Mol Genet Genomics (2009) 281:193–206
DOI 10.1007/s00438-008-0407-5

ORIGINAL PAPER

Gene expression changes during asexual sporulation by the late blight agent *Phytophthora infestans* occur in discrete temporal stages

Howard S. Judelson · Reena D. Narayan · Audrey M. V. Ah-Fong · Kyoung Su Kim

Received: 22 September 2008 / Accepted: 15 November 2008 / Published online: 3 December 2008

Abstract Transcriptional changes during asexual sporangia formation by the late blight pathogen *Phytophthora infestans* were identified using microarrays representing 15,646 genes and RNA from sporulation time-courses, purified spores, and sporulation-defective strains. Results were confirmed by reverse transcription-polymerase chain reaction analyses of sporulation on artificial media and infected tomato. During sporulation, about 12% of genes were up-regulated compared to vegetative hyphae and 5% were down-regulated. The most prevalent induced genes had functions in signal transduction, flagella assembly, cellular organization, metabolism, and molecular or vesicular transport. Distinct patterns of expression were discerned based on the kinetics of mRNA induction and their persistence in sporangia. For example, most flagella-associated transcripts were induced very early in sporulation and maintained in sporangia, while many participants in metabolism or small molecule transport were also induced early but had low levels in sporangia. Data from this study are a resource for understanding sporogenesis, which is critical to the pathogenic success of *P. infestans* and other oomycetes.

Microarray data reported in this paper are deposited at NCBI GEO as experiment series GSE13580.

Communicated by J. Perez-Martin.

H. S. Judelson (✉) · R. D. Narayan · A. M. V. Ah-Fong · K. S. Kim
Department of Plant Pathology and Microbiology, University of California, Riverside, CA 92521, USA
e-mail: howard.judelson@ucr.edu

Keywords Developmental biology · Late blight · Microarray · Oomycete pathogen · Sporulation · Transcriptome · Zoospore

Introduction

The eukaryotic microbes known as oomycetes cause myriad destructive diseases of plants, insects, and animals. They are most infamous for their effects on plants, as they are considered to represent the most significant pathogens of dicots. *Phytophthora infestans,* for example, limits the production of potato and tomato worldwide (Fry 2008). *Phytophthora* and other plant-colonizing oomycetes such as *Albugo*, *Pythium*, and downy mildews exhibit patterns of growth and pathogenicity that resemble those of many true fungi, at least superficially. However, oomycetes have little taxonomic affinity to fungi, and instead belong to the kingdom Stramenopila along with organisms such as brown algae and diatoms (Adl et al. 2005).

The spatial dissemination of most oomycetes relies to a large degree on the production of copious amounts of asexual sporangia. Relatively little is known of the molecular biology of how these form or germinate, although a few participant genes have been studied (Ah Fong and Judelson 2003; Latijnhouwers et al. 2004; Walker et al. 2008). Sporangia develop upon the termini of specialized aerial hyphae, provided that humidity and oxygen levels are high. Most species must grow for several days before sporulation can initiate, indicating that "competence" as well as environmental inputs are required as in many true fungi (Axelrod et al. 1973). However, there are fundamental cellular and molecular differences between oomycete and fungal spores (Hardham and Hyde 1997; Judelson and Blanco 2005). Oomycete hyphae are aseptate, with hyphal

cytoplasm flowing into sporangiophores and then sporangia without delimitation by specialized intermediary cells such as the foot cells or phialides of true fungi. Indeed, young oomycete sporangiophores are generally indistinguishable from vegetative hyphae until the incipient sporangia appear as terminal or intercalary bulbous swellings. A burst of nuclear division, cytoplasmic expansion, and organelle synthesis helps to fill the developing sporangia, which in most species are multinucleate (Marks 1965; Maltese et al. 1995). Only relatively late in development, when a plug forms at the base of each sporangium, do these become partitioned from the vegetative thallus.

Oomycete spores are fully hydrated and metabolically active at maturation, unlike most fungi where desiccation is part of normal development. This facilitates active sensing of the environment, which is needed to regulate germination. In most phytopathogenic oomycetes this occurs through the direct extension of germ tubes or by zoosporogenesis. The latter is stimulated by cool and moist conditions, and is important since it extends the range in which infections occur. Zoosporogenesis involves cleavage of the multinucleate sporangial cytoplasm by nucleus-enveloping membranes, assembly of flagella, dissolution of the sporangial papilla, and expulsion of uninucleate zoospores (Hardham 2001). In response to chemical signals, zoospores swim towards potential hosts and then form walled cysts, which germinate to form appressoria for breaching plant cells (Deacon and Donaldson 1993; Hardham 2001). The proteins needed for zoosporogenesis and encystment may be preformed within mature sporangia (Clark et al. 1978; Penington et al. 1989).

Phytophthora infestans has emerged as a model for studying oomycete spores. Unlike some oomycetes, sporulation occurs profusely without requiring cultural manipulations such as starvation (Ribeiro 1983). Also, *P. infestans* sporangia are deciduous and thus separable from hyphae, which facilitates molecular analysis. In a recent study, microarrays representing the majority of *P. infestans* genes were employed to characterize the mRNA content of hyphae, mature sporangia, zoospores, and germinated cysts (Judelson et al. 2008). While this identified many potential participants in spore biology, much information about early steps in sporulation were likely missed since stages intermediate to hyphae and mature sporangia were not characterized. To fill this gap, here we report new data on changes to the transcriptome during sporulation by also analyzing sporulating hyphae, sporulation time-courses performed on artificial media and *in planta,* and a mutant blocked early in sporulation. This work identifies several hundred new sporulation-induced genes and whether they are induced at early or late stages of development. Therefore, this study establishes a basis for detailed examinations of specific genes, molecular dissection of the pathways regulating sporulation, and identification of targets for crop protection strategies.

Materials and methods

Growth conditions and RNA extractions

Isolates were cultured on nonclarified rye-sucrose media containing 40 U/ml nystatin at 18°C in the dark. Strains employed included isolates 88069 and 1306, and derivatives of 1306 obtained by silencing the *Cdc14* gene (Ah Fong and Judelson 2003). Nonsporulating hyphae were obtained by inoculating 25 ml of rye-sucrose broth (clarified by centrifugation) in a 100-mm plastic petri dish with 10^3 sporangia. Alternatively, sporangia were spread on a 0.4-µm pore polycarbonate membrane laid on the surface of rye-sucrose media containing 1.5% agar. After about 72–96 h, tissue from such cultures was examined to ensure that new sporangia had not yet formed, prior to harvesting. Sporulating hyphae were obtained from 10-day cultures grown on polycarbonate membranes, using a rubber policeman to recover the entire hyphal mat. Sporangia were also purified from such cultures by adding 10-ml of water to each plate, rubbing with a bent glass rod, and passing the resulting fluid through a 50-µm mesh to remove hyphal fragments; the sporangia were then pelleted by centrifugation for 5 min and frozen in liquid nitrogen as rapidly as possible. To induce sporulation in liquid-grown hyphal mats, these were removed from the broth using forceps, spread on 1.5% agar, and incubated for the times stated in "Results"; for gene expression studies the agar contained rye-sucrose media, while for studying potential effects of starvation on sporulation only water agar was used and the mats were briefly rinsed in water prior to placement on the agar.

Tomato inoculations were performed by dipping leaflets of cultivar New Yorker in a suspension of zoospores obtained by incubating sporangia at 10^4/ml in water at 10°C for 2 h. Leaflets were placed adaxial-side up in sealed plastic boxes containing water-saturated blotting paper. The boxes were then incubated at 16°C using a 12 h day–12 h night cycle, with leaflets located 10 cm below a Sylvania Gro-Lux F20T12 lamp.

Before harvesting tissues destined for RNA extraction, they were examined microscopically to either verify the presence or absence of sporangia, depending on the desired type of tissue. To quantitate sporulation, a minimum of three measured blocks (typically 4 cm^2 each) were excised from culture plates or leaflets, and added to water. These were then vortexed to release sporangia, which were counted using a hemacytometer. RNA was obtained after grinding in liquid nitrogen using the RNAEasy Plant Mini kit (Qiagen, Valencia, CA, USA). RNA quality was assessed spectrophotometrically and by gel electrophoresis.

