 0.34	11
C	ham37 (390)	6	650	9.0 ± 1.9	2
D	ham27 (860)	4	4,400/4,500	2.2 ± 0.14	1
E	ham34 (690)	7	900	6.2 ± 0.10	1

^aClones were first selected as showing moderate to high hybridization with a radiolabeled total cDNA probe and then cross-hybridized to allow classification. Indicated in parentheses is the insert size of the clone in base pairs.

^bIn nucleotides, determined by hybridizing the cDNAs to RNA resolved by denaturing gel electrophoresis.

^cDetermined for mRNA in germinated spores using RNA dot blots; values are averages derived from counts of four dots in each of two experiments.

^dDetermined by DNA dot blotting, rounded to the nearest integer; values are averages derived from counts of four dots in each of two experiments.

lacked introns and contained 70 and 82 bp of 5' and 3' untranslated regions. A "TATA-like" AT-rich region is located 40 bp 5' to the transcriptional start site. Several probable full-length cDNA clones were also isolated and characterized; their DNA sequences were consistent with the conclusions from the transcript mapping experiments.

A hydropathy plot was calculated for the deduced 16.5-KDa Ham34 protein using the algorithm of Kyte and Doolittle (1982) (Fig. 5). The predominant features identified by this analysis were hydrophobic regions at each end of the protein that flanked a long, moderately polar internal domain. This central domain comprising amino acids 18 to 157 was unusually rich in uncharged, polar amino acids, especially threonine (31%). The amino terminus of the presumptive protein contained the basic, hydrophobic, and polar regions that characterize leader sequences (Von Heijne 1985). Sites for N-linked glycosylation were absent. Significant similarities ($z > 15$; Lipman and Pearson 1985) were detected between the Ham34 protein (minus the signal peptide) and a number of extracellular or cell surface structural proteins (for example, salivary glue from *Drosophila melanogaster*, collagen, and herpes simplex surface antigens). This, however, could be an artifact of the high content of threonine and other polar residues in the *Ham34* gene product.

DISCUSSION

We have characterized a discrete group of unusually abundant transcripts that was detected in germinating

spores of *B. lactucae*. The mRNAs studied accounted for more than 20% of the mRNA population in germinating spores and were transcribed from five genes or gene families. These observations are in contrast with previous studies of stage-specific gene expression in the saprophytic fungi that used cascade hybridization and R_{ot} analyses to quantitate the expression of stage-specific transcripts during vegetative growth and differentiation (Blumberg and Lodish 1980; Timberlake 1980). These earlier studies did not indicate the presence of mRNAs at concentrations similar to the abundant transcripts in *B. lactucae*.

Our studies suggest that the transcriptional activities of the germinating spore of *B. lactucae* are focused on a limited number of structural and other proteins necessary for germ tube growth, penetration, and establishment of a primary vesicle in the host. The major features of spore germination in *B. lactucae* are the rapid extension of a germination tube and the hydrolysis of stored lipids. There is little increase in net cytoplasmic volume, and nuclear division does not occur until secondary vesicles are formed within a host (Sargent 1981; Tommerup 1981). Efficient and rapid colonization may be required since *B. lactucae* is an obligate biotroph that can only derive its nutrition from living host tissues, and since the spores of *B. lactucae* are hydrated, metabolically active, and short-lived unlike the dormant spores typical of filamentous ascomycetes and basidiomycetes. The deduced amino acid sequence of the *Ham34*-encoded protein suggests that it may be a structural protein, which is consistent with the type of gene products required in high amounts for infection. In *B. lactucae*, as in other

