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Sporangium-Specific Gene Expression in the Oomycete Phytopathogen *Phytophthora infestans*

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The oomycete genus *Phytophthora* includes many of the world's most destructive plant pathogens, which are generally disseminated by asexual sporangia. To identify factors relevant to the biology of these propagules, genes induced in sporangia of the potato late blight pathogen *Phytophthora infestans* were isolated using cDNA macroarrays. Of ~1,900 genes known to be expressed in sporangia, 61 were up-regulated >5-fold in sporangia versus hyphae based on the arrays, including 17 that were induced >100-fold. A subset were also activated by starvation and in a nonsporulating mutant. mRNAs of some genes declined in abundance after germination, while others persisted through the germinated zoospore cyst stage. Functions were predicted for about three-quarters of the genes, including potential regulators (protein kinases and phosphatases, transcription factors, and G-protein subunits), transporters, and metabolic enzymes. Predominant among the last were several dehydrogenases, especially a highly expressed sorbitol dehydrogenase that accounted for 3% of the mRNA. Sorbitol dehydrogenase activity also rose during sporulation and several stress treatments, paralleling the expression of the gene. Another interesting metabolic enzyme resembled creatine kinases, which previously were reported only in animals and trypanosomes. These results provide insight into the transcriptional and cellular processes occurring in sporangia and identify potential targets for crop protection strategies.

Sporulation is central to the life cycles of most plant-pathogenic lower eukaryotes, including true fungi and oomycetes. The latter group encompasses important pathogens, such as *Phytophthora*, *Pythium*, and the downy mildews, which infect many economically significant hosts (14). The molecular biology of sporulation has been characterized in detail for several true fungi (1, 11, 28, 43). However, little information exists on oomycetes, which despite their "fungus-like" appearance are more closely related to organisms such as diatoms and brown algae (3).

Asexual sporangia play the major role in spreading *Phytophthora infestans*, which causes late blight of potato and tomato (17). The roles of nutrients, pH, aeration, light, and humidity in sporulation are well described (33, 41). In older portions of plant lesions and in laboratory cultures, sporangia form upon aerial sporangiophores branched from hyphae. In some members of the genus, such as *P. infestans*, this occurs spontaneously as cultures age, but in others significant sporulation requires washing nutrients from media (41). In both cases, nuclear divisions rapidly occur within sporangiophores, and then nuclei and cytoplasm quickly flow into terminal swellings, which develop into sporangia (7, 32). These mature by forming a basal plug, which delimits the multinucleate sporangium from the coenocytic hypha, and an apical papilla, which is a future site of germination.

Two modes of germination are possible in most oomycetes, including *P. infestans.* Indirect germination by way of zoospores predominates at cool temperatures, typically below 12°C, while direct germination is favored at higher temperatures (41). Indirect germination is considered most critical for plant infection. This involves the cleavage of sporangial cytoplasm into six or more zoospores, which swim, encyst, form germ tubes, and develop appressoria on plants or hydrophobic surfaces (20). These are rapid steps, with cytoplasmic cleavage typically visible 30 min after cold induction, zoospore liberation 15 min later, and zoospore encystment occurring seconds after chemical or mechanical stimulation. The factors required for germination and encystment are thought to be preformed, since these stages are not blocked by actinomycin D or cycloheximide (9, 36).

Some themes in sporulation appear to be conserved between oomycetes and true fungi, including the role of starvation as an inducer, the necessity of an aerial environment, a period required for sporulation competence, and the reliance of germination upon preformed RNA and DNA (11, 31, 42). However, most morphological, genetic, and biochemical features are very different. In true fungi, spores (conidia) usually form upon differentiated stalk cells separated from hyphae (sterigmata), while oomycete sporangiophores are contiguous with vegetative hyphae and similar in structure (21). In addition, unlike the desiccated conidia of true fungi, oomycete sporangia remain metabolically and transcriptionally active and are consequently short-lived (35). Acyclic polyols are generally abundant in fungal conidia but not in oomycete sporangia (27, 38). Oomycete sporangia are also distinct in their two modes of germination, although a few true fungi produce zoospores (31, 44).

Studies of sporulation in oomycetes have lagged behind those of true fungi, in part due to the uniqueness and challenges of applying genetic and molecular tools to oomycetes. A few loci that are up-regulated in sporangia have been identified (26, 34), but no comprehensive search for such genes has been reported. Nevertheless, transcripts present in sporangia but not vegetative hyphae of an oomycete such as *P. infestans*

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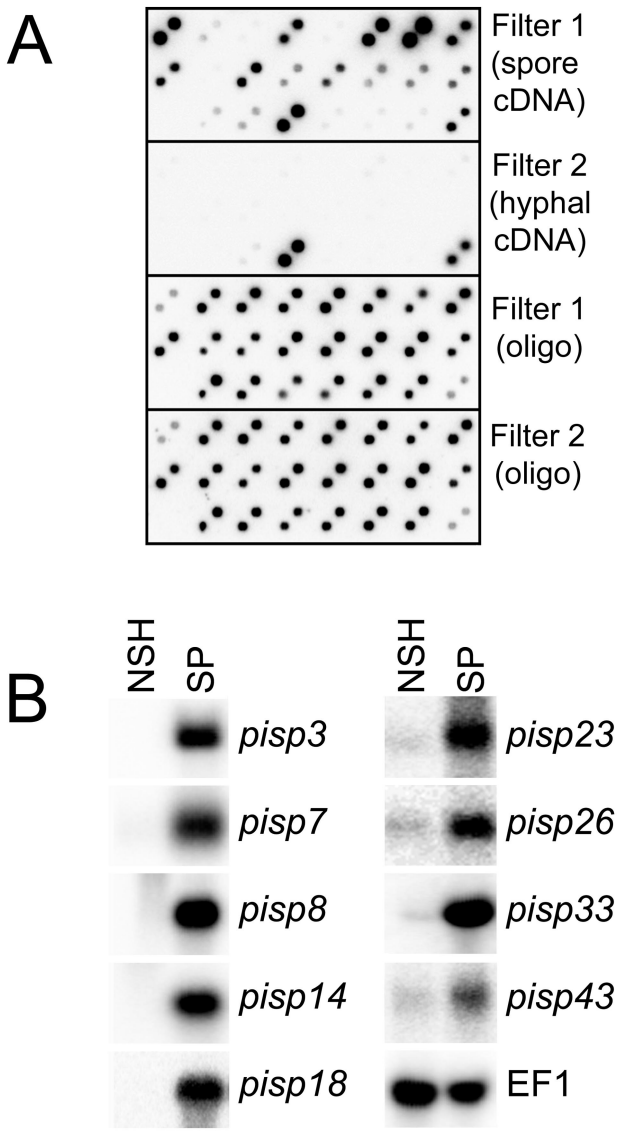


would be predicted to fall into several categories. One class would participate in both early and late stages of sporangium formation, since cytoplasm migrates rapidly into sporangia from sporangiophores and no intervening cell types analogous to sterigmata exist. A second class would help maintain the viability of sporangia and detect signals for germination. Other classes of transcripts would function in forming zoospores and enabling their behaviors, such as chemotaxis and encystment. To identify genes that transact these functions, we performed a cDNA macroarray screen for mRNAs present in sporangia but not hyphae. This resulted in the identification of 61 up-regulated genes and the characterization of their expression during different stages of growth and in a sporulation-defective mutant. One of the most common types of sporulation-induced genes, a putative sorbitol dehydrogenase, was examined in detail.

