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Elevated amino acid biosynthesis in *Phytophthora infestans* during appressorium formation and potato infection

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Abstract

Appressorium formation is believed to be an important event in establishing a successful interaction between the late blight pathogen, *Phytophthora infestans*, and its host plants potato and tomato. An understanding of molecular events occurring in appressorium development could suggest new strategies for controlling late blight. We used parallel studies of the transcriptome and proteome to identify genes and proteins that are up-regulated in germinating cysts developing appressoria. As a result, five distinct genes involved in amino acid biosynthesis were identified that show increased expression in germinating cysts with appressoria. These are a methionine synthase (*Pi-met1*), a ketol-acid reductoisomerase (*Pi-kari1*), a tryptophan synthase (*Pi-trp1*), an acetolactate synthase (*Pi-als1*), and a threonine synthase (*Pi-ts1*). Four of these *P. infestans* genes were also up-regulated, although to lower levels, during the early, biotrophic phase of the interaction in potato and all five were considerably up-regulated during the transition (48 hpi) to the necrotrophic phase of the interaction. Real-time RT-PCR revealed that expression of potato homologues of the amino acid biosynthesis genes increased during biotrophic and necrotrophic infection phases. Furthermore, we investigated levels of free amino acids in the pre-infection stages and found that in most cases there was a decrease in free amino acids in zoospores and germinating cysts, relative to sporangia, followed by a sharp increase in germinating cysts with appressoria. Amino acid biosynthesis would appear to be important for pathogenicity in *P. infestans*, providing a potential metabolic target for chemical control.

Keywords: Proteomics; SSH; Real-time RT-PCR; Oomycete; Late blight; Amino acid biosynthesis; Pathogenicity

1. Introduction

The oomycete pathogen *Phytophthora infestans* (Mont.) de Bary is responsible for potato late blight. It is best known as the causal agent of the Irish potato famine in the 1840s. Despite the use of major genes for resistance and chemical control measures, this disease still causes significant crop losses the world over, with an estimated cost, due to losses and control measures, of over US $5 billion per annum (Duncan, 1999). Recently, late blight destroyed more than 15% of the potato crop in Russia, the world's second largest potato producing nation (Schiermeier, 2001).

Although molecular processes underlying disease establishment and development are poorly understood, the infection cycle of *P. infestans* is well characterized (Birch and Whisson, 2001; van West and Vleeshouwers, 2004). An understanding of the earliest molecular events during infection is important to inform strategies for disease control and our studies are therefore focused principally around the appressorial stage of the interaction. The cell types that differentiate prior to infection of a host cell, germinated cysts and appressoria, can be

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produced in a synchronized fashion from in vitro grown mycelia, making *P. infestans* amenable to laboratory studies (Kramer et al., 1997; van West et al., 1998). We anticipate that these (pre-) infection structures are likely to be rich in molecules involved in establishing infection or in triggering disease resistance, since it is also during their development that plant defense responses are initiated (Vleeshouwers et al., 2000).

Phytophthora infestans grows as a diploid coenocytic mycelium in its vegetative state. Asexual sporangia are formed on sporangiophores that emerge from infected plant tissue, or sporulating hyphae on artificial media. Once sporangia detach and encounter liquid, either direct or indirect germination occurs. Direct germination is favored at higher temperatures and in the presence of nutrients. Indirect germination involves production of motile, biflagellate zoospores that are released by cleavage of the multinucleate sporangial cytoplasm in conditions typically below 12 °C and in the absence of nutrients. When zoospores are released they swim in search of a host and encyst in response to chemical, electrical, and physical cues (van West et al., 2002, 2003). Cysts subsequently elaborate a germ tube. Penetration of the host cell occurs when the tip of the germ tube differentiates into an appressorium and forms a penetration peg. Once the plant cuticle has been breached, an infection vesicle is produced in the epidermal cell. Hyphae grow into the mesophyll cell layers, both inter- and intra-cellularly, occasionally producing haustoria as a means of obtaining nutrients from host cells. After 1–2 days, *P. infestans* switches from biotrophic growth to a necrotrophic mode of growth. After 3–4 days, large areas of the leaf are fully colonized and sporulating hyphae emerge from stomata. Sporangiophores develop on the leaf underside and release sporangia, allowing aerial dissemination of the pathogen (van West et al., 1998; van West and Vleeshouwers, 2004).

During the initial stages of the infection cycle of fungal and oomycete plant pathogens, external nutrient sources are likely to be scarce and therefore storage compounds must be degraded by the pathogen to provide the energy and materials for the differentiation of structures required for host penetration. Genomic studies of the obligate pathogen *Blumeria graminis* have revealed a large number of proteases during these early stages, suggesting that protein degradation may provide a source of amino acids prior to host cell penetration (Thomas et al., 2001, 2002).

Little is known about physical or chemical signals and nutritional or metabolic requirements for progression of oomycetes through cell differentiation to successful plant colonization. Prior to host colonization, *P. infestans* is reliant on stored energy reserves such as lipids (Bimpong, 1975) and carbohydrates (Wang and Bartinicki-Garcia, 1980). The zoospore lacks a cell wall and maintains cell volume and turgor by means of a water expulsion vacuole (Mitchell and Hardham, 1999). A cell wall containing β-1-3 glucan is produced during encystment and it is believed that at this point the cell switches from total dependence on internal energy reserves to the uptake and metabolism of external carbon and nitrogen. Early studies of the metabolism of *P. palmivora* showed that the transition from zoospore to cyst was characterized by the appearance of disaccharides and a reduction in phosphate. Germinating cysts contained a large soluble amino acid pool with aminobutyrate and alanine increasing at the onset of germination (Grant et al., 1988).

