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Gene expression during oosporogenesis in heterothallic and homothallic *Phytophthora*

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Abstract

A large-scale screen for genes induced during sexual development was performed in the heterothallic oomycete *Phytophthora infestans*, the potato blight agent. Of 15,644 unigenes on an Affymetrix chip, 87 were induced >10-fold during mating, with 28 induced >100-fold. This was validated in independent matings using RNA blots and RT-PCR. Only 44 genes resembled sequences in GenBank. These encoded regulators such as protein kinases, protein phosphatases, and transcription factors, plus enzymes with metabolic, transport, or cell-cycle activities. Several genes were induced during both mating and asexual sporogenesis, suggesting crosstalk between those pathways. In the homothallic species *P. phaseoli*, 20% of the 87 genes were expressed at higher levels during conditions conducive to oosporogenesis than non-conducive conditions, while the rest were at similar levels. Many of the latter exhibited higher mRNA concentrations in *P. phaseoli* than in any non-mating culture of *P. infestans*, suggesting that part of the sexual pathway is active constitutively in homothallics.

Keywords: Heterothallism; Homothallism; Microarray; Oomycete; *Phytophthora*; Sexual development

1. Introduction

Sexual reproduction is an important feature of the life cycles of oomycetes, an important group of fungus-like eukaryotes. These include both saprophytes and many significant pathogens, such as those causing foliar blights or root, crown, or fruit rots on crops, ornamentals, and native plants (Agrios, 2004). Sexual reproduction by oomycetes generates thick-walled sexual spores called oospores that play key roles in many of these diseases. Oospores are capable of surviving in soil or plant debris for many years, resisting harsh environmental conditions such as cold, degradation by microbes, or chemical fumigation. They are particularly important for phytopathogenic oomycetes since their hyphae and asexual sporangia are usually incapable of long-term survival apart from a host plant. In many pathosystems, oospores germinating at the start of a growing season represent the initial inoculum for epidemics (Grunwald and Flier, 2005). The sexual cycle also enhances pathogen fitness by generating recombinant genotypes that may be more pathogenic or resistant to crop protection chemicals (Gavino et al., 2000).

Many of the most important oomycetes belong to the genus *Phytophthora*, which encompasses more than 60 species that are either heterothallic (with A1 and A2 mating types) or homothallic (Erwin and Ribeiro, 1996). For example, the heterothallic species *Phytophthora infestans* is notorious for its role in causing the Irish potato famine in the 1840's, and is still responsible for major losses on potato and tomato (Smart and Fry, 2001). In many regions, a soil-borne reservoir of oospores is a source of primary inoculum for the late blight disease (Flier et al., 2004; Grunwald and Flier, 2005). Oospores also play important functions in diseases caused by other *Phytophthora* spp., including heterothallics such as the cucurbit pathogen *P. capsici* (Lamour and Hausbeck, 2002) and

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homothallics such as the bean pathogen *Phytophthora phaseoli* (Schwartz, 1991).

Sexual development in *P. infestans* resembles that of other heterothallic oomycetes, with mating occurring in response to hormones specific to each mating type (Ko, 1988). The first known response to the hormones is a swelling of hyphal tips, which develop into male and female gametangial initials that become antheridia and oogonia, respectively (Fabritius et al., 2002; Hemmes, 1983). Each mating type is dioecious, capable of forming male and female gametangia (Judelson, 1997). After physical contact occurs, an oogonial initial penetrates an antheridial initial and subsequently expands into an oospore. The male is thought to release an adhesive needed for mating, while the female provides most cytoplasm and nutrient reserves for the oospore. Nuclei within initials are diploid as in vegetative hyphae, but then undergo meiosis. Migration of an antheridial nucleus into an oogonium is followed by formation of a diploid zygote and enrichment of the oogonial cytoplasm with lipid bodies, proteins, and β-linked glucose polymers. A thick multilayered wall develops as oospores mature, which in combination with a cytoplasmic matrix rich in carbon reserves makes oospores well-adapted resting structures (Hemmes, 1983). Oosporogenesis by homothallic species of *Phytophthora* occurs by a similar process, with male and female organs capable of developing from the same thallus (Erwin and Ribeiro, 1996). Oospores later germinate to produce mycelia or a germ sporangium capable of releasing zoospores, similar to asexual sporangia.

Understanding the molecular basis of mating in oomycetes may hasten the development of new strategies for controlling disease, for example by identifying targets for chemicals interfering with oospore formation or germination. Relatively little molecular data are available concerning sexual development in any oomycete, although in *Phytophthora* nine mating-induced genes have been identified by subtraction cloning (Akino et al., 2003; Fabritius et al., 2002) and one of the mating hormones was recently shown to be a diterpene (Qi et al., 2005). Molecular details about mating in other oomycete taxa are limited (Silver et al., 1993). Due to a lack of taxonomic affinity between the fungus-like oomycetes and true fungi, knowledge of sexual processes obtained from the latter is unlikely to be directly relevant to *Phytophthora* (Baldauf, 2003).

To increase our understanding of sexual development in oomycetes, here we describe a large-scale screen for mating-induced genes in *Phytophthora.* Using Affymetrix GeneChips targeted against 15,644 *P. infestans* unigenes, 87 genes up-regulated by more than 10-fold during sexual development were identified and validated by reverse transcription-polymerase chain reaction (RT-PCR). Expression patterns were measured in several different developmental stages and in a homothallic relative, *P. phaseoli*, providing insight into relationships between the different sporogenesis pathways and mating systems.

2. Materials and methods

2.1. Culture conditions and RNA extractions

Wild-type *P. infestans* strains of the A1 mating type were 8811 and 88069, which had been isolated from the United Kingdom and the Netherlands, respectively. A2 isolates were 618 and E13, from Mexico and Egypt. Also employed were two *P. infestans* strains generated by silencing the *PiCdc14* gene in an A1 background, causing a block in asexual sporulation (Ah Fong and Judelson, 2003), and a wild-type isolate of a homothallic species, *P. phaseoli* (373, provided by P. Tooley). Stock cultures were maintained in the dark at 18 °C on rye A containing 1.5% agar (Caten and Jinks, 1968).

For plates destined for RNA extraction, a 0.4 μm-pore polycarbonate membrane was placed on top of the agar which was then overlaid with inoculum; the use of the membrane allowed all *Phytophthora* tissue to be readily recovered, since otherwise many oospores would form beneath the agar surface. Mating cultures were established by placing strips of A1 and A2 inoculum in parallel, 2 cm apart (Cvitanich and Judelson, 2003). After 3 and 10 days, RNA was harvested from a 1-cm zone centered at the junction between strains, taking care to obtain similar amounts of material originating from the A1 and A2 parents.

Two methods were used to establish non-mating cultures. For agar cultures of *P. infestans* or *P. phaseoli*, strips from a single strain were applied to a polycarbonate membrane laid upon rye agar. Rye broth cultures were initiated from small plugs from stock plates and incubated without shaking.

RNA was extracted using the Plant Mini RNA kit (Qiagen, Valencia, CA USA) after grinding in liquid nitrogen, following the manufacturer's instructions. Mating and non-mating cultures of *P. infestans* were harvested after 3 or 10 days. *P. phaseoli*, which grows at approximately 50% the rate of *P. infestans*, was harvested after 15 days. RNA was treated with DNase prior to use in microarray or reverse transcription-polymerase chain reaction (RT-PCR) studies. The quality of RNA was assessed based on its gel electrophoresis profile and its A_{260}/A_{280} ratio, with a minimum acceptable value of 1.90.

2.2. Affymetrix Genechip analysis

Total RNA was provided to a core facility for labeling and hybridization to custom Affymetrix GeneChips provided by Syngenta Corporation. These arrays will be described in more detail elsewhere (Judelson et al., in preparation). Briefly, over 18 K *P. infestans* unigenes identified from expressed sequence tags (ESTs) and partial genome sequence data (Randall et al., 2005) were submitted to Affymetrix for probe computation. Satisfactory probe sets, comprised of 13 pairs of 25-mer perfect match and mismatch oligonucleotides, were designed for 15,644 unigenes. Validation tests using a hyphal RNA sample indicated >98% correlation between technical replicates.