MATERIALS AND METHODS

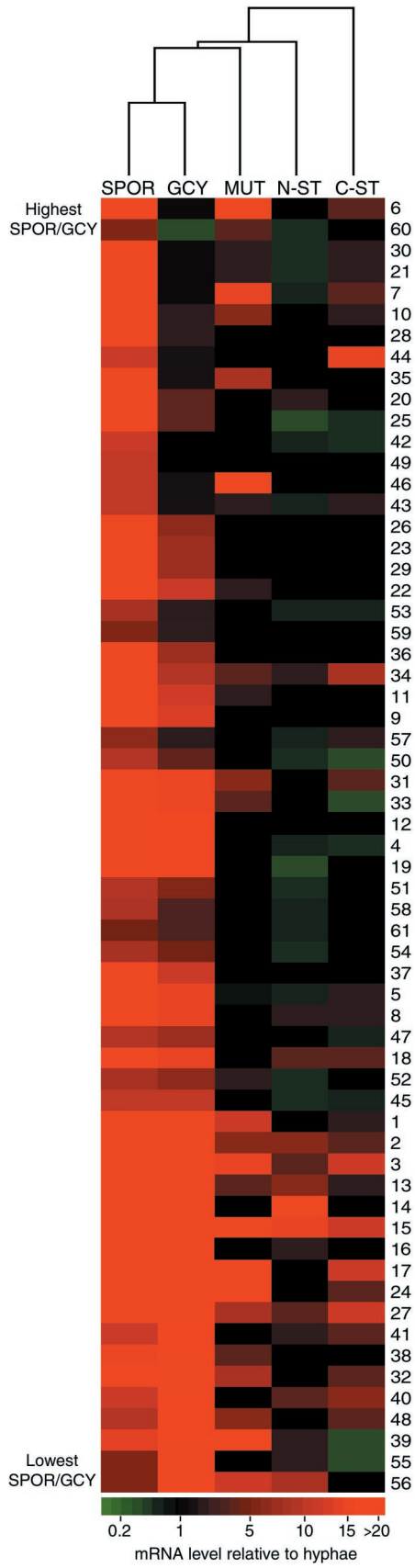
Growth of *P. infestans*. Developmental stages of isolate 88069 were prepared as described previously (24). Briefly, this involved nonsporulating hyphae from 5-day-old liquid rye broth cultures; sporulating hyphae or purified sporangia from 8- to 18-day-old rye agar cultures; zoospores released from sporangia after incubation in 10°C water for 90 min; germinated zoospore cysts, prepared by vortexing zoospores for 1 min followed by 6 h of incubation in rye broth at 18°C; and hyphae from liquid cultures of defined media (52) either lacking glucose but retaining fumaric acid (carbon starvation) or with 1/10 the normal amount of ammonium sulfate (nitrogen starvation).

Also used was the *P. infestans* transformant AF193, which shows a 99% reduction in sporangiophore and sporangium formation. It was generated by transformation of a homology-dependent silencing construct of the *PiCdc14* gene and will be described separately (2).

Enzyme assays. A Polytron homogenizer (Brinkmann, Westbury, N.Y.) was used at speed 7 for 30 s to make extracts, which were clarified at $10 \times g$ for 1 min and assayed at 25°C for alcohol dehydrogenase activity as described previously (50). This involved measuring changes in absorbance at 340 nm due to the reduction of NAD in the presence of 1 M ethanol, xylitol, sorbitol, or glycerol. One unit is defined as the enzyme forming 1 μmol of NADH per min. A Coomassie dye-binding method was used to determine protein concentrations.

cDNA library construction. RNA extracted as described previously (24) from sporangia from rye agar cultures was used to construct a directional cDNA library in the *Not*I and *Sal*I sites of pSPORT1 (Invitrogen, Carlsbad, Calif.). Plasmids were electrotransformed into *Escherichia coli* DH5α, and the transformants were stored in 384-well plates.

FIG. 1. Identifying differentially expressed genes of *P. infestans* using cDNA macroarrays. (A) Representative portions of macroarrays from the secondary screen. Filters containing two spots of each cDNA on the diagonal were prepared and hybridized with ^{32}P-cDNA from sporangia (spore; filter 1) or nonsporulating hyphae (filter 2). The filters were then stripped and rehybridized with radiolabeled SPORT-L (oligo). Not all spots in this illustration represent up-regulated genes, including the lower right-hand spot pair (EF1) and the lower left-hand pair (a control for nonspecific hybridization) in each portion. (B) RNA blot analysis of representative *pisp* genes. RNAs from nonsporulating hyphae (NSH) and purified sporangia (SP) were hybridized with probes for the indicated genes. (C) Relative expression of EF1 and 18S RNA in hyphae and sporangia of *P. infestans*. RNAs from nonsporulating hyphae (5-day-old cultures [NSH]) and from sporangia from cultures 8, 13, and 18 days after inoculation (SP8, SP13, and SP18, respectively) were electrophoresed in the presence of ethidium bromide (EtBr), blotted, and hybridized with probes for EF1 and 18S RNA. Shown at the base of the blot are the ratios of EF1 to 18S, relative to nonsporulating hyphae, as calculated by phosphorimager analysis.



RNA, DNA, and array analyses. cDNA inserts were amplified by two-step PCR using SPORT-L (5′-ACGTCGCATGCACGCGTACGTAAGC) and SPORT-R (5′-ACGACTCACTATAGGGAAAGCTGGTACG), a denaturation temperature of 94°C (30 s), an annealing-extension temperature of 72°C (60 s), and a 35-cycle reaction. A fraction of the reaction products were checked on a gel for purity and concentration. The remainder were spotted on nylon membranes using a slotted 96-pin tool delivering 0.5 μl per pin (V and P Scientific, San Diego, Calif.). Also spotted were cDNAs from actin (*actA*) and elongation factor 1α (EF1) plus controls for nonspecific hybridization. Membranes were treated with 0.4 M NaOH for 10 min and with 3× SSPE (540 mM NaCl, 30 mM $NaHPO_4$, 3 mM EDTA, pH 7.4) for 10 min, UV cross-linked to 1,200 μJ/cm^2, boiled for 1 min in 0.1% sodium dodecyl sulfate, and then hybridized with ^{32}P-labeled cDNA as described previously (15). Primary screens for genes up-regulated in sporangia were performed using replicate filters, with each clone represented once. After removal of the probe by heating the membrane to 95°C, reciprocal hybridizations were performed to confirm the expression patterns. Secondary filters which included two replicates of each clone were then prepared and hybridized in the same manner. To control for the quality of each filter and the amount of DNA per spot, the filters were probed with ^{32}P-labeled SPORT-L, using a hybridization temperature of 45°C and washing conditions of 6× SSPE–0.1% sodium dodecyl sulfate at 40°C.