We have recently demonstrated the effectiveness of proteomics for the analysis of secreted proteins from *P. infestans* (Torto et al., 2003), and asexual development of *Phytophthora palmivora* (Shepherd et al., 2003). Reproducible protein profiles were obtained for each of the mycelial, sporangial, cyst, and germinating cyst stages of the lifecycle of *P. palmivora* and approximately 1% of the proteins appeared to be specific for each of these stages. Here, we extend this approach to identify proteins in *P. infestans* that are specific or more abundant in germinating cysts developing appressoria in vitro, when compared with mycelium, sporangia, swimming zoospore, and cyst lifecycle stages. In a parallel analysis of the transcriptome, suppression subtraction hybridization (SSH) was used to identify transcripts up-regulated specifically during the germinating cysts with appressoria stage; SSH is a method for generating cDNA libraries enriched for differentially expressed genes that has been used for studying potato–*P. infestans* interactions (Birch et al., 1999, 2003). Employing both approaches, genes involved in amino acid biosynthesis were identified. Amino acid profiling and real-time quantitative RT-RCR from pre-infection cell types and during infection were thus used to provide a unique insight into the metabolism of *P. infestans* prior to and during the first 72 h of potato infection.

2. Materials and methods

2.1. Growth of P. infestans, potato plants, and plant inoculation

Phytophthora infestans strain 88069 (A1 mating type, race 1.3.4.7) was maintained on rye medium supplemented with 2% sucrose as described by (Caten and Jinks, 1968). Potato cultivar (cv.) Bintje, lacking *R* gene-mediated resistance and with a field resistance rating of only 2 on the 1–9 scale (NIAB, 1999) was chosen to study late blight disease development. *P. infestans* strain 88069 used for plant inoculation gave a clear compatible interaction with cv. Bintje. To allow for

variation in infection, five non-flowering glasshouse-grown plants per time-point were spray inoculated with a suspension of zoospores (5×10^4 per ml) to ensure a massive and synchronous infection as described by Stewart et al. (1983). Samples of mature leaves were taken before inoculation (B0), and at 12 h post-inoculation (hpi) (B12), 24 hpi (B24), 33 hpi (B33), 48 hpi (B48), and 72 hpi (B72), and leaf material from each time point combined prior to RNA extraction. These time points correspond to the biotrophic interaction (B12–B33), a transition phase between biotrophy and necrotrophy (B48), and hyphal ramification and sporulation in host tissue during the necrotrophic phase (B72). Collected leaf samples were frozen in liquid nitrogen and then stored at −70 °C prior to RNA extraction. Additional plants (inoculated and uninoculated) kept for 5 days after inoculation showed high levels of infection and no infection, respectively (results not shown).

2.2. Preparation of samples for protein and RNA extraction

Non-sporulating mycelium of *P. infestans* was grown in still submerged culture in rye broth for 4 days before harvesting, freezing, and storage at −70 °C prior to RNA extraction. Mycelium for protein isolation was grown for 6 days in liquid rye broth, culture filtrate was removed using a vacuum manifold and the resulting mycelial tissue was stored at −70 °C until use. In vitro formed sporangia were obtained from twelve 11-day-old agar cultures. These were flooded with cold water and the sporangia scraped from the surface of the plates immediately. The material was filtered through a 50 μm mesh to remove mycelial fragments and the sporangia were collected by centrifugation. Sporangia were also washed off infected potato leaves 7 dpi. Samples of zoospores were derived from one hundred and twenty 11-day-old agar cultures. These were flooded with cold water and incubated at 4 °C, for 2–3 h. Zoospore material was carefully decanted from the surface of the plates and filtered to remove mycelial and sporangial fragments, from which the cells could be collected using gentle centrifugation. Cysts were induced by continuously shaking a zoospore suspension for 2 min, followed by centrifugation. Newly formed cysts germinated at room temperature after incubation for 1–2 h. To produce appressoria, encystment of zoospores was induced and the resulting suspension was poured immediately into empty Petri dishes and left to germinate at 11 °C. About 50% of the germinating cysts had produced an appressorium after 16–18 h and these were harvested from the Petri dish surfaces with a cell scraper. The suspension was gently centrifuged, which resulted in a pellet containing the germinated cysts with appressorial structures.