Results were returned by the core facility after preprocessing with Affymetrix MAS5.0 software. This included global normalization and scaling, and calculations of "present" and "absent" calls that indicate whether signal intensities were significantly above background and specific to the gene of interest. The hybridization signals for 5′ and 3′ probe sets for actin and elongation factor-1α genes were also checked to ensure good quality of the RNA and labelling reactions. Data were then reanalyzed using GeneSpring 6.1 (Agilent Technologies, Santa Clara, CA USA). This involved importing the MAS data, after removing genes from species other than *P. infestans* that were on the chips and approximately 2500 *P. infestans* genomic sequences which had been predicted to have high coding potential, yet which did not give a present call when hybridized to any RNA from approximately 20 different stages of growth and development including mating. The data were then renormalized to the 50th percentile, using per-chip normalization but not per-gene normalization. Mating-induced genes were then identified by filtering for fold-changes, including genes containing a present call in at least one mating sample. Searches for mating-repressed genes excluded those lacking a present call in the 10-day mating. For samples with technical replicates, mean values are reported in Results. The MAS 5.0 data for the mating samples and non-mating controls are deposited in the GEO database (http://www.ncbi.nlm.nih.gov/geo/) as experiment series GSE6343.

2.3. Semiquantitative RT-PCR

DNase-treated RNA (100 ng) was reverse-transcribed using the SuperScript III First-strand Synthesis System (Invitrogen, Carlsbad, CA USA). cDNA was then amplified with primers for mating-regulated genes or elongation factor 1α (EF-1); these primers are presented as supplementary material as Table S1. Preliminary experiments using EF-1 were used to ensure that cDNA from all tissues yielded identical amplification profiles. Reactions were performed in 25 μl and contained 10 mM KCl, 20 mM Tris–HCl pH 8.8, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Triton X-100, 125 μM dNTPs, 0.01% bovine albumin, and 1 unit of *Taq* DNA polymerase. These were heated for 2 min at 94 °C; cycled between 94 °C for 1 min, 55 °C for 1 min and 72 °C for 2 min; and held at 72 °C for 7 min. Separate reactions were performed for 21, 25, and 30 cycles for all primer sets, and in some cases for 17 and 35 cycles. Controls lacking reverse transcriptase were included for each primer set at the maximum number of cycles, to ensure that amplicons were not derived from genomic or contaminating DNA.

Products were electrophoresed in 1% agarose, stained with ethidium bromide, and photographed. The gels were also imaged using the Fluor-S system (Biorad, Richmond, CA, USA) to quantitate relative expression levels. This involved analyses with Biorad Quantity One v. 4.6 or ImageJ v. 10.2 software, the latter from the National Institutes of Health (Bethesda, MD, USA). Calculations were based on band intensity ratios measured within the logarithmic portion of the amplification curve, typically at 21 or 25 cycles.

2.4. Blot analysis

Ethidium bromide-stained total RNA was separated on 1.2% agarose-6.6% formaldehyde gels, transferred to nylon membranes by capillary blotting in 10× SSPE (1.8 M NaCl, 0.1 M $NaHPO_4$, 0.01 M EDTA, pH 7.7), fixed by UV cross-linking, and hybridized with ^{32}P-labeled randomly primed probes made from amplified fragments of the targeted genes. High-stringency washes were in 0.2× SSPE (1.8 M NaCl, 0.1 M $NaHPO_4$, 0.01 M EDTA, pH 7.7), 0.2% sodium dodecyl sulfate, and 0.1% sodium pyrophosphate at 65 °C. Low-stringency washes were in 1× SSPE, 0.2% sodium dodecyl sulfate, and 0.1% sodium pyrophosphate at 55 °C. Signals were detected by phosphorimager analysis and quantified using Quantity One software.

2.5. DNA sequence analysis

Predictions of gene function were made by comparing the *P. infestans* sequences to GenBank using BLASTX. Initially, comparisons used the original unigene sequences reported by Randall et al. (2005). However, just prior to submission of this paper raw sequences became available from a *P. infestans* genome project being performed by the Broad Institute (Massachusetts Institute of Technology, Cambridge, MA USA), which could be downloaded from the NCBI Trace Archive (National Institutes of Health, Bethesda, MD USA). The unigene sequences, which were typically incomplete fragments of genes, were therefore extended up to 2-kb in the 5′ and 3′ directions and compared again to GenBank. *E* values shown in Table 1 reflect these expanded sequences, which may include introns.

3. Results

3.1. Overview of Genechip experiment

A preliminary identification of *P. infestans* genes up-regulated during sexual development was achieved by expression-profiling unigenes obtained from an EST and partial genome sequencing study (Randall et al., 2005). 15,644 of those unigenes were represented on custom Affymetrix GeneChips made available generously by Syngenta. The genes will be referred to in this paper by their names on the chips, which start with a "Pi" prefix. The corresponding unigene sequences, as identified by Randall et al. (2005), are downloadable as web supplements from that journal's web site.

Candidate mating-induced genes were revealed by comparing matings between isolates 88069 and 618 (A1 × A2) with non-mating cultures. Several non-mating tissues were tested in the GeneChip experiments, and later RT-PCR and RNA blot analyses, due to uncertainty about which

