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Genes expressed in zoospores of *Phytophthora nicotianae*

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Abstract The genus *Phytophthora* includes many highly destructive plant pathogens. In many *Phytophthora* species, pathogen dispersal and initiation of plant infection are achieved by motile, biflagellate zoospores that are chemotactically attracted to suitable infection sites. In order to study gene expression in zoospores, we have constructed a cDNA library using mRNA from zoospores of *Phytophthora nicotianae*. The library was arrayed and screened using probes derived from mycelium or zoospore mRNA. More than 400 clones representing genes preferentially expressed in zoospores were identified and sequenced from the 5′ end of the insert. The expressed sequence tags (ESTs) generated were found to represent 240 genes. The ESTs were compared to sequences in GenBank and in the Phytophthora Genome Consortium database, and classified according to putative function based on homology to known proteins. To further characterize the identified genes, a colony array was created on replicate nylon filters and screened with probes derived from four *Phytophthora* developmental stages including zoospores, germinating cysts, vegetative mycelium and sporulating hyphae, and from inoculated and uninoculated tobacco seedlings. Data from sequence analysis and colony array screening were compiled into a local database, and searched to identify genes that are preferentially expressed in zoospores for future functional analysis.

Keywords Gene expression · Oomycetes · Plant-pathogen interactions · *Phytophthora* · Zoospores

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Introduction

Zoospores are flagellate motile cells used for dispersal. They are wall-less and chemotactic and can swim for hours, relying on endogenous food reserves (Carlile 1986). After reaching a suitable surface, they become immobile and adhesive, and secrete a cell wall to form cysts that germinate and attempt to penetrate the substrate or the host organism (Carlile 1986; Deacon and Saxena 1997; Callow et al. 2001). Zoospores are found in a number of taxonomic groups including some common pathogens of plants, insects, fish and occasional human pathogens. In pathogenic species, zoospores can serve as infectious agents and may be important in spreading disease.

The genus *Phytophthora* is one of the most economically significant among the zoosporic organisms, since it contains over 50 species of extremely destructive pathogens of plants, including a number of major crops (Erwin and Ribeiro 1996). It belongs to the oomycetes, a taxonomic group previously classified as fungi due to similarities to true fungi in morphology and growth habits. More recently, recognition of important differences in structure and metabolic pathways (reviewed by Hardham et al. 1994), as well as gene sequence comparisons (Gunderson et al. 1987; Förster et al. 1990), have indicated that the oomycetes are not true fungi, but belong to the stramenopiles, a diverse but distinct group which also includes diatoms and brown algae. Recognition of the correct phylogeny of the oomycetes is important, not only for our understanding of the fundamental biology of these organisms, but also because the structural and physiological differences between members of the group and true fungi have broader implications; for example, they may help to explain the lack of response by *Phytophthora* to some traditional antifungal agents (reviewed by Erwin and Ribeiro 1996). The significant genetic difference between stramenopiles and other eukaryotes means that the recent explosion of sequence information from a number of model

organisms is of limited use in describing the molecular basis of *Phytophthora* physiology. This makes the Phytophthora Genome Consortium (PGC) database (http://xgi.ncgr.org/pgc) an extremely valuable resource. The database compiles information from several genomic projects focusing on two species, *P. infestans* and *P. sojae* (Waugh et al. 2000; reviewed by Tyler 2001), and includes sets of expressed sequence tags (ESTs) derived from *P. infestans* mycelium (Kamoun et al. 1999), *P. sojae* mycelium and infected soybean tissue, as well as *P. sojae* zoospores (Qutob et al. 2000). More recently, a draft sequence of the whole genome of *P. sojae*, produced by the US Department of Energy Joint Genome Institute (JGI), became publicly available (http://www.jgi.doe.gov/).

The development, ultrastructure and behaviour of zoospores have been extensively studied, and are well understood at the cellular level (Hickman 1970; Hemmes 1983; Hardham et al. 1991; Hardham and Hyde 1997; Gow et al. 1999; reviewed by Tyler 2002). In contrast, there is little information about the genes that control zoospore biology. A few studies (van West et al. 1998; van't Klooster et al. 2000; Ambikapathy et al. 2002; Judelson and Roberts 2002; Laxalt et al. 2002), including an EST project (Qutob et al. 2000), have documented the presence of particular gene transcripts in zoospores. Not surprisingly, many of the ESTs were novel and showed no significant similarities to other publicly available sequences (Qutob et al. 2000). Due to the paucity of sequence data from other stramenopiles, it is difficult to determine whether a particular novel sequence among the zoospore-specific ESTs is a unique feature of zoospores or a species characteristic unrelated to zoospore biology.

As part of our research on pathogenicity determinants in *Phytophthora*, we initiated a small-scale EST project focusing on genes from zoospores of the pathogenic oomycete *P. nicotianae*. Our aim was to identify a number of zoospore-specific genes that could then be studied in detail. In order to generate data on a number of genes simultaneously, we used a combination of differential library screening, sequencing and colony blot analysis. Here we summarize data from sequence analysis and colony blot screening with probes derived from four *P. nicotianae* developmental stages and from inoculated and uninoculated host-plant tissue.