2.3. Extraction of proteins, 2D electrophoresis and protein identification

Phytophthora infestans samples were ground to a fine powder in liquid nitrogen and lyophilized by freeze-drying overnight. A 0.3 ml aliquot of 2D lysis buffer (7.5 M urea, 2.5 M thiourea, 1.25 mM EDTA, pH 8.0, 625 mM DTT, 250 mM Tris–HCl, pH 10.8, 20% w/v Chaps, 50% v/v glycerol, and 10% v/v carrier ampholytes (Bio-Lyte pH 4–6)) was added to each sample. Samples were placed on ice for 2 h and then centrifuged at 10,000*g* for 5 min in a bench top Eppendorf 5804R centrifuge. After centrifugation, the supernatant containing solubilized proteins was collected and stored at −70 °C. Samples were firstly run on pre-cast one dimensional (1D) Novex NuPAGE 12% Bis-Tris mini-gels (Invitrogen), according to the manufacturer's recommendations, to estimate the quantity and quality of protein material prior to two dimensional (2D) electrophoresis. Approximately 50 μg protein was separated in the first dimension by isoelectric focusing of 7 cm Immobiline dry polyacrylamide gel strips with an immobilized pH 4–7 gradient (Amersham Biosciences) using an IPGphor (Amersham Biosciences). Strips were focused for a total of 8000 V/h. Proteins were separated in the second dimension on Novex NuPAGE 12% Bis-Tris mini-gels (Invitrogen) according to the manufacturer's recommendations. Gels were stained using the colloidal Coomassie based GelCode Blue Stain Reagent (Pierce) according to the manufacturer's protocol. Protein spots were excised and digested in-gel with sequencing grade modified trypsin (Promega, Madison, WI) according to Anderson et al. (1991) and Schevchenko et al. (1996). Mass spectra were acquired on a PerSeptive Biosystems Voyager-DE STR MALDI-TOF mass spectrometer. Positive-ion MALDI mass spectra were obtained in the reflected-ion mode and were calibrated internally using observed trypsin autolytic digestion product ions. Peptide fingerprints were obtained from MALDI-TOF analysis and putative protein identities were acquired after using MS-Fit (http://prospector.ucsf.edu) to compare the tryptic fragments obtained from MALDI-TOF MS data with hypothetical tryptic digests of EST clones from both the public *Phytophthora* functional genomics database at http://www.pfgd.org and a private *Phytophthora* EST database maintained by Syngenta.

2.4. Extraction and analysis of free amino acids

Samples of mycelium, sporangia, zoospores, germinating cysts, germinated cysts with appressoria, uninfected potato (cv. Bintje) leaves, and infected potato leaves from 12, 24, 32, 48, and 72 hpi were ground to a fine powder in liquid nitrogen and lyophilized overnight in a freeze drier. Amino acids were precipitated using 100% ethanol and centrifuged at 6000*g* for 10 min.

Norleucine (2.5 mM) was added to each sample as an internal standard. An aliquot of supernatant was dried under vacuum, re-dissolved in 0.1 M hydrochloric acid and placed into a 10 kDa molecular weight cut off filter. Free amino acids were automatically derivatized using PITC under alkaline conditions to generate phenylthiocarbomyl (PTC) amino acids. These were separated using a 130 Å separation system employing a C18 reverse phase narrow bore cartridge 200 mm × 2.1 mm at 38 °C on a PE Applied Biosystems 420 Amino Acid Analyzer. PTC amino acids were separated, identified, and quantified by a UV absorbance detector at 254 nm and a Spectra Physics WINner on a Windows Integration System at an attenuance of 32 mV. Samples were run in triplicate and for each sample standard errors of the means were obtained to allow comparisons to be made between samples.

2.5. RNA extraction and cDNA synthesis

Total RNA was extracted from frozen samples using a Qiagen RNeasy Plant mini kit, following the protocol supplied by the manufacturer. Integrity of the RNA was tested by agarose gel electrophoresis. RNA yield was measured using a NanoDrop Micro Photometer (NanoDrop Technologies). Prior to cDNA synthesis, RNA samples were DNase I treated using the Ambion DNA-free kit, following the manufacturer's protocol. mRNA was purified from total RNA extracted from inoculated leaf samples using Dynabeads oligo$(dT)_{25}$ (Dynal Biotech ASA). First strand cDNA was synthesized from 20 μg total RNA or 150 ng mRNA by oligo(dT) priming using the first strand cDNA synthesis kit (Amersham–Pharmacia Biotech), following the manufacturer's protocol.

2.6. Suppression subtractive hybridization

Suppression subtractive hybridization (SSH), using Clontech's PCR-Select cDNA subtraction kit, was performed to generate a cDNA library enriched for sequences specifically expressed during appressoria formation. cDNA generated from *P. infestans* germinating cysts with appressoria was used as a tester. cDNA generated from *P. infestans* cysts was used as a driver. A 1:200 ratio of tester:driver was used in the subtraction. Following SSH, amplification products from 100 bp to over 600 bp were generated, purified using the Promega Wizard PCR Preps kit, directly cloned using the Promega pGEM-T Easy Vector System I kit, and transformed into electromax DH10B cells (Invitrogen Life Technologies) by electroporation. Recombinant transformants were transferred into 384-well plates (AB Gene) containing 50 μl sterile freezing medium (LB-medium, 10% (v/v) 10× freezing medium, 1.1 mM $MgSO_4 \cdot 7H_2O$, 50 μg/ml ampicillin) and grown for 24 h at 37 °C before storing at −70 °C. Plasmid DNA for sequence determination was isolated from bacterial clones by alkaline lysis (Sambrook et al., 1989). Sequences of cloned DNA were determined using SP6 and T7 promoter primers, and BigDye v3.1 sequencing chemistry (Applied Biosystems, UK).

2.7. SYBR green real-time RT-PCR assays

Primer pairs (Table 2) were designed and their amplification efficiency was optimized as described in Avrova et al. (2003). cDNA from uninoculated leaves of potato cv. Bintje was used as a template for optimization of all potato primer pairs. Primer concentrations giving the lowest threshold cycle (C_t) value were selected for further analysis. Previously designed and optimized primers for the *P. infestans* actin A (*actA*) gene, as a constitutively expressed endogenous control (Avrova et al., 2003), primers for *P. infestans* elongation factor 2 (*ef2*), also constitutively expressed (Torto et al., 2002), and the *P. infestans ipiO1* gene, known to be up-regulated in invading hyphae during infection (van West et al., 1998), were also used. For SYBR green real-time RT-PCR assays, all calculations and statistical analyses were carried out as described by Avrova et al. (2003). Amplification efficiency of all genes of interest and the endogenous controls were shown to be equivalent, allowing the use of the comparative C_t method for relative quantification of *P. infestans* gene expression levels in sporangia, zoospores, germinating cysts, germinating cysts with appressoria and in planta in relation to their expression in mycelium, and potato gene expression levels at the same time points post-inoculation of potato cv. Bintje with *P. infestans* in relation to their expression in uninoculated Bintje. β-Tubulin was used as an endogenous control for relative quantification of potato gene expression.

