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Gene expression in germinated cysts of *Phytophthora nicotianae*

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SUMMARY

The life cycle of *Phytophthora* species contains several distinct asexual developmental stages that are important for plant infection and disease development. These include vegetative growth of filamentous hyphae, production of multinucleate sporangia, motile, uninucleate zoospores and germinated cysts, the stage at which plant colonization is initiated. To understand mechanisms regulating molecular and cellular processes in germinated cysts, differential hybridization analysis of a cDNA library was used to identify genes up-regulated after cyst germination in *P. nicotianae*. Arrays of 12 288 random cDNA clones derived from a germinated cyst cDNA library were screened with ^{32}P-labelled cDNA probes synthesized from mRNA isolated from four different developmental stages and tobacco tissues infected with *P. nicotianae*. The resultant expression profiles for each cDNA clone led to the identification of over 300 clones showing up-regulated expression in germinated cysts compared with the other three stages. Sequencing of the 5′ end of 382 selected clones yielded 355 sequences representing 146 putative unigenes. Sequence analysis revealed similarities to genes encoding proteins involved in energy production, protein biosynthesis, signalling, cell-wall biogenesis and transcription regulation. Novel genes putatively involved in cell adhesion, cell-wall biogenesis and transcriptional regulation were identified. Thirty-one cDNA clones were analysed by Northern blotting and for 28 the pattern of expression in the Northern blots was the same as that indicated by the macroarray screening, verifying the fidelity of the colony hybridization data.

INTRODUCTION

The oomycetes include a diverse and unique group of fungus-like organisms that have recently been confirmed by molecular analysis of mitochondrial and ribosomal DNA sequences as being phylogenetically distant from true fungi (Förster *et al.*, 1990; Gunderson *et al.*, 1987; Paquin *et al.*, 1997; Sachay *et al.*, 1993). They are fundamentally different from true fungi in terms of cell-wall composition, reproductive biology and genetics (Judelson, 1997). Most fungicides against major fungal pathogens are ineffective in controlling diseases caused by oomycetes. These features suggest that distinct biochemical and genetic mechanisms may be involved in plant infection and colonization.

Oomycetes include many obligate and facultative parasites that cause disease on a wide range of plants worldwide. Among the notable oomycetes, nearly all 60 members of the genus *Phytophthora* are plant pathogens and cause destructive diseases on thousands of plant species. These include *P. infestans*, which caused the potato famine in Ireland in the 1840s and which is still a worldwide problem in potato-growing regions (Birch and Whisson, 2001). They also include *P. sojae*, which causes major problems for soybean production in North America (Tyler, 2001), *P. ramorum*, which causes Sudden Oak Death in California (Rizzo *et al.*, 2002), and *P. cinnamomi*, which causes severe damage to forests and heathlands in Australia (Podger, 1972).

Phytophthora species have several distinct asexual developmental stages, such as vegetative growth, sporulation, flagellate zoospores and cysts, and all are important for plant infection and disease development (Hardham, 2001). The disease cycle is initiated by the release of motile, wall-less zoospores, which, following a chemotactic stage, encyst on plant surfaces before penetration occurs. The cyst germinates quickly to form a germ tube that either, for some species such as *P. sojae* (Enkerli *et al.*, 1997), invades the host directly or, more frequently for some other species such as *P. nicotianae* (Perrone *et al.*, 2000), *P. infestans* (Pristou and Gallegly, 1954) and *P. boehmeriae* (Shan *et al.*, 1995), forms a specialized infection structure, the appressorium, prior to entering host tissues.

Spore germination and germling growth is an essential step in the disease cycle of most fungal and oomyceteous pathogens and has been extensively characterized using cytological and cell biological approaches (Hardham, 1992), yet little is known of the molecular mechanisms regulating this process. It is likely that with the germination of cysts, a complex programme of differential gene expression is initiated in which genes required for the development of infection structures and invasion of the host plants may be activated. A better understanding of the molecular mechanisms underlying these developmental processes could lead

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to the development of new strategies to reduce *Phytophthora* infections and thereby benefit agricultural and forestry practices. One approach to identifying genes important for the biology of a given developmental stage involves the identification of transcriptional changes occurring therein. Generally, this is based on the assumption that genes highly expressed in a given stage are important for the establishment of that stage. The identification of these genes is greatly facilitated by the development of various differential screening approaches, such as the differential screening of cDNA (Kim and Judelson, 2003; Skalamera *et al.*, 2004; Tani *et al.*, 2004) or genomic (Pieterse *et al.*, 1993) libraries and subtractive hybridization (Fabritius *et al.*, 2002), which have been successfully used for the identification of infection- or developmental stage-specific genes from *Phytophthora* species.

P. nicotianae is an important pathogen capable of infecting species of more than 72 plant genera (Hickman, 1958). We use *P. nicotianae* as a model species to investigate genes important for development and plant infection. Unlike highly specific *P. infestans* and *P. sojae*, *P. nicotianae* has a broad host range. EST analysis is a useful tool to investigate both common and unique mechanisms required for interaction with host plants. In our laboratory, we have used differential hybridization of cDNA libraries to identify genes that are up-regulated in motile zoospores (Skalamera *et al.*, 2004). In order to identify genes that are specifically expressed in germinated cysts of *P. nicotianae*, in the present study a cDNA library derived from germinated cysts of *P. nicotianae* was constructed and screened by differential hybridization with total cDNA probes created from mRNA of four different asexual developmental stages and of tobacco roots infected with *P. nicotianae*. The resulting information will contribute valuable genetic resources for functional characterization of *Phytophthora* genes, data that will ultimately be vital for the development of durable and environmentally friendly strategies to control notorious plant diseases initiated by *Phytophthora*.

RESULTS

Generation of germinated cyst cDNA library

A cDNA library was constructed with mRNA isolated from germinated cysts harvested after a 4-h incubation of *P. nicotianae* H1111 cysts in the presence of pectin. At this time point, > 90% of cysts had germinated and the average length of germ tubes was approximately ten times the diameter of the cyst. The λ cDNA library was converted into plasmid clones by mass excision for library screening and analysis. Analysis of 50 random cDNA clones indicated that 90% of the recombinant clones had inserts and the average insert size was 0.85 kb. A total of 12 288 plasmid cDNA clones were transferred to 384-well microtitre plates and stored at –80 °C.

Identification of stage-specific genes by differential hybridization

To identify genes up-regulated in germinated cysts, the 12 228 random cDNA clones derived from the germinated cyst cDNA library were arrayed in 384-well microtitre plates (Nunc) and replicated on to N+ nylon membranes so that each contained 1536 cDNA clones. Two sets of colony filters were prepared and hybridized sequentially with ^{32}P-labelled cDNA probes synthesized with mRNA isolated from sporulating hyphae, vegetative hyphae, zoospores, germinated cysts and tobacco tissues infected with *P. nicotianae*. Analysis of two membranes (3072 colonies) indicated that 45.4% of clones hybridized to the germinated cyst cDNA probe, 28.8% to vegetative hyphae, 18.7% to sporulating hyphae, 38.5% to zoospores and 0.6% to tobacco tissue infected with *P. nicotianae*. Screening of the first half of the 12 228 clones revealed that some genes, such as *translation elongation factor 1α* (*TEF1α*), *GAPDH* (glyceraldehyde 3-phosphate dehydrogenase) and several ribosomal protein sequences were being selected many times. Thus during screening of the second half of the cDNA clones, attempts were made to exclude these over-represented genes by re-hybridizing the membranes with probes for these genes and avoiding the subsequent selection of clones that were positive. This reduced the selection of *GAPDH* and *TEF1α* clones by 60% but not the sequences encoding ribosomal proteins. Approximately 20% of clones hybridized to both germinated cyst and zoospore cDNA probes, whereas only ~8% of the clones appeared to be expressed in all four asexual developmental stages.

Sequence analysis

In total, 314 cDNAs expressed predominantly in germinated cysts, 37 cDNAs showing up-regulated expression in both zoospores and germinated cysts, 21 cDNAs showing expression in all four asexual developmental stages, and ten cDNAs displaying expression in infected plant material were subjected to 5′ end sequencing using the T3 primer. Although our emphasis was on genes that were expressed predominantly in germinated cysts, the additional 68 clones were included because of their up-regulated expression in both zoospores and germinated cysts, and their potential value in the verification of expression profile data and in future improvements to *P. nicotianae* transformation methods. Readable partial sequences larger than 200 bp were obtained from 355 of the 382 cDNA clones submitted for sequencing.