Table 1
Eighty-seven genes induced during mating in *Phytophthora infestans*

Gene[a]	Fold-induction in mating[b]			Overall pattern[c]		Best match in GenBank[d]	*E* value
	88069 × 618 (GeneChip)	88069 × 618 (RT-PCR)	8811 × E13 (RT-PCR)	*P. infestans*	*P. phaseoli*		
Pi000192	>100	>50	>50	M, NS	U, 18 ± 5	Elicitin-like protein M25,*P. infestans* AF507054	6e-69
Pi000193	>100	>50	>50	M, NS	C	Elicitin-like protein M25, *P. infestans* AF507054	1e-58
Pi001986	10	12 ± 2	8 ± 2	M, NS	C	Chitin synthase, *Saprolegnia monoica* AAC49743	2e-72
Pi002500	10	9 ± 0	12 ± 3	M, NS, SP	C	Nitrate transporter, *Skeletonema costatum* AAL85928	5e-79
Pi002501	>100	>50	>50	M	C	Elicitin-like protein, *P. sojae* AY183414	1e-31
Pi002983	26	12 ± 3	13 ± 4	M, SP	C	Calcium-ATPase, *Homo sapiens* AAA08376	0.0
Pi002989	12	9 ± 2	7 ± 2	M	U, 5 ± 1	Chloroperoxidase-like, *Neurospora crassa* CAD71220	2e-12
Pi003049	13	18 ± 5	16 ± 2	M	C	Crinker-like CRN8, *P. infestans* AAY43402	3e-25
Pi003363	13	11 ± 1	15 ± 3	M	C	Maltase-glucoamylase, *Homo sapiens* NP_004659	1e-67
Pi003398	28	36 ± 11	>50	M	C	β-Ketoacyl reductase, *Pseudomonas aeruginosa* AAG06775	7e-17
Pi003432	14	11 ± 2	9 ± 2	M, NS	C	No hit	
Pi003811	53	18 ± 1	8 ± 1	M	C	CDPK kinase-like,*Solanum tuberosum* AAM29184	1e-22
Pi003858	82	41 ± 12	37 ± 9	M, SP	U, 6 ± 1	PvLEA1 protein, *Polypedilum vanderplanki* BAE92616	1e-23
Pi004097	28	>50	>50	M	C	Acid phosphatase, *Aspergillus ficuum* AAB31768	5e-27
Pi004167	68	>50	>50	M	U, >50	Laccase I, *Hortaea acidophila* AAY33970	4e-53
Pi004382	55	39 ± 12	>50	M	U, >50	Phosphate carrier, *Strongylocentrotus purpuratus* XP_801890	4e-51
Pi004695	>100	48 ± 13	>50	M	C	No hit	
Pi004734	>100	>50	>50	M	C	No hit	
Pi004801	23	15 ± 6	18 ± 8	M	C	Sulfatase, *Azotobacter vinelandii* EAM05877	8e-15
Pi004848	14	17 ± 7	5 ± 1	M, NS, SP	U, >50	Pumilio protein, *Homo sapiens* AAH13398	3e-84
Pi004919	48	37 ± 10	27 ± 9	M	C	No hit	
Pi005054	>100	>50	>50	M	C	Zinc finger protein, *Danio rerio* AAI17601	4e-15
Pi005197	>100	>50	>50	M	C	No hit	
Pi005700	26	10 ± 3	7 ± 2	M	C	No hit	
Pi005710	27	11 ± 4	34 ± 11	M, NS, SP	C	No hit	
Pi005794	60	>50	38 ± 14	M, SP	C	No hit	
Pi005879	>100	43 ± 14	>50	M	C	No hit	
Pi006440	15	7 ± 2	36 ± 13	M	C	No hit	
Pi006590	>100	48 ± 6	>50	M	U, 11 ± 2	No hit	
Pi006656	26	17 ± 7	>50	M	C	No hit	
Pi006831	>100	>50	>50	M	U, 29 ± 11	No hit	
Pi006879	12	16 ± 4	8 ± 2	M, NS, SP	C	Crinker protein CRN9, *P. infestans* AAY43403	3e-35
Pi006907	>100	>50	>50	M	C	No hit	
Pi007072	16	32 ± 8	19 ± 4	M	C	Protein phosphatase 2C epsilon, *Mus musculus* AAO4355	4e-28
Pi007197	23	>50	48 ± 6	M, NS	C	No hit	
Pi007830	64	16 ± 5	21 ± 5	M	U, 8 ± 0	No hit	
Pi007954	62	>50	39 ± 13	M	C	11R-lipoxygenase, *Gersemia fruticosa* AAY98506	5e-24
Pi008183	17	14 ± 5	12 ± 4	M, NS	C	No hit	
Pi008199	10	9 ± 3	7 ± 2	M	C	Nno hit	
Pi008231	54	42 ± 5	>50	M	A	No hit	
Pi008242	15	28 ± 9	16 ± 3	M, SP	C	ABC transporter, *Oryza sativa* NP_916719	5e-79
Pi008771	66	>50	>50	M	A	No hit	
Pi008890	>100	>50	>50	M	C	Loricrin-like protein, *Oryza sativa* XP_464880	1e-25
Pi008898	>100	>50	>50	M	U, 40 ± 10	No hit	
Pi009317	94	>50	>50	M, NS	C	No hit	
Pi009459	35	15 ± 5	19 ± 3	M	C	GAF sensor, *Magnetospirillum magnetotacticum* ZP_00048086	4e-06
Pi009644	76	>50	>50	M	C	No hit	
Pi009906	>100	>50	>50	M	C	No hit	
Pi010024	43	>50	>50	M, NS	D, >50	No hit	
Pi010125	>100	>50	>50	M, SP	C	Elicitin-like protein INL6, *P. infestans* ABB55941	3e-72
Pi010145	31	31 ± 3	8 ± 3	M	C	DNA repair protein, *Strongylocentrotus purpuratus* XP_79764	1e-128
Pi010538	10	9 ± 1	13 ± 2	M, NS	C	No hit	
Pi010846	>100	>50	>50	M	C	No hit	
Pi010913	53	>50	>50	M	C	No hit	
Pi011096	96	>50	>50	M, SP	C	No hit	
Pi011132	>100	>50	>50	M	A	No hit	
Pi011181	15	9 ± 3	12 ± 3	M	C	No hit	
Pi011393	>100	>50	>50	M	C	No hit	
Pi011478	15	>50	>50	M	C	No hit	
Pi011511	58	48 ± 20	>50	M, NS, SP	C	Recombination initiation protein, *Trypanosoma cruzi* EAN84841	4e-05
Pi011744	65	34 ± 7	22 ± 10	M, NS, SP	C	No hit	

(*continued on next page*)

Table 1 (*continued*)

Gene[a]	Fold-induction in mating[b]			Overall pattern[c]		Best match in GenBank[d]	*E* value
	88069 × 618 (GeneChip)	88069 × 618 (RT-PCR)	8811 × E13 (RT-PCR)	*P. infestans*	*P. phaseoli*		
Pi011975	66	25 ± 8	15 ± 4	M, NS, SP	C	mRNA decapping enzyme, *Debaryomyces hansenii* CAG89700	9e-33
Pi012205	52	>50	>50	M, SP	C	Elicitin protein, *P. sojae* AAO24644.1	2e-51
Pi012417	47	31 ± 7	12 ± 4	M	C	No hit	
Pi012583	26	8 ± 2	5 ± 0	M	C	No hit	
Pi012654	>100	>50	>50	M	A	No hit	
Pi012846	>100	>50	>50	M, NS, SP	C	mRNA decapping enzyme, *Danio rerio* AAP47149	1e-24
Pi012968	>100	>50	>50	M	A	No hit	
Pi013489	27	10 ± 2	12 ± 1	M	C	DNA helicase, *Arabidopsis thaliana* CAC14867	1e-82
Pi013531	44	16 ± 8	12 ± 4	M	C	Mutator transposon, *Oryza sativa* ABA99201	1e-25
Pi013579	75	32 ± 18	11 ± 1	M	C	No hit	
Pi013584	32	25 ± 5	8 ± 3	M, SP	C	No hit	
Pi013787	12	11 ± 2	14 ± 6	M	C	No hit	
Pi013922	18	10 ± 3	13 ± 1	M	C	ABC transporter, *Arabidopsis thaliana* AAD39650	3e-123
Pi014084	>100	43 ± 7	31 ± 12	M	C	Hypothetical protein, *Gluconobacter oxydans* AAW60014	7e-08
Pi014092	>100	>50	>50	M, NS	U, >50	Mating-induced protein M96, *P. infestans* DQ196155	0.0
Pi014241	13	10 ± 2	14 ± 3	M	C	Zinc-finger protein, *Coprinellus disseminatus* AAZ14935	2e-09
Pi014253	11	9 ± 0	5 ± 1	M	A	Crinkler protein CRN9, *P. infestans* AAY43403	8e-128
Pi014347	>100	>50	>50	M	U, 21 ± 2	Alkaline phosphatase, *Clostridium acetobutylicum* AAK80385	2e-06
Pi014363	15	16 ± 4	13 ± 3	M, SP	C	Ankyrin-like protein, *Dictyostelium discoideum* EAL61012	8e-27
Pi014365	>100	>50	>50	M, NS	U, >50	No hit	
Pi014503	67	67	>50	M, NS, SP	C	No hit	
Pi015281	>100	>50	>50	M	U, >50	Dynamin GTPase, *Epinephelus coioides* ABD95980	1e-40
Pi015297	69	17 ± 6	8 ± 2	M, NS, SP	C	Myosin light chain kinase, *Eimeria tenella* CAK51490	2e-57
Pi015306	13	8 ± 2	9 ± 1	M	C	CDC20 protein, *Ostreococcus tauri* AAV68609	6e-69
Pi015539	>100	>50	>50	M	A	No hit	
Pi015704	>100	>50	>50	M	C	Zinc finger protein BRCA1, *Bos taurus* AAO92399	3e-06

[a] Number refers to unigene represented on the Affymetrix GeneChip; the assembly sequence or EST anchoring each gene as defined by Randall et al. (2005) are shown in Supplementary Table S1. A few lines represent data merged from unigenes found later to represent a single locus; in such cases, expression data are the average of the value for each gene. Redundant unigenes were Pi003237 and Pi013922; Pi003858 and Pi008242; Pi004167, Pi002961, and Pi004496; and Pi007954, Pi008568, and Pi013658.

[b] Induction ratios from the microarray study are calculated as the expression in the 10-day mating sample (MAT10; 88069 × 618) divided by the mean of non-mating A1 (88069) and A2 (618) agar cultures. RT-PCR data compares band intensity in the 10-day mating sample (88069 × 618 or 8811 × E13) by the mean of 10-day non-mating A1 (88069 or 8811) and A2 (618 or E13) agar cultures. The 88069 and 618 samples were independent of those used for the GeneChip experiment. Data represent means and ranges from duplicate determinations.