3. Results

3.1. Identification of amino acid biosynthesis genes in germinated cysts with appressoria

Soluble proteins were extracted from *P. infestans* mycelium, sporangia, zoospores, germinating cysts, and germinating cysts with appressoria. Samples were run on separate 2D gels, each in triplicate, and were compared to zoospores and mycelium as controls; spots appearing specific to the germinating cyst with appressoria stage and several common spots, present in all stages, were targeted for identification by peptide fingerprinting. Fig. 1 shows 2D gels of protein samples isolated from mycelium, zoospores, and germinated cysts with appressoria. Indicated are four spots corresponding to amino acid biosynthesis proteins. Spot S1 and S2 were found to

be more abundant in the germinating cysts with appressoria protein samples compared to mycelial and zoospore samples based on visual observations (three independent replicates). Protein levels of spots S3 and S4 were always very low in abundance and increases in protein volume could not be observed from the two-dimensional electrophoresis experiments. Table 1 shows putative identities of the proteins highlighted in Fig. 1. Once EST sequences matching peptide fingerprints were identified, BLASTX searches of these against public databases were performed to predict protein identity and comparisons were made to the SSH data also shown in Table 1.

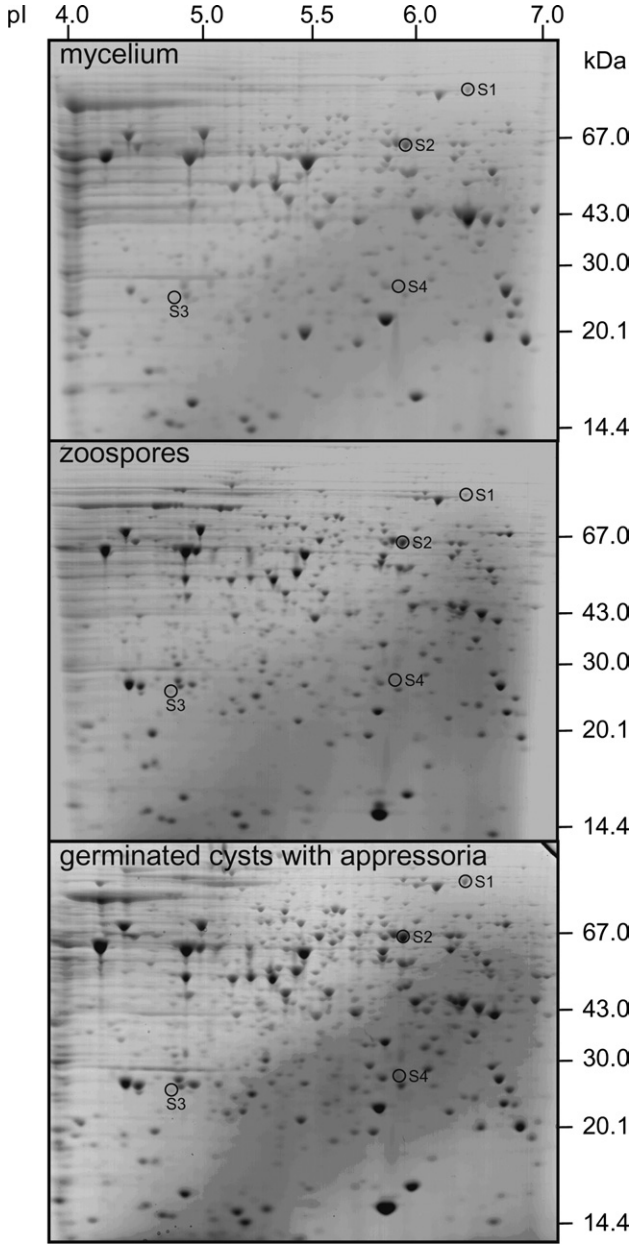

Fig. 1. Two-dimensional polyacrylamide gels containing proteins from mycelium, zoospores, and germinating cysts with appressoria. Spots that correspond to amino acid biosynthetic enzymes: *Pi-met1* (S1), *Pi-kari1* (S2), *Pi-als1* (S3), and *Pi-trp* (S4), are indicated. Equal amounts of protein have been loaded in each gel. The spots highlighted were excised from the germinating cyst with appressoria gel and analyzed by tryptic digestion and MALDI-TOF MS.

Spot S1 (Fig. 1) showed similarities to EST clones predicted to encode a methionine synthase (MET1) protein, cobalamin-independent 5-methyltetrahydropteroyltriglutamate-homocysteinemethyltransferase (EC 2.1.1.14), which catalyzes the formation of methionine from homocysteine. Spot S2 showed similarity to ketol-acid reductoisomerase (EC 1.1.1.86), an enzyme that catalyzes the formation of hydroxy-2-methyl-3-oxobutanoate using 2,3-dihydroxy-3-methylbutanoate as a substrate. Spot S3 was similar to acetolactate synthase (EC 4.1.3.18), which catalyzes the formation of acetolactate from pyruvate. The S2 and S3 proteins are both part of the major pathway of branched chain amino acid biosynthesis, which results in the production of valine, isoleucine, and leucine. Spot S4 has homology to tryptophan synthase (EC 4.2.1.20), which catalyzes the conversion of indole to tryptophan.