Self-blast analysis indicated that the 355 ESTs formed 146 clusters putatively representing 146 different genes. Among the 146 putative different genes, 60 were represented by two or more ESTs (Table 1). Blast searches against NCBI databases identified 13 genes corresponding to known *Phytophthora* genes and 105 genes (represented by 111 ESTs) corresponded to homologues of known genes (*E* value generally less than 10^{-10}, although for

Table 1 BLASTX results with the *Phytophthora nicotianae* ESTs identified in this study.

Clone ID (accession number)	Size (bp)	No. of ESTs	Best hit in GenBank (source species)	Cellular function	Blastx *E* value
WS3 (CK859400)	500	2	Fructose-bisphosphate aldolase (*Haemophilus influenzae*)	Metabolism	7e-43
WS6 (CK859401)	> 500	25	Translation elongation factor 1α (*Phytophthora infestans*)	Protein synthesis	1e-95
WS9 (CF891671)	500	4	Hypothetical (*Phytophthora capsici*)	Unknown	4e-11
WS15 (CF891672)	540	2	Hypothetical (*Phytophthora capsici*)	Unknown	4e-11
WS16 (CF891673)	> 540	3	42 kD glycoprotein elicitor (*Phytophthora sojae*)	Cell wall/defense	3e-52
WS17 (CF891674)	> 370	2	No hit	Unknown	—
WS20 (CK859402)	> 420	6	40S ribosomal protein S4 (*Dictyostelium discoideum*)	Protein synthesis	6e-31
WS21 (CF891675)	> 540	2	40S ribosomal protein S3A (*Arabidopsis thaliana*)	Protein synthesis	4e-61
WS24 (CF891676)	> 480	3	G protein beta subunit (*Chlamydomonas reinhardt*)	Signal transduction	1e-129
WS25 (CK859403)	> 480	2	40S ribosomal protein P40 (*Urechis caupo*)	Protein synthesis	9e-56
WS26 (CK859404)	> 450	1	40S ribosomal protein S5 (*Cicer arietinum*)	Protein synthesis	6e-50
WS28 (CK859405)	268	1	60S ribosomal protein L11b (*Arabidopsis thaliana*)	Protein synthesis	8e-16
WS29 (CK859406)	> 520	1	60S ribosomal protein L15 (*Picea mariana*)	Protein synthesis	4e-58
WS32 (CK859407)	> 550	3	Phosphoglycerate kinase (*Aplysia californica*)	Metabolism	2e-53
WS41 (CF891677)	560	1	No hit	Unknown	—
WS42 (CK859408)	615	1	30S ribosomal protein S15 (*Thermus aquaticus*)	Protein synthesis	1e-10
WS43 (CF891678)	> 600	3	No hit	Unknown	—
WS45 (CK859409)	> 640	2	DnaK-type chaperone GRP78/BiP (*Phytophthora cinnamomi*)	Stress defense	1e-101
WS47 (CK859410)	219	1	No hit	Unknown	—
WS48 (CK859411)	> 580	2	60S ribosomal protein L7 (*Argospecten irradians*)	Protein synthesis	1e-42
WS51 (CK859412)	437	1	40S ribosomal protein S8 (*Oryza sativa*)	Protein synthesis	2e-40
WS52 (CK859413)	> 560	2	Dihyrdolipoamide acetyltransferase (*Podospora anserine*)	Metabolism	6e-29
WS53 (CK859414)	> 560	6	40S ribosomal protein S3 (*Arabidopsis thaliana*)	Protein synthesis	1e-67
WS54 (CK859415)	382	1	No hit	Unknown	—
WS56 (CF891679)	400	3	Translation elongation factor 2 (*Filobasidiella*)	Protein synthesis	1e-26
WS57 (CF891680)	> 600	2	No hit	Unknown	—
WS59 (CK859416)	> 560	1	Nitrate transporter (*Cylindrotheca fusiformis*)	Transport	7e-24
WS63 (CK859417)	412	2	Cytochrome c oxidase subunit Via (*Homo sapiens*)	Metabolism	2e-5
WS64 (CF891681)	600	1	Peptidylprolyl isomerase (*Phytophthora infestans*)	Metabolism	2.1e-73
WS66 (CF891682)	> 600	6	Plasma membrane H^+-ATPase (*Lycopersicon esculentum*)	Transport	1e-16
WS72 (CK859418)	> 500	2	Translation elongation factor eEF-3 (*Yarrowia lipolytica*)	Protein synthesis	2e-55
WS73 (CK859419)	> 560	1	40S ribosomal protein S18 (*Mus musculus*)	Protein synthesis	3e-49
WS74 (CK859420)	> 550	1	40S ribosomal protein S2 (*Drosophila melanogaster*)	Protein synthesis	1e-60
WS75 (CK859421)	> 520	3	60S ribosomal protein L10A (*Arabidopsis thaliana*)	Protein synthesis	4e-38
WS77 (CK859422)	> 400	2	60S ribosomal protein L14 (*Cucumis sativus*)	Protein synthesis	1e-26
WS78 (CK859423)	> 500	1	No hit	Unknown	—
WS79 (CK859424)	> 500	2	60S ribosomal protein L34 (*Saccharomyces cerevisiae*)	Protein synthesis	8e-30
WS80 (CK859425)	> 600	1	20S proteasome alpha 2 subunit (*Oryza sativa*)	Protein synthesis	7e-62
WS84 (CF891683)	> 530	1	Cellulose synthase catalytic subunit (*Arabidopsis thaliana*)	Cell wall	1e-5
WS86 (CK859426)	> 600	11	Aquaporin (*Toxocara canis*)	Transport	1e-23
WS91 (CK859427)	> 570	5	40S ribosomal protein S15 (*Spodoptera frugiperda*)	Protein synthesis	4e-52
WS98 (CK859428)	> 450	6	60S ribosomal protein L9 (*Arabidopsis thaliana*)	Protein synthesis	2e-41
WS100 (CF891684)	> 520	1	MYB-related transcription factor (*Arabidopsis thaliana*)	Transcription	3e-8
WS101 (CK859429)	> 420	1	No hit	Unknown	—
WS102 (CK859430)	> 550	1	Arsenite-resistance protein (*Cricetulus griseus*)	Metabolism	2e-12
WS103 (CK859431)	265	1	No hit	Unknown	—
WS104 (CK859432)	480	1	60S ribosomal protein L37a (*Caenorhabditis elegans*)	Protein synthesis	8e-32
WS105 (CF891685)	550	1	No hit	Unknown	—
WS107 (CK859433)	630	3	60S ribosomal protein L7A (*Arabidopsis thaliana*)	Protein synthesis	2e-48
WS108 (CK859434)	> 540	4	60S ribosomal protein L18A (*Arabidopsis thaliana*)	Protein synthesis	1e-48
WS109 (CK859435)	> 480	3	60S ribosomal protein L13a (*Cyanophora paradoxa*)	Protein synthesis	7e-49
WS110 (CK859436)	520	1	No hit	Unknown	—
WS112 (CK859437)	598	4	60S ribosomal protein L23a (*Homo sapiens*)	Protein synthesis	3e-45