[c] *P. infestans* patterns are defined as M, induced >10-fold during mating; NS, induced in mating and also >5-fold in 10-day agar cultures of the nonsporulating *PiCdc14*-silenced mutant compared to the 4-day nonsporulating rye broth cultures; and SP, induced in mating and also >5-fold in 10-day sporulating agar cultures compared to the 4-day rye broth cultures. *P. phaseoli*patterns are defined as A (absent), expression not detected in both RT-PCR and GeneChip studies; C (constitutive), expression at similar levels in oosporogenesis-conducive agar and non-conducive broth cultures; U (upregulated), higher in the agar versus broth cultures at the indicated ratios, e.g. U21 means induced 21-fold during oosporogenesis-conducive conditions; and D (downregulated), lower mRNA levels in the agar versus broth cultures.

[d] Indicated are matches in BLASTX analyses with $E < 10^{-5}$. Shown is the best non-oomycete match, except for oomycete-specific proteins such as members of the crinkler and elicitin families.

represented the best control to compare with sexual development. This is because cultures grown for equivalent amounts of time with and without the opposite mating type vary in features besides sexual structures. Several characteristics of normal growth are arrested during mating, such as the formation of aerial hyphae and asexual sporangia (Fabritius et al., 2002).

Sexual interactions examined were 3-day and 10-day 88069 × 618 pairings. These were established by allowing each to approach each other from opposite sides of a rye agar plate. After three days their hyphae are still separated by about 1.5-mm, and it was hoped that this timepoint would reveal events mediated by mating hormones. Present at ten days are a mixture of young gametangial interactions and oospores approaching maturity. At 3 and 10 days, hyphae in the mating zones are flat on the surface of the medium, and contain few aerial hyphae, asexual sporangiophores, or sporangia. The non-mating samples included 3-day cultures of 88069 and 618 grown separately on rye agar, in which hyphal mats are relatively flat and contain few asexual spores; 10-day agar cultures of 88069, in which aerial, fluffy hyphae containing asexual sporangia are abundant (these develop typically after four days); and 10-day agar cultures of strains blocked in asexual sporulation due to silencing of *PiCdc14* (Ah Fong and Judelson, 2003).

To reduce biological variation in the GeneChip studies, RNAs used in each hybridization were pooled from three cultures grown during different weeks with separate batches of media. As technical replicates, each type of RNA pool was hybridized against two chips. Exceptions were the 3 and 10-day matings, which were tested on one chip each since only a limited number of the custom *Phytophthora*

chips were made available for this study. Due to this constraint a decision was made to focus only on genes showing major changes during mating, and to validate all of their expression patterns by semiquantitative RT-PCR using RNA from a 88069 × 618 mating (distinct from the cultures used for the GeneChip study), as well as by RT-PCR using a mating between two other isolates, 8811 and E13.

3.2. Candidates for mating-regulated genes

Comparisons between the 3 or 10-day matings and the 3-day non-mating agar cultures were considered most appropriate for identifying mating-regulated genes. This is because the hyphal mats of each are relatively flat and largely lacking in sporangiophores and asexual sporangia. Using a mating culture older than the control (10 versus 3-days) was recognized to be a potential complication, but as will be discussed later this turned out to be a minor issue.

Substantial changes in gene expression patterns were observed after 10 days (Fig. 1). Of 10,356 genes showing signal intensities significantly above background in at least one of the tissues, 1663 showed a greater than 2-fold increase in the 10-day mating compared to the non-mating A1 and A2 cultures, with 94 expressed more than 10-fold higher. However, no genes showed greater than a 2-fold increase in the 3-day mating compared to the 3-day non-mating control.

A smaller number of genes showed a reduction in mRNA levels during mating, with 349 dropping more than 2-fold in the 10-day mating versus the control. Other experiments indicated that their mRNA levels were also reduced in 10-day non-mating cultures (not shown), so most are probably not relevant specifically to mating. Based on DNA sequence many appeared to have roles in growth and metabolism, frequently having functions in amino acid biosynthesis and transport.

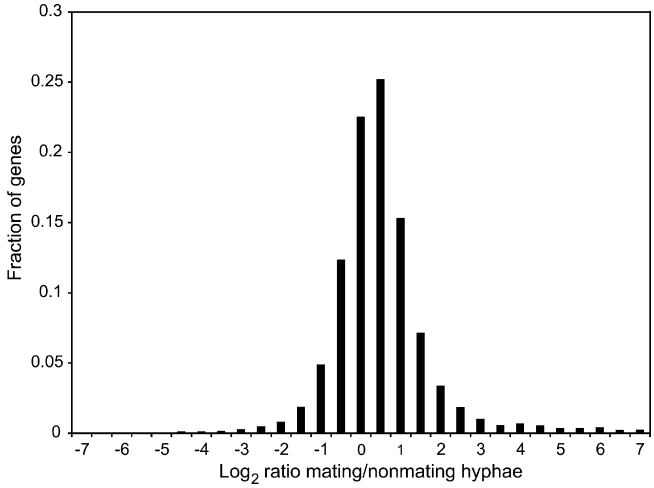

Fig. 1. Global changes in gene expression during mating in *P. infestans*. Indicated are the relative expression levels of genes in the 10-day mating compared to the mean of 3-day A1 and A2 cultures grown separately. Data are shown only for the 10,356 genes showing "present" calls in either the mating or non-mating samples.

The calculations used in the above analyses were performed assuming that tissue of A1 and A2 origin were represented equally within the mating zones excised for RNA extraction; non-mating levels of expression were therefore set as the mean of separate non-mating A1 and A2 cultures. This assumption proved to be quite accurate. By measuring the relative abundance within the mating RNA of polymorphisms detected between the A1 and A2 parent in three constitutively expressed genes, it was calculated that $47 \pm 15\%$ and $42 \pm 13\%$ of RNA from the 3 and 10-d matings, respectively, were derived from the A1 parent.

3.3. Validation of array data

Subsequent examinations focused on the 94 genes induced by at least ten-fold during mating. This involved analyses of the 88069 × 618 cross and a mating between isolates 8811 (A1) and E13 (A2). The latter two parents are geographically and genetically distinct from 88069 and 618 based on RG57 fingerprinting (not shown; Goodwin et al., 1992), and were selected to test whether the genes are universally mating-induced in *P. infestans*. To further assess whether the genes are induced by mating, as opposed to other factors such as culture age, 10-day mating cultures were compared to 10-day non-mating (asexually sporulating) cultures.

RNA blot studies of a subset of the genes confirmed that they are induced in both the 88069 × 618 and 8811 × E13 matings (Fig. 2). Blot analyses of many of the remaining genes proved challenging due to low expression levels. Consequently, a semiquantitative RT-PCR method was applied to all of the 94 genes, using RNA from both 88069 × 618 and 8811 × E13 matings (in both cases, distinct from the RNA used on the GeneChips). After standardizing RNA levels based on the amplification of elongation factor 1∝ (EF-1), primers for candidate genes were tested against RNA from 10-day matings and 10-day cultures of the A1 and A2 strains grown separately. Reaction products were analyzed after 21, 25, and 30 cycles of amplification; 17 and 35 cycles were also examined for some genes. To calculate *approximate* induction ratios, a CCD camera was used to quantify band intensities during the exponential phase of the reaction. As illustrated in Fig. 3A for representative genes, this usually persisted through 21 or 25 cycles.