Materials and methods

Organisms and culture conditions

Phytophthora nicotianae B. de Haan isolate H1111 (ATCC MYA-141, kindly supplied by Dr. D. Guest, University of Melbourne) was maintained on V8 nutrient agar (Chen and Zentmyer 1970). Mycelium was obtained from liquid cultures initiated from small agar plugs and grown in V8 broth (Chen and Zentmyer 1970) in the dark at 25°C for 4 days. Mycelium was collected by vacuum filtration, and agar plugs were removed prior to freezing in liquid nitrogen. Zoospores were produced on mycelium-containing Miracloth (Calbiochem, La Jolla, Calif.) discs (Ambikapathy et al. 2002) incubated in V8 broth under light at 23°C for 7–21 days. To release the zoospores, discs were rinsed three times in cold sterile water and incubated in water in the light at 15°C for 1 h. Zoospores were collected by centrifugation at 2000× *g* for 2 min. Pellets were frozen in liquid nitrogen. Sporulating hyphae and germinating cysts were obtained as described previously (Ambikapathy et al. 2002).

Seeds of *Nicotiana tabacum* cv. Petit Havana (a kind gift from Dr. D. Jones, Australian National University) were sterilized by immersion in 3% bleach for 5 min and rinsed in sterile water. They were germinated on moist filter paper in large petri dishes in the dark at 23°C for 14 days. Seedlings were inoculated by adding 5 ml of zoospore suspension (3×10^4 to 2.5×10^5 zoospores per ml) to each dish, and incubated in the dark. Inoculated and uninoculated seedlings were harvested by plunging into liquid nitrogen.

RNA isolation and cDNA probe synthesis

Total RNA was isolated from frozen ground tissue as described previously (Logemann et al. 1987). Poly(A)$^+$ RNA was purified from total RNA using Oligo $(dT)_{25}$ Dynabeads according to the manufacturer's (Dynal, Oslo, Norway) recommendations. Digoxigenin (DIG)-labelled cDNA probes were made from poly(A)$^+$ RNA using SuperScript II reverse transcriptase (Life Technologies, Melbourne, Australia) in the presence of alkali-stable DIG-11-dUTP (Roche Diagnostics, Mannheim, Germany) and the oligo(dT) primer. The reaction was stopped by addition of 0.5 M EDTA and 1% SDS, and RNA was removed by hydrolysis in 0.3 M NaOH for 1 h at 65°C. The mixture was neutralized by addition of an equal volume of 1 M TRIS-HCl (pH 8) and added directly to the hybridization buffer (described below).

cDNA library construction and screening

The cDNA library was constructed in the lambda Uni-ZAP XR vector (Stratagene) from approximately 6 μg of zoospore poly(A)$^+$ RNA. The Stratagene cDNA synthesis kit, ZAP-cDNA Synthesis kit and ZAP-cDNA Gigapack III Gold Cloning kit were used as recommended in the instruction manual (#200401-12, revision #119011). The primary library contained 1.4 million clones and was amplified once. Average insert size in the library was 1.2 kb, with sizes ranging from 0.2 to 4.8 kb. A portion of the amplified library was mass-excised and transduced into the SOLR strain. Single colonies were either manually arrayed on Luria-Bertani (LB) agar plates containing ampicillin (100 μg/ml), grown overnight at 37°C and lifted onto HybondN+ filter membranes (Amersham Pharmacia Biotech, Sydney, Australia) or picked into 384-well plates, grown overnight at 37°C without shaking, and transferred to filter membranes using a multipin replicator. In the latter case membranes were laid on top of LB agar plates containing 100 μg/ml ampicillin, and incubated overnight at 37°C. All membranes were treated by standard procedures (Sambrook and Russell 2001) prior to hybridization. Prehybridization and hybridization were performed in Church buffer (Sambrook and Russell 2001), except that BSA was omitted. DIG-probe-containing hybridization buffer was reused up to four times during screening to reduce the incidence of replicate clones. Signal was detected using alkaline phosphatase-conjugated anti-DIG antibody according to the manufacturer's (Roche Diagnostics) instructions.

Plasmid isolation and sequencing

Plasmid DNA was isolated from selected clones grown overnight in LB broth containing 100 μg/ml ampicillin using the QIAprep Spin Miniprep kit (Qiagen, Clifton Hill, Australia). Inserts were sequenced using the T3 promoter primer (corresponding to the 5′ end of cloned RNA) and Big Dye terminators at the Australian Genome Research Facility (Brisbane, Australia; detailed protocols are available at http://www.agrf.org.au). After manual removal of

vector sequences, ESTs were compared to sequences in GenBank using the BLASTX program and the Phytophthora Genome Consortium database and the JGI *P. sojae* database using the BLASTN program (Altschul et al. 1997), via the database websites (http://www.ncbi.org, http://www.xgi.ncgr.org/pgc/; http://genome.jgi-psf.org/physo00/physo00.home.html). Matches were considered significant if the expect value (E) was 10^{-4} or less. Replicate clones were identified by aligning putative clusters using BLASTN (Altschul et al. 1997), and the Search Local Database function in the DNAtools program (http://www.dnatools.dk/, now discontinued). Before assigning clusters, all alignments were individually evaluated to eliminate errors. Clones were considered redundant if their sequences matched with at least 98% identity over regions of 45 bp or longer. All data were manually entered into a customized database created in Microsoft Access. Sequences can be accessed in dbEST (Genbank Accession Nos. CD051362–CD051765).