Following SSH to generate a cDNA library enriched for sequences up-regulated in the germinating cyst with appressoria stage, 320 clones were sequenced and three of these showed similarities to amino acid biosynthesis genes (Table 1). One of these (Pia1) exhibited similarity to the *Pi-met1* gene, also identified using proteomics. The other two identical clones, called Pia2, were similar to a threonine synthase from fission yeast. This enzyme catalyzes the last step in the formation of threonine; the conversion of phosphohomoserine to threonine. All five sequences have been submitted to GenBank (Accession Nos.: *Pi-met1*, AY702345; *Pi-kari1*, AY702346; *Pi-als1*, AY702347; *Pi-trp1*, AY702348; and *Pi-ts1*, AY702349).

3.2. Real-time RT-PCR assays of amino acid biosynthesis genes in pre-infection stages

To determine whether the five genes identified using proteomics and SSH are up-regulated during appressorium formation, primer pairs (Table 2) were designed to anneal to the five transcripts from *P. infestans* for real-time RT-PCR analysis. Template cDNA was derived from non-sporulating mycelium grown in rye broth, in vitro produced sporangia, zoospores, germinating cysts, and germinating cysts with appressoria. The *actA* gene from *P. infestans* was used as a constitutively expressed endogenous control and the expression of each gene in mycelium was determined relative to *actA* expression. Expression of all genes in different samples was compared to the level of their expression in a calibrator sample, which was cDNA from mycelium. The expression of each gene in the mycelium cDNA sample was assigned the value of 1.0.

The *Pi-als1*, *Pi-kari1*, *Pi-met1*, and *Pi-ts1* genes were strongly up-regulated in germinating cysts and germi-

Table 1
Identification of protein spots S1–S4 and SSH clones Pia1–Pia2

Protein spot or SSH clone	Gene name	EST match in SPC database	Mowse score	Number and (%) masses matched	Peptide masses matched	Theoretical peptide masses of EST matched	Percentage of protein matched	BLASTX of EST	BLASTX score
S1	*Pi-met1*	CON_005_05820	1.17e+004	7/49 (14)	1232.6725 1334.6869 1345.7487 1462.7963 1626.7974 1804.9529 2031.9396	1232.5509 1334.6891 1345.6864 1462.7840 1626.7916 1804.9420 2031.9677	28	NP_660391 5-(methionine synthase) [*Buchnera aphidicola* str. Sg (*Schizaphis graminum*)]	$5e^{-91}$
S2	*Pi-kari1*	CON_011_07040	2.91e+007	9/37 (24)	1003.5249 1173.6007 1544.7369 1770.0274 1926.0929 2004.0014 2009.9564 2524.4336 2566.4842	1003.5114 1173.5363 1544.6837 1769.9736 1926.0747 2003.9602 2009.9126 2524.3200 2566.2394	29	ILV5_SPIOL Ketol-acid reductoisomerase [*Spinacia oleracea*]	$5e^{-96}$
S3	*Pi-als*1	CON_015_07273	1.61e+005	5/54 (9)	1192.6200 1753.8145 2072.9414 2078.1556 2547.3879	1192.6226 1753.7768 2073.1391 2078.2014 2547.3870	35	Q57625 Acetolactate synthase small subunit [*Methanococcus jannaschli*]	$8e^{-30}$
S4	*Pi-trp1*	CON_002_02446	9.18e+003	7/43 (16)	1313.7466 1542.9087 1769.9059 1883.9888 2322.1719 2416.2491 2468.2456	1313.7429 1542.8855 1769.9325 1883.9867 2322.1916 2416.2585 2468.2421	50	NC001139 Tryptophan synthase [*Saccharomyces cerevisae*]	$8e^{-53}$
Pia1 (194 bp)	*Pi-met*1	CON_003_04588, CON_005_05820						NP_660391 5- (methionine synthase) [*Buchnera aphidicola* str. Sg (*Schizaphis graminum*)]	$3e^{-40}$ $5e^{-91}$
Pia2 (189 bp)	*Pi-ts1*	CON_001_20553						T42748 probable threonine synthase (EC 4.2.3.1)—fission yeast (*Schizosaccharomyces pombe*)	$3e^{-23}$

Table 2
Oligonucleotide primers used in real-time RT-PCR expression analysis and amplicon sizes