Such RT-PCR analyses of both the 88069 × 618 and 8811 × E13 crosses confirmed that 87 genes are mating-induced. Typical reactions are shown in Fig. 3B, and presented in Table 1 are induction ratios for the 87 genes, defined as the signal from the 10-day mating divided by the mean of 10-day A1 and A2 signals, based on the mean of two replicate reactions. Also shown in Table 1 are the induction ratios from the GeneChip experiment, representing the expression level in the 10-day 88069 × 618 mating divided by the mean of 3-day A1 and A2 levels. There was good agreement between the assays, although some quantitative variation was observed. For example, the induction of gene Pi006440 in the 88069 × 618 mating was 15-fold based on the

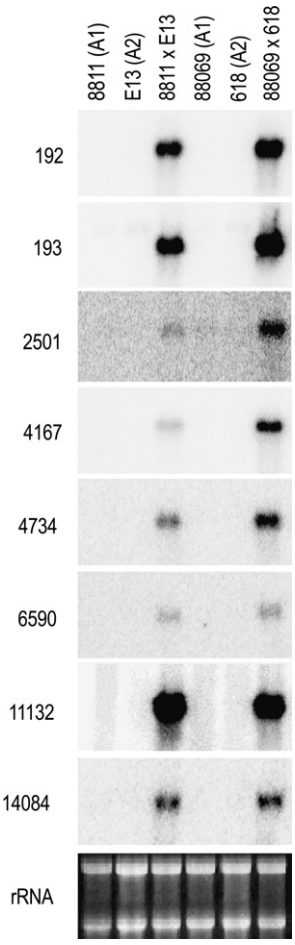

Fig. 2. RNA blot analysis of mating-induced genes in *P. infestans*. Ten micrograms of RNA from 10-day agar cultures of the individual A1 and A2 strains, or 10-day matings, were electrophoresed, blotted, and hybridized with probes for the indicated genes (in this and other figures, the "Pi" prefix is omitted from the gene names).

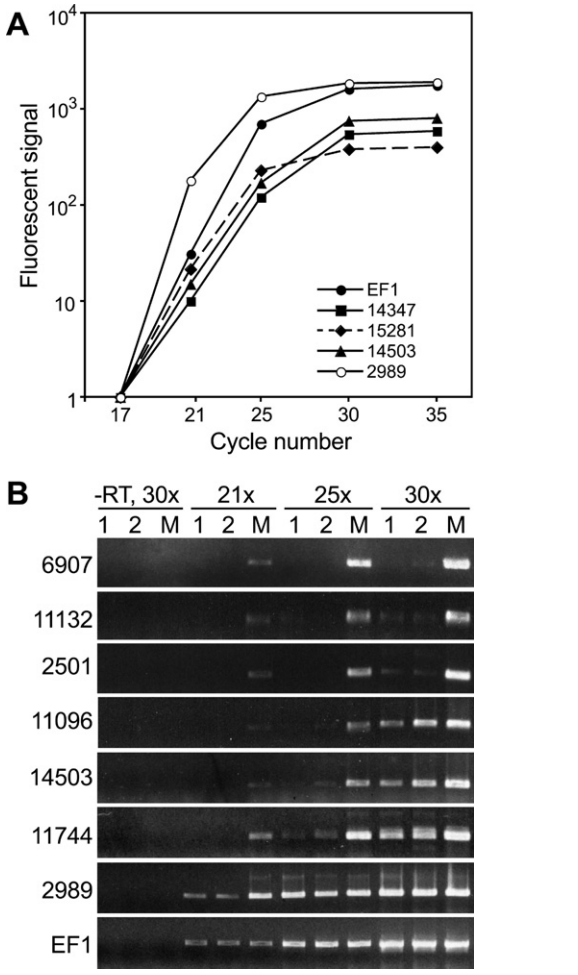

Fig. 3. Semiquantitative RT-PCR in *P. infestans*. (A) Band intensities for representative genes obtained after amplification for the indicated number of cycles. In most cases reactions are exponential up to 21 or 25 cycles, as indicated by the slope of the lines. For genes such as Pi002989, calculations of approximate expression levels were based on the 21-cycle value. For genes such as Pi014347, 25-cycle values were used. (B) RT-PCR products for representative mating-induced genes. Reactions were performed for 21, 25, and 30 cycles after reverse transcriptase treatment, or without reverse transcriptase for 30 cycles. Templates were from a 10-day 8811 × E13 mating (M), a 10-day culture of A1 strain 8811 (1), or a 10-day culture of A2 strain E13 (2).

GeneChip and 7-fold based on RT-PCR, and 36-fold based on RT-PCR of the 8811 × E13 pairing. Such differences may be attributed to either of several technical and biological issues. These include variation in mating intensity between the two crosses, variation between the 3-day and 10-day non-mating controls, and error in measuring the weak non-mating signals used in the denominator of each calculation. Due to the latter issue, the maximum induction ratios for GeneChip and RT-PCR assays are listed conservatively as ">100×" and ">50×", respectively; empirical data indicated that RT-PCR had about half the dynamic range of the GeneChip assays. In the case of the RT-PCR experiments, for example, a 50× value might mean that a strong band was detected in the mating reaction and no band, or one that was extremely faint, in non-mating reactions.

Of the seven genes not validated by RT-PCR, most had very low and unreliable expression levels in the GeneChip studies. The exception was Pi011633, which the chip study indicated was induced modestly during mating (using the 3-day non-mating control) but RT-PCR showed was non-induced (using the 10-day non-mating, asexually sporulating control). This difference was later explained by finding that this gene was induced >100-fold during asexual sporulation (not shown). The corollary of the situation with Pi011633 is that the other 87 genes appeared mating-induced regardless of whether 3-day or 10-day non-mating cultures were the control. As will be described later, this should not be construed to indicate that these 87 genes are unexpressed during asexual sporulation; some are expressed during asexual sporulation, but generally at much lower levels than during mating.

3.4. Expression of genes under different conditions

Relative RNA levels of the 87 validated mating-induced genes as determined by the chip studies are shown in Fig. 4. The four left-most columns of each panel correspond to

growth conditions previously described. These are the 3-day A1 and A2 cultures (3d NSH; mean of replicated A1 and A2 cultures), 3 and 10-day matings (3d MAT, 10d MAT), and 10-day non-mating cultures containing hyphae exhibiting asexual sporulation (10d SPHY; mean of two replicates). The fifth column portrays nonsporulating mutants of *P. infestans* generated by silencing *PiCdc14* in the A1 background (10d NSMU; mean of two silenced lines). The mutants were tested since they might indicate gene expression patterns in a 10-day culture that are independent of asexual sporulation and oosporogenesis. Like mating cultures, the *PiCdc14*-silenced strains lack fluffy aerial hyphae and asexual sporangia (Ah Fong and Judelson, 2003); they also mate normally. The sixth column in the figure represents *P. phaseoli*, a homothallic species that will be discussed in a later section.

Fig. 4 is partitioned into four panels based on whether the genes, compared to the 3-day control, are up-regulated in *P. infestans* during mating alone or also by silencing *PiCdc14* or during asexual sporulation. In each panel, genes are presented in descending order of their induction ratio during mating; the numeric ratios are listed in Table 1. Of the 87 genes, 57 are induced exclusively during mating (left column), so it is reasonable to assume that these play specific roles in sexual development. Ten genes are up-regulated during both mating and asexual sporulation, and in theory might function in activities common to both pathways such as the need to establish nuclear dormancy and nutrient reserves in spores. Twelve genes are induced during mating and in the nonsporulating *PiCdc14* mutants, and eight by all three conditions; such patterns may simply be typical of mature (10-day) cultures and not related directly to sexual development, but this is not necessarily true as will be addressed in Section 4.