Colony-blot screening

Putative zoospore-specific clones identified after two rounds of differential screening with zoospore- and mycelium-derived probes were arrayed into a single 384-well plate and replicated in duplicate (two colonies/clone) onto filter membranes as described above. Blots were hybridized with developmental stage-specific probes as described for library screening, except that in one series of experiments DIG Easy Hyb (Roche Diagnostics) hybridization buffer was used. Signal intensity for each colony was visually assessed by two individuals independently, and assigned a value between 0 (low) and 3 (high) based on comparisons with controls represented by clones without inserts. Final values represent averages of values assigned by the two observers for two independent hybridization experiments at each stage. Visual assessment was chosen because colonies could be individually examined so that variability due to colony size and smearing of signal could be taken into account, since these factors could be easily perceived with colorimetric detection of the DIG-labelled probes.

ImageQuant quantification software was also used to measure signal intensities on the colony blots, and the results were compared to those obtained using visual scoring. ImageQuant afforded increased precision, in that smaller differences in signal intensity between clones could be distinguished. However, the observed increase in precision was masked by the variability observed between signals from different hybridization experiments, and in some cases by the variability between different clones representing the same gene in a single experiment. Observed levels of variability were similar with the two observation methods used (data not shown).

Northern analysis

Total RNA for each sample was treated with glyoxal (Sambrook and Russell 2001) and fractionated on a 1% agarose gel in 20 mM HEPES (pH 7) containing 1 mM EDTA. RNA was transferred to Nytran-N (Amersham) membranes by standard procedures (Sambrook and Russell 2001). Probes were synthesized from gel-purified insert DNA using the DIG-High Prime kit (Roche) according to the manufacturer's instructions. Membranes were hybridized in Church buffer, as described above.

Results

cDNA library screening and sequencing of clones

A cDNA library was constructed from mRNA isolated from zoospores of *P. nicotianae*. This species produces large numbers of zoospores in culture, and has a much wider host range than the two species of *Phytophthora* whose sequences have been lodged in the PGC database. Bacterial colonies containing library clones as excised plasmids were arrayed on replicate nylon filters and screened with cDNA probes derived from either zoospores or 4-day-old mycelium. Observation by microscopy confirmed that there were no visible signs of sporulation in the 4-day-old mycelium. We screened 10,430 clones and identified 434 clones that gave a stronger signal with the zoospore-derived (Z) probe than with the mycelium-derived (M) probe in two rounds of screening. Sequencing of these clones was done using the T3 promoter primer, which generated ESTs corresponding to the 5′ end of the cloned cDNA. After removal of vector sequences from 408 sequences that were readable, ESTs were compiled into a local database and compared using the BLASTN program (Altschul et al. 1997) to identify redundant clones. It was determined that the ESTs represent 240 genes. The majority (192; i.e. 47%) of the clones were unique, i.e. most genes (79%) were represented by a single clone. Some of the genes identified were represented by up to 24 clones, indicating that some genes were highly expressed.

Comparison of ESTs with sequences from public databases

To help elucidate the putative functions of identified genes, ESTs generated from the library screen were compared to sequences in the nr database (containing non-redundant sequences) in GenBank using the BLASTX program (Altschul et al. 1997). ESTs were assigned a putative function if the degree of similarity to a known protein gave rise to an E value of e^{-4} or less. For the redundant clones, the EST with the lowest E value was used. Fifty percent of the identified genes had matches with E values higher than e^{-4} or matched uncharacterized sequences only, and were classified as unknowns. Genes to which a putative function could be assigned were divided into four broad categories (Table 1). As some gene functions could arguably be assigned to more than one category, categories were assigned primarily to facilitate further tracking and characterization of the clones rather than to imply real functional divisions. The proportions of genes assigned to each category were: 21% metabolic, 10% signalling, 3% structural and 16% ribosomal proteins (Table 1). All sequences were also compared to the EST database in GenBank and to the PGC public database to identify matches with E value of e^{-4} or less with genes from other *Phytophthora* species. Genes with unknown function (50%) were grouped according to whether they showed matches with *P. sojae* zoospore ESTs only (14%) or with any other *Phytophthora* sequence (8%), or had no matches (28%). In total, 50% of all *P. nicotianae* ESTs had matches among the public *P. sojae* zoospore-derived ESTs. Of the sequences that had both *P. infestans* and *P. sojae* matches that differed in E value, 82% had a lower E value for the *P. infestans* match. Comparison of

Table 1 List of clones assigned to functional categories based on matches with characterized proteins in GenBank