Blot analysis of RNA and DNA electrophoresed on agarose gels was performed as described previously (24). For RNA blots, EF1 and 18S ribosomal DNA probes were used as loading controls.

Hybridization signals were captured by phosphorimager analysis and analyzed using Quantity One version 4.1 software for Macintosh (Bio-Rad, Richmond, Calif.). For arrays, several exposure times were used to ensure that all signals were captured within the linear range of the phosphorimager, and then circular zones were matched to each spot for quantitation. After correction for background, the data were filtered based on the signal-to-noise ratio (a minimum of 3.0 was deemed acceptable to assume expression), normalized to EF1 or 18S rRNA, and analyzed for significant differences by Student's *t* test. Hierarchical clustering based on average distance (unweighted pair group method with averaging) was performed using EPCLUST (European Bioinformatics Institute [http://ep.ebi.ac.uk/EP/EPCLUST]).

Sequence analysis. Data from automated DNA sequencing were trimmed of vectors and assembled using Seqman software for Macintosh (DNAStar, Madison, Wis.). GenBank searches were performed using a local implementation of BLASTX. Alignments of protein sequences were performed using a version of ClustalW, as described below.

Nucleotide sequence accession numbers. The DNA sequences have been deposited in GenBank under accession numbers CF106689 to CF106749.

RESULTS

Identification of genes up-regulated in asexual sporangia. Sixty-one unique genes expressed at higher levels in sporangia than in nonsporulating hyphae were identified by hybridizing differential probes to cDNA macroarrays. The arrays were made from 5,184 clones from a sporangial cDNA library, some of which had also been used in an expressed sequence tag (EST) project (S. T. Lam, abstract from the *Phytophthora* genomics consortium, Phytopathology **91:**S158, 2001). Se-

FIG. 2. Abundance of *pisp* mRNA based on macroarray data. Arrays were hybridized with radiolabeled cDNAs from nonsporulating hyphae, sporangia (SPOR), germinated cysts (GCY), a nonsporulating mutant (MUT), and nitrogen and carbon-limited cultures of hyphae (N-ST and C-ST). Each column shows the ratio of mRNA abundance in the indicated tissue relative to hyphal grown on rich rye medium, normalized to 18S rRNA and scaled as shown at the base of the figure. Black represents no change, and the maximum ratio shown is 20-fold induction. The 61 *pisp* genes (right) are ranked in decreasing order of their expression in sporangia versus germinated cysts. At the top of the figure is a tree based on hierarchical clustering that portrays the similarity of the results from each probe set.

quence assemblies suggested that these clones represented 1,927 genes.

In a primary screen, 329 of the 5,184 clones appeared to display significant up-regulation in sporangia versus hyphae. These candidates were then subjected to a secondary screen using new filters containing replicate spots; representative portions of the arrays are shown in Fig. 1A. To identify and eliminate culture-to-culture variation, the primary and secondary screens employed cDNA probes from independent cultures of *P. infestans*. After redundant clones revealed by cross-hybridization and comparisons of 5′ and 3′ sequence data were removed, 61 unique up-regulated genes were identified that fit the following criteria: ≥5-fold induction in sporangia versus hyphae as calculated on the final filters, consistent up-regulation in each round of screening (±30%), signal-to-noise ratios of >5.0 in sporangia, and induction that was significant ($P = 0.05$) based on *t* test analysis. The genes were named *pisp* genes (for *P. infestans* sporangia) (Table 1 and Fig. 2).

Of the 61 *pisp* genes, 47 showed >10-fold induction in sporangia based on the arrays, and 17 were induced >100-fold. The induction ratios of the latter are recorded in Table 1 as >100-fold, since accurate ratios usually could not be determined because the hyphal probe yielded a signal near background. RNA blot analysis was performed for nine representative genes, which yielded data in good agreement with the array results (Fig. 1B). These blots employed preparations of RNA separate from those used with the arrays, thus serving as a third biological replicate of the data.

Several strategies for normalizing the array data were tested, resulting in the choice of standardization to 18S rRNA. Previous RNA blot analyses from our laboratory indicated that traditional standards, such as actin and EF1, varied relative to rRNA in sporangia versus hyphae and thus would be inappropriate for normalization (10). Levels of EF1 were lower in sporangia than in hyphae, for example (Fig. 1C). There was also concern about normalizing to the average of all spots, even after excluding outliers, since the array was enriched for genes induced in sporangia. Consequently, a two-step approach that normalized the level of each mRNA to EF1 was employed, which was then corrected for the 18S rRNA/EF1 ratio in each RNA sample used for probe generation. For example, the EF1 level in RNA from sporangia of the 13-day-old culture used as a probe in the secondary screen was 0.53 times that of EF1 in hyphal RNA (Fig. 1C). Other preparations of hyphal and sporangial RNAs yielded similar ratios (0.48 to 0.55).

The abundance of *pisp* mRNAs ranged from 1 to 440% of EF1, with a median of 4% (Table 1). This was determined by comparing the hybridization signals from the sporangial cDNA probe with that of ^{32}P-labeled SPORT-L, which was a primer used to amplify each cDNA insert. This also confirmed that the replicated spots usually contained equal amounts of DNA (±5%); in the few exceptions, the SPORT-L signal was used to make the appropriate correction. Based on the frequency of EF1 in a public database of hyphal ESTs (25), and correcting for the reduction of EF1 mRNA in sporangia versus hyphae, the 61 *pisp* genes accounted for 14% of the mRNA in sporangia. This represents a transcript population with complexity similar to that of conidia of the downy mildew pathogen *Bremia lactucae*, another oomycete. In *B. lactucae*, the 39 most highly expressed genes comprised 20% of the mRNA (23) versus 13% for the top 39 from *P. infestans.*

Gene functions predicted by sequence comparisons. BLASTX analysis revealed matches against GenBank for 53 of the 61 genes, using an *E* value threshold of 10^{-5} (Table 1). This utilized an average of 937 nucleotides (nt) of sequence data per clone, which was obtained by 5′ and 3′ sequencing and primer walking. This relates to a typical full-length *P. infestans* mRNA of ~1,200 nt, as predicted from the public EST database for *Phytophthora* (25).

Classification of the matches revealed hits against proteins involved in a range of functions, including cellular regulation (protein kinases and phosphatases, and transcriptional activators), transport (ion pumps and ABC transporters), and metabolism. The most common group of genes included those for several distinct NADH-dependent ketose reductases (*pisp3*, *-8*, *-9*, and *-17*), which in BLASTX analysis typically matched proteins called xylitol or sorbitol dehydrogenases. One of the latter (*pisp3*) was expressed at particularly high levels, accounting for >300 positives on the primary arrays, and is discussed in detail below.