For genes showing some induction during asexual sporulation, their expression was much higher during mating which explains why they scored as mating-induced in RT-PCR using the 10-day non-mating controls. For example,

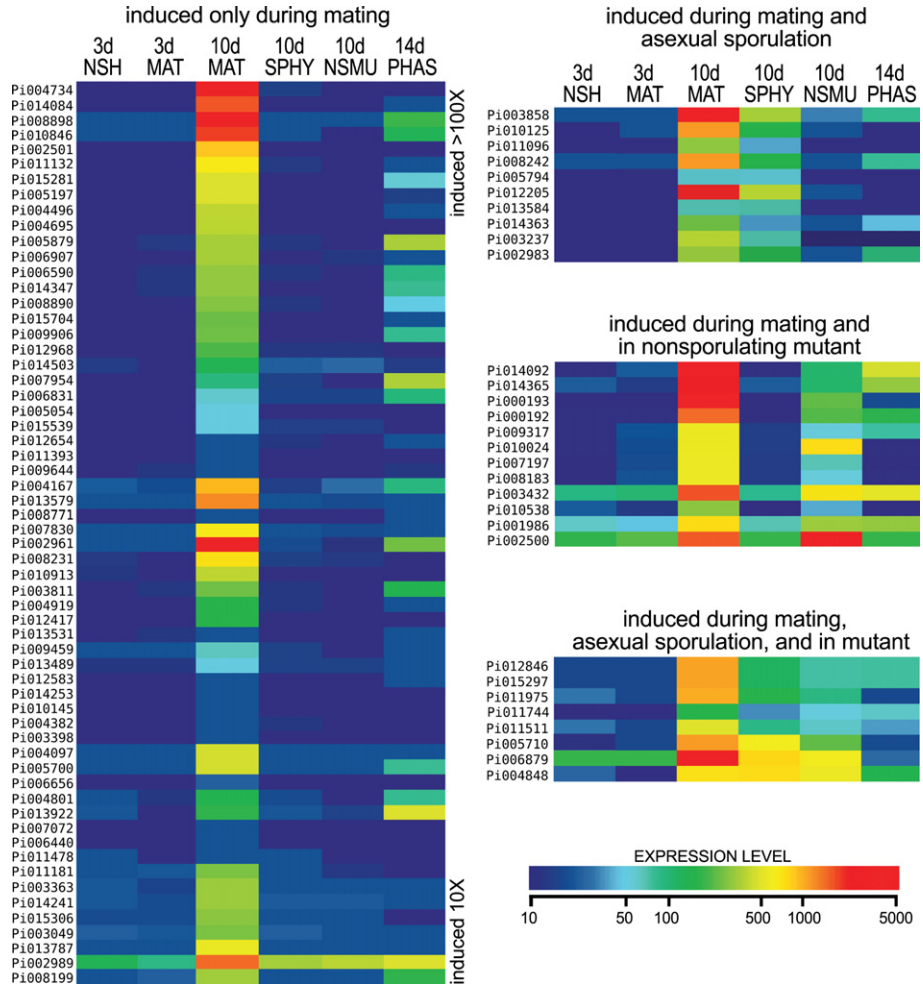

Fig. 4. Relative mRNA levels of the 87 mating-induced genes. Expression levels determined from the GeneChip experiments are shown for the 3-day A1 and A2 agar cultures of *P. infestans* (3d NSH), 3 and 10-day agar matings (3d MAT, 10d MAT), 10-day agar cultures exhibiting asexual sporulation (10d SPHY), 10-day agar cultures of nonsporulating mutants of *P. infestans* generated by silencing *PiCdc14* (10d NSMU), and a 14 day agar culture of *P. phaseoli* (14d PHAS). Except for the 3d MAT and 10d MAT samples, data represent the mean of replicate hybridizations, representing a total of 10 chips. The genes are split into four panels based on their pattern of expression in the different types of *P. infestans* samples. Within each panel, genes are ranked according to their magnitude of induction during mating, compared to the 3d NSH samples.

from the chip data Pi003858 was calculated as induced 82-fold during mating compared to the 3-day cultures, but only 12-fold in matings versus the 10-day asexually sporulating cultures; it follows that the gene may be induced about 7-fold during asexual sporulation. Similarly, genes showing higher expression in the nonsporulating mutant were usually expressed much more during mating. For example, Pi014092 was expressed >100-fold higher during mating compared to 3-day cultures, and 7-fold higher in mating than in the *PiCdc14* mutants.

3.5. Functional annotation of genes

Of the 87 mating-induced genes, 44 have significant matches to proteins in GenBank, using a threshold *E* value in BLASTX of 10^{-5}. These are listed in Table 1. The best non-oomycete matches are shown, except in the case of oomycete-specific proteins such as elicitins. The lack of matches to the other 43 genes is not an artifact of using partial gene sequences for database searching. It is true that the initial searches of GenBank were performed using the unigene sequences, many of which did not represent the entire gene (Randall et al., 2005). However, just before completion of this manuscript, sequence reads from the publicly funded genome sequencing project for *P. infestans* became available through the NCBI Trace Archive. By walking in this database, the unigene data were extended in both the 5′ and 3′ directions by 2 kb. It is these sequences, untrimmed of potential introns, that were used for the GenBank search results shown in Table 1. If a match was obtained with the extended sequence but not the unigene sequence, care was taken to ensure that the similarity was in the mating-induced gene and not an adjoining gene.

Four of the genes resembled, or matched, genes previously identified as being mating-induced in *P. infestans* (Fabritius et al., 2002). Pi000192 and Pi000193 were related but not identical to gene *M25*, which was previously suggested to be an elicitin-like sterol binding protein. A recent study showed that *Phytophthora* species encode diverse families of such proteins, although most of their expression patterns are unknown (Jiang et al., 2005). Pi004848 was the same gene as *M90*, which encodes a RNA binding protein (Cvitanich and Judelson, 2003). Pi014092 was related to *M96*, which was already known to be a member of a larger family (Cvitanich et al., 2006). The other five genes shown previously to be mating-induced were not represented on the GeneChip.

For genes not similar to sequences in GenBank, their predicted proteins were checked for motifs in the Conserved Domain Database at NCBI. Only Pi008242 resulted in a match, which was against pfam04658 (TAFII55_N; $E = 10^{-14}$) defining a sequence in factors that bind TATA-binding protein. However, several of the predicted proteins were rich in low-complexity regions or repeated motifs, which are suggestive of roles as structural proteins. Many of the genes lacking relatives in GenBank also appear to have diverged substantially during speciation in *Phytophthora*. For example, no significant hits in the *P. ramorum* database (available on-line from the Joint Genome Institute of the US Department of Energy) were obtained in BLASTN searches using Pi005151, Pi005197, Pi006907, Pi008771, Pi010846, Pi010913, Pi011096, Pi011511, Pi014084, Pi014092, and Pi014365, although in some cases weak matches could be detected by TBLASTX.

When the unigene sequences were expanded using the genome data from the NCBI Trace Archive, it became apparent that in three cases separate "genes" on the chips actually represented the same locus; different unigenes may have represented non-overlapping ESTs from the 5′ and 3′ ends of a gene, for example. Such cases of redundancy were eliminated from all parts of the analyses in this paper. Therefore, the 87 mating-induced sequences represent unique genes.

3.6. Expression of genes in P. phaseoli

In phylogenetic studies, the homothallic species *P. phaseoli* clusters very closely to *P. infestans* (Tooley et al., 1996). DNA sequence analysis of housekeeping genes currently in GenBank (cytochrome oxidase, elongation factor 1α, F1 ATPase, β-tubulin) reveals identity of 98-99% between the species, although 10% divergence was detected for a mating-induced gene (Cvitanich et al., 2006). It was therefore predicted that many *P. phaseoli* genes would be assayable on the GeneChips, enabling expression patterns to be compared between this homothallic and its close heterothallic relative *P. infestans*.

Fifty-four of the 87 genes showed significant signals on the chips when hybridized with RNA from rye agar cultures of *P. phaseoli* (Fig. 4). Calculated levels of RNA abundance were frequently higher in *P. phaseoli* than in non-mating *P. infestans*, and often similar to the 10-day *P. infestans* mating. For example, normalized expression values for Pi004801 were 326 and 349 in the *P. infestans* mating and *P. phaseoli*, respectively. For Pi013922 values of 178 and 395 were recorded in *P. infestans* matings and *P. phaseoli*, respectively.

It was more typical for expression calls to be lower in *P. phaseoli*, however. For example, the expression value for Pi010846 was 1916 in the *P. infestans* mating and 172 in *P. phaseoli*. This *P. phaseoli* signal is one-tenth of that seen in *P. infestans*, but oospore densities within *P. phaseoli* cultures are lower than in A1 × A2 matings of *P. infestans* (1000/m^2 versus 3000 to 10,000/cm^2 for *P. infestans*, depending on isolate). Therefore, the signal is proportional to the extent of sexual development. DNA sequence differences between *P. phaseoli* genes and targets on the chip could also explain the lower values. Nevertheless, it is notable that of the 57 genes induced only during mating in *P. infestans*, of which 27 gave a clear signal using *P. phaseoli* RNA, 22 showed higher levels in *P. phaseoli* than in any non-mating sample of *P. infestans*.