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1   AGTGGCGATA AGTCGTGTCT TACCGGGTTG GACTCAAGAC GATAGTTACC GGATAAGGCG CACGGTCGGG CTGAACCGGG GGTTCGTGCA
91  CACAGCCCAG CTTGGAGCGA ACGACCTACA CCGAACTGAG ATACCTACAG CGTGCCTCTG ATGGACAAGG GGTCGCCTCT ACGTACCCGC
181 GACAATCTCG GCTTTTCCAA TGTCGACAGC TGGCATATCG CACGCGAGGT TAGGCGTCTT CAGCCGGCGT AACCACAAC CTCGAGTCAA
271 GTACCCTTTT TCAGACCTTA ATTTCCGTGT CCATTCCCTC GCGTTTACAC GAAGGTTTCT GAGAATACCA CATGTCACAG AGAGAGAGAA
361 GATTGGAGAA CCTTCTCAAC CTGACCCGTG CAGAGCGCTT TTGCGTCTTA CCATCCGTTA ATTATAGCAC CACCACCACA TGCTCGCCCG
451 GAACTCTCTT ATCCGCTCTT TTTGATCGTC TTTTTCGCG CCCATTAAGG ACATCTCTTG CACAGGGATC GTTTCTGATT GGCCATTTCA
541 CTTCTCTTTT TACATCCGAA AACCACCTGT TTGTGGGGTT GTGAAGCCAC AACCGGACGC TCAAAAAGCC TCGCCCGACT CGCCACCGAT
631 CGGAAGGCTC ATTCTCCTT TCACCTCTAC GTGCCAAAGT CCCAACCGAC TCTTTTTCGA CTTCACTCT CACCGACAAC AATGAAGTTC
      oo
721 TCCCAGATTC TCGTACTCGC TGCCATTGCG GTGGCTGCCA TTAGCGTCA AGACGCTGCG CCTGCCACGA CGCCAGACAC TGCGACCCGG
      S Q I L V L A A I A V A A I S A Q D A A P A T T P D T A T A
811 ACGACTCCTG CTGCTGCCAC GACCACCAGC ACTACGACAA CACCCGACG CGATGCTGGC ACTGCCAGTA CGGAACAGAC GACCAGTCT
      T T P A A A T T T T T T T P A A D A G T A S T E Q T T T A
901 GGACCAGAGG CGGCTGGGGC GACGAATGGC ACGACTACGA CACCTCCGGC GGATGGTACG CAGACGGCCA CCGCTCCTCT TGATGCAACG
      G P E A A G A T N G T T T T P P A D G T Q T A T A P L D A T
991 GCGACGGAAG AATCTTCCGC ATCGGGAGAA ATGACTCCTA CAGTTGGCAC GGACACGTCC GACCAGGTAT CGGACTCGAC TGCTGCCGGG
      A T E E S S A S G E M T P T V G T D T S D Q V S D S T A A G
1081 CCATCGACAC CAGAGGGGTC TATGACCGGT ACTTCGACGC CCAAGGCCCT GGACAGCAGC TCGTCTGCCA CCGACACGAC AAGCGGTGCT
      P S T P E G S M T G T S T P K A S D S S S S A T D T T S G A
1171 TCGCACACCA CGATGGCCGT TCGGTGCGCG ACGGTATGA CTGTTGGTGC CTACTTCTTG TAAGCCGAAG GTCGAGCTGT GTGCACTGAG
      S H T T M A V A C A T V M T C G A Y F L
1261 TGCGAAGTTG CAGATTTTGT GTCTATGCAT TTAATACAAT TTCTTTTTT CCATTTTCGT TGTTTTTTTT TGTGAAAGAA CGATTGTTTT
1351 CAAAAGAAAG CCGTTTGTAG AACAAATTTT TTTTATACT GCATTTGGTA TTCGAGTGTG ACGAATCCGT GTGGCGCTT TACAATGAA
1441 ATATAACGCT GAACAAGCGC AGTGACAGCT TTATTTTATA ACAGAGAATA AAGACCAAAG TAGAGTAGAC TGATGGCGGC TTGAAACAAA
1531 ACTACGTGAC TTCTAATGCA AAGAACCAT CCCCCACGCT ACTTAGAATT GTTTCTCTT GGTGTAGTAC CGCTATAGTC GTATGGAGTG
1621 ATCCCTTTTT GTCAATATT CGATTCTTGC TAAACCCTG ACTATTTTGA GGCAACTGTG TGACTTAAAC ATGGTAGAAA TTTCTATGTT
1711 TAAGCCAGCT GACAATCATT CTTTATTTAT CGTAAACCAA CCATAACAAT GGCAATGCGC GTGTTCCAAT GTCCGGCAAC AGCTCGCATA
1801 GGGGCTCTA TTGAGGGCTT GT