Table 1 *continued.*

Clone ID (accession number)	Size (bp)	No. of ESTs	Best hit in GenBank (source species)	Cellular function	Blastx *E* value
WS113 (CK859438)	470	1	60S ribosomal protein L35a (*Ictalurus punctatus*)	Protein synthesis	2e-30
WS126 (CK859439)	> 640	1	60S ribosomal protein L17 (*Arabidopsis thaliana*)	Protein synthesis	4e-51
WS135 (CK859440)	580	2	40S ribosomal protein S19 (*Arabidopsis thaliana*)	Protein synthesis	9e-46
WS136 (CK859441)	> 580	2	40S ribosomal protein S27a (*Ictalurus punctatus*)	Protein synthesis	2e-34
WS138 (CK859442)	> 320	2	Triosephosphate isomerase (*Phytophthora infestans*)	Metabolism	1e-57
WS143 (CK859443)	> 450	6	Pyruvate dehydrogenase (*Rattus rattus*)	Metabolism	7e-54
WS147 (CK859444)	> 450	1	Ubiquinol—cytochrome-c reductase-like (*Arabidopsis thaliana*)	Metabolism	5e-10
WS148 (CK859445)	> 480	3	Annexin A13 isoform b (*Homo sapiens*)	Metabolism	2e-10
WS155 (CK859446)	401	2	60S ribosomal protein L13 (*Danio rerio*)	Protein synthesis	2e-39
WS157 (CK859447)	340	8	40S ribosomal protein S20 (*Homo sapiens*)	Protein synthesis	2e-46
WS158 (CK859448)	473	4	60S ribosomal protein L22 (*Arabidopsis thaliana*)	Protein synthesis	3e-20
WS159 (CK859449)	506	2	60S ribosomal protein L28 (*Ictalurus punctatus*)	Protein synthesis	9e-14
WS163 (CK859450)	> 520	2	Voltage-dependent anion channel (*Squalus acanthias*)	Transport	1e-6
WS167 (CK859451)	200	1	60S ribosomal protein L39 (*Branchiostoma belcheri*)	Protein synthesis	2.1
WS175 (CK859452)	> 520	1	Glutamine synthetase (*Zea mays*)	Metabolism	8e-34
WS176 (CK859453)	> 530	1	No hit	Unknown	—
WS178 (CK859454)	350	3	No hit	Unknown	—
WS182 (CK859455)	> 490	1	Cysteine synthase (*Solanum tuberosum*)	Metabolism	3e-51
WS183 (CK859456)	> 520	1	Methionine synthase (*Catharanthus roseus*)	Metabolism	3e-39
WS184 (CK859457)	> 530	2	40S ribosomal protein S10 (*Branchiostoma belcheritsin*)	Protein synthesis	6e-37
WS187 (CF891694)	> 490	1	DnaK-type molecular chaperone (*Bremia lactucae*)	Stress response	4e-59
WS188 (CK859458)	> 570	1	40S ribosomal protein S27 isoform (*Homo sapiens*)	Protein synthesis	4e-32
WS189 (CK859459)	> 580	3	40S ribosomal protein S23 (*Arabidopsis thaliana*)	Protein synthesis	7e-44
WS191 (CK859460)	402	1	Similar to elongation factor 1-gamma (*Arabidopsis thaliana*)	Protein synthesis	8e-14
WS197 (CK859461)	546	7	40S ribosomal protein S26 (*Octopus vulgaris*)	Protein synthesis	3e-28
WS198 (CK859462)	> 430	1	Translationally controlled tumor protein (*Plasmodium yoelii yoelii*)	Unknown	3e-10
WS201(CK859463)	> 530	2	Aspartate aminotransferase (*Panicum miliaceum*)	Metabolism	2e-49
WS202 (CK859464)	> 530	1	60S ribosomal protein L11 (*Arabidopsis thaliana*)	Protein synthesis	1e-68
WS203 (CK859465)	455	4	60S ribosomal protein L30 (*Argopecten irradians*)	Protein synthesis	1e-38
WS205 (CK859466)	600	4	Actin depolymerizing factor (*Lilium longiflorum*)	Cell organization	5e-27
WS210 (CK859467)	500	1	Putative senescence-associated protein (*Pisum sativum*)	Cell defense	2e-25
WS215 (CK859468)	> 530	1	Translation elongation factor 1 (*Saccharomyces cerevisiae*)	Protein synthesis	1e-73
WS220 (CF891689)	500	1	Peptidylprolyl isomerase (cyclophilin) (*Drosophila melanogaster*)	Metabolism	9e-45
WS222 (CK859469)	> 530	3	Glutathione peroxidase (*Arabidopsis thaliana*)	Defense	5e-16
WS225 (CF891690)	> 540	1	Phosphate transport protein G7 (*Glycine max*)	Transport	4e-25
WS227 (CF891691)	> 480	3	Phosphoglyceromutase 1 (*Salmonella typhimurium*)	Metabolism	5e-54
WS235 (CF891692)	> 400	1	ADP, ATP carrier protein T1 (*Mus musculus*)	Transport	3e-22
WS238 (CK859470)	> 560	8	No hit	Unknown	—
WS240 (CK859471)	> 390	2	Translation elongation factor 1 gamma (*Rana sylvatica*)	Protein synthesis	6e-25
WS242 (CK859472)	> 510	1	Cytochrome c oxidase subunit 6b (*Oryza sativa*)	Metabolism	1e-15
WS248 (CK859473)	> 600	1	Hypothetical protein (*Deinococcus radiodurans*)	Unknown	1e-4
WS253 (CK859474)	> 490	1	Ubiquitin-activating enzyme (*Physarum polycephalum*)	Metabolism	4e-25
WS257 (CK859475)	587	1	Cytochrome c (*Zea mays*)	Metabolism	2e-35
WS261 (CK859476)	> 540	1	60S ribosomal protein L10 (*Drosophila melanogaster*)	Protein synthesis	6e-66
WS264 (CK859477)	575	1	60S ribosomal protein L36 (*Arabidopsis thaliana*)	Protein synthesis	7e-21
WS271 (CK859478)	> 340	1	60S ribosomal protein L12 (*Caenorhabditis briggsae*)	Protein synthesis	2e-32
WS272 (CK859479)	485	3	60S ribosomal protein L35 (*Gallus gallus*)	Protein synthesis	6e-39
WS273 (CK859480)	> 300	2	40S ribosomal protein S13 (*Zea mays*)	Protein synthesis	9e-24
WS275 (CK859481)	> 550	1	60S ribosomal protein L5 (*Oryza sativa*)	Protein synthesis	2e-27
WS277 (CK859482)	> 490	1	Cytochrome P450 monooxygenase (*Zea mays*)	Cell defense	2e-22
WS279 (CK859483)	200	1	60S ribosomal protein L9-A (*Pisum sativu*)	Protein synthesis	9e-7
WS282 (CK859484)	> 570	2	60S ribosomal protein L3 (*Arabidopsis thaliana*)	Protein synthesis	8e-67
WS291 (CK859485)	374	1	Hypothetical protein (*Mus musculus*)	Unknown	4e-12