Microarray hybridizations and data analysis

These were performed as described using custom Affymetrix GeneChips assembled from 76,000 ESTs and sequences of high coding potential from a 1× genome skim (Randall et al. 2005). The GeneChips were generously provided by Syngenta, but are no longer available due to a limited production run. They were designed to detect 15,646 unigenes; by comparison, about 18,155 genes are currently predicted from the draft sequence of the *P. infestans* genome developed by the Broad Institute of MIT and Harvard (http://www.broad.mit.edu). GeneChip data were preprocessed using Affymetrix MAS 5.1 software, and reanalyzed using GeneSpring GX (Agilent Technologies, Santa Clara, CA, USA) after further background correction, 50th percentile normalization, and per-gene normalization. Genes showing differential expression were identified by filtering for fold-changes, using replicates to establish a $P < 0.05$ threshold based on a t test. Also, only genes for which "present" flags were called in one of the treatments were considered to be differentially expressed. Hierarchical clustering was performed using the Pearson correlation method with average linkage. Annotation was performed by matching unigene sequences (which are often not full length) with the corresponding putative full-length proteins predicted by the Broad Institute. These were then compared with GenBank using BLASTP.

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

RNA was treated with DNAse, reverse-transcribed using oligo-dT with a first-strand synthesis kit from Invitrogen (Carlsbad, CA, USA), and subjected to PCR in an iCycler (Bio-Rad, Richmond, CA, USA). Hot-start *Taq* polymerase (Roche, Indianapolis, IN, USA) was used in amplifications, with the intercalation of SYBR Green as a reporter for amplification. Reactions were performed in duplicate using the following conditions: one cycle of 95°C for 8 min, and 35 cycles of 95°C for 20 s, 55°C for 20 s, and 72°C for 30 s. cDNA concentrations were normalized based on primers for the constitutively expressed gene encoding ribosomal protein S3a (Judelson et al. 2008). Relative expression levels were determined by the $\Delta\Delta C_T$ method assuming a PCR efficiency of 1.9. Controls lacking reverse transcriptase were also performed, and after PCR the end-point products were checked to confirm amplification of the desired bands.

Radioactive hybridization

Dot-blot and RNA blot hybridizations were performed as described (Kim and Judelson 2003). For dot-blot studies, two nylon membranes were each spotted with purified PCR products representing different genes. These were hybridized with ^{32}P-labeled cDNA prepared from sporangia from 8- to 13-day cultures of isolate 1306. Each filter contained two spots per gene, and each filter was sequentially hybridized with each probe, resulting in four data points per gene per probe. Signal intensities were determined by phosphorimager analysis and normalized to spots from elongation factor-1α (EF-1).

Results

Culture conditions for studying asexual sporulation

Several approaches were tested for obtaining biological material for examining gene expression during sporulation. Cultures grown on rye-sucrose agar sporulated well, but infiltration of the agar by hyphae impaired the harvesting of tissue. A more useful strategy involved growth on polycarbonate membranes laid upon the rye-sucrose agar, as hyphae and sporangia were easily recoverable from the filters. In both types of cultures, however, sporulation was asynchronous. Even when uniformly distributed sporangia were used as inoculum, sporulation typically began at day 6 and continued through day 11.

More synchronous samples were obtained by transferring submerged hyphal mats from rye-sucrose broth to the surface of rye-sucrose agar. As in many true fungi, sporulation by *P. infestans* is blocked in submerged culture. However, sporangia began to appear 6 h after transferring the mats to the aerial environment on the agar (Fig. 1a). Sporulation was semi-synchronous, occurring over 48 h, and not enhanced by starvation as similar kinetics were observed in hyphae shifted to rye-sucrose and water agar.

Based on these findings, several tissues were employed for transcriptome studies. Nonsporulating tissue was from submerged hyphal mats (typically 3–4 days old), or young cultures grown on the surface of polycarbonate filters (typically 3 days old); 10-day polycarbonate cultures were used as a source of sporulating tissue, which include both developing and mature sporangia, sporangiophores, and associated hyphae. Sporangia purified from the 10-day cultures were also analyzed. To help identify early events in sporulation, hyphal mats shifted from submerged rye-sucrose broth to agar (aerial) media for 2–24 h were used. Infected plants were also examined to check if genes induced on artificial media were also activated during the natural life cycle.

Initial microarray studies of gene expression using isolate 88069

Analyses were performed with isolate 88069 (A1 mating type, from potato in The Netherlands) and custom Affymetrix

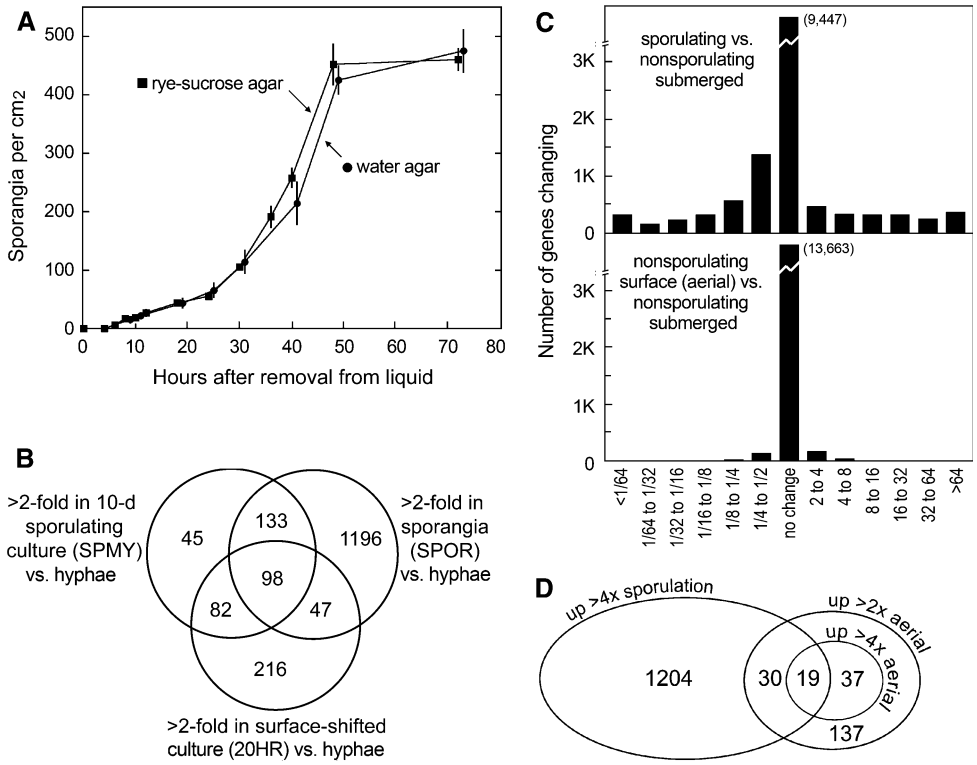


Fig. 1 **a** Time-course of *P. infestans* sporulation. Hyphae were shifted from submerged liquid cultures to either rye-sucrose agar or water agar. Sporangial density was counted at the indicated times. **b** Numbers of genes induced by >twofold ($P < 0.05$) during sporulation based on comparing nonsporulating submerged mycelia (NSMY) with the same shifted to agar for 20-h to induce sporulation (20HR), a 10-day agar culture (SPMY), or purified sporangia (SPOR). **c** Numbers of genes up- or down-regulated in comparisons of sporulating hyphae with submerged hyphae (*top*) or comparisons of 3-day nonsporulating aerial cultures with 3-day submerged cultures (*bottom*). **d** Diagram indicating whether the sporulation-induced set of 1,253 genes showed either minor (twofold) or greater (fourfold) induction in nonsporulating aerial cultures versus nonsporulating submerged tissues

GeneChips representing 15,646 *P. infestans* unigenes (Judelson et al. 2008). Of the genes represented on the array, 13,363 had detectable expression in the samples used in this study as determined by Affymetrix MAS 5.1 software. Several strategies were used to control for error. First, replicate hybridizations were used for each tissue type, with differentially expressed genes identified using a *P*-value threshold of 0.05. Second, RNA for each hybridization was pooled from two to three independent cultures grown during different weeks using different batches of media. Consequently, each gene was studied using RNA from four to six biological replicates. Third, as will be described later, a subset of sporulation-activated genes were validated by quantitative RT-PCR using a separate isolate, 1306 (A1 mating type, tomato, USA).