To understand in more detail the expression of the genes in *P. phaseoli*, blot assays were tested using RNA from rye

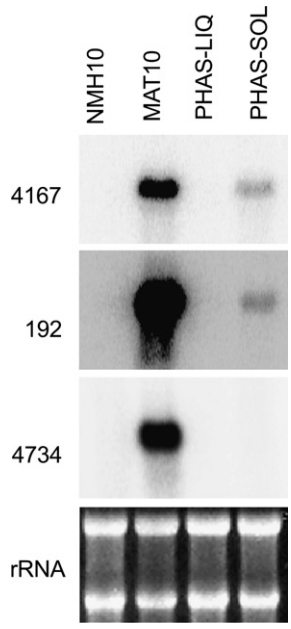

Fig. 5. RNA blot analysis of *P. infestans* and *P. phaseoli*. Probes from genes Pi004167, Pi000192, and Pi004734 were hybridized to RNA from 10-day non-mating hyphae of *P. infestans* grown on agar (NMH10; 5 μg of total RNA from a culture of 8811 and 5 μg from E13); 10 μg of RNA from a 10-day 8811 × E13 mating (MAT10); or 10 μg of RNA from 15-day *P. phaseoli* cultures grown in liquid media not conducive to oospore formation (PHAS-LIQ) or on solid rye media in which oospores form (PHAS-SOL).

agar cultures, in which oospores are made spontaneously, and submerged liquid cultures in which oospores do not form. This developmental difference enables an assessment of whether the genes are expressed constitutively or only during oosporogenesis. Typical results are in Fig. 5. As shown for Pi000192 and Pi002961, many genes are expressed only in the oosporogenesis-conducive conditions. However, transcripts were frequently not detected in the blots, as illustrated for Pi004734. This may simply reflect their low abundance in *P. phaseoli*, as was also the case for many genes in *P. infestans*, or sequence divergence between the *P. infestans*-derived hybridization probe and *P. phaseoli*.

To better measure all 87 genes in *P. phaseoli*, the more sensitive semiquantitative RT-PCR approach was employed. This indicated that 14 genes are up-regulated during the oosporogenesis-conducive conditions (on agar), one is down-regulated on agar, and 57 are expressed at similar levels during agar and liquid culture. These patterns are summarized in Table 1, including the extent of up- or down-regulation calculated by RT-PCR. Seven genes amplified poorly from *P. phaseoli* and were not examined further.

Representative RT-PCR data are shown in Fig. 6. Typical of constitutive genes are Pi002983, Pi007954, and Pi002501, which were expressed at similar levels on agar and liquid cultures (Fig. 6A). In the GeneChip experiment the first two had high expression in *P. phaseoli* compared to non-mating *P. infestans*, while the latter did not yield a

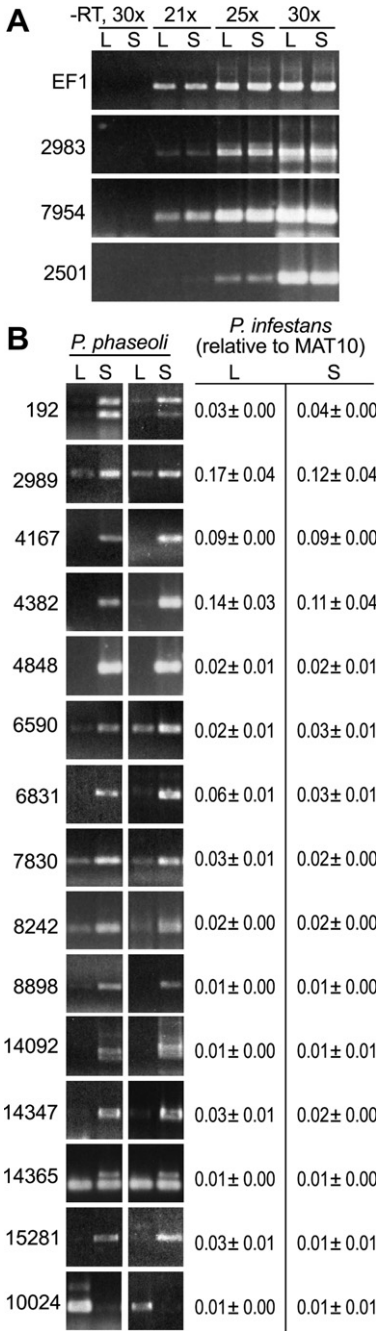

Fig. 6. Semiquantitative RT-PCR in *P. phaseoli*. (A) Representative reactions performed as shown in Fig. 3, except that the RNA used was from liquid (L) or solid agar (S) cultures of *P. phaseoli*. (B) Fifteen genes regulated during oosporogenesis in *P. phaseoli*. Photographs on the left portray replicated reactions using *P. phaseoli* RNA from liquid (L) or solid agar (S) cultures. The ratio of band intensities in lanes S and L was used to calculate approximate induction ratios for each gene, which are listed in Table 1. Numbers on the right show the expression levels of the genes in liquid and solid agar cultures in *P. infestans* relative to a 10 day mating culture of 88069 × 618, based on two replicate GeneChip studies each.

significant expression call against *P. phaseoli* presumably due to sequence differences between the two species. Shown in Fig. 6B are the 15 genes that showed significant differences between oosporogenesis-conducive (agar) and non-conducive (broth) cultures. Fourteen are up-regulated by

the oosporogenesis-conducive conditions by 8 to >50-fold, while one (Pi010024) is down-regulated by >50-fold. This curious result with Pi010024 was seen in several independent assays. The gene did not have any match in GenBank that might suggest a function.

The most likely explanation of the overall results from *P. phaseoli* is that some mating-associated genes are constitutively expressed due to homothallism, while others are activated only when oosporogenesis is enabled during growth on agar. However, factors unrelated to sexual development might be involved since liquid and surface (agar) growth vary in several ways, such as the relative degree of oxygenation. Asexual sporulation also only occurs on agar, although oospores outnumber asexual spores by a ratio of 4 to 1 in the isolate of *P. phaseoli* that was used. Nevertheless, it is suggested that the up-regulation of the 14 genes in *P. phaseoli* is related to oosporogenesis, since in *P. infestans* they are expressed similarly in liquid and agar non-mating cultures (Fig. 6B, right portion).

For the seven genes that amplified poorly during RT-PCR in *P. phaseoli*, the same primers functioned well in PCR against *P. infestans* cDNA. Therefore, either these genes are unexpressed in *P. phaseoli* or diverged in sequence. In BLASTX searches of GenBank, six of these genes lacked matches while Pi012453 belonged to a rapidly evolving class of genes, the Crinklers (Kamoun, 2006). Interestingly, it was shown previously that genes involved in sexual development in *Phytophthora*, as in other taxa, evolve more rapidly than others (Cvitanich et al., 2006).

4. Discussion

This work has expanded our understanding of sexual development in *Phytophthora*, increasing the number of genes known to be induced during mating by an order of magnitude compared to our previous study (Fabritius et al., 2002). Altogether, about 100 genes are induced >10-fold, of which one-third are induced >100-fold and have little expression in non-mating cultures. About 1600 genes, or 10% of the transcriptome, are induced >2-fold. By comparison, in *Schizosaccharomyces pombe* adding mating pheromones induces 175 genes >2-fold, and starving diploids to stimulate meiosis up-regulates 2000 genes (Mata et al., 2002). Our data likely underestimate the extent of induction in *P. infestans* matings, however. As gametangia typically account for less than 10% of tissue mass within mating cultures, our measurements were affected by the dilution of sexual structures by non-mating hyphae. Macroscopic sexual entities that might be purified for precise analyses of gene expression, analogous to the perithecia of ascomycetes, are not formed by *Phytophthora*.

Major transcriptional changes were detected in the *P. infestans* mating culture after 10 days, but genes up-regulated at the 3-day timepoint were not detected. This was disappointing as it was hoped that hormone-induced genes might be identified at that stage. The results could be explained if physical contact between A1 and A2 is a prerequisite for gene induction. Alternatively, changes may have been below the detection limit since only a fraction of tissue may have been hormone-stimulated, or due to the general preponderance of non-mating hyphae within mating cultures. The latter may also explain why few down-regulated genes were identified, other than a small number seemingly related to the aging of cultures.