Table 1 *continued.*

Clone ID (accession number)	Size (bp)	No. of ESTs	Best hit in GenBank (source species)	Cellular function	Blastx *E* value
WS292 (CK859486)	> 500	51	GAPDH (*Saccharomyces cerevisiae*)	Metabolism	7e-76
WS294 (CK900548)	> 430	1	Cytochrome P450 monooxygenase (*Zea mays*)	Defense	2e-25
WS296 (CK859487)	662	1	60S ribosome protein L19 (*Arabidopsis thaliana*)	Protein synthesis	7e-53
WS297 (CK859488)	530	1	60S ribosomal protein L27 (*Drosophila melanogaster*)	Protein synthesis	9e-32
WS304 (CK859489)	> 600	1	Glycine hydroxymethyltransferase (*Arabidopsis thaliana*)	Metabolism	4e-69
WS306 (CK859490)	> 680	1	14-3-3-like protein (*Phytophthora infestans*)	Transcription	2e-81
WS309 (CK859491)	> 550	1	Eukaryotic translation initiation factor 5A (*Arabidopsis thaliana*)	Protein synthesis	1e-44
WS310 (CK859492)	475	1	40S ribosomal protein S25 (*Drosophila melanogaster*)	Protein synthesis	5e-13
WS314 (CK859493)	674	1	Ubiquitin-conjugating enzyme (*Phytophthora infestans*)-	Metabolism	2e-68
WS315 (CK859494)	360	1	tubulin (*Nicotiana tabacum*)	Cell organization	3e-20
WS317 (CK859495)	> 670	1	60S ribosomal protein P0 (*Neurospora crassa*)	Protein synthesis	9e-43
WS318 (CK859496)	> 550	1	Pyrophosphatase (*Phytophthora infestans*)	Signal transduction	3e-60
WS322 (CK859497)	504	1	No hit	Unknown	—
WS323 (CK859498)	442	1	Prefoldin subunit 2 (*Mus musculus*)	Stress response	9e-22
WS327 (CK859499)	537	2	60S ribosomal protein L35 (*Branchiostoma belcheri*)	Protein synthesis	6e-39
WS336 (CK859500)	473	1	Inositol polyphosphate-4-phosphatase (*Drosophila melanogaster*)	Metabolism	1e-19
WS338 (CK859501)	> 520	1	Elongation factor-1β (*Saccharomyces cerevisiae*)	Protein synthesis	4e-11
WS339 (CK859502)	512	1	NEU1 protein (*Homo sapiens*)	Regulation	0.12
WS340 (CK859503)	> 517	1	40S ribosomal protein S21 (*Oryza sativa*)	Protein synthesis	4e-20
WS341 (CK859504)	> 480	3	40S ribosomal protein S16 (*Euphorbia esula*)	Protein synthesis	3e-61
WS344 (CK859505)	107	3	Transcript antisense to ribosomal RNA (*Saccharomyces cerevisiae*)	Transcription	3e-9
WS359 (CK859506)	> 550	1	Hypothetical protein (*Phytophthora infestans*)	Unknown	1e-125
WS364 (CK859507)	517	1	Orf122, hypothetical protein (*Chlorobium tepidum*)	Unknown	8e-6
WS370 (CK859508)	406	1	Cysteine proteinase precursor (*Geodia cydonium*)	Metabolism	1e-21
JM300 (CK859509)	484	1	No hit	Unknown	—
JM305 (CK859510)	270	1	No hit	Unknown	—
JM310 (CF891693)	280	2	No hit	Unknown	—
JM311 (CK859511)	> 600	1	GTP-binding nuclear protein spi1 (*Schizosaccharomyces pombe*)	Signal transduction	1e-95
JM312 (CK859512)	612	1	High mobility group protein (*Plasmodium yoelii yoelii*)	Unknown	4e-10
JM315 (CK859513)	610	1	No hit	Unknown	—
JM316 (CK859514)	> 550	1	H^+-transporting ATPase beta subunit (*Hemicentrotus Pulcherrimus*)	Transport	2.1e-59
JM317 (CK859515)	> 540	1	No hit	Unknown	—
JM318 (CK859516)	603	1	60S Ribosomal protein L2 (*Phytophthora infestans*)	Protein synthesis	1e-109
JM326 (CK859517)	> 600	1	Hypothetical protein (*Azotobacter vinelandii*)	Unknown	1e-4
JM329 (CK859518)	> 640	1	D-lactate dehydrogenase (*Nostoc* sp. *PCC 7120*)	Metabolism	4.1e-43
JM335 (CK859519)	> 530	1	No hit	Unknown	—
JM337 (CK859520)	> 620	1	No hit	Unknown	—
JM348 (CK859521)	> 480	1	Receptor tyrosine kinase (*Caenorhabditis elegans*)	Signal transduction	7e-5
JM343 (CK900549)	> 450	1	Hydroxyacid dehydrogenase (*Nitrosomonas europaea*)	Metabolism	2e-12
JM350 (CK859522)	> 390	1	Hypothetical protein Y37A1B.2d (*Caenorhabditis elegans*)	Unknown	2e-4

some short sequences the *E* value was less than 10^{-5}) from other organisms (particularly plants and green and red alga). Six genes represented by 17 ESTs had a weak hit (*E* value less than 10^{-4}) with hypothetical proteins in the NCBI database and 22 genes (represented by 34 ESTs) were categorized as having no hit in the NCBI databases. A Blast search was also performed against ESTs in the *Phytophthora* Functional Genomics Database (PFGD; http://www.pfgd.org/pfgd/) and revealed that all but three ESTs had hits in this database. A further search against the Joint Genome Initiative (JGI) draft *P. sojae* genome sequence (http://genome.jgi-psf.org/physo00) indicated that all *P. nicotianae* ESTs in the present study have homologous sequences in *P. sojae*. Partial sequence alignment revealed varying similarities (about 40% to over 90% identities) between *P. nicotianae* and *P. sojae* or *P. infestans* genes, suggesting varying degrees of conservation of these genes.

Cyst germination requires active protein synthesis (Penington *et al.*, 1989). Among the genes highly expressed in germinated

cysts, there were many genes involved in energy generation, protein turnover and biosynthesis (e.g. *GAPDH* and other genes involved in the glycolic pathway), translation elongation factors and various ribosomal proteins that are involved in protein biosynthesis. Of 196 ESTs sequenced from the screening of the first 6144 cDNA clones, 27 (14%), 17 (9%) and 70 (36%) encoded *GAPDH*, *TEF1*α and ribosomal proteins, respectively. In total, 51 ESTs encoding *GAPDH*, 25 ESTs encoding *TEF1*α and 123 ESTs encoding 54 different ribosomal proteins were identified. In addition, 22 ESTs encoded pyruvate dehydrogenase, phosphoglycerate kinase, phosphoglyceromutase and other proteins involved in glycolysis and energy production.

Genomic organization analysis

The copy number and genomic organization of 37 genes encoded by the ESTs were determined by genomic and bacterial artificial chromosome (BAC) DNA blot analysis (Shan and Hardham, 2004). Thirty-three of the 37 genes were single-copy genes. For the majority of these genes, only single hybridizing bands were detected in the Southern blots; in a few cases, two or more hybridizing bands arose through possession of polymorphic restriction sites between the alleles. For the remaining four genes, multiple hybridizing bands on Southern blots were due to these genes being members of small multigene families. These included *WS187*, encoding a homologue to heat shock protein 70 (*Hsp70*) and *WS238*. BAC analysis showed that members of these two gene families were physically clustered (see Fig. 1 for *WS238*). Northern blot analysis showed that in all four cases in which ESTs represented members of gene families, single hybridizing bands were obtained in RNA blots. Partial sequence alignment indicated that all eight ESTs represented by *WS238* appear to be derived from the same member of the gene family.

Northern blot analysis of selected genes

The expression pattern of a selection of 31 (21% of the 146 unigenes) of the cloned genes was analysed on RNA gel blots of total RNA isolated from the four different stages of the asexual life cycle and from infected tobacco seedlings. The main criterion on which the cDNAs clones were selected was their potentially important role in *P. nicotianae* pathogenesis and development. Many (26) of these 31 genes were expressed predominantly in germinated cysts and at relatively high levels, but in order to assess the performance of the macroarray screening, we also included cDNAs with other expression patterns and with a range of hybridizing intensities. Careful comparison of hybridization intensities with RNA loading revealed in replicate experiments that one cDNA, *WS41*, which had been randomly selected in the macroarray screening from clones that showed constitutive expression in all four developmental stages, was found to carry a gene whose even expression profile was confirmed by Northern blot analysis. This gene, which encodes an unknown *P. nicotianae* protein, is thus a useful standard for analysis of gene transcription levels and was used to normalize hybridization signals relative to amounts of *P. nicotianae* RNA loaded in Northern blot analysis. Hybridization of *P. nicotianae*-infected tobacco samples suggests that *WS41* may also be constitutively