Category[a]	DS No.	GenBank ID	Closest putative homologue (species of origin)	Accession No. of homologue	E value
MET	37	CD051396	Possible quinolinate synthetase (*Chlamydomonas reinhardtii*)	AAK54345.1	2e-23
	42	CD051400	dUTP pyrophosphatase (*Arabidopsis thaliana*)	AAL34163.1	2e-27
	52	CD051410	NADH ubiquinone oxidoreductase (*Bos taurus*)	Q02369	3e-11
	68	CD051426	RNA binding protein (*Drosophila melanogaster*)	AAB61993.1	3e-14
	74	CD051432	Nascent polypeptide-associated complement (*Arabidopsis thaliana*)	AF370545	3e-35
	77	CD051435	GEL1, beta-1,3-glucanosyl transferase (*Aspergillus fumigatus*)	AAC35942.1	2e-05
	82	CD051439	isocitrate dehydrogenase (*Bos taurus*)	Q04467	5e-24
	101	CD051457	Tryptophan synthase (*Neisseria meningitidis* Z2491)	NP_283692	3e-08
	105	CD051461	Elongation factor 1-gamma (*Artemia salina*)	P12261	3e-36
	110	CD051466	Translation factor sui1 (*Homo sapiens*)	NP_005866	3e-18
	114	CD051470	Ribonucleotide reductase M2 subunit (*Trypanosoma brucei brucei*)	CAA71741	9e-69
	116	CD051472	RNA polymerase III (*Mus musculus*)	Q921X6	3e-18
	117	CD051473	Elongation factor 1 beta (*Saccharomyces cerevisiae*)	P32471	5e-06
	120	CD051476	Cytochrome *c* peroxidase (*Euglena gracilis*)	BAC05484.1	8e-22
	121	CD051477	Cellulase (*Piromyces rhizinflata*)	AAF34679.1	8e-11
	124	CD051480	3-Isopropylmalate dehydratase (*Methanosarcina acetivorans*)	Q8TLF1	2e-21
	134	CD051490	Mitochondrial solute carrier (*Arabidopsis thaliana*)	AAM65239.1	2e-14
	155	CD051511	Lyase, carboxylase [*Oryza sativa* (*japonica*)]	BAB64236.1	3e-46
	171	CD051526	Nucleotide sugar epimerase (*Mesorhizobium loti*)	BAB53985.1	2e-23
	216	CD051563	Prenylated SNARE protein (*Nicotiana tabacum*)	AAD00116.1	5e-42
	217	CD051564	RNA helicase (*Saccharomyces cerevisiae*)	CAA93395	6e-26
	222	CD051569	ABC transporter [*Oryza sativa* (*japonica*)]	CAD59565.1	3e-24
	253	CD051598	Thaumatin-osmotin like (*Cicer arietinum*)	CAA09229.1	9e-05
	255	CD051600	Transitional ER ATPase (*Arabidopsis thaliana*)	T48355	3e-17
	276	CD051620	Ubiquitin-specific protease (*Homo sapiens*)	NP_006667	2e-07
	288	CD051632	Putative purple acid phosphatase (*Glycine max*)	AF236108	2e-04
	290	CD051634	Serine hydroxymethyl-transferase (*Leishmania major*)	CAB72302	7e-52
	291	CD051635	Cellobiohydrolase precursor (*Neocallimastix patriciarum*)	AAC49315.1	5e-15
	292	CD051636	Ribonucleotide reductase M1 subunit (*Mus musculus*)	P07742	1e-68
	296	CD051640	Diacylglycerol acyltransferase (*Homo sapiens*)	NP_115953	7e-24
	299	CD051643	Ammonium transporter (*Phytophthora infestans*)	AAN31513.1	6e-67
	301	CD051645	Acyl-CoA binding (*Caenorhabditis elegans*)	NP_491412.1	5e-15
	302	CD051646	Beta-1,3-glucanase (*Avena sativa*)	AAF80276	2e-05
	311	CD051654	Vacuolar type H + -translocating pyrophosphatase (*Arabidopsis thaliana*)	C86303	3e-59
	322	CD051665	NADPH dependent 2 cyclohexene reductase (*Azotobacter vinelandii*)	BAA88211	2e-38
	327	CD051670	Acyl-CoA dehydrogenase (*Pseudomonas fluorescens*)	CAC34855	2e-52
	335	CD051678	DNA ligase I (*Xenopus laevis*)	P51892	2e-27
	337	CD051680	Mitochondrial carrier (*Arabidopsis thaliana*)	NP_198104	1e-10
	339	CD051682	Ubiquitin (*Branchiostoma belcheri*)	AAK91296	9e-34
	352	CD051695	Glutathione s-transferase (*Glycine max*)	AAG34813.1	2e-17
	356	CD051699	S-adenosyl methionine synthase (*Phytophthora infestans*)	AAN31489.1	3e-94
	363	CD051706	Putative molybdenum cofactor synthesis (*Mus musculus*)	AAH24371.1	3e-30
	369	CD051712	Elongation factor 1 alpha (*Phytophthora infestans*)	CAB65347.1	1e-86
	380	CD051720	Glycerol-3-phosphate dehydrogenase (*Xenopus laevis*)	AAH43631.1	2e-52
	385	CD051725	Probable plasma membrane ATPase (*Dictyostelium discoideum*)	T30580	2e-14
	388	CD051726	Flavohemoglobin (*Bacillus halodurans*)	B83782	4e-20
	396	CD051733	Glucokinase (*Thermosynechococcus elongatus*)	NP_682285	1e-20
	409	CD051743	Translation elongation factor 3 (*Yarrowia lipolytica*)	BAA33895	7e-69
	417	CD051751	Ubiquitin conjugating enzyme (*Phytophthora infestans*)	AAN31466.1	5e-75
	427	CD051757	Xylitol dehydrogenase (*Hypocrea jecorina*)	AAO42466.1	6e-61
SIG	39	CD051398	Nek2 serine/threonine kinase (*Xenopus laevis*)	AB019557	1e-31
	81	CD051438	IMP dehydrogenase (*Homo sapiens*)	BC033622	1e-26
	140	CD051496	GMP synthase (*Schizosaccharomyces pombe*)	CAB88269.1	4e-37
	143	CD051499	Prohibitin (*Toxocara canis*)	AAB53231.1	3e-38
	146	CD051502	Ca-lipid binding (*Arabidopsis thaliana*)	CAC05504	1e-10
	159	CD051515	Diacylglycerol kinase (*Mesocricetus auratus*)	Q64398	7e-04
	170	CD051525	Tubby homolog (*Homo sapiens*)	CAC14586.1	4e-05
	177	CD051531	WD40-repeat containing (*Dictyostelium discoideum*)	P54686	8e-23
	212	CD051559	GTP-binding regulatory (*Chlamydomonas reinhardtii*)	S11904	4e-51
	268	CD051613	DG1148 (*Dictyostelium discoideum*)	AAD28548.1	8e-46
	269	CD051614	Ran-binding, suppressor of FUS1 (*Saccharomyces cerevisiae*)	NP_010285.1	3e-06
	273	CD051617	G-protein, beta (*Danio rerio*)	O42248	2e-75
	332	CD051675	CDC14 homolog A (*Homo sapiens*)	AF122013	6e-51
	333	CD051676	Serine/threonine/tyrosine kinase [*Oryza sativa* (*japonica*)]	AAM22712.1	1e-09