Genes expressed differentially during asexual sporulation were identified by comparing nonsporulating submerged hyphae with three types of sporulating or spore-containing samples. These were the 10-day polycarbonate culture, purified sporangia, and liquid-grown hyphae shifted to surface culture for 20 h. This identified 1,817 genes up-regulated by >twofold in at least one of the sporulating samples compared to the control, with 1,253 induced >fourfold ($P < 0.05$). Genes down-regulated by >twofold numbered 840 (449 by >fourfold). The Venn diagram in Fig. 1b shows the experimental comparisons in which the genes were detected as being >twofold induced. The top portion of Fig. 1c quantifies the number of genes at different fold-change thresholds compared to submerged hyphae. Of the 1,817 genes induced by >twofold, 343 had not been identified in the prior study which only compared sporangia with nonsporulating hyphae (Judelson et al. 2008). A retrospective analysis indicated that the reason for this is that most of the 343 had low mRNA levels in purified sporangia.

Distinguishing sporulation versus aerial growth-associated patterns

In the above analysis, submerged hyphae had been chosen as a more reliable nonsporulating tissue than young hyphae grown on polycarbonate membrane surfaces. This is because parts of the latter could have been entering the initial stages of sporulation, which lack distinguishable morphological features. A potential artifact of using submerged tissue, however, is that some genes appearing as

sporulation-induced might instead reflect differences between surface (aerial) and liquid growth.

To test this, a microarray experiment was performed that compared submerged hyphae with young, nonsporulating polycarbonate surface cultures. The results indicated that the vast majority of genes classified as "sporulation-induced" were not artifacts. The mRNA for 220 genes was more abundant in surface as compared to the submerged cultures (Fig. 1c, bottom); however, their fold-changes were modest unlike most genes induced in the sporulating samples (Fig. 1c, top). Sequence analysis indicated that such genes commonly had roles in metabolism. For example, of the five genes showing the highest induction in surface as compared to the submerged conditions, two were predicted to encode alcohol dehydrogenases (Pi001544, Pi002318), two encoded lipases (PI001136, Pi002898), and one encoded a putative organic acid antiporter.

Of the 1,253 genes defined as being sporulation-induced by fourfold, only 49 were also induced by the surface (aerial) growth conditions (Fig. 1d). Even these might not be artifacts, since the natural sporulation cycle of *P. infestans* involves the transition of hyphae from the moist internal spaces of plants to the outside of plant surfaces. As will be shown later, many such genes are induced during sporulation *in planta.*

Cluster analysis of sporulation-induced genes

Expression patterns of the 1,253 genes up-regulated fourfold or more during sporulation are shown in Fig. 2. Tissues portrayed are submerged nonsporulating mycelia (NSMY), cultures shifted from submerged to surface growth for 20 h (20 h), 10-day surface-grown sporulating cultures (SPMY), and sporangia purified from 10-day cultures (SPOR). The genes are ordered by hierarchical clustering on the left side of Fig. 2, with red and green representing up- and down-regulation, respectively, compared to the mean. The right side of the figure illustrates average expression within eight major clusters (A–H), which exhibit several notable patterns. For example, some clusters appear to be induced early in sporulation, with signals in the 20-h sample exceeding or matching those in sporangia or the 10-day culture (C, D, E, G, H). In contrast, others show higher signals in sporangia (A, B, F). Varying stability of transcripts or tissue-specific partitioning may also explain such patterns, in addition to changes to de novo transcription. For example, cluster A exhibits strong signals in sporangia purified from the 10-day cultures yet weak levels in the entire 10-day cultures, which suggests they accumulate preferentially in sporangia. In contrast, clusters such as G encode mRNAs that accumulate poorly in the 10-day sporangia, but are at relatively high levels in the 10-day culture. Cluster D genes exhibit similar levels in sporangia and the entire 10-day culture.

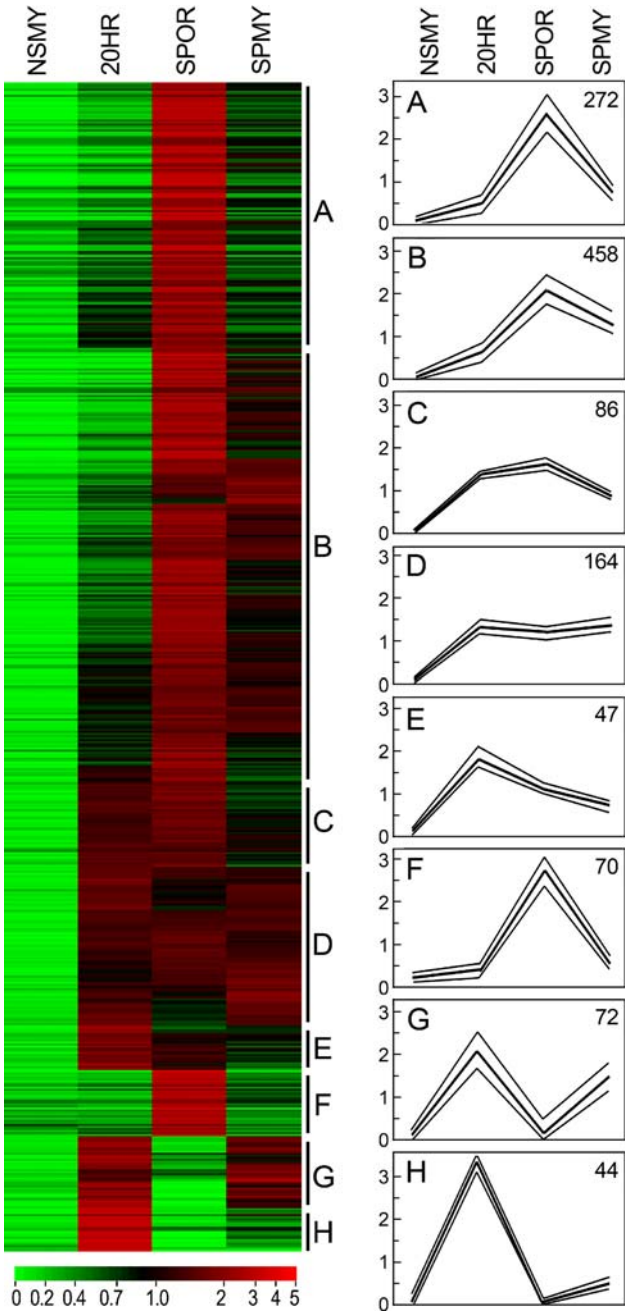


Fig. 2 Hierarchical clustering of microarray data for the 1,253 genes induced by >fourfold during sporulation. Tissues are submerged *NSMY*, submerged cultures shifted to surface growth for 20 h (*20HR*), 10-day sporulating cultures (*SPMY*), and purified sporangia (*SPOR*). The *left panel* shows clustering based on Pearson correlations with average linkage, using data subjected to per-chip and per-gene normalization. *Red* and *green* represent up- and down-regulation compared with the mean (1.0) of the four conditions. *Lines A–H* denote notable groups, which are shown in more detail in the right column along with the number of included genes. In the *right panels*, *center lines* represent mean levels of expression and outer lines stand for 75 and 25% values

Validation of GeneChip data by qRT-PCR using isolate 1306

The robustness of the GeneChip data was demonstrated by qRT-PCR using a different isolate of *P. infestans,* 1306. This involved testing 65 genes from clusters A to H using RNA from nonsporulating submerged hyphae and 10-day sporulating cultures. The resulting fold-change values are graphed in Fig. 3. General agreement was observed between the GeneChip (88069) and qRT-PCR data (1306), as all 65 genes were consistently sporulation-induced. However, the induction of certain genes varied quantitatively between the two types of experiments. For example, a few that were about 80-fold induced in the 88069 experiment only showed fivefold activation in 1306. While such genes are in the minority, such discrepancies might be attributed to technical issues related to microarrays versus RT-PCR, or strain differences. Cultural variation may also be a cause, since induction ratios are highly dependent on factors such as the time of sampling and age of culture. Indeed, experiments to be discussed later demonstrate that the abundance of many mRNAs in sporangia is age-dependent.