Related to the aging issue is whether 10-day asexually sporulating cultures or 3-day non-sporulating cultures were the best controls for matings. The lack of an ideal control is not unique to *Phytophthora*. For example, nutrient limitation is commonly used to complete the sexual cycle in yeasts, which complicates the interpretation of meiosis-associated expression patterns (Mata et al., 2002; Schaber et al., 2006). Data from both the 3 and 10-day non-mating cultures of *P. infestans*, as well as the *PiCdc14*-silenced strains which like 10-day matings contain few asexual spores, were therefore useful since they indicated the specific involvement in mating of the 57 genes shown in the left panel of Fig. 4 ("M" class in Table 1).

The roles of the other 30 mating-induced genes, which were also up-regulated by asexual sporulation and/or *PiCdc14* silencing are less clear, since their expression patterns may relate to culture aging and not sexual development. However, arguments can be made for the importance of such genes in mating. For example, it would be quite surprising if some patterns of expression were not shared between the sexual and asexual sporogenesis pathways, since oospore germination involves the emergence of germ sporangia that are similar to asexual sporangia (Ribeiro, 1983). Also, studies of transgenic *P. infestans* in which the Pi004848 promoter ("M, NS, SP" class; Table 1) was fused to β-glucuronidase (GUS) showed that its expression occurs specifically in gametangia and asexual sporangia, and not in aged or starved hyphae (Cvitanich and Judelson, 2003); Pi004848 may help repress nuclear division to establish dormancy in both kinds of spores, based on the activity of its homologue against cyclin B in other organisms (Nakahata et al., 2003). That some mating-induced genes were expressed in the *PiCdc14*-silenced strains does not necessarily exclude them from roles in sexual development. The stage blocked by the *PiCdc14* defect is not well-defined, so genes activated early in sporulation could still be expressed (Ah Fong and Judelson, 2003).

Nearly all of the 87 genes were expressed in *P. phaseoli*, and some were induced in the oosporogenesis-conducive agar cultures compared to liquid cultures. Why oospores are absent in the latter is unknown, but could relate to differences in oxygen levels or the instability of hormone gradients in liquid. We also observe poor oosporogenesis in liquid matings of *P. infestans*, although others report different results (Akino et al., 2003). Nevertheless, the *P. phaseoli* data provide insight into the roles of the genes and differences between homothallism and heterothallism. The genetic basis of homothallism in oomycetes is unknown. It is reasonable to suggest that homothallics contain both A1 and A2 genes, since trisomy at the mating type locus leads

to homothallism in *P. infestans* (Mortimer et al., 1977), homothallic species make both mating hormones (Ko, 1988), and homothallics are found within clades of heterothallics in molecular phylogenies of *Phytophthora* (Cooke et al., 2000). Expression calls from the Affymetrix chips using *P. phaseoli* may be inaccurate, but about half of the 87 genes appear to be expressed higher in *P. phaseoli* than in non-mating *P. infestans*, in both liquid and agar. This suggests that part of the mating pathway is continually activated in *P. phaseoli*, either by mating hormones or other regulators such as the heterodimeric transcription factors that control mating in basidiomycetes (Brachmann et al., 2001). The 14 genes up-regulated in *P. phaseoli* by the oosporogenesis-conducive conditions may participate in late stages of development, possibly needing input from other factors such as the nutritional status of the culture (Leal et al., 1967).

Future studies of *P. infestans* and *P. phaseoli* transformants expressing promoter-GUS fusions should help illuminate the roles of the genes. This will indicate if they are expressed in male or female gametangia (or both), in hyphae within mating zones, or in structures related to asexual sporogenesis. Since well-synchronized mating methods do not exist, this will also reveal if the genes are expressed early or late in mating.

In the meantime, sequence information provides insight into the function of many of the mating-induced genes. Several appear to encode proteins with regulatory activities. These include zinc finger transcription factors (Pi015704, Pi014241, and Pi005054), protein kinases (Pi003811, Pi015297), and a protein phosphatase (Pi007072). Induction of a calcium-ATPase (Pi002983) is also of interest since calcium was shown to regulate oosporogenesis in *Lagenidium* and *Saprolegnia* (Fletcher, 1988; Kerwin and Washino, 1986a). It is also notable that several proteins participate in RNA metabolism or regulation, including the Puf RNA binding protein (Pi004848), two distinct mRNA decapping enzymes (Pi011975, Pi012846), and the *M24* RNAse identified in our previous study (Fabritius et al., 2002). These could be involved in the destabilization of specific mRNAs, or sequestering or recycling mRNA as the oospore enters dormancy. As oospores mature, their ribosomes disassemble so transcripts required for germination may need to be stored within other ribonucleoprotein complexes (Leary et al., 1974). Candidates for participants in nuclear division and meiosis were also identified, such as potential DNA repair and modification enzymes (Pi010145 and Pi011511), and a Cdc20 relative (Pi015306) which in other species regulates cell cycle transitions by directing cyclin ubiquination (Irniger, 2002).

Several of the mating-induced genes encode proteins with low-complexity amino acid regions or other features associated with wall and matrix proteins, and thus may encode part of the sexual structures such as the adhesive between antheridia and oogonia, or the thick protein–glucan oospore wall. For example, the Pi014092 protein contains repetitive glycine and tyrosine-rich domains, and oxidative cross-linking of the latter structure is commonly used to fortify cell walls (Cassab, 1998). Pi003858 encodes an alanine and lysine-rich protein that resembles a family thought to strengthen cells by hydrogen bonding to membranes and other macromolecules (Rajesh and Manickam, 2006). The Pi008890 protein is glycine and cysteine-rich, like many proteins found in extracellular matrices; its abundance of cysteine suggests a capacity to form extensive disulfide bridges and its glycine loops are often flanked by glutamines that are potential targets for transglutaminase-mediated crosslinking. Notably, mating-induced gene *M81* was shown to be a transglutaminase that might act on a protein like Pi008890 (Fabritius and Judelson, 2003).

Other genes appear to participate in various aspects of metabolism, especially lipid modification or transport. These include Pi003398 and Pi007954, which resemble β-ketoacyl reductase and lipoxygenase, respectively. These are of interest since oxidative lipid metabolism was shown necessary for antheridial induction, gametangial fusion, meiosis, and oospore maturation in *Lagenidium* (Kerwin and Washino, 1986b). The lipoxygenase could also be generating signaling molecules needed for mating, as in plants and animals (Feussner and Wasternack, 2002). Also related to lipid activities are several extracellular elicitin-like proteins (Pi000192, Pi000193, Pi002501, Pi012205), which are proposed to encode sterol- and lipid-transport proteins and modifying enzymes (Nespoulous et al., 1999; Osman et al., 2001). Their induction during mating is of interest since sterols are required for sexual development, but can not be synthesized by oomycetes (Ayers and Lumsden, 1975; Kerwin and Duddles, 1989). The ABC transporters encoded by Pi008242 and Pi013922 might be involved in efflux of the mating hormones, as in true fungi (Christensen et al., 1997).

As more is learned about the activities of these genes in mating, by studying those also induced during asexual sporulation a better understanding should emerge of the relationship between the two spore-forming pathways. Some inputs to the pathways are apparently conserved, since both are suppressed in liquid and require a period of culture aging, like the competence phenomenon described for many fungi (Dahlberg and Van Etten, 1982). On the other hand, some outputs from the pathways apparently diverge since asexual sporulation is suppressed in zones undergoing mating in *P. infestans* (Fabritius et al., 2002). A negative correlation between sexual and asexual sporulation is also described for other oomycetes, such as downy mildews (Bonde, 1982). Cross-talk between developmental pathways is also common in other taxa (Casas-Flores et al., 2006; Schaber et al., 2006; Swarup et al., 2002). This ensures that cells trigger the appropriate developmental response based on multiple data inputs, and allows efficiency in cellular design by having sensory or regulatory pathways serve multiple functions.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.fgb.2006.11.011.

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