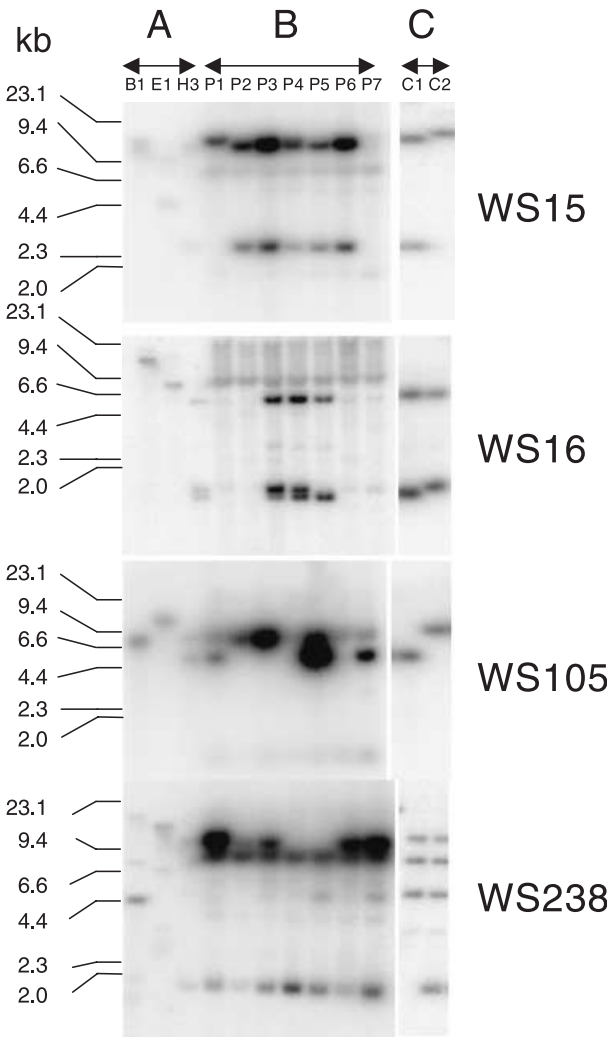

Fig. 1 Southern blot analysis of selected *Phytophthora nicotianae* cDNA clones. The copy number of selected genes was determined by genomic Southern blot (A) and BAC analysis (B,C). Lanes P1 to P7 (B) are seven BAC pool DNA each containing 1536 BAC clones equivalent to ~1.5 *P. nicotianae* haploid genome coverage. The BAC pool DNA was digested with *Hin*dIII to release all inserts and probed with selected genes to facilitate identification of BAC clones carrying hybridizing fragments corresponding to all hybridized *Hin*dIII bands in the genomic Southern blot. For analysis of genomic organization of gene families, at least three positive BAC clones were used to determine physical linkage of gene family members. Data shown in C are two overlapping BAC clones (C1 and C2) that indicate the presence of different alleles for the single-copy genes (clones *WS15*, *WS16* and *WS105*) or physical linkage of gene family members (*WS238*). E1, *Eco*RI; B1, *Bam*HI; H3, *Hin*dIII.

expressed during plant infection as similar transcript levels are seen in both early and late plant colonization (see Fig. 3).

Northern blot analysis confirmed that the expression patterns of 28 out of the 31 genes were as initially indicated by the macroarray colony hybridization. For 14 out of 25 genes in which differential hybridization had shown up-regulation in germinated cysts, quantification of signal intensity in the RNA blots indicated a two-fold or more increase in expression levels in germinated cysts compared with vegetative hyphae. Among those genes that were up-regulated more than ten-fold in germinated cysts compared with vegetative mycelia were *WS9*, *WS15*, *WS17*, *WS105* and *WS238*, which encode proteins of unknown function; *WS16*, a homologue of the 42-kDa glycoprotein of *P. sojae* (Parker *et al.*, 1991; Sacks *et al.*, 1995); and *WS100*, a gene with a weak homology to *Myb*-related transcription factors.

TEF1α and *GAPDH* are two genes whose transcripts are highly represented in the ESTs selected by differential hybridization. They are both single-copy genes in the *P. nicotianae* genome (Table 2) and their transcript accumulation is significantly increased in germinated cysts (Table 2).

Although most of the genes analysed were significantly up-regulated in germinated cysts, they were generally expressed at lower levels in other developmental stages, in particular in sporulating hyphae, compared with their mRNA levels in vegetative hyphae (Table 2). For example, *WS86*, a gene encoding a glycerol uptake facilitator protein, was down-regulated substantially in sporulating hyphae but was up-regulated nearly ten-fold in germinated cysts compared with vegetative hyphae (Table 2).

Clone *WS105* is a single-copy gene (Fig. 1); however, Northern blots indicated that it has two distinct messages in germinated cysts, suggesting that differential splicing occurs for *WS105* expression. Expression of only one of these transcripts predominates in zoospores.

Characterization of selected genes

Based on their high level of transcription and the function of their homologues in other pathogens, several genes identified may be directly involved in plant infection. Several ESTs represented by cDNA *WS16* encode a secreted protein with 530 amino acid residues that shares 76% sequence identity to the 42-kDa glycoprotein elicitor, *GP42*, of *P. sojae* (Parker *et al.*, 1991; Sacks *et al.*, 1995) and that was designated *PnGP42*. Unlike *P. sojae GP42*, which is a member of a multigene family (Sacks *et al.*, 1995), the *PnGP42* gene (*WS16*) appears to be a single-copy gene in the *P. nicotianae* genome (Fig. 1) under our hybridization conditions and is highly up-regulated in germinated cysts (Fig. 2). As in *P. sojae GP42*, genomic sequencing indicated that *PnGP42* (*WS16*) contains no introns.

cDNAs *WS105* and *WS238* also encoded putative secreted proteins and their expression was highly up-regulated in germinated cysts (Figs 2 and 3). Their predicted proteins contain motifs potentially associated with cell-wall synthesis and cell adhesion, respectively. *WS238*, which represents seven ESTs, is a member of a small gene family (Fig. 1). The predicted protein contains many hydrophobic residues (Gly, Val, Pro and Ala), which constitute 63.9% of the 185 residues in the mature polypeptide. It contains hydrophobic repetitive motifs, such as GVGVP at the C-terminus, which are characteristic of tropoelastin.

Several ESTs represented by *WS250* encode a protein with significant similarities to various phospholipid hydroperoxide glutathione peroxidases (PHGPX). This is a single-copy gene in the *P. nicotianae* genome (Table 2) whose expression is highly up-regulated in germinated cysts (Fig. 2). Unlike many PHGPX proteins that are generally approximately 20 kDa in size, the *P. nicotianae* homologue has a much larger molecular mass, with 561 amino acid residues and a predicted molecular weight of 63.3 kDa (data not shown). The full-length cDNA was obtained by 5′-RACE (rapid amplification of cDNA ends) using a gene-specific primer (5′-CGATTTCTCGTGCAGCTGCTG-3′) and was consistent with the mRNA size in the Northern blot. PHGPX is an antioxidant enzyme that can directly reduce intracellular phospholipid hydroperoxide, and is presumed to be involved in self-defence against an oxidative burst or in response to environmental stresses in other organisms.

WS66 represents seven ESTs encoding a putative plasma membrane H^+-ATPase protein. Two genes encoding putative transporters were also identified. *WS59* encodes a nitrate transporter protein and *WS225* encodes a protein similar to phosphate transport proteins. Several genes involved in amino acid biosynthesis were also identified by differential hybridization. *WS175* encodes a glutamine synthetase, *WS182* encodes a cysteine synthase, and *WS183* encodes a methionine synthase protein.

Several genes encoding putative transcription factors were identified. *WS17* and *WS100* encode proteins with weak similarity to *Myb*-related transcription factors. All of these genes are single-copy genes in the *P. nicotianae* genome and displayed highly up-regulated expression in germinated cysts (Table 2).

WS84 encodes a protein with moderate similarity to bacterial and plant cellulose synthases. Unlike its homologues in plants, it is a single-copy gene in the *P. nicotianae* genome. Its expression pattern in the *P. nicotianae* life cycle, however, has not been confirmed yet.

DISCUSSION

Isolation of development-specific genes

For plant pathogens with little or no history of genetic research, single-pass sequencing of random cDNA clones, as in EST projects, represents a relatively inexpensive and rapid procedure for finding genes and information regarding their expression (Skinner *et al.*, 2001). Since it was first introduced for human study (Adams *et al.*, 1991), EST analysis has been applied to a variety of organisms, including fungal plant pathogens such as *Blumeria graminis* (Thomas *et al.*, 2001), *Fusarium graminearum*

Table 2 Quantitative analysis of expression of selected genes in *Phytophthora nicotianae*.

Selection of ESTs[a]	EST clones	Putative function	Copy number[b]	mRNA size (kb)	Relative expression[c]		
					S	Z	G
G	WS15	Unknown	1	0.8	–	+	++++
	WS238	Unknown	M	0.9	–	+	++++
	WS16	42 kD glycoprotein	1	2.2	2.9	54.3	87.3
	WS9	Unknown	1	0.8	0.6	3.3	20.1
	WS17	Unknown	1	1.1	2.8	8.0	15.1
	WS66	Plasma membrane H^+-ATPase	2	3.1	0.3	5.8	12.7
	WS86	Glycerol uptake facilitator protein	M	1.4	0.1	0.5	9.7
	WS250	Glutathione peroxidase	1	1.8	1.0	1.0	6.9
	WS39	GAPDH	1	1.7	0.4	0.6	6.3
	WS227	Phosphoglyceromutase	1	1.5	2.2	0.8	3.4
	WS63	Cytochrome c oxidase subunit Via	1	0.8	0.9	1.5	3.2
	WS40	TEF1α	1	1.5	1.7	1.1	2.7
	WS43	Unknown	1	1.1	0.6	0.7	2.5
	WS225	Phosphate transporter protein G7	1	1.6	0.9	2.0	2.3
	JM310	Unknown	1	1.4	0.1	0.4	1.9
	WS56	TEF2	1	3.5	0.5	0.5	1.9
	WS235	ADP/ATP carrier protein T1	1	1.6	0.8	0.4	1.8
	WS64	Cyclophilin	1	1.5	0.4	0.1	1.0
	WS151	Annexin	1	1.2	0.7	0.1	0.1
G and Z	WS105	Unknown	1	2.8	1.7	27.8	43.4
	WS100	Myb-related transcription factor	1	1.5	0.0	2.6	18.4
	WS24	G protein β subunit	1	0.9	2.2	5.1	4.9
	WS196	Unknown	1	0.8	0.7	0.8	2.8
	WS187	Hsp 70	M	2.4	0.6	2.2	2.6
	WS192	TEF3	1	4.2	7.1	11.2	2.5
Infection	WS364	Unknown	1	3.0	1.2	1.9	1.6
	WS294	Senescence-associated protein	1	5.1	1.1	1.0	1.0
	WS359	Unknown	1	4.4	1.2	1.4	0.8
	WS370	Cathepsin	1	3.2	0.8	0.7	0.6
All stages	WS21	S-phase-specific protein	1	1.2	1.5	3.5	2.7
	WS41	Unknown	1	1.2	1.0	1.0	1.0