Three genes from *Phytophthora* had previously been identified. These were α and β G-protein subunits (25) and a *cdc14* cell cycle phosphatase (2). The mucin-like gene (*pisp15*) was distinct from the mucin-like cDNA previously identified in germinated cysts of *P. infestans* (19).

Expression of genes under other conditions. To better address the roles of the *pisp* genes, the arrays were used to measure mRNA levels in germinated zoospore cysts, hyphae undergoing nutrient-limited growth in defined media, and a nonsporulating mutant generated by introducing a homology-dependent silencing construct containing the *cdc14* gene of *P. infestans* (2). In yeasts and metazoans, Cdc14 regulates cytokinesis and the exit from mitosis (51), and we have proposed that the *P. infestans* orthologue regulates nuclear behavior at an early stage of sporulation (2).

One interesting point of differentiation among *pisp* genes was their relative expression in ungerminated sporangia versus germinated zoospore cysts (germlings). This criterion was used to order the genes in Fig. 2, where those at the top have the highest ratio of mRNA in sporangia versus germinated cysts, and those at the base have the lowest. Most genes were expressed at higher levels in sporangia than in germlings (i.e., *pisp6* and *-30*), but others were at similar levels (i.e., *pisp1* and *-2*) or higher in germlings (i.e., *pisp55* and *-56*).

Many *pisp* genes were induced in nonsporulating hyphae grown on media containing limiting amounts of nitrogen or carbon (Fig. 2), although the abundances of their mRNAs were usually lower in starved hyphae than in sporangia. Induction of some *pisp* genes was expected, since starvation is considered a trigger of sporulation in *P. infestans* and since some degree of starvation may be experienced by the sporangia once they become delimited from hyphae. Some genes were induced in both nitrogen- and carbon-limited cultures (i.e., *pic2* and *-3*), while others were induced by only one of the treatments (i.e., *pic14* and *-44*). Differences in genetic response to nitrogen and carbon starvation have also been observed in other species (18). However, while it was interesting to check for overlaps between sporulation- and starvation-induced genes, excessive conclusions about the specific responses of *P. infestans* to the

TABLE 1. *P. infestans* genes upregulated >5-fold in asexual sporangia

Gene	Sporangia vs hypha induction ratio[a]	Relative mRNA abundance[b]	Best BLASTX match[c]	Cellular function[d]	BLAST E
pisp1	>100	4.40	No hit	Unknown	
pisp2	>100	3.25	Hypothetical protein (*C. elegans* CAA98279)	Unknown	2e-10
pisp3	>100	2.80	Sorbitol dehydrogenase (*N. crassa* EAA34038)	Metabolism	1e-99
pisp4	>100	1.82	Protein phosphatase 2C (*C. elegans* NP505702)	Signal transduction	7e-42
pisp5	>100	0.79	No hit	Unknown	
pisp6	>100	0.49	No hit	Unknown	
pisp7	>100	0.46	Hypothetical protein (*C. elegans* T23512)	Unknown	2e-12
pisp8	>100	0.32	Sorbitol dehydrogenase (*N. crassa* EAA34038)	Metabolism	5e-65
pisp9	>100	0.29	Sorbitol dehydrogenase (*Neurospora crassa* EAA34038)	Metabolism	1e-77
pisp10	>100	0.22	Hypothetical protein (*A. thaliana* AAL59988)	Unknown	6e-08
pisp11	>100	0.15	Glucose-inhibited protein A (*Fusobacterium nucleatum* EAA23857)	Unknown	5e-18
pisp12	>100	0.14	Cdc14 cell-cycle phosphatase (*Homo sapiens* NP201569)	Cell cycle	2e-84
pisp13	>100	0.05	Hypothetical protein (*A. thaliana* AF436836)	Unknown	4e-28
pisp14	>100	0.03	Nuclear LIM domain interactor (*Dictyostelium discoideum* AF111941)	Transcription	2e-39
pisp15	>100	0.02	Mucin-like protein (*Heterodera glycines* AF092449)	Cell organization	4e-19
pisp16	>100	0.02	Endo-1,4-beta-glucanase (*Pyrococcus horikoshii* X62582)	Metabolism	2e-15
pisp17	>100	2.61	NADH-ubiquinone oxidoreductase (*Bos taurus* Q02366)	Metabolism	7e-12
pisp18	87 ± 21	0.03	Serine/threonine protein kinase (*H. sapiens* Q9HBY8)	Signal transduction	1e-56
pisp19	83 ± 11	2.35	Hypothetical protein (*H. sapiens AAN37911*)	Unknown	7e-61
pisp20	78 ± 13	0.05	Leucine aminopeptidase (*Vibrio cholerae* B82414)	Protein fate	2e-17
pisp21	74 ± 1	0.36	No hit	Unknown	
pisp22	68 ± 8	0.60	Aldehyde dehydrogenase (*Zea mays* AF467541)	Metabolism	1e-123
pisp23	61 ± 11	0.07	Suppressor of actin mutations (*H. sapiens* AAH16559)	Cell organization	5e-46
pisp24	59 ± 19	0.05	No hit	Unknown	
pisp25	58 ± 6	0.24	Hypothetical protein (*Mus musculus* XP236938)	Unknown	1e-06
pisp26	37 ± 4	0.13	Protein phosphatase 2B (*Paramecium tetraurelia* AAB80918)	Signal transduction	3e-09
pisp27	36 ± 3	0.02	Hypothetical protein (*A. thaliana* NM101737)	Unknown	2e-09
pisp28	36 ± 2	0.03	Diphosphonucleotide phosphatase (*Oryzae japonica* BAB86413)	Metabolism	2e-27
pisp29	35 ± 2	0.14	Cathepsin D (*M. musculus* AF197479)	Protein fate	1e-8
pisp30	34 ± 9	0.01	Homocysteine *S*-methyltransferase (*P. putida* NP744920)	Metabolism	2e-31
pisp31	32 ± 10	0.02	No hit	Unknown	
pisp32	30 ± 8	0.03	Complement receptor (*Papio hamadryas* AAA62170)	Unknown	2e-07
pisp33	28 ± 4	0.06	Suppressor of bimD6 (*H. sapiens* NP0038220	Cell cycle	3e-34
pisp34	26 ± 3	0.18	PEP carboxykinase (*Leptospira interrogans* AAN47450)	Metabolism	e-138
pisp35	25 ± 4	0.50	Elongation factor 3A (*S. cerevisiae* S65245)	Protein synthesis	3-178
pisp36	22 ± 2	0.01	Hypothetical protein (*D. discoideum* AAM34324)	Unknown	5e-15
pisp37	19 ± 2	0.02	Origin recognition complex subunit (*Schizosaccharomyces pombe* U92539)	Cell cycle	7e-08
pisp38	17 ± 1	0.01	Glycoprotein-*N*-acetylgalactosamine galactosyltransferase (*C. elegans* AAG36940)	Protein fate	4e-27
pisp39	14 ± 1	0.01	No hit	Unknown	
pisp40	13 ± 3	0.01	Sexually induced protein 3 (*Thalassiosira weissflogii* AF154501)	Unknown	2e-36
pisp41	12 ± 2	0.01	Creatine kinase (*Ictalurus punctatus* AAO25756)	Energy	2e-94
pisp42	11 ± 1	0.65	Glutathione *S*-transferase (*Xenopus laevis* AAM82563)	Defense	4e-28
pisp43	11 ± 0	0.04	G protein beta subunit (*P. infestans* AY050538)	Signal transduction	2e-77
pisp44	10 ± 2	0.02	Growth arrest-specific 11 (*H. sapiens* AAC69519)	Unknown	2e-31
pisp45	10 ± 0	0.02	Calcium/calmodulin-dependent protein kinase (*X. laevis* BAC19848)	Signal transduction	2e-72
pisp46	10 ± 2	0.04	Calcium-transporting ATPase (*A. thaliana* P92939)	Transport	0
pisp47	10 ± 1	0.01	Methionine aminopeptidase (*H. sapiens* BAA07679)	Protein fate	7e-26
pisp48	9 ± 1	0.08	Mitochodrial succinate-fumarate carrier (*A. thaliana* NP195754)	Energy	2e-68
pisp49	9 ± 2	0.01	ABC transporter (*N. crassa* EAA36439)	Transport	3e-33
pisp50	9 ± 0	0.04	Nodulin MtN3 family protein (*A. thaliana* BAA97235)	Unknown	8e-21
pisp51	9 ± 2	0.02	Vacuolar proton-translocating ATPase (*D. discoideum* AAO51473)	Transport	7e-84
pisp52	8 ± 2	0.02	Na^+ K^+ ATPase alpha subunit (*Callinectes sapidus* AF327439)	Transport	1e-33
pisp53	8 ± 2	0.03	Hypothetical protein (*A. thaliana* AAM62828)	Unknown	2e-12
pisp54	8 ± 1	0.01	G protein alpha subunit (*P. infestans* AY050536)	Signal transduction	2e-91
pisp55	7 ± 0	0.02	Phosphoribosylglycinamide synthetase (*S. cerevisiae* X04337)	Metabolism	2e-42
pisp56	7 ± 1	0.02	Mitochondrial ribosomal protein S13 (*P. infestans* U17009)	Protein synthesis	2e-66
pisp57	6 ± 1	0.14	Adenosine kinase (*Anopheles gambiae* EAA02798)	Metabolism	6e-44
pisp58	6 ± 0	0.01	Hypothetical protein (*Oryza sativa* BAA83585)	Unknown	3e-40
pisp59	6 ± 1	0.01	No hit	Unknown	
pisp60	5 ± 1	0.01	Ribosomal protein S9 (*A. thaliana* NP198801)	Protein synthesis	4e-69
pisp61	5 ± 1	0.01	Actin-depolymerizing factor (*A. thaliana* NP194289)	Cell organization	5e-19