Gene name, Accession no.	Amplicon size (bp)	Real-time RT-PCR forward and reverse primers
Phytophthora infestans		
Pi-met1, CON-005-05820	70	5′-AACCTGCCAAGAAGGACGAGTAC-3′ 5′-TTGGCCACAGCAGTTGACA-3′
Pi-kari1, CON-010-383425	67	5′-TCAATTCGAGTGTGGCTATCCA-3′ 5′-CTCCAGCCCAAAGCAATCTC-3′
Pi-als1, CON-015-07273	64	5′-TGGTCGCATTGAGGCATTT-3′ 5′-ACTGCGGTGGATCTCTGTGAT-3′
Pi-trp1, CON-002-02446	83	5′-ACGCAGCTAAGCACGGACTT-3′ 5′-CACCGAGTCGATGAGCTTCAT-3′
Pi-ts1, CON_001_20553	83	5′-CGTAGAAACCGGCAAGGATGT-3′ 5′-TGCTCACGACGAGGGTGTTAG-3′
IpiO1, gi:397690	84	5′- TGCGTTCGCTC CTGTTGA-3′ 5′-CGGTGTTGAGATTGGATGAAAC-3′
Actin A, gi:169301	69	5′-CATCAAGGAGAAGCTGACGTACA-3′ 5′-GACGACTCGGCGGCAG-3′
Elongation factor 2	73	5′- TCGTGTGGTGTACGCTTGTCA-3′ 5′-CCTGGATGTCGGCAAGGA-3′
Solanum tuberosum		
Methionine synthase, gi:8439544	84	5′-CAGCCCTGCAAAACATGGT-3′ 5′-CTCCAGTGGCAGATTCAACTCA-3′
Ketol-acid reductoisomerase, gi:13607458	93	5′-TTGCGGCTCGCATGGT-3′ 5′-GGGTTACTTTCTCCTTCTTAAAAACG-3′
Acetolactate synthase, gi:11529360	87	5′-TTTCTCTTCAAATGTTGGGTATGC-3′ 5′-TCACCCCAAATGCAAGCAA-3′
Tryptophan synthase α1-like protein, gi:9250760	78	5′-ACTCTGATCCGTTGGCTGATG-3′ 5′-TGGCAAAGTTGGTTCCTTTACTC-3′
Threonine synthase, gi:8439546	99	5′-GGCATTGACTGCACTGTTCAA-3′ 5′-GAGTAAACTTCAATCCATGAGCAGTAC-3′
Putative-tubulin-β1-chain, gi:13607341	66	5′-CAAATGTGGGATGCCAAGAA-3′ 5′-AGCTGTCAGGTAACGTCCATGA-3′

nating cysts with appressoria. *Pi-trp1* was also strongly up-regulated at these stages but showed a similar level of expression in zoospores (Fig. 2).

The *P. infestans ipiO1* gene, previously shown to be up-regulated in invading hyphae during infection (van West et al., 1998) was highly up-regulated in germinating cysts and germinating cysts with appressoria (Fig. 2). Constitutively expressed *P. infestans* elongation factor 2 (*ef2*) showed levels similar to those of *actA* in all pre-infection stages, supporting the use of *actA* as an endogenous control gene for comparing relative gene expression levels (data not shown).

3.3. Real-time RT-PCR assays of amino acid biosynthesis genes during infection

To determine whether the amino acid biosynthesis genes were also up-regulated during infection, real-time RT-PCR was also performed using cDNAs from uninfected potato cv. Bintje, and infected Bintje (12, 24, 33, 48, and 72 hpi). Expression of *P. infestans* genes was again compared to that of *actA* and *ipiO1*. Constitutively expressed *P. infestans ef2* again showed levels similar to those of *actA* in all the samples tested (results not shown).

As expected, *ipiO1* was highly up-regulated early in the interaction (12–48 hpi) with its expression level decreasing by 72 hpi (Fig. 3). In addition, all of the *P.infestans* sequences tested were also up-regulated by the end of the biotrophic phase of the interaction (48 hpi), with *Pi-als1*, *Pi-ts1*, *Pi met1*, and *Pi-trp1* expression levels increasing as early as 12 hpi (Fig. 3). As was the case for *ipiO1*, the expression of all five *P. infestans* amino acid biosynthesis genes was down-regulated by 72 hpi.

It was noted that all the amino acid biosynthesis genes tested showed higher relative levels of expression in germinated cysts with appressoria (Fig. 2), than in the early, biotrophic stages (12–33 hpi) of infection (Fig. 3). This apparent down-regulation of the biosynthesis genes following host penetration could be due to a ready access of amino acids from the plant. We thus investigated the expression of the potato homologues of the amino acid biosynthesis genes during infection. The primer pairs used are shown in Table 2. Expression of the plant genes was normalized using a potato β-tubulin gene and was compared to expression in uninfected Bintje, which was assigned the value of 1.0.

The potato genes encoding acetolactone synthase, ketol-acid reductoisomerase and threonine synthase were up-regulated throughout the biotrophic phase (12–