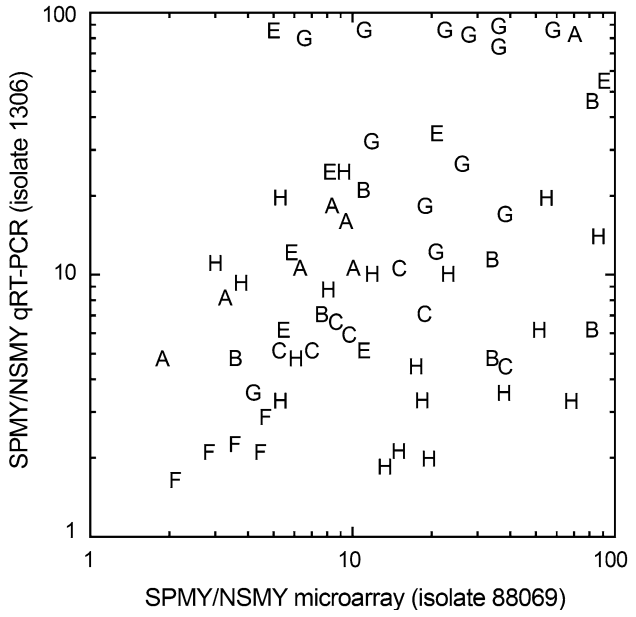


Fig. 3 Validation of microarray data by quantitative reverse transcription-polymerase chain reaction (*qRT-PCR*). Graphed are fold-change values for 75 genes, with each represented by a letter corresponding to its source cluster as shown in Fig. 2. Fold-change values represent comparisons of 10-day sporulating hyphae (*SPMY*) and submerged hyphae (*NSMY*) in the microarray study using isolate 88069 or the qRT-PCR assay using isolate 1306. For qRT-PCR, samples were normalized against ribosomal protein S3a. Error bars from replicates are omitted for clarity

In planta validation

To further authenticate the sporulation-induced patterns of the genes, samples were obtained from tomato infected by isolate 1306. Leaflets were inoculated with zoospores and incubated with a 12 h day–12 h night cycle. Visible sporulation began in the middle of the fourth night after infection (94 h, Fig. 4). Sporulation continued until the following dawn, and resumed during the fifth night.

RNA was then measured by qRT-PCR at 48 and 72 h (pre-sporulation) and 96 h (post-sporulation), using primers for 80 genes. These were selected to represent a range of expression patterns based on the microarray data, but were somewhat biased towards genes induced early in sporulation since these were under-represented in the earlier study (Judelson et al. 2008). Of the 80 genes, signals were not detected for seven *in planta,* probably due to weak signals resulting from the large excess of plant RNA. However, good data were obtained for 73 genes (Fig. 5, left). All but two showed significant induction in the 96-h sample as compared to the pre-sporulation time points, further validating the microarray data. The two exceptions were Pi002695, which is predicted to encode a glycosyl transferase, and Pi005604, which has no predicted function.

The *in planta* assays also suggested that most of the sporulation-induced genes that were also up-regulated in surface-grown nonsporulating cultures as compared to submerged cultures were authentically sporulation-induced. Six of the genes tested *in planta* fell into the surface growth-induced class, and all six were up-regulated in the sporulating plants. One example is Pi012879 (fourth from the top in Fig. 5). The levels of its mRNA were sixfold

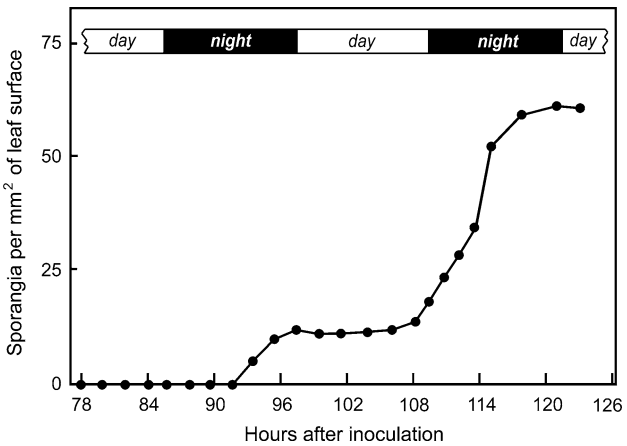
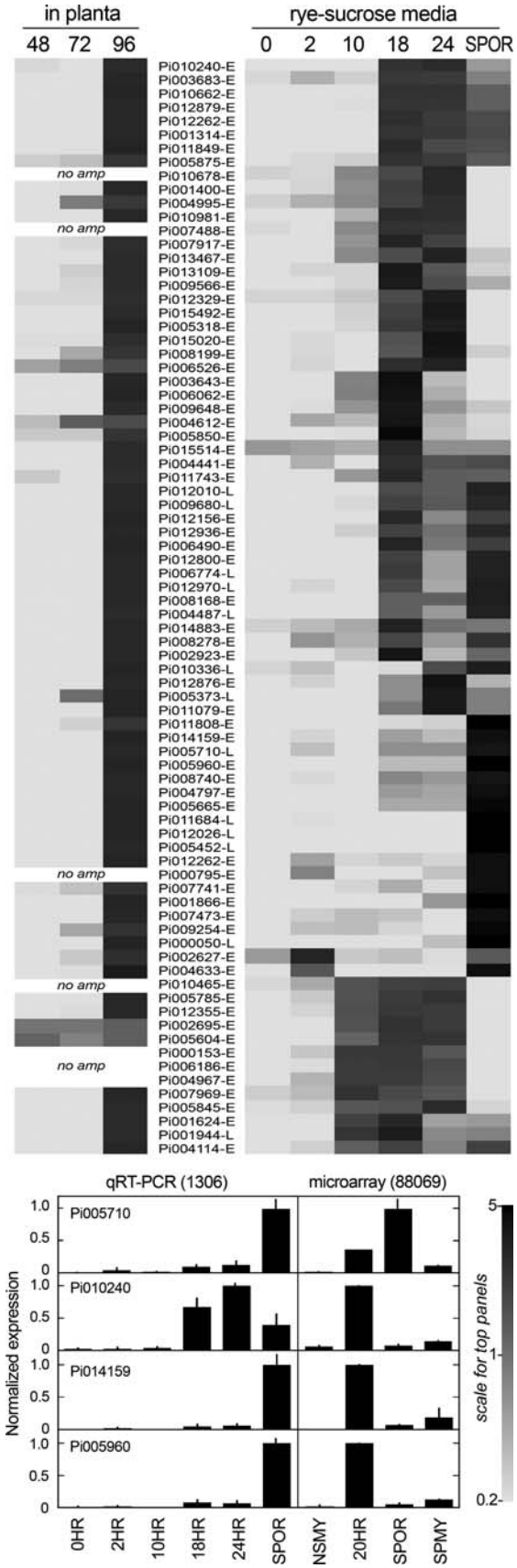


Fig. 4 Sporulation of *P. infestans* in infected tomato leaflets. Leaflets were inoculated with zoospore suspensions from isolate 1306, incubated with a day–night cycle, and sporulation quantitated as described in "Materials and Methods". Values are averages determined from approximately five leaflets