Table 1 (Continued)

Category[a]	DS No.	GenBank ID	Closest putative homologue (species of origin)	Accession No. of homologue	E value
	338	CD051681	Transcriptional coactivator-like (*Nicotiana tabacum*)	T49151	3e-12
	354	CD051697	Serine-protease like (*Arabidopsis thaliana*)	AAM13204.1	5e-35
	358	CD051701	cAMP-dependent kinase (*Aspergillus niger*)	CAA64172.1	9e-06
	383	CD051723	Ran gene product (GTP binding) (*Caenorhabditis elegans*)	CAB07240.1	1e-84
	384	CD051724	CCCH zinc-finger protein C3H-2 (*Xenopus laevis*)	AF061980	1e-15
	390	CD051728	Proliferating cell nuclear antigen (*Brassica napus*)	Q43124	3e-59
	401	CD051737	Adenylate kinase (*Bordetella pertussis*)	S43016	5e-27
	404	CD051740	Putative cAMP-dependent kinase (*Arabidopsis thaliana*)	AAD24392.1	1e-31
STR	4	CD051364	Sperm mitochondrial capsule selenoprotein (*Mus musculus*)	P15265	2e-04
	11	CD051371	Ankyrin (*Homo sapiens*)	AAC79841.1	3e-16
	163	CD051519	Actin depolymerizing factor (*Lilium longiflorum*)	S30935	9e-20
	173	CD051528	Extensin (*Triticum aestivum*)	BAA89307	4e-06
	244	CD051590	Alpha tubulin (*Reclinomonas americana*)	AAK37433.1	2e-98
	266	CD051611	Calponin homolog (*Echinococcus granulosus*)	S40075	1e-23
	348	CD051691	Vitellogenein (*Cyprinus carpio*)	AAL07472	6e-06

[a]MET, metabolism; SIG, signalling; STR, structural. Clones representing 37 ribosomal proteins (RIB) were omitted

ESTs with sequences from the JGI *P. sojae* database, revealed that only 19 of the identified *P. nicotianae* genes had no significant matches among the *P. sojae* genomic sequences. Most of them represented unknown proteins, except for ds255, which may code for a transitional ER ATPase.

A large proportion of our ESTs was found to represent genes with cellular housekeeping roles whose transcripts are found in most eukaryotic cell types. These genes may be indicative of metabolic pathways that are up-regulated in zoospores. However, since it is also possible that some of these genes were either not consistently differentially expressed, or represented false positives after two rounds of screening, a further evaluation of the expression levels of identified genes was performed on a number of developmental stages by arrayed colony blot screening.