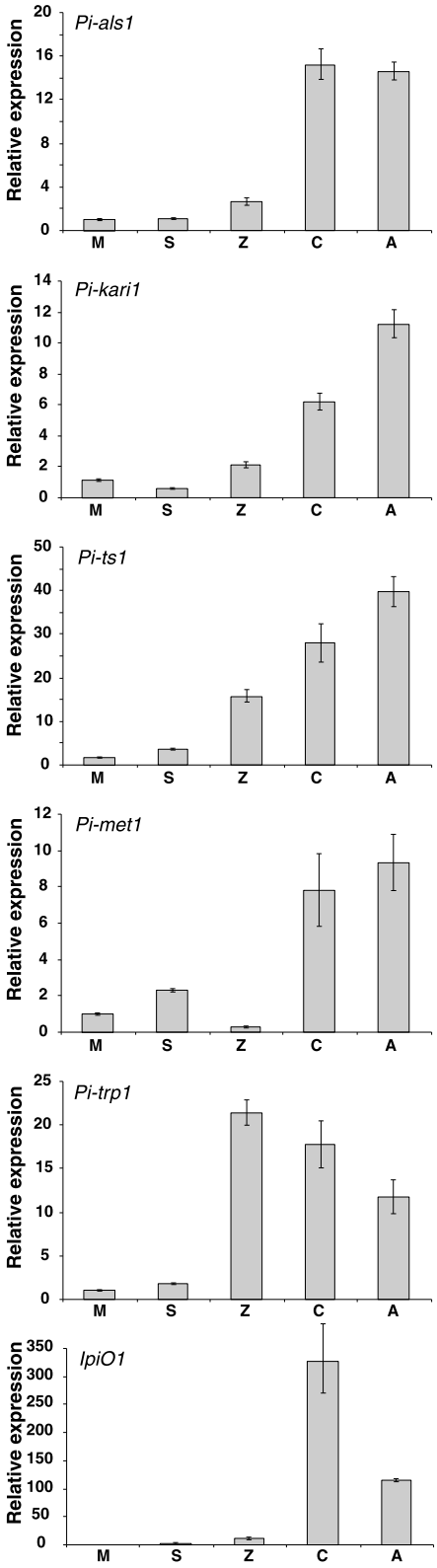

Fig. 2. Real-time RT-PCR expression profiles of *P. infestans* amino acid biosynthesis genes and *ipiO1* in pre-infection stages (sporangia, S; zoospores, Z; germinating cysts, C; and germinating cysts with appressoria, A) relative to their expression in vegetative non-sporulating mycelium (M).

33 hpi). All five of the potato sequences were up-regulated at 48–72 hpi relative to uninfected tissue (Fig. 3). Repeated amplifications, on independent occasions and with different starting RNA and cDNA samples, resulted in similar expression profiles for all transcripts studied in this work.

3.4. Free amino acid analysis

Several *P. infestans* amino acid biosynthesis genes were shown to be up-regulated at the transcriptional and translational levels during the development of appressoria and other pre-infection cell types and during the later stages of infection. We therefore investigated the abundance of free amino acids in each of the pre-infection stages of the lifecycle, and during the first 72 h of infection, at the same time points as real-time RT-PCR analyses were conducted. Table 3 shows levels of free amino acids present in both sporulating and non-sporulating mycelium, sporangia formed either in vitro or in planta, zoospores, germinating cysts, and germinating cysts with appressoria.

The levels of many of the amino acids appeared to have increased in the germinating cyst with appressoria stage, compared to the preceding pre-infection stages. In particular, glutamine, alanine, and proline were highly elevated and together accounted for 59% of the total free amino acids present in germinating cysts with appressoria. In contrast, methionine showed a statistically significant decrease in germinating cysts with appressoria where it can no longer be detected. However, there were no significant changes in the levels of aspartic acid, glutamic acid, histidine, ornithine, tryptophan or phenylalanine, between germinating cysts and germinating cysts with appressoria. Our observations suggested that approximately 50% of germinating cysts had produced an appressorium after 16–18 h, and therefore transferred their cytoplasm from the cyst to the appressorium, whilst approximately 50% of cysts had produced a germ tube and did not differentiate further. With this consideration, it is probable that levels of most free amino acids are higher in the appressorium itself.

We also investigated amino acid levels in sporangia grown in vitro and sporangia isolated from infected leaves. In almost all cases, except for cysteine, amino acid levels were lower in sporangia harvested from infected leaves. Furthermore, we investigated amino acid levels between young non-sporulating mycelium and sporulating mycelium and found that levels of most amino acids were significantly higher in young, non-sporulating mycelium. The exceptions were proline and tryptophan, which were more abundant in sporulating mycelium.

Table 4 shows free amino acids present in uninfected potato cv. Bintje, and infected Bintje (12, 24, 33, 48, and 72 hpi). Of the 23 amino acids tested, all were detected