[a] Hybridization signal with sporangia-cDNA probe divided by hyphal signal, normalized by a two-step procedure to 18S rRNA. This involved normalization to internal EF1 controls, followed by correction for differences in 18S and EF1 signals as determined from Northern blot analysis of RNAs from three independent preparations of hyphae and sporangia. Accumulated error (E_a) in the induction ratio (R) was calculated from error (e) in measurements from hyphal (h) and sporangial (s) cDNA hybridizations as follows: $E_a = R \times \sqrt{([e_h/h]^2 + [e_s/s]^2)}$.

[b] Compared to EF1 mRNA.

[c] Only matches with an E of $<10^{-5}$ are shown.

[d] Using functional categories from Munich Information Center for Protein Studies (http://mips.gsf.de/proj/yeast/catalogues/funcat/).

carbon- and nitrogen-limited media should not be drawn from this study. A proper analysis of starvation responses should include additional nutrient manipulations and a less biased set of genes.

The nonsporulating mutant (Fig. 2) provided further information about the staging of gene expression during spore development. This strain was grown under conditions that matched those of the wild-type cultures used to harvest sporangia, except that the mutant produced few if any sporangia or sporangiophores. Despite this sporulation defect, 16 of the *pisp* genes still were induced >5-fold.

Sorbitol dehydrogenase-like proteins are the most highly expressed. Of several putative dehydrogenases induced during sporulation, the most abundant was *pisp3*; its RNA rose in abundance >100-fold upon the formation of sporangia and was 2.8 times more plentiful than that of EF1. In BLASTX analyses, *pisp3* best matched a known xylitol dehydrogenase from *Candida albicans* and a putative sorbitol dehydrogenase from *Neurospora crassa* (both *E* values equaled 10^{-99}). These are members of a family of dimeric, zinc-containing NAD-dependent ketose reductases (EC 1.1.1.14) that are typically ~360 amino acids in size. Such enzymes generally have broad substrate specificity, especially against sugar alcohols. Hence, a protein called a xylitol dehydrogenase is also a sorbitol, mannitol, or glycerol dehydrogenase (29). For simplicity, they will henceforth be described as sorbitol dehydrogenases.

In consideration of their high frequency of detection, the sorbitol dehydrogenases from *P. infestans*, and in particular *pisp3*, were examined in more detail. Low-stringency hybridizations of a full-length cDNA clone of *pisp3* against genomic DNA digested with *Bam*HI, *Eco*RI, *Kpn*I, *Sal*I, *Xba*I, and *Xho*I (Fig. 3) revealed that it was a member of a small family, since three or four bands were detected in each digest; *pisp3* does not contain sites for any of these enzymes. Sequence comparisons of *pisp3* and the other sorbitol dehydrogenases (*pisp8*, *-9*, and *-17*) indicated that the former lacked significant similarity to the others (<50% DNA identity), and consequently, the others did not account for the weak bands seen in Fig. 3. *pisp8* and *pisp9* appeared to be members of the same family, however.

The weak bands related to *pisp3* appeared to represent pseudogenes. Sequencing of >300 *pisp3*-related cDNA clones from the sporangium library suggested that each cDNA was derived from a single highly expressed locus, since only three sequence differences were detected within the 1,167-nt full-length cDNA. All polymorphisms were C-to-T changes, which probably represent differences between alleles of a single-copy gene. Nevertheless, it is possible that the weak bands represent loci transcribed at an untested developmental stage.

Structure and expression of *pisp3*. A comparison of the *pisp3* cDNA to genomic clones showed that the 1,178 transcription units contained 45- and 46-nt 5′ and 3′ untranslated regions, a 1,167-nt open reading frame, and no introns. The predicted 389-amino-acid gene product aligned closely with other members of the sorbitol dehydrogenase family (Fig. 4). These included residues shown by X-ray crystallography to contact the NAD and zinc cofactors (L and M, respectively [Fig. 4]) (12). A phylogenetic analysis of the *P. infestans* protein and orthologues from animals, plants, true fungi, and bacteria revealed the highest affinity to proteins from true fungi (Fig. 5). This was surprising, since previous analyses of *Phytophthora* genes usually revealed better affinity to plant genes, although there were exceptions (49).