[a]EST clones for Northern gel blot analysis were selected according to their expression profiles obtained by colony filter hybridization. Representative EST clones that hybridized highly and specifically with the cDNA probes of germinated cyst (G), both G and zoospores (Z), infected tobacco tissues (Infection), or all four asexual stages (All stages): vegetative hyphae, sporulating hyphae (S), Z and G were chosen.

[b]Copy number determined by genomic Southern blot and BAC analysis. M, multiple copies.

[c]Relative expression value was calculated in relation to expression in vegetative hyphae and normalized using EST *WS41* to standardize loading of RNA samples. In cases where there were no detectable signals in the vegetative hyphae lanes, expression levels in other lanes are indicated as '–' for no detectable signal, '+' for low-level expression, and '++++' for high-level expression.

(Kruger *et al.*, 2002), *Mycosphaerella graminicola* (Keon *et al.*, 2000) and *Heterobasidion annosum* (Karlsson *et al.*, 2003), and oomycete plant pathogens such as *P. infestans* (Kamoun *et al.*, 1999) and *P. sojae* (Qutob *et al.*, 2000).

To learn more about genes involved in different developmental stages and plant infection, however, either multiple cDNA libraries made from various developmental stages or specific screening strategies are required. In the present study, we used a differential screening strategy to identify genes involved in a specific developmental stage of the *P. nicotianae* life cycle, namely germinated cysts. Our results show that screening of a cDNA library by differential hybridization is a highly effective method for the isolation of a large number of developmentally regulated genes in plant pathogens. Genes whose expression is regulated during infection have been isolated by differential screening for a number of plant pathogenic fungi and oomycetes. Examples include the isolation of *in planta*-induced genes from *Uromyces fabae* (Hahn and Mendgen, 1997), *Puccinia triticina* (Thara *et al.*,

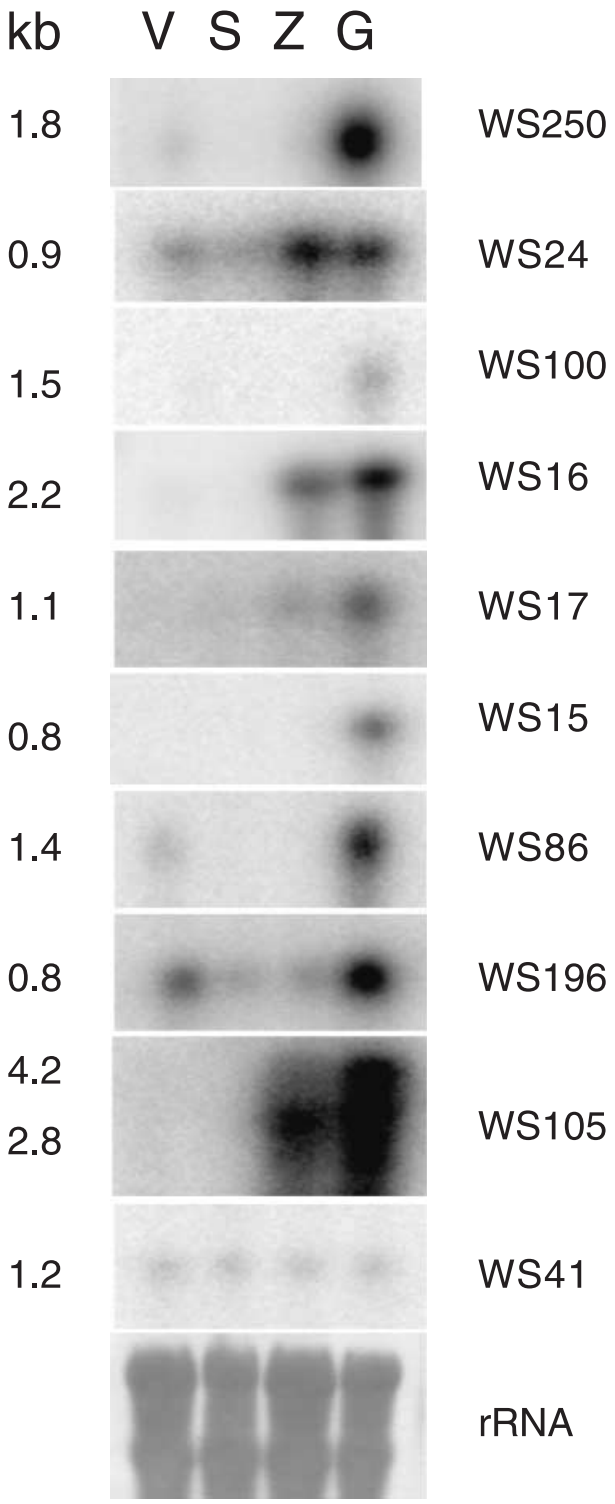

Fig. 2 Examples of Northern blot analysis of selected *Phytophthora nicotianae* cDNA clones. Total RNA (10 μg) from vegetative hyphae (V), sporulating hyphae (S), motile zoospores (Z) and germinated cysts (G) was separated, blotted and probed with selected genes. In the lower panel, ribosomal RNAs are stained with methylene blue to show quality and quantity of RNA samples. Clone *WS41* was used as a standard to normalize loading of RNA samples.

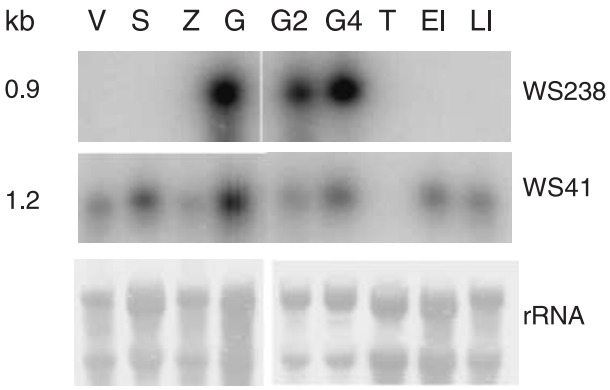

Fig. 3 Clone *WS238* is specifically expressed in germinated cysts. Each lane contains 10 μg total RNA except infection RNA samples for which 20 μg was loaded. V, vegetative hyphae; S, sporulating hyphae; Z, motile zoospores; G, germinated cysts; G2 and G4, cysts prepared by vortexing motile zoospores so that over 95% had encysted and germinated in 2.5% V8 broth for 2 h and 4 h at 23 °C, respectively; T, uninfected tobacco; EI, newly infected tobacco (biotrophic phase); LI, late infected tobacco tissue (necrotrophic phase). The same blot was subsequently probed with clone *WS41*, a standard used to normalize loading of RNA samples. rRNA: ribosomal RNA stained with methylene blue to show quality and quantity of RNA samples transferred.

2003), *Magnaporthe grisea* (Kim *et al.*, 2001; Rauyaree *et al.*, 2001) *Colletotrichum graminicola* (Sugui and Deising, 2002) and *P. infestans* (Beyer *et al.*, 2002; Pieterse *et al.*, 1993).