◀ **Fig. 5** Relative levels of mRNA during sporulation in tomato and rye-sucrose media. In the *left top panel*, RNA was from tomato 48, 72, or 96 h after inoculation with isolate 1306; as shown in Fig. 4, sporulation had occurred in the 96 h sample. In the *right top panel*, RNA was from tissue of isolate 1306 shifted from submerged rye-sucrose broth to rye-sucrose agar for 0, 2, 10, 28, or 24 h, or purified sporangia (SP). Data are presented as per-gene normalized mRNA levels according to the scale in the *lower right-hand corner*, as determined by qRT-PCR. Reactions that failed in planta are labeled "*no amp*." Genes are ordered based on hierarchical clustering of the data from the rye-sucrose agar experiment. Gene numbers are appended with "*E*" or "*L*" to denote genes in early or late induction classes as described in the main text. In the *bottom panel*, qRT-PCR and microarray data are shown for selected genes

higher in nonsporulating surface as compared to nonsporulating submerged cultures in the microarrays; 11–33-fold higher in the various sporulating rye-sucrose cultures (the sporulation time-course and 10-day culture) as compared to the submerged cultures in the microarrays; and based on qRT-PCR were 94-fold higher in 96-h sporulating leaflets compared to 48- and 72-h nonsporulating leaflets. In the same comparisons, Pi012262 (fifth from the top in Fig. 5) exhibited induction ratios of 76, 35–52, and 66. Pi013109 (16th from the top) showed induction ratios of 7.2, 9.4–47, and 15. Similar results were observed for Pi006774, Pi010662, and Pi011684. Consequently, it would be incorrect to dismiss genes induced during aerial versus submerged growth conditions as being irrelevant in the biology of sporulation.

Identifying early and late genes by microarray and qRT-PCR

To discover genes playing roles at different stages of development, the 1,253 genes induced >fourfold during sporulation were divided into 286 "early" and 967 "late" genes. Early genes were defined as those with a higher microarray signal in the 20-h sporulation sample than purified sporangia. This classification is somewhat arbitrary but may help identify distinct developmental patterns. For example, early genes might be used to form spore structures while late genes might encode products acting after germination.

To better understand the different types of expression, a sporulation time-course involving isolate 1306 was analyzed by qRT-PCR. This involved cultures shifted from submerged to surface growth for 2, 10, 18, and 24 h to induce sporulation, as well as purified sporangia; qRT-PCR was performed against the same 80 genes that had been tested *in planta*.

The results confirmed that all 80 genes were induced during sporulation and revealed distinct patterns such as activation at early, intermediate, or late stages (Fig. 5, right).

Pi004612 (encoding histidine ammonia-lyase) and Pi004995 (no match in GenBank) are notable since they were induced earlier than most genes, both in artificial media and *in planta*. Interestingly, mRNAs for these and most other genes up-regulated at early stages failed to persist in sporangia. Other genes were induced at later stages, such as around 18 h. Some show little persistence in sporangia such as Pi005318, which encodes a mucin-like protein, while others are maintained such as Pi009680, which encodes a mastigoneme protein. Others showed relatively minor levels of transcripts in sporulating hyphae, but very strong levels in sporangia, and thus may be induced after sporangia begin to mature. An example of such a gene is Pi011079, which encodes a major facilitator group transporter.

Most genes classified as "early" or "late" from the 88069 microarray data showed a similar pattern in the 1306 qRT-PCR study, based on the ratio between 18 h and sporangia samples. This was true for 52 of 66 early genes, and 12 of 14 late genes; to aid the reader, gene names in Fig. 5 are appended with "E" or "L" denoting their early or late classification from the microarray data. About half of the 16 discrepancies are attributable to slight variation between experiments which likely have little biological significance; for example, ratios between the 24 h and sporangia samples could have been 0.9 and 1.1 in the 88069 and 1306 experiments, respectively. However, several genes showed striking differences, as illustrated at the base of Fig. 5. Genes Pi005700 (late) and Pi010240 (early) are presented as examples of genes yielding consistent results in the 1306 and 88069 experiments. In contrast, Pi014159 and Pi005960 exhibited major differences. For example, in the 1306 experiment, Pi014159 had negligible signals in the 18-h samples and a strong signal in sporangia, but a low signal in 88069 sporangia in the microarray analysis. As will be discussed later, it is suggested that such differences may be due to differential mRNA stability in sporangia.

Functions of early and late sporulation-induced genes

Roles of the genes were predicted by database searches, using full-length gene models from the *P. infestans* genome project. Functions could be assigned to approximately half of the sporulation-induced genes based on BLAST searches against GenBank using an *E*-value threshold of 10^{-10}. Functional classifications of the 1,253 genes induced >four-fold during early and late sporulation are shown in Fig. 6. The 50 genes from the early class with the highest fold-change values and informative matches in GenBank are described in Table 1. BLAST matches of genes from the late-induction class (i.e. most abundant in sporangia) generally correspond to those listed in the prior study (Judelson et al. 2008).

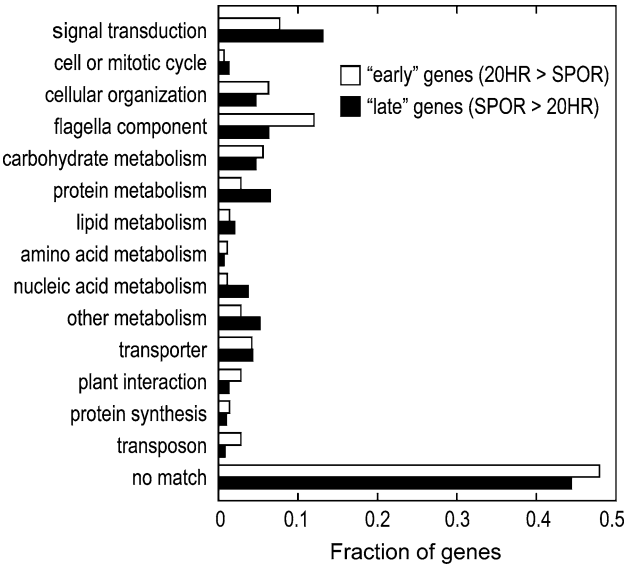


Fig. 6 Functional classification of the 286 "*early*" and 967 "*late*" genes. Results from database searches were used to classify the genes into the indicated functional classes

Predominant classes in both early and late gene sets included participants in signal transduction, transport, and cellular organization or structure, with the latter including constituents of flagella. Specific points about many such genes are reserved for "Discussion". However, as can be seen from Table 1 for early-induced genes, transporter activities were diverse and included members of ABC transporter, amino acid permease, cation transport ATPase, nucleoside transporter, sodium:solute symporter, and major facilitator groups. Their prevalence in the early class forced consideration of whether their induction might be an artifact of the experimental methods. However, two of these genes had been tested *in planta* where both were found to be associated with sporulation (Pi003683 and Pi011079; Fig. 5).

Many proteins induced during sporulation lacked significant matches against GenBank but had features suggestive of structural roles. For example, Pi012156 which showed the highest induction ratio of any gene in the early class is predicted to encode a protein containing 20 tandem collagen triple helix repeats. Gene Pi006769, which had the third-highest induction ratio during early sporulation, encodes a low-complexity protein comprising many threonine and serine-rich motifs, which are typical of glycosylated structural or adhesion proteins. Another example is Pi003110 which contains extensive serine and glycine-rich repeats. While such repeats could reflect nucleic acid adaptations, it is suggested that these proteins may form part of sporangia or cyst walls, adhesion proteins, barriers to desiccation, or similar functions.