RNA blot analysis confirmed that *pisp3* mRNA was absent from nonsporulating hyphae (Fig. 6A) but was expressed in sporulating hyphae, purified sporangia, and zoospores. Greater-than-10-fold reductions in mRNA abundance occurred once hyphae began to reform, after 16 h of direct germination of sporangia (Fig. 6B) or 6 h of germination of zoospore cysts.

As predicted by the arrays, the gene was also induced in nonsporulating cultures by starvation (Fig. 6C). Growing *P. infestans* at 26°C, which appears to cause heat stress, since growth is reduced 30% relative to that at 21°C, also induced the gene (Fig. 6B). While the starvation conditions eventually led to sporulation 3 days after tissue was harvested for RNA extraction, the heat-stressed cultures did not sporulate.

The pattern of expression in planta also indicated that *pisp3* expression was associated with sporulation (Fig. 6A). No *pisp3* RNA was detected in tomato leaflets 4 days after inoculation with zoospores, at which time all *P. infestans* organisms were within the plant and sporulation had not yet occurred. EF1 expression was easily observed in the 4-day sample, indicating that substantial pathogen RNA was present. At 7 days, when

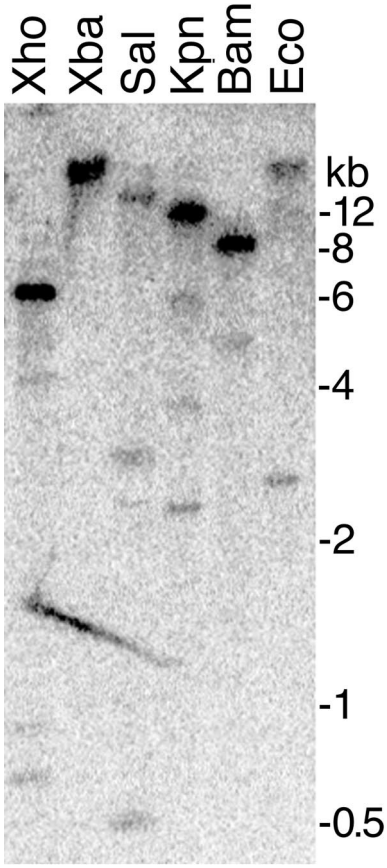


FIG. 3. DNA blot analysis of *pisp3*. Genomic DNA of *P. infestans* was digested with *Xho*I, *Xba*I, *Sal*I, *Kpn*I, *Bam*HI, or *Eco*RI and hybridized with a full-length *pisp3* cDNA. Washes were performed at low stringency (melting temperature, −20°C). Size markers are shown on the right.

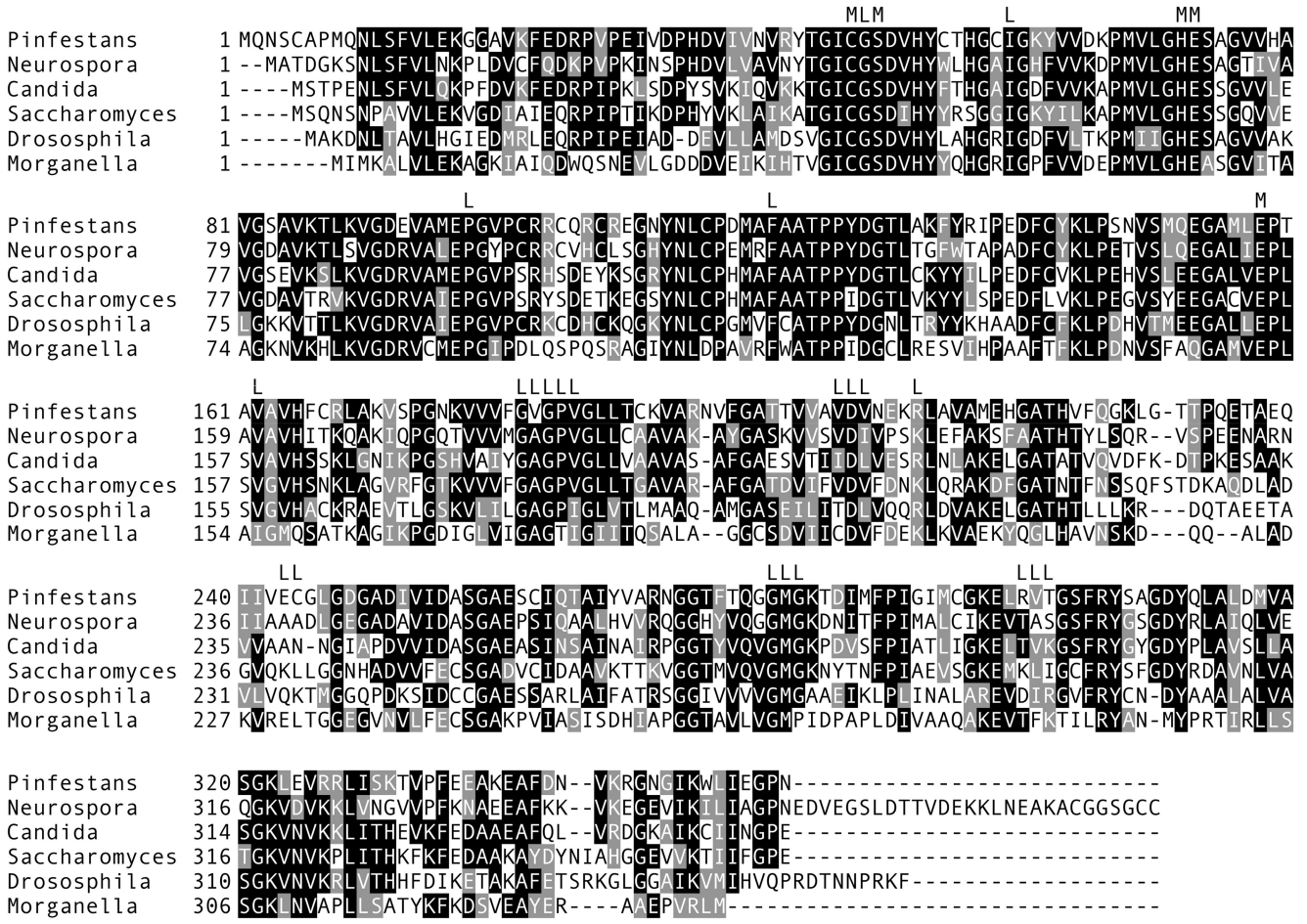


FIG. 4. Alignment of sorbitol dehydrogenase proteins. Shown are the *P. infestans* protein (from *pisp3*) and proteins from *N. crassa* (GenBank accession no. NCU00891), *C. albicans* (AAC24597), *S. cerevisiae* (NP0100035), *Drosophila melanogaster* (AAD00902), and the bacterium *Morganella morganii* (AAA25324.1). Indicated at the top of the alignment are regions in contact with metal ions (M) or the NAD ligand (L). The solid squares represent blocks of identity, and the shaded squares represent blocks of similar amino acids.

the leaflets showed profuse sporulation, *pisp3* mRNA was clearly detected (despite the underloading of RNA in the sample).