Colony blot screening and analysis

Clone-containing colonies representing 208 of the identified genes were arrayed and replicated in duplicate on multiple nylon membranes for further screening. Genes represented by clones that were difficult to subculture were omitted. Forty of the arrayed genes were represented by two or more independent clones, so that there were 265 colonies in total, not including the background controls which contained plasmids with no inserts. The membranes were screened using cDNA probes derived from four developmental stages of *Phytophthora*, as well as infected and uninfected tobacco seedlings. Signals from the colonies were visually assessed and assigned an arbitrary value between 0 and 3, so that higher numbers represent stronger signals compared to the background controls.

To assess the level of variability of signal intensity values we compared colony blot signals derived from different clones representing the same gene. The standard error in signal intensity at a given developmental stage for five genes that were represented by three or more independent clones varied between 0.15 and 1.5. The difference in values obtained for individual clones in two separate experiments at a given developmental stage resulted in an overall standard error of 0.3–1.1.

To identify clones that were preferentially expressed in zoospores compared to mycelium the difference between the average values for signal intensity between the two stages (Z-M) was calculated. Although 115 clones had Z-M values greater than 0, indicating that they might be differentially expressed, in view of the level of variability described above, we focused on clones for which (Z-M)≥1. Some 29 clones, representing 22 genes, exceeded this cut-off level (Table 2). Among these, 15 genes, including seven of unknown function, had matches among *P. sojae* zoospore ESTs in the PGC database. Colony-blot screening with probes from other developmental stages revealed that transcripts for 12 of the 22 genes could also be detected in sporulating hyphae (i.e. S≥1), while 10 were represented in germinating cysts (G≥1). The difference between the value for inoculated and uninoculated tissue (I-U) was greater or equal to 1 for only one of the 22 identified genes, which represented an unknown protein (Table 3).

Northern analysis

To verify the expression patterns derived from the colony blots, Northern analysis was performed for 10 clones for which (Z-M)≥1, and for two clones for which (Z-M) = 0.75 (Fig. 1). In agreement with the colony array data, transcripts corresponding to all of the tested clones were present in zoospores and absent in mycelium. There was also general agreement between the Northern-blot signals and the colony-blot data for sporulating hyphae and germinating cysts. However, for four clones (ds121, ds160, ds51 and ds158) the signal

Table 2 Summary of the data for clones expressed in zoospores at higher levels than in mycelium

DS No.	GenBank ID	Closest putative homologue	Z-M[a]	PsZ[b]	PsM[b]	PsI[b]	PiM[b]	E-s[c]	E-i[c]	Number of clones[d]
51	CD051409	30 S ribosomal S12 (mitochondrial)	2	+	+	+	+	e-122	e-133	1
279	CD051623	None	1.75	+	-	-	-	2e-18		12
121	CD051477	Cellulase, exoglucanase	1.75	-	-	-	-			1
134	CD051490	Mitochondrial solute carrier protein	1.5	+	-	-	+	2e-99	e-118	1
12	CD051372	None	1.5	-	-	-	-			1
114	CD051470	Ribonucleotide reductase M2 subunit	1.5	+	-	-	-	e-178		21
356	CD051699	S-Adenosyl methionine synthase	1.5	+	+	-	+	0	0	5
43	CD051401	CBEL protein	1.5	+	+	+	-	0		4
23	CD051383	None	1.5	+	-	-	-	9e-21		2
18	CD051378	None	1.5	+	-	-	-	3e-15		5
158	CD051514	None	1.25	-	-	-	-			11
314	CD051657	None	1.25	-	-	-	-			1
32	CD051391	None	1.25	+	-	-	-	3e-79		1
65	CD051423	None	1	+	-	-	-	e-107		3
111	CD051467	None	1	+	-	-	-	e-144		3
166	CD051522	Unknown	1	+	-	-	-	4e-17		6
213	CD051560	Tubby homolog	1	+	-	-	-	7e-37		8
222	CD051569	ABC transporter	1	+	+	+	+	e-123	e-153	3
269	CD051614	Ran-binding, suppressor of FUS1	1	+	+	-	+	3e-79	4e-94	2
276	CD051620	Ubiquitin-specific protease	1	-	-	-	-			1
291	CD051635	Cellobiohydrolase precursor	1	-	-	-	-			1
338	CD051681	Transcriptional coactivator-like	1	+	+	+	-	e-50		1
349	CD051692	Oxidant induced cell-cycle arrest	1	-	-	-	-			1

[a]Z-M represents the difference between average assigned signal intensity observed in two rounds of screening with zoospore and mycelium-derived probes
[b]Presence of homologous sequences among public ESTs derived from *P. sojae*is indicated: PsI (inoculated soybean tissue), PsM (mycelium), PsZ (zoospores); homologues in *P. infestans* mycelium ESTs are indicated in column PiM
[c]BLASTN E-values for best sequence match with *P. sojae* and*P. infestans* are shown in columns E-s and E-i, respectively
[d]Number of clones in the library that belong to the same EST cluster