Table 1 The 50 early-induced genes having the highest induction ratios during sporulation and informative BLAST matches

Gene[a]		Induction rank[b]	Normalized expression level[c]				Best BLASTP match[d]	*E* value
Unigene	Gene model		NSMY	20HR	SPOR	SPMY		
Pi001305	17315	4	0.01	2.18	1.05	0.76	Caltractin, *Zea mays* ACF87029	1e-16
Pi000059	12361	5	0.01	3.66	0.06	0.27	Cutinase, *Kineococcus radiotolerans* YP_001363838	5e-43
Pi003747	03600	6	0.01	1.64	1.48	0.88	BBS2 flagellar protein, *Strongylocentrotus purpuratus* XP_001176601	3e-166
Pi001544	10290	7	0.02	3.06	0.00	0.91	Mannitol-1-phosphate dehydrogenase, *Laccaria bicolor* XP_001881024	2e-88
Pi005373	07085	8	0.02	3.31	0.08	0.58	ABC transporter, *Dictyostelium discoideum* XP_641766	2e-21
Pi010535	17766	9	0.02	3.31	0.01	0.66	Amino acid permease, *Tetrahymena thermophila* XP_001024395	1e-10
Pi003683	07085	10	0.02	3.44	0.02	0.51	Sugar phosphate transporter (MFS superfamily), *D. discoideum* XP_641766	2e-21
Pi002923	16981	12	0.03	3.63	0.00	0.33	F-spondin adhesion protein, *Hydra vulgaris* CAJ65510	6e-95
Pi002744	02320	15	0.02	2.05	0.92	1.01	Phosphogluconolactonase, *Stigmatella aurantiaca* ZP_01463836	2e-47
Pi013604	00719	16	0.02	2.28	0.95	0.75	Aminophospholipid ATP-dependent transporter, *Mus musculus* NP_056618	0.0
Pi013653	03213	19	0.04	2.51	0.59	0.87	β-Glucosidase, *Salinibacter ruber* ABC43952	9e-92
Pi015853	05682	26	0.03	1.51	1.31	1.15	Dynein light chain, *Paramecium tetraurelia* CAK88700	13-11
Pi012504	08755	27	0.06	3.11	0.30	0.53	*Myb* transcription factor, *Oryzias latipes* NP_001098159	4e-31
Pi011079	00722	29	0.06	2.72	0.34	0.88	Major facilitator superfamily protein, *Bdellovibrio bacteriovorus* NP_969699	2e-32
Pi004441	20212	35	0.04	1.58	1.29	1.09	Intraflagellar transport 172 protein, *Danio rerio* NP_001002312	0.0
Pi004588	06336	36	0.09	3.51	0.04	0.36	Sugar transporter (MFS superfamily), *Trichomonas vaginalis* XP_001322131	2e-21
Pi007488	07663	42	0.09	3.08	0.14	0.70	Oxalate transporter (MFS superfamily), *Physcomitrella patens* XP_001760648	3e-82
Pi001783	13567	44	0.07	2.29	0.80	0.84	β-Glucanase, *Aspergillus fumigatus* XP_750173	2e-86
Pi012473	06748	45	0.07	2.31	1.23	0.38	*Myb* transcription factor, *Homo sapiens* CAI20200	9e-42
Pi002695	17508	49	0.12	3.41	0.14	0.33	β-Glucosidase, *Nematostella vectensis* XP_001630873.1	2e-93
Pi012807	03673	51	0.05	1.38	1.25	1.32	Ankyrin, *Candidatus Amoebophilus asiaticus* YP_001957322	1e-22
Pi010336	18709	52	0.06	1.53	1.27	1.15	Coiled-coil domain flagellar protein, *Nematostella vectensis* XP_001628027	3e-63
Pi002409	00912	59	0.09	2.17	0.88	0.87	3-Hydroxyisobutyrate dehydrogenase, *Bos taurus* NP_001039571	3e-80
Pi001579	07023	60	0.14	3.30	0.06	0.51	GDP-mannose 4,6 dehydratase, *Methanosarcina barkeri* YP_303605	2e-135
Pi010240	00245	62	0.14	3.25	0.19	0.42	Cathepsin, *Suberites domuncula* CAH04633	4e-56
Pi002898	08004	67	0.12	2.38	0.05	1.45	Glucosylceramidase, *Caldicellulosiruptor saccharolyticus* YP_001181281	7e-68
Pi003263	09906	72	0.13	2.34	0.04	1.49	Arabinogalactan endogalactosidase, *Botryotinia fuckeliana* XP_001556606	6e-82
Pi013852	10050	75	0.10	1.76	1.05	1.08	Alpha-*N*-acetylglucosaminidase, *D. rerio* XP_688608	1e-140
Pi015676	07911	77	0.08	1.32	1.25	1.35	Glutamate-cysteine ligase, *H. sapiens* NP_001489	1e-160
Pi013169	03886	78	0.09	1.46	1.46	1.00	Nucleoside transporter, *S. purpuratus* XP_783862	3e-15
Pi007247	06778	80	0.11	1.82	1.32	0.75	Qilin-like flagellar protein, *H. sapiens* XP_001697203	3e-76
Pi008066	15387	82	0.14	2.14	0.58	1.14	Ankyrin, *Trichomonas vaginalis* XP_001315403	8-26
Pi008109	02050	84	0.18	2.83	0.21	0.78	Chitin synthase, *Aphanomyces euteiches* ACA96933	1e-168

Table 1 continued

Gene[a]		Induction rank[b]	Normalized expression level[c]				Best BLASTP match[d]	*E* value
Unigene	Gene model		NSMY	20HR	SPOR	SPMY		
Pi005266	03379	86	0.13	2.03	0.28	1.56	Nuclear protein importer, *Triticum aestivum* AAY84877	1e-15
Pi010229	01936	93	0.11	1.53	1.42	0.93	Serine/threonine protein kinase, *Paramecium tetraurelia* XP_001423533	8e-109
Pi005642	14533	98	0.17	2.34	0.05	1.44	Calponin-containing monooxygenase, *H. sapiens* EAW4834	3e-28
Pi014337	02080	99	0.11	1.52	0.90	1.47	Serine/threonine protein kinase, *Leishmania braziliensis* XP_001562591	8e-32
Pi009985	12195	100	0.14	1.89	0.35	1.61	Katenin p60 ATPase, *P. patens* XP_001768074	1e-100
Pi005060	02329	101	0.24	3.20	0.05	0.52	*N*-glycanase, *Trichoplax adhaerens* EDV26937	1e-75
Pi014935	17343	102	0.11	1.47	1.23	1.18	Presenilin protease, *Vitis vinifera* CAO45408	3e-34
Pi011719	19957	104	1.64	1.49	0.74	0.12	Hydroxyphenylpyruvate dioxygenase, *Chlamydomonas reinhardtii* CAD24031	1e-154
Pi001136	01186	105	0.17	2.17	0.00	1.66	Lysosomal lipase, *Canis familiaris* XP_853280	3e-73
Pi001002	05806	106	0.11	1.49	1.38	1.01	Clathrin coat protein, *D. discoideum* AAO51241	5e-40
Pi012432	07905	107	0.22	2.77	0.05	0.96	Xylose isomerase, *Tetraodon nigroviridis* CAF93733	3e-148
Pi012572	00806	109	0.13	1.65	1.05	1.17	Flagella-asssociated tetratricopeptide protein, Mus musculus EDL08825	2e-11
Pi001744	00719	114	0.14	1.63	1.01	1.22	Acetyl-CoA synthetase, *D. discoideum* EAL68581	0.0
Pi003847	02651	115	0.25	2.96	0.13	0.66	Sodium-sugar symporter, *T. nigroviridis* CAF99317	2e-94
Pi008090	02254	119	0.12	1.43	0.97	1.48	Pre-acrosome protein, *D. rerio* XP_001339858	3e-27
Pi003607	16507	124	0.18	2.00	1.13	0.69	β-Xylosidase, *Monosiga brevicollis* XP_001745192	6e-106

[a] Unigene numbers correspond to the assemblies of ESTs and partial genome sequences used to design the array, and are the codings deposited in NCBI GEO. Gene model numbers denote the corresponding gene identifier, with the PITG prefix omitted, from the Broad Institute's *P. infestans* genome sequence (version 1, March 2007). In about 4.9% of cases, two unigene sequences matched the same gene model; such redundant sequences are eliminated from the analysis

[b] Genes are listed in order of their fold-change during early sporulation, in descending order. Ranks not shown (for example, numbers 1–3, 11) lacked significant hits in GenBank using a threshold *E* value of 10^{-10}

[c] Based on per-chip and per-gene normalization. Values are from nonsporulating submerged mycelia (NSMY), hyphae shifted from submerged to surface cultures for 20 h (20HR), purified sporangia (SPOR), and 10-day sporulating cultures (SPMY)

[d] Indicated are the best hits against non-oomycete proteins in GenBank, based on full-length gene sequences predicted from the genome project

Analysis of sporulation-defective strains

The temporal staging of genes based on the microarray and qRT-PCR data described above is one approach for identifying changes in early sporulation. As an alternative strategy for detecting early genes, two *P. infestans* transformants defective in sporulation were analyzed, which had been developed by silencing the *Cdc14* gene (Ah Fong and Judelson 2003). These are blocked at an early stage of sporulation, as they produce neither the aerial hyphae typical of sporulating cultures or sporangia.