To identify genes highly and differentially expressed in germinated cysts, we constructed a cDNA library using mRNA isolated from germinated cysts of *P. nicotianae*. Cyst germination and germling growth represents an early stage of the interaction of *Phytophthora* with host plants. In infected plant tissues, total RNA is composed of a combination of plant and pathogen RNAs, each of which may change considerably over time (Mahe *et al.*, 1992). However, the proportion of pathogen biomass in host tissue is relatively low, particularly at early stages of infection, and it is difficult accurately to analyse changes in expression patterns of pathogen genes within hours of exposure to the host because of the overwhelming representation of genes of plant origin. Thus, only a few studies have identified pathogen genes expressed in early stages of plant infection (e.g. Sánchez-Torres and González-Candelas, 2003).

Genes regulated during late infection when disease lesions are visible may be different from those active in early stages of the interaction with host plants. *Phytophthora* infection and colonization of host plants can be achieved in a short time period. During *P. nicotianae* infection of the hypocotyl region of tobacco seedlings, zoospore encystment, cyst germination, appressorium formation and hyphal penetration of the plant surface occurs within 3 h of inoculation; sporangia begin to differentiate within 28 h of inoculation (Perrone *et al.*, 2000). Similarly, the differentiation of sporangia and oospores *in planta* occurs within 24 h of inoculation of cotton leaves with *P. boehmeriae* zoospores (Shan *et al.*, 1995) and within 48 h after inoculation of chestnut roots

with *P. cinnamomi* (Chambers *et al.*, 1995). In our differential screening, only low numbers of clones hybridized with cDNA probes synthesized with mRNA isolated from *P. nicotianae*-infected tobacco root tissues 24 h post inoculation. Although the low proportion of pathogen mRNA in the probe will contribute to the difficulty in detecting *P. nicotianae* transcripts in the infected tissue samples, the results may also be indicative of striking changes in patterns of gene expression as infection progresses. It is also possible that inoculation of tobacco roots with very large numbers of zoospores as was used to prepare the infected tissue may result in material resembling late infection in which sporulation may have initiated. Northern analysis of selected EST clones indicated that nearly all genes up-regulated in germinated cysts displayed down-regulation in sporulating hyphae.

Northern hybridization experiments with cDNAs identified in the macroarray screening as being expressed predominantly in germinated cysts confirmed this expression pattern for most of the selected cDNAs. However, genes that are differentially expressed but at lower levels may not have been detected. Under our hybridization conditions, repeated experiments indicated that about 50% of the germinated cyst cDNA clones showed signal intensities that were not above background levels when hybridized with cDNA probes synthesized from germinated cyst mRNA or total RNA. Quantitative analysis of gene expression levels by real-time PCR and microarray-based approaches will be valuable for identification of genes differentially expressed at lower levels. Nevetheless, our analysis has successfully identified more than 100 genes that are highly expressed in germinated cysts.

Genes with potential pathogenicity functions

Little is known about the genes that are expressed in *Phytophthora* during early stages of plant infection. In the present study, we have identified 146 unigenes from the germinated cyst cDNA library, and most of these are up-regulated after cyst germination. It seems likely that genes that are highly expressed in germinated cysts play an important role in these cells and are potentially involved in plant infection. Sequencing of the selected cDNAs and database searches for similar genes has revealed relevant information for 124 of the 146 genes.

PnGP42 (*WS16*) encodes a secreted protein sharing 76% sequence identity with the 42-kDa glycoprotein elicitor, GP42, of *P. sojae* (Parker *et al.*, 1991; Sacks *et al.*, 1995). The *P. sojae* GP42 protein is a Ca^{2+}-dependent transglutaminase capable of inducing defence responses in the non-host parsley (Brunner *et al.*, 2002). In *P. sojae*, the gene is constitutively expressed and the protein has been immunolocalized in the cell wall of hyphae growing in the host. The GP42 protein contains a domain of 13 residues that are conserved among *Phytophthora* species; this domain possibly functions as a genus-specific recognition determinant for the activation of defence responses in host and non-host plants (Brunner *et al.*, 2002). The high level of sequence similarity may reflect conservation in the mechanism of action and fundamental importance of *PnGP42* in the infection process.

Two other cDNAs, *WS238* and *WS105*, encode putative secreted proteins. They are highly and specifically expressed in germinated cysts and the predicted proteins contain motifs characteristic of proteins involved in cell adhesion and cell-wall biogenesis, respectively. *WS238* encodes a secreted, highly hydrophobic protein, containing multiple copies of the GVGVP motif, which is characteristic of tropoelastin, a highly conserved protein identified in all vertebrates studied to date—except the primitive cyclosomes—but not in invertebrates (Davison *et al.*, 1995). Tropoelastin is a soluble precursor to elastin, which is an extremely insoluble protein due to the extensive cross-linking at lysine residues. Elastin is among the most hydrophobic proteins known. *WS105* encodes a large (1282 amino acid residues) highly hydrophilic protein containing repetitive motifs. Its expression pattern suggests that it may play an important role in *P. nicotianae* development and in an early stage of interaction with host plants.

Additional genes that are highly expressed in germinated cysts include several genes encoding proteins with unknown function. Most of these are possible transcription factor genes, such as *WS9*, *WS15*, *WS17* and *WS100*, because they contain motifs similar to those found in transcription factors. The appearance of these transcripts in germinated cysts suggests that novel proteins are produced to regulate genes associated with cyst germination.

Genes involved in amino acid biosynthesis have also been identified in this study and are potential pathogenicity factors. In *M. grisea*, insertional mutagenesis experiments suggested that methionine biosynthesis is essential for pathogenicity (Balhadère *et al.*, 1999) and a histidine-deficient mutant is significantly less pathogenic than a wild-type strain (Sweigard *et al.*, 1998). The up-regulated expression of a methionine biosynthesis gene in *Cladosporium fulvum* colonizing host plant tomato has also been observed (Solomon *et al.*, 2000), and in the soil-borne pathogen *Fusarium oxysporum* f. sp. *melonis*, mutation of an arginine biosynthesis gene led to reduced pathogenicity (Namiki *et al.*, 2001). In fact, it has been recognized for many years that there is a link between deficiency in amino acid biosynthesis and lack of pathogenicity in several fungal pathogens, including *Ustilago maydis* (Kahmann and Basse, 1999) and *Venturia inaequalis* (Boone *et al.*, 1958). These findings suggest that nutrient levels are limited during plant infection and can affect the ability of pathogens to cause diseases on host plants.

EXPERIMENTAL PROCEDURES

P. nicotianae strain and culture conditions

P. nicotianae strain H1111 (ATCC MYA-141) was a gift from Dr David Guest (University of Melbourne) and was originally

isolated from tobacco plants in Queensland, Australia (his isolate M4951). Under appropriate conditions, it can synchronously produce large numbers of zoospores and is used as a model strain in our laboratory. In the present study, the strain H1111 was used for all experiments, including tobacco infection, RNA isolation and construction of the germinated cyst cDNA library.

P. nicotianae cultures were routinely maintained on 10% (v/v) cleared V8 juice agar (1.5%, w/v) supplemented with 0.002% (w/v) β-sitosterol and 0.01% (w/v) $CaCO_3$. Preparation of mycelia for DNA isolation was as described previously (Shan and Hardham, 2004).

Zoospore production followed the method described by Mitchell and Hardham (1999) with minor modifications in which the liquid cultures for sporangia production were maintained at 23 °C for at least 14 days without shaking. To prepare germinated cysts, zoospores were chilled at −20 °C until some encystment was apparent. Once a few cysts were present, cultures were warmed to 4 °C and agitated until approximately 90% of zoospores had encysted. The cysts were mixed in an equal volume of 0.2 mg/mL pectin prepared in 40 mM $CaCl_2$ (Irving and Grant, 1984) and shaken for 4 h at 23 °C. The cysts and 4-h germlings were collected by centrifugation and frozen in liquid nitrogen and stored at −80 °C. Alternatively, motile zoospores were vortexed to induce encystment and the germinated cysts were prepared by mixing an equal volume of cyst suspension with 5% V8 broth and incubating at 23 °C with shaking (100 r.p.m.) for 2 or 4 h.

RNA isolation and cDNA library construction

For RNA isolation, vegetative hyphae, sporulating hyphae, zoospores, germinated cysts and tobacco leaves infected with *P. nicotianae* H1111 were frozen in liquid nitrogen and ground into a fine powder in a mortar, dispersed in denaturing solution and purified by the guanidinium/caesium chloride gradient method (Chirgwin *et al.*, 1979).