Table 3 Results from the colony array screening for clones described in Table 2

DS No.	Signal intensity[a]				
	M	S	Z	G	I-Ub
51	0.25	1.0	2.25	1.0	0.5
279	0.5	0.0	2.25	0.5	0.25
121	0.25	0.25	2.0	1.0	0.25
134	0.25	2.0	1.75	0.75	0.75
12	0.25	2.75	1.75	0.0	0.75
114	1.25	1.0	2.75	0.5	0.0
356	1.0	2.5	2.5	1.0	0.25
43	0	3.0	1.5	0.5	0.5
23	0.5	1.0	2.0	1.0	0.25
18	1.25	1.5	2.75	1.25	0.5
158	0.25	0.5	1.5	1.5	0.0
314	0.75	1.0	2.0	0.5	0.5
32	0.25	0.5	1.5	0.5	-0.5
65	1.25	1.0	2.25	0.25	0.75
111	1.25	0.5	2.25	1.0	1.25
166	0.0	0.0	1.0	0.0	0.0
213	0.5	0.25	1.5	1.0	-0.5
222	1.0	1.75	2.0	0.75	-0.5
269	1.25	0.25	2.25	1.5	0.75
276	1.0	0.0	2.0	1.25	0.25
291	0.5	0.0	1.5	0.0	0.25
338	0.5	1.0	1.5	0.75	0.75
349	0.5	0.0	1.5	1.0	0.25

[a]Numbers represent average assigned signal intensity (0–3) observed in two rounds of screening with stage-specific probes (Z, zoospores; M, mycelium; G, germinating cysts; S, sporulating hyphae). I-U is the difference in values observed for inoculated and uninoculated tobacco seedlings)

intensity on the Northern blots hybridized with the germinating cyst probe was greater than indicated by the colony-blot hybridization, and for three clones (ds160, ds51, ds23) no bands were detected on Northern blots hybridized with sporulating hyphae probe, although the average signal intensity on the colony array blots was ≥1. No signal could be detected for any of the clones in Northern blot lanes containing up to 15 μg of total RNA from inoculated seedlings, even for the clone for which the I-U value from colony array was 1.25 (the highest value observed; data not shown).

Discussion

After differentially screening over 10,000 cDNA clones, we have identified more than 200 genes expressed in *P. nicotianae* zoospores. Most of the genes (79%) were represented by single clones, indicating that the screen was not exhaustive. Of the 240 *P. nicotianae* zoospore genes, 138 (57%) had matches in the PGC database. The majority of *P. nicotianae* ESTs that had homologues in both *P. infestans* and *P. sojae* libraries showed a higher degree of similarity with transcripts from *P. infestans* than with *P. sojae* sequences. This observation is consistent with the reported phylogenetic relationship between these three species based on the analysis of the ITS sequences of genomic rDNA (Cooke et al. 2000).