Using the GeneChips, mRNA from 10-day cultures of the two mutants was compared to that of nonsporulating hyphae. Using a *P* value threshold of 0.05, 147 genes were up-regulated in the mutants by >twofold and 92 by >fourfold. Of the latter, 35 were represented in the set of 1,235 genes previously characterized as being >fourfold induced during sporulation in wild-type (Fig. 7). It is suggested that such genes may be activated at the earliest stages of sporulation. Twenty had significant matches in GenBank (Table 2). Common predicted functions include roles in regulation (protein kinases, ADP-ribosylation factor, phosphatidylinositol kinase), vesicle and organelle movement (α-adaptin, clathrin coat assembly protein, katanin p60), and flagella (ciliary outer arm dynein heavy chain, axonemal dynein regulatory protein).

Differential stability of mRNAs in sporangia

Earlier it was noted that certain transcripts had low abundance in 88069 sporangia based on the microarray data, but higher levels in 1306 sporangia based on the qRT-PCR experiment. A hypothesis presented to explain this was that some mRNAs might be unstable, resulting in varying

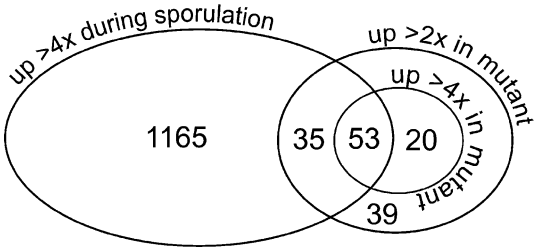


Fig. 7 Relationships between genes induced in wild-type *P. infestans* and nonsporulating mutants. The 1,253 genes induced >fourfold during sporulation are those induced in one of the sporulating samples compared to submerged, nonsporulating cultures. The 147 genes induced in the *Cdc14*-silenced mutants have higher mRNA levels in their 10-day polycarbonate cultures compared to submerged cultures of wild-type. mRNA levels in the two Cdc14-silenced strains were very similar ($R = 0.93$)

measurements from different batches of sporangia. To test this, a dot-blot assay was performed against 18 genes induced during sporulation and 18 expressed at equal levels in hyphae and sporangia. These were hybridized with labeled cDNA from sporangia from 8- to 13-day old cultures of isolate 1306, in which sporulation had started on day 6.

The results indicated that the abundance of many sporulation-induced mRNAs change substantially as the spores aged. This is shown in Fig. 8a, where ratios of mRNA levels in the 13-day versus 8-day samples are graphed along with fold-change values from the microarray data. Most sporulation-induced mRNAs (normalized to EF-1α) fell in the aged sporangia, as the average 13-day/8-day signal ratio was 0.44 ± 0.45. In contrast, most genes that were not sporulation-induced showed minor changes (ratio of 0.89 ± 0.46). Not all genes within each group showed equal patterns, for example one sporulation-induced gene (Pi002822) had a stronger signal in the 13-day material. Nevertheless, on average the sporulation-induced mRNAs were much less stable.

Table 2 Genes induced >fourfold in both wild-type and nonsporulating mutant compared to hyphae

Gene[a]		Fold-change ratio[b]		Best BLASTP match[c]	*E* value
Unigene	Gene model	NSMY/SPMY	NSMY/MUTANT		
Pi000624	04957	15	5	Clathrin coat assembly protein, *Paramecium tetraurelia* XP_001445722	3e-41
Pi001624	20230	5	7	Amino acid transporter, *Tetrahymena thermophila* XP_001024395	1e-07
Pi002616	12195	8	6	Katanin p60 microtubule regulator, *Physcomitrella patens* XP_001768074	1e-100
Pi003671	12577	141	87	Ciliary outer arm dynein beta heavy chain, *Paramecium tetraurelia* AAA61680	0.0
Pi004089	00332	16	6	Mitochondrial sodium/hydrogen exchanger, *Gallus gallus* NP_001034364	1e-71
Pi004175	13011	6	40	Nitrate transporter, *Skeletonema costatum* AAL85928	6e-98
Pi005726	17342	15	7	Axonemal dynein regulator PF2, *Chlamydomonas reinhardtii* XP_001697295	1e-93
Pi005728	20413	238	59	Elicitin-like protein INL13, *Phytophthora infestans* ABB55934	3e-62
Pi006895	05392	44	9	Peptidase-like gliding motility protein, *Myxococcus xanthus* YP_634459	8e-67
Pi007127	20221	42	9	Phosphatidylinositol 3- and 4-kinase, *Oryza sativa* EAZ00815	73-40
Pi007550	08612	11	4	Cellulase, *Pyrococcus horikoshii* NP_143072	3e-33
Pi007620	13269	292	64	ADP-ribosylation factor binding protein, *Gallus gallus* NP_001007971	9e-20
Pi010255	04335	151	44	Alpha-adaptin, *Drosophila melanogaster* NP_476819	2e-160
Pi011975	00288	5	6	Scavenger mRNA decapping enzyme, *Drosophila sechellia* EDW55013	8e-23
Pi012145	08795	28	19	Collagen triple helix repeat protein	–
Pi012205	06908	128	113	Elicitin-like protein INL6, *Phytophthora infestans* ABB55941	3e-56
Pi012903	09999	5	11	Highly conserved hypothetical protein, *Aspergillus oryzae* XP_001727558	2e-63
Pi013642	18644	5	5	Calcium-binding protein, *Chlamydomonas reinhardtii* XP_001694726	1e-08
Pi013713	18366	9	27	Carnitine O-palmitoyltransferase, *Xenopus tropicalis* NP_001107300	1e-175
Pi015565	15616	21	4	Serine/threonine protein kinase, *Tribolium castaneum* XP_971409	6e-21

[a] Genes are listed in numerical order based on the corresponding unigene number. Also shown is the gene model number from the Broad Institute database, with the PITG prefix omitted. Not shown are 15 genes for which the predicted proteins lacked significant hits in GenBank using a threshold *E* value of 10^{-10}

[b] Fold-change ratios calculated from nonsporulating submerged mycelia (NSMY), 10-day sporulating agar cultures (SPMY), and 10-day cultures of nonsporulating strains obtained by silencing Cdc14 (MUTANT). Values are averages based on replicate tissue samples, or two independent silenced transformants in the case of the nonsporulating strains

[c] Best hits against non-oomycete proteins in GenBank, based on full-length gene sequences predicted from the genome project

RNA blots confirmed the trends seen in the dot-blot data (Fig. 8b). Pi001642, which encodes a component of the flagellar apparatus (Bardet-Biedl syndrome 4 protein), showed a 2.5-fold decay in abundance in the older sporangia. A greater decline was exhibited by Pi004638, which encodes a member of the MTN3 family of transmembrane proteins. In contrast, little change was seen for Pi003684, which encodes malic oxidoreductase. The EF-1α control showed similar levels in the samples.