Sorbitol dehydrogenase activity parallels *pisp3* expression. The enzymatic activities of sorbitol dehydrogenases during the growth and development of *P. infestans* paralleled the abundance of *pisp3* mRNA and that of its relatives. In nonsporulating hyphae, very little activity was detected (Fig. 7). Activity rose greatly in sporulating hyphae and in purified sporangia, peaking at levels between 3 and 5 U per pg of protein. Enzyme levels also rose in response to nutrient stress. This included growth in defined minimal medium, in which growth is reduced one-third compared to rye medium, and the defined media in which nitrogen and carbon sources were reduced to levels that retarded growth by an extra one-third relative to that in rye medium. Minimal medium appeared sufficient to induce the gene to near-maximal levels.

The enzyme assays also showed that the predominant activity in extracts of sporangia exhibited the same biochemical features described for other members of the sorbitol dehydrogenase family (29). This included optimal activity at alkaline pH (pH 9 to 10), a preference for NAD as opposed to NADP as a cofactor, and broad substrate specificity. The induced enzyme displayed similar activities when sorbitol, xylitol, sorbitol, or glycerol was used as a substrate.

DISCUSSION

This study presents the first large-scale screen for genes induced in the asexual sporangia of oomycetes, which is a critical phase in their life and disease cycles. Of the estimated 1,927 genes examined, 3% displayed >5-fold increases in mRNA abundance in sporangia, accounting for 14% of the mRNA, and many appeared to be specific for sporangia. This can be compared to studies of sporogenesis in true fungi, even though the processes are not biologically equivalent. In *Saccharomyces cerevisiae*, 7.7% of all genes were up-regulated during sporulation, with an average of 1.5% induced >5-fold at any given time (8). In *N. crassa*, 3% of genes were induced by blue light, which stimulates conidiation (28). The proportion of *P. infestans* genes that change in expression is certainly higher than the 3% value reported in this study, however. Our focus was on genes showing substantial increases, not decreases, in mRNA abundance in sporangia compared to that in

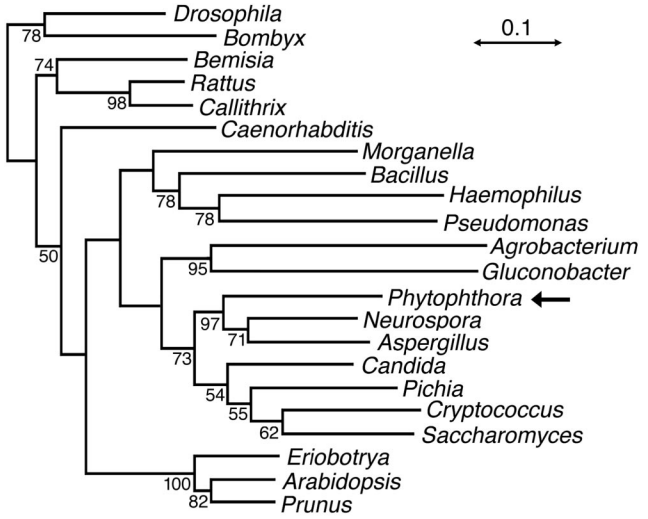


FIG. 5. Neighbor-joining tree of NAD-dependent sorbitol dehydrogenases. An alignment was developed using a version of ClustalW as implemented in the VectorNTI Suite for Macintosh (Informax, Bethesda, Md.), which was exported to Phylip version 3.57c for Macintosh (16). Bootstrap replicates were generated using SEQBOOT, distances were determined using the PAM option of PROTDIST, neighbor-joining trees were developed using NEIGHBOR, and a consensus tree was made with CONSENSE. The numbers at the nodes indicate the percentages of occurrence in 500 replicates, and the scale represents 0.1 PAM units. In addition to the proteins shown in Fig. 3, others were from *Bombyx mori* (BAA02634), *Bemisia argentifolii* (AAD02817), *Rattus norvegicus* (S38363), *Callithrix* sp. (AAB69288), *Caenorhabditis elegans* (CAA94841), *Bacillus subtilis* (AAA22508), *Haemophilus influenzae* (Q57517), *Pseudomonas putida* (AAB58982), *Agrobacterium tumefaciens* (NP357437), *Gluconobacter suboxydans* (AAE66066), *Aspergillus fumigatis* (The Institute for Genomic Research contig 4937), *Pichia stipitis* (P22144), *Cryptococcus neoformans* (from the Stanford Genome Technology Center *C. neoformans* Genome Project, contig cneo011005.C1135, and The Institute for Genomic Research *C. neoformans* EST k4g08j2.r1), and the plants *Eriobotrya japonica* (BAA95897), *Arabidopsis thaliana* (BAB11045), and *Prunus persica* (BAA94084).

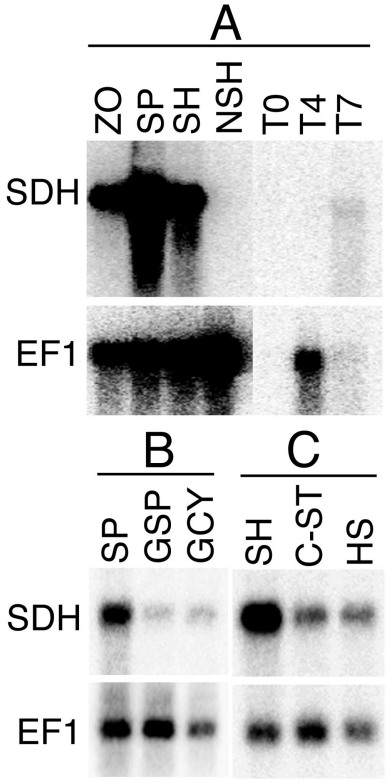


FIG. 6. RNA blot analysis of *pisp3.* Total RNA was electrophoresed and hybridized with a probe for *pisp3* or EF1. (A) RNAs from zoospores (ZO), sporangia (SP), sporulating 10-day-old hyphae (SH), nonsporulating hyphae from a 5-day broth culture (NSH), uninfected tomato leaflets (T0), and tomato 4 or 7 days after inoculation with *P. infestans* zoospores (T4 and T7). (B) RNAs from sporangia (SP), directly germinated sporangia (GSP), and germinated cysts from zoospores (GCY). (C) RNAs from sporulating hyphae (SH), carbon-starved hyphae (C-ST), and heat-stressed hyphae (HS).

hyphae, even though subtle increases or reductions can also be biologically relevant. Genes declining in mRNA abundance were uncommon in the data; however, these would have been underrepresented in the arrays due to the use of cDNAs from sporangia.