For germinated cyst cDNA library construction, poly (A+) RNA was isolated from total germinated cyst RNA using a Dynabeads mRNA Purification kit (DYNAL A. S., Oslo, Norway). The cDNA was synthesized from poly (A+) RNA using oligo (dT) as a primer for first-strand cDNA synthesis. The double-stranded cDNA was ligated into the phage Uni-ZAP XR vector between the *Eco*RI and *Xho*I adapters (Stratagene, La Jolla, CA). This allowed 5′- and 3′-directional cloning of the cDNA product into the vector. *In vitro* packaging of the DNA and transfection of *E. coli* strain XL1 blue was performed following the manufacturer's protocol. The total primary titre of the library in recombinant plaque forming units was 1.5×10^6 pfu/mL. After amplification, samples of the cDNA library were used to subclone inserts by mass excision for conversion of phage to plasmid form (pBluescript KS), according to the procedure described by Stratagene.

Creation and screening of macroarrays

Over 12 000 colonies were picked from the cDNA library and arrayed in 28 384-well microtitre plates. Clones from sets of four 384-well microtitre plates (Nunc) were replicated by hand on to single Hybond N+ nylon membranes (Amersham) using a 384-pin replicator (VP scientific, San Diego, CA), off-setting the placement of the clones from each plate to give a total of 1536 clones on each membrane. Prior to colony transfer, the nylon membranes were prewetted on LB agar (1% bacto-tryptone, 0.5% bacto-yeast extract, 10 g/L NaCl, 1.5% agar, pH 7) plates as described (Shan and Hardham, 2004) except that the medium was supplemented with 50 μg/mL ampicillin. The membranes were incubated with the colony side up on LB plates at 37 °C for about 20 h or until the colony reached an appropriate size. The membranes were treated sequentially with solutions to lyse cells and release and fix plasmid DNA on the membrane surface, following a procedure described by Wang *et al.* (1995).

Because of uneven growth of bacterial colonies on nylon membranes and uneven hybridization as revealed by pilot experiments, two sets of colony filters were prepared and hybridized sequentially with ^{32}P-labelled cDNA probes. In general, one filter was labelled with probes synthesized with mRNA isolated from sporulating hyphae, vegetative hyphae and germinated cysts, while the other was labelled with probes made from mRNA from zoospores, germinated cysts and tobacco tissues infected with *P. nicotianae*. In cases where there were problems with hybridization on one filter, the duplicate filter was also used. Hybridization conditions were as described for Southern blotting. The intensity of hybridizations to colony spots was assessed by visual inspection of the colony filters. For genes putatively up-regulated in germinated cysts, clones were selected in cases where there was a clear increase in signal intensity after hybridization with the germinated cyst probe compared with that for the vegetative hyphae probe.

DNA isolation and Southern blot analysis

For isolation of total genomic DNA for Southern blot analysis, *P. nicotianae* H1111 mycelia were ground into a fine powder in liquid nitrogen in a mortar before dispersing into DNA extraction buffer (Dudler, 1990). BAC pool DNA was isolated using a PlasmidMidi kit (Qiagen, Germany) and screened as described (Shan and Hardham, 2004). For Southern blot analysis, 2 μg of total *P. nicotianae* genomic DNA or 3 μg of BAC pool DNA was digested with appropriate restriction enzyme(s), resolved in 0.8% agarose and transferred to Hybond N+ nylon membranes (Amersham Pharmacia) using 0.4 N NaOH as the transfer agent following a standard procedure (Sambrook *et al.*, 1989).

Hybridizations were done at 65 °C for 16 h using either 6× SSPE or 6× SSC with 5× Denhardt's solution (1× Denhardt's

solution contains 0.02% Ficoll 400, 0.02% PVP, 0.02% BSA) and 0.1% SDS as the hybridization buffer as described by Sambrook *et al.* (1989). Following hybridization, the membranes were washed at 65 °C twice in 2× SSC and 0.1% SDS each for 15 min, and twice in 0.2× SSC and 0.1% SDS each for 15 min. Signals were detected by exposing the filters to a PhosphoImager screen for 2 h to 3 days (Molecular Dynamics, CA).

DNA sequencing and analysis

Plasmid DNA templates of cDNA clones for sequencing were prepared using an ethidium bromide-based procedure. In brief, cDNA clones were grown overnight in 1.6 mL of LB broth. The plasmid DNA was isolated by standard alkali lysis, precipitated with isopropanol and re-dissolved in 250 μL TE (10 mM Tris-HCl, 1 mM EDTA, pH 9) supplemented with 100 μg/mL RNAse A and incubated at 37 °C for 1 h before ethidium bromide extraction, in which the DNA solution was mixed with 15 μL ethidium bromide (10 mg/mL) and 140 μL 7.5 M ammonium acetate, followed by extraction with 420 μL phenol/chloroform (1 : 1). The DNA was precipitated with 360 μL isopropanol, washed with 80% ethanol, air dried and finally dissolved in 30 μL H_2O. Prior to sequencing, all plasmids were checked for concentration and presence of an insert by *Eco*RI digestion and electrophoresis on agarose gels.

The 5′ end DNA sequencing was conducted at the Australian Genome Research Facility, Brisbane, Queensland, by using the T3 primer, Big Dye Terminator chemistry (Applied Biosystems, Foster City, CA), and an ABI3700 sequencer (Applied Biosystems). Selected cDNA clones were also sequenced from the 3′ end using the T7 primer.

The cDNA sequences were edited manually to remove vector sequences and to correct ambiguous base calls by comparison with the electropherograms to improve the quality and reliability of the data. cDNA sequences larger than 200 bp were compared with the international databases at the protein level using the gapped BlastX algorithm (Altschul *et al.*, 1997). *P. nicotianae* sequences were considered to be homologous to previously reported genes if the *E* value was less than 10^{-5}.

The redundancy of cDNA sequences was determined by comparing all sequences with one another using the BlastN algorithm on an NCBI local nucleotide database generated with the Formatdb program. The self-score values were used to normalize the score values of matches to other sequences as described by Kamoun *et al.* (1999). Normalized score values greater than 10% were taken as an indication that the sequences represented the same gene.

Northern analysis

For Northern hybridizations, 10 μg total RNA were mixed in 20 μL 1× RNA-treating buffer (15 μL DMSO, 3 μL glyoxal, 2 μL 10× Hepes buffer) and heat-treated at 70 °C for 5 min before being separated in 1% agarose gels in 1× Hepes buffer (20 mM Hepes, 1 mM EDTA, pH 7) and transferred to Hybond N nylon membranes (Amersham Pharmacia) by 10× SSC (1× SSC contains 0.15 M NaCl, 15 mM sodium citrate) following a standard procedure (Sambrook *et al.*, 1989). The membranes were baked at 80 °C for 2 h in a vacuum to fix RNA samples and were stained with 0.05% methylene blue (w/v, in 0.3 M sodium acetate) to check for RNA integrity and equal loading of RNA samples. The probes were released from cDNA clones by *Eco*RI and *Xho*I double digestion. Random priming using a Megaprime kit was used to prepare ^{32}P-labelled radioactive probes. Conditions for hybridization and blot washing were as described above for Southern blot analysis. Blots were exposed to a PhosphoImager screen for 2 h to 3 days and signals were detected and quantified using ImageQuant software (Version 5.0) (Molecular Dynamics). Probing with *WS41* was used to normalize RNA loading. Steady levels of constitutive expression of this gene were confirmed in several pilot experiments in which RNA was quantified spectrophotometrically and verified by ethidium bromide staining of RNA separated in gels.

PCR conditions

Gene-specific primers derived from partial cDNA sequences and T3 primer were used to amplify cDNA using serial diluted germinated cyst cDNA library as the template. VentR DNA polymerase (NEB) was used in this study following the manufacturer's recommendation. The following thermal cycle condition was used: initial 94 °C for 5 min, 30 cycles of 94 °C for 30 s, 55–60 °C for 40 s and 72 °C for 2–3 min, followed by a final extension at 72 °C for 10 min. PCR product was cleaned by PCR purification columns (Qiagen, Germany), tailed by *Taq*DNA polymerase (Promega) at 72 °C for 20 min, and cloned into pGEM-T cloning vector (Promega), following the manufacturer's suggestion.

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