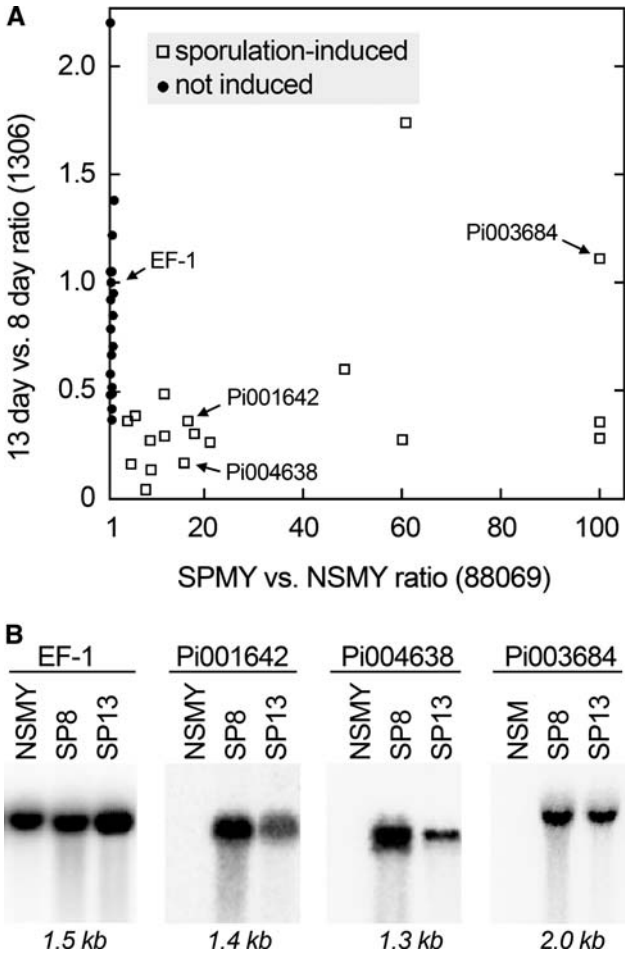


Fig. 8 Stability of mRNA during aging of sporangia. **a** RNA from sporangia purified from 8- to 13-day old cultures of isolate 1306 were converted to ^{32}P-labeled cDNA and hybridized to replicate filters containing duplicate spots of DNA corresponding to selected genes. Hybridization signals were normalized to elongation factor-1α (*EF-1*). The ordinate shows the ratio of signals between 13- and 8-day probes. The abscissa shows the fold-change values from the 88069 microarray experiment, comparing sporulating (*SPMY*) with nonsporulating (*NSMY*) mycelia. *Open squares* represent sporulation-induced genes and *closed circles* represent constitutively expressed genes. **b** Blot analysis of RNA from nonsporulating mycelia of isolate 1306 (*NSMY*), or sporangia from 8- to 13-day cultures (*SP8*, *SP13*). Hybridizations were performed using probes for *EF-1*, *Pi001642*, *Pi004638*, and *Pi003684*

Discussion

The transcriptional changes identified by this study can be placed in context with prior cytological analyses of spore development in *Phytophthora*. Sporangia accumulate many organelles absent from hyphae such as large peripheral vesicles, encystment vesicles, kinetosomes, and flagella (Christen and Hohl 1972; Hardham and Hyde 1997). Several of the early-induced genes encode participants in vesicle movement that may aid in assembling these structures, such as α-adaptin, clathrin-associated factors, and katanin p60 (Pi000624, Pi001002, Pi005726, Pi007620, Pi009985). Other early-induced genes encode components of the spore-specific vesicles or organelles themselves including over 70 flagella-associated proteins (Pi003671, Pi003747, and others) as well as a thrombospondin-like encystment protein (Pi002923; Robold and Hardham 2005).

In addition to forming sporulation-specific organelles, a range of metabolic and physiological changes also occurs during sporulation. For example, the trafficking of small compounds is apparently stimulated, based on the induction of genes encoding transporters for amino acids, nucleosides, organic acids, phospholipids, and sugars. This is consistent with observations that many free amino acids are more concentrated in sporangia than hyphae. One amino acid in particular, glutamate, was shown to be 20 times more abundant in sporangia than surrounding hyphae (Grenville-Briggs et al. 2005). This may explain the induction of a gene encoding histidine ammonia-lyase (Pi004612), which is a rate-limiting step in glutamate synthesis.

Relative levels of mRNAs in sporulation time-courses and sporangia, and in the non-sporulating mutants, were used to predict which genes participate in early versus late sporulation events. However, such distinctions may be deceptive as a gene could be partially activated early and induced more highly at a later stage. The classifications could also be misleading for genes that are auto-activated, as is the case for conidiation regulators in fungi such as *brlA* (Yu et al. 2006). Nevertheless, the diverse modes of accumulation of transcripts identified by this study (early vs. late sporulation, sporangia-persistent vs. non-persistent, etc.) demonstrate that *P. infestans* establishes a complex hierarchy of expression patterns during spore development.

Dissecting this hierarchy needs to be a priority in future studies of sporulation in *Phytophthora* as well as other oomycetes. This will be a challenge since sporulation is not highly synchronous and does not involve forming a series of easily distinguished cell types, unlike the case in some true fungi such as *Aspergillus* (Yu et al. 2006). However, sporulation-defective strains of *P. infestans* generated by silencing regulators besides Cdc14 may be of use in identifying discrete stages of development. The use of fusions

between sporulation-induced genes and reporters such as GUS or GFP should also aid in discerning components of the sporulation program.

Future studies will also need to establish the relative contributions of de novo transcription and RNA turnover to development. A role of mRNA instability was suggested by our analysis of the differential stability of transcripts in aging sporangia. Several genes potentially involved in mRNA instability are induced during sporulation, such as a deadenylation protein (Pi004848) and decapping enzyme (Pi011975). Transcripts acting only during early sporulation may have evolved to decay once sporangia mature, while mRNAs used to maintain viability through germination would persist. Under favorable field conditions, sporangia can maintain viability for as much as a week, although the dynamics of germination change during this period (Wallin 1953; Sato 1994) in addition to the expected changes in mRNA levels. How then should the sporangium stage be defined for molecular studies?

A related challenge involves defining non-sporulating hyphae. Submerged cultures served this purpose in our study instead of young surface-grown hyphae due to fear that portions of the latter might be in the midst of initiating sporulation. Submerged cultures are also common sources of nonsporulating tissue in studies of true fungi (Roncal and Ugalde 2003). However, it is possible that some *P. infestans* genes classified as sporulation-induced were actually stimulated by aerial growth. However, <50 were in this class, and most were also associated with sporulation *in planta*.

Understanding how submerged growth blocks sporulation is important, since this may be exploitable for crop protection. Surface growth may trigger sporulation by causing desiccation, localized hyperoxidant domains, or conformational changes in wall proteins. Similar phenomena may explain why sporulation occurs on plant surfaces, which is ecologically advantageous. Why *P. infestans* preferentially sporulates *in planta* during dark periods is also of interest. Previous studies showed that continuous light inhibits sporulation (Cohen et al. 1975), although variable results are reported for other members of the genus (Ribeiro 1983). As there is no evidence for a light-regulated clock in *Phytophthora*, this suggests that a plant signal generated by the day-night cycle influences sporulation. Similar phenomena are reported for other oomycetes (Raffray and Sequeira 1971).

Identifying the *Phytophthora* proteins that regulate sporulation is a priority. The two *myb*-like transcription factors induced early in sporulation (Pi012473 and Pi012504) are candidates for regulators of the early or intermediate stages. Transcription factors that may control late stages, such as the HMG box protein Pi008690, were mentioned in the prior study (Judelson et al. 2008). Additional regulation is likely conferred by protein kinases induced during sporulation, such as Pi010229, Pi014337, and Pi015565. However, the initial triggers of sporulation will likely not be found on any list of sporulation-induced genes since they are already in hyphae. Classical mutagenesis schemes for finding such regulators, which have proved fruitful in studies of true fungi, would be difficult in oomycetes which are normally diploid. An alternative would be to identify the transcription factors that turn on genes during sporulation, and then the upstream activators of the transcription factors. This study sets the stage for such research by identifying genes induced during early sporulation.

Acknowledgments We are grateful to Syngenta Corporation for providing the Affymetrix GeneChips, and to T. Zhu and G. Aux for technical assistance in analysis of the arrays. This work was funded by the United States Department of Agriculture-National Research Initiative program of the Cooperative State Research, Education, and Extension Service.

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