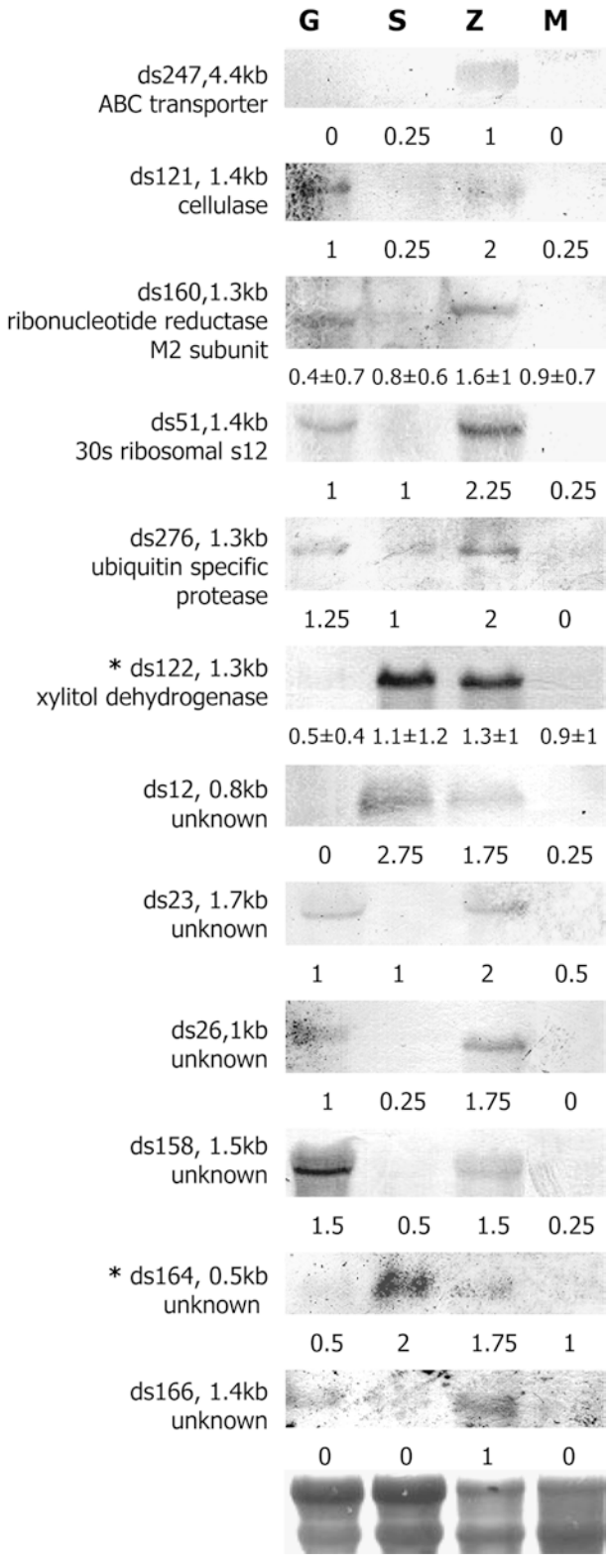

Fig. 1 Comparison of Northern and colony blot analyses. Assigned database numbers followed by estimated transcript size in kilobases and putative gene function are shown to the *left* of the Northern blots. The average value for the colony blot signal is given *below* each lane. For clones ds160 and ds122 the values represent means (±S.D.) from, respectively, 8 and 4 independent clones representing the same gene. The developmental stage from which the RNA was isolated is indicated at the *top*: G, germinating cysts; S, sporulating hyphae; Z, zoospores; M, mycelium. The blot at the bottom shows methylene blue staining of the total RNA on the blot. Clones marked with *asterisks* had colony array values (Z-M) = 0.75, for all others (Z-M) ≥ 1. ds26 belongs to the same gene cluster as ds279 shown in Tables 2 and 3

Zoospores of different *Phytophthora* species have been shown to differ in ultrastructure, antigenic properties (reviewed by Hardham et al. 1991), chemotactic response to specific compounds (reviewed by Deacon and Donaldson 1993) and host range (Erwin and Ribeiro 1996). Thus, some of the *P. nicotianae* genes not found among the *P. sojae* zoospore ESTs or genomic sequences currently available could be related to these species-specific differences. Comparison with the GenBank non-redundant database enabled us to assign a putative function to half of the genes identified. At the time of writing, 28% of the identified *P. nicotianae* genes were novel, since they had no matches in either GenBank or PGC databases.

Since some of the observed differences in gene expression between zoospores and mycelium could have been due to physiological differences between tissue samples unrelated to developmental stage, identified clones were arrayed and screened again with probes derived from zoospores, mycelium, germinating cysts, sporulating hyphae and infected and uninfected tobacco seedlings. After two additional rounds of screening, 115 clones consistently produced stronger signals with zoospore- compared to mycelium-derived probes, making them strong candidates for representatives of truly differentially expressed genes. However, before concluding that selected genes were differentially expressed, it was necessary to assess the variability in signal intensity observed between redundant clones, or between repeated experiments for a particular clone at a given developmental stage. The considerable level of variability observed in this and other arrayed transcript profiling studies (e.g. Hughes et al. 2001; Mills et al. 2001; Carson et al. 2002) is not surprising, given that the signal intensity is dependent on parameters that are hard to standardize—including the amount of target DNA in each spot or colony and the degree of cross-hybridization between a cloned gene segment and other non-specific sequences present in the labelled probe. In view of the observed variability levels, we have chosen to focus on the clones for which (Z-M)≥1. This criterion was satisfied by 29 clones, representing 22 genes. Seven of these genes represent previously uncharacterized proteins with homologues among the *P. sojae* zoospore ESTs, and therefore could be involved in conserved zoospore-specific functions. The screen also showed that 12 of the 22 genes were also expressed in sporulating hyphae, indicating that they may function during zoospore development as well as in zoospores. On the

other hand, 10 of the genes that were also expressed in germinating cysts may be involved in cyst germination and the development of infection structures. In addition, at least one gene may also be associated with plant colonization, since it was detected among transcripts from inoculated tissue.

Northern analysis confirmed differential expression of 10 of the genes discussed above, and also revealed differential expression of two additional genes for which the difference in signals observed on colony blots was slightly below our cut off value (Z-M = 0.75). This suggests that the chosen cut off value was fairly stringent and that further expression analysis of the clones from the colony blot array may show differential expression for additional genes. Some discrepancies were observed between signals obtained from Northern blots and colony arrays, particularly with probes derived from germinating cysts and sporulating hyphae. Since the RNA samples used to generate the probes for colony array were collected independently from those used in Northern blots, it is possible that the different results observed by two methods arise from physiological differences in the tissues from which the RNA was extracted.

In summary, we have identified a set of genes, a number of which are novel, that are expressed in zoospores but not in the mycelium of *P. nicotianae,* suggesting that they may be involved in asexual reproduction of this and possibly other oomycetes. Future experiments will focus on determining the function of these genes and gene regulatory sequences through analysis of genomic clones, transgenic constructs and exogenously expressed proteins. The results of these experiments should lead to a better understanding of the infection process in *Phytophthora* as well as a number of other zoospore-forming pathogens.

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