The nature of sporangial development in *P. infestans* facilitated some aspects of our experiments but may have limited the genes identified. *P. infestans*, unlike many members of the genus, produces abundant sporangia in culture without requiring starvation treatments. Its sporangia are also decidious, i.e., detachable from sporangiophores. This aided our comparisons of hyphae and sporangia, but genes transiently induced at the onset of sporulation may not have been detected if their mRNAs failed to persist in sporangia. *P. infestans* does not represent a good system for synchronously inducing sporulation, at least in laboratory culture (32). Better synchrony is observed in species like *Phytophthora cinnamomi*, in which starvation induces sporangia, although they are not deciduous (34). There may be value in studying the expression of the *pisp* genes in such species.

Previous observations of the biology of oomycete sporangia can be connected to the predicted functions of many *pisp*

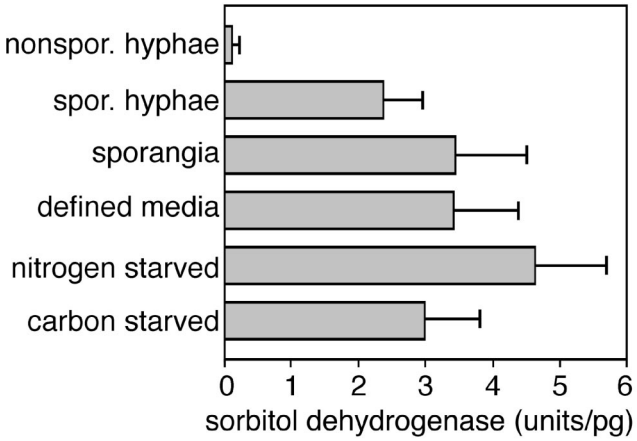


FIG. 7. Sorbitol dehydrogenase activity in *P. infestans.* Assays were performed using 1 M sorbitol and tissue extracts from nonsporulating (nonspor.) hyphae, sporulating (spor.) hyphae, purified sporangia, defined media, and defined media with reduced levels of nitrogen (nitrogen starved) and carbon (carbon starved). Activity is expressed as the number of micromoles of sorbitol oxidized per minute per picogram of protein. The data are averages (plus standard deviations) of assays performed on at least two replicate tissue samples made on different days.

genes. Several genes participate in calcium signaling, which regulates zoosporogenesis and encystment (20). These include *pisp45*, which encodes a putative calcium-regulated protein kinase, and *pisp46*, which encodes a calcium pump. As purines were shown also to regulate germination (9), it was interesting to find that *pisp55* encodes phosphoribosylglycinamide synthetase, an enzyme in the purine biosynthetic pathway, and that *pic57* encodes adenosine kinase, part of the purine salvage pathway.

Many *pisp* genes were also induced by nutrient limitation. This may reflect the stimulatory effect of starvation on sporulation (41) or a shift to the utilization of nutrient reserves once sporangia form. However, only a subset of genes induced in the nutrient-limited cultures were expressed at significant levels in the nonsporulating mutant, and vice versa. Sporulation is clearly not determined by a simple linear dependent pathway activated by starvation.

After sporulation, the uptake of nutrients does not appear to be possible until after zoospores encyst and germinate (36). Until germinated spores contact laboratory media or establish a feeding relationship with a plant, stored lipids and a β-1,3-glucan called mycolaminarin are used for energy (21). *pisp34* and *pisp48* may help mobilize carbon stored in the lipids through the citric acid cycle. *pisp48* encodes an enzyme involved in transporting citric acid cycle intermediates across the mitochondrial membrane, while *pisp34* encodes phosphenolpyruvate carboxykinase, which converts oxaloacetate from the citric acid cycle to phosphoenolpyruvate. Interestingly, phosphoenolpyruvate metabolism was recently suggested to play a role in sporulation in *P. cinnamomi* (34).

Three other sporulation-induced genes with potential roles in catabolism are *pisp20*, *-29*, and *-47*, which encode proteases. Only *pisp20* was induced by starvation, however. Such proteases may cycle proteins during transitions between developmental stages. This would provide a source of amino acids during germination, as suggested for some true fungi (48). Alternatively, these proteases may be formed in anticipation of a role in plant penetration.

One intriguing group of genes induced by both sporulation and starvation, such as *pisp3*, encoded sorbitol hydrogenases. These are typically broad-spectrum reversible oxidoreductases, in which conversions, such as mannitol to mannose or sorbitol to fructose, are coupled to NAD reduction (29). In true fungi, polyols, such as mannitol, arabitol, and glycerol, accumulate and exhibit multiple cellular functions. These include maintaining osmotic balance (40), generating turgor in appressoria (47), and quenching reactive oxygen generated within stressed fungal tissue or by plants during defense reactions (6, 22). Sugar alcohols may form in plant-pathogenic fungi when their β-fructosidases transform plant sucrose to fructose, which is converted to sorbitol by sorbitol dehydrogenase; biosynthesis rather than direct uptake is more likely for colonizers of the majority of plants, including potato and tomato, which produce little of these compounds (45).

Assessing the role of the sorbitol dehydrogenases in *Phytophthora* is challenging, since oomycetes were reported in several studies not to accumulate any sugar alcohols. This is a classic criterion used to demonstrate that oomycetes are unrelated to true fungi (4, 30, 38, 39). However, a recent paper reported that *P. infestans* spores contain arabitol (46). This was at only 7% of the level of glucose, which seems too low to explain why genes like *pisp3* account for 5% of the mRNA in sporangia. Consequently, alternative roles for the enzyme must be considered, such as whether it acts on an atypical substrate. Alternatively, the enzyme may shuttle carbon from glucose to fructose, which through the action of aldehyde oxidoreductase (*pisp22*?), ketohexokinase, and fructose-bisphosphate aldolase would produce dihydroxyacetone phosphate for entry midway through the glycolytic pathway or for lipid biosynthesis. The difficulty in detecting sugar alcohols might be explained if flux through this pathway is rapid.

A final *pisp* gene worthy of special comment also appears to play an intriguing role in *P. infestans* metabolism. *pisp41*, which was also induced during starvation, exhibited high similarity in database searches to genes for phosphagen kinases, with *E* values in the 10^{-90} range. Such enzymes transfer phosphate to guanidino group-containing compounds, such as creatine and arginine (13). Creatine kinase in muscles, for example, catalyzes the transfer of a phosphoryl group from phosphocreatine to ADP to form creatine and ATP. This buffers ATP levels in cell types with high energy demands, a class into which zoospores would certainly fall. However, with one exception, this type of pathway has been found only in animals, not in plants or true fungi (5). The exception is interesting: *Trypanosoma cruzi*, a unicellular parasite that contains flagella, like *Phytophthora* (37). Addressing the function of this putative phosphogen kinase, as well as the sorbitol dehydrogenases, will require integrating genetics and biochemistry into future studies. Until then, these enzymes will be considered orphans with uncertain substrates and roles in sporulation.

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