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Fig. 4. Primary structure of the *Ham34* gene, transcription unit, and amino acid translation of the open reading frame. Indicated are the two transcriptional start points (each underscored with "o"), the two 3' termini of the mRNA (double underlines), and potential "CAAT" (underline) and "TATA"-like (broken underline) elements.

pathogens, the synthesis of proteins required for recognition, penetration, and colonization of the host is likely superimposed upon patterns of gene expression in similar saprophytic species. Abundant transcripts are probably present in many fungi. The previous studies that used R_{ot} analysis may have failed to detect highly abundant transcripts; the R_{ot} approach provides only average mass values for kinetic classes of mRNA that cannot be directly compared to our data. Studies of proteins synthesized during the sporulation of *Botryodiplodia theobromae* Patouillard have identified species of protein that accumulate to high levels (Van Etten *et al.* 1979).

Our results also showed the feasibility of studying the expression of genes during the growth of pathogens *in planta* and thus enable molecular investigations of pathogen and disease development. This is particularly pertinent to obligate biotrophic fungi, such as *B. lactucae*. In contrast to the saprophytic fungi, where numerous studies of stage-specific expression of genes have been performed, our knowledge of gene expression in the plant pathogenic fungi is limited. Several analyses of physiological and morphological changes during differentiation in pathogens have been performed (Freytag *et al.* 1988; Kunoh *et al.* 1988; Wanner *et al.* 1985); however, only limited studies of the expression of genes have been executed (Ruiters *et al.* 1988; Staples *et al.* 1986), and some of these studies involved changes occurring during sexual development rather than during the disease process *per se*.

Very abundant mRNAs in higher eucaryotes are often transcribed from multigene families (Blumenberg 1988; Casey *et al.* 1986). Only one of the five highly expressed mRNA species that we identified in *B. lactucae* was derived from a multigene family. The other mRNA species could have been stable or derived from genes with a highly active promoter. Those transcripts found in both spores and germinating spores could have been synthesized during sporogenesis and could have accumulated in stable RNA-protein complexes until germination, as has been described for RNAs in another of the Oomycetes, *Blastocladiella emersonii* Cantino & Hyatt (Lovett 1975). The *Ham9* and *Ham12* mRNAs, however, must be synthesized *de novo* since they are specific to germination.

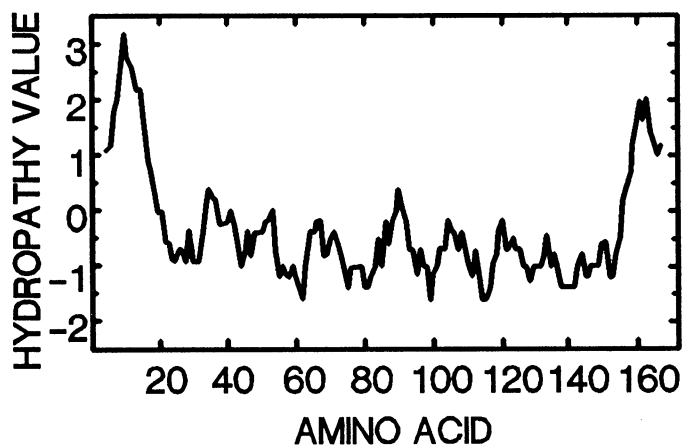


Fig. 5. Hydropathy plot of the deduced Ham34 protein. Profiles were determined using a window of nine amino acids.

A procedure for transformation is being developed for *B. lactucae* as part of our strategy to identify avirulence genes. Most of the fungi in the Oomycotina are currently recalcitrant to transformation. This may be due in part to the lack of transformation vectors that utilize regulatory sequences from this group of fungi to express selectable marker genes. In this study we have identified a single gene, *Ham34*, that is expressed constitutively and at a high level and was therefore a good source of endogenous regulatory sequences. We have consequently fabricated vectors for transformation by fusing promoter and polyadenylation regions of the *Ham34* gene to bacterial genes that confer drug resistance, and we are using them in our transformation experiments.

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