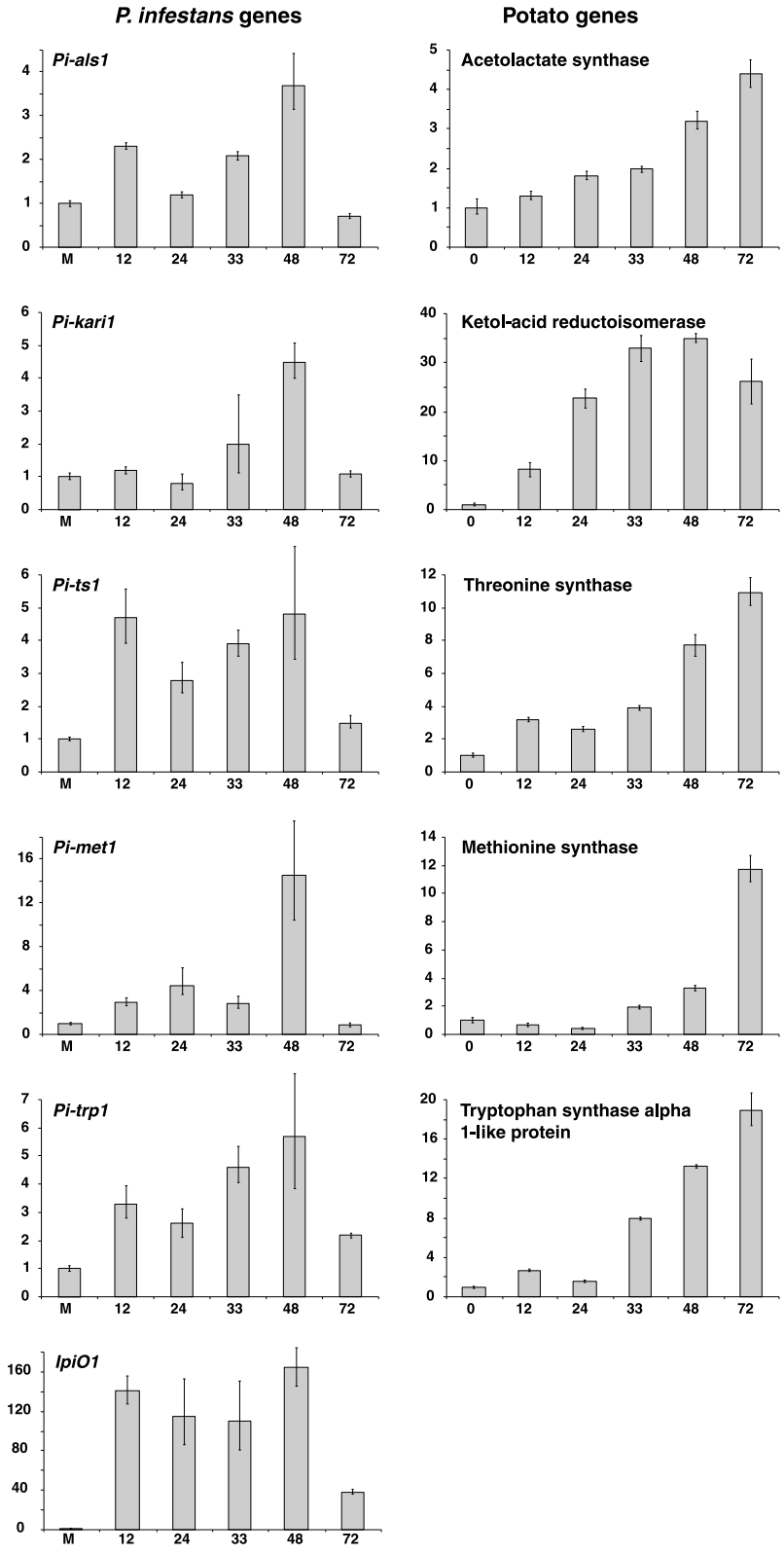

Fig. 3. Real-time RT-PCR expression profiles of *P. infestans ipiO1*, and *P. infestans* and potato amino acid biosynthesis genes in uninfected (B0) potato cv. Bintje and 12 (B12), 24 (B24), 33 (B33), 48 (B48), and 72 (B72) hours post-inoculation with a compatible race of *P. infestans*.

except for hydroxyproline, methionine, and cysteine. Homocysteine was detected only at very low levels. Ornithine could not be determined in this test and is most likely to be co-precipitating with tryptophan. Therefore, the levels recorded for tryptophan may be higher than the actual values present within the leaf. The levels of all

Table 3
Free amino acids present in *P. infestans* tissue from pre-infection stages of the life cycle

Amino acid	Non-sporulating mycelia	Sporulating mycelia	Sporangia washed from leaves	In vitro grown sporangia	Zoospores	Germinating cysts	Germinating cysts with appressoria
Asp	180.61 ± 7.60	32.58 ± 9.45	37.20 ± 1.60	167.07 ± 35.89	23.17 ± 12.18	214.30 ± 24.68	191.65 ± 19.39
Hyp	—[a]	38.83 ± 3.45	31.55 ± 4.82	328.70 ± 78.84	11.33 ± 5.70	—[a]	—[a]
Glu	1165.52 ± 40.41	272.68 ± 37.50	1378.70 ± 93.81	5885.09 ± 789.34	816.17 ± 90.87	2529.85 ± 322.67	2199.65 ± 333.25
Asn	307.11 ± 9.82	107.45 ± 16.70	127.22 ± 9.60	442.64 ± 57.23	33.23 ± 13.28	133.23 ± 23.50	334.40 ± 66.62
Ser	187.45 ± 7.33	103.42 ± 12.43	95.31 ± 9.20	394.75 ± 84.65	50.17 ± 6.81	123.68 ± 10.00	267.68 ± 34.30
Gln	1692.06 ± 52.62	517.77 ± 51.94	255.90 ± 30.21	4272.89 ± 585.42	426.27 ± 65.26	1810.68 ± 196.48	5930.35 ± 862.52
Gly	120.94 ± 4.50	107.87 ± 11.51	203.8 ± 13.61	684.87 ± 132.27	58.87 ± 6.24	210.98 ± 27.26	345.88 ± 21.53
His	216.28 ± 7.51	236.38 ± 23.82	—[a]	386.34 ± 80.47	149.30 ± 22.78	263.93 ± 35.57	286.35 ± 98.79
Tau	87.36 ± 3.58	16.92 ± 5.64	—[a]	64.22 ± 34.94	12.33 ± 6.49	—[a]	—[a]
Arg	1069.03 ± 33.10	167.63 ± 21.26	235.40 ± 11.12	495.68 ± 87.30	136.63 ± 14.77	535.90 ± 67.50	773.10 ± 95.61
Thr	183.76 ± 7.02	147.93 ± 17.57	85.71 ± 10.06	540.99 ± 110.67	69.90 ± 1.90	192.60 ± 32.81	352.40 ± 37.03
Ala	1277.62 ± 31.55	452.23 ± 33.78	546.25 ± 37.12	3275.87 ± 694.78	285.63 ± 12.47	1536.08 ± 209.30	3076.10 ± 333.62
Pro	878.91 ± 24.71	1234.37 ± 215.39	4323.01 ± 403.12	6206.88 ± 2249.34	941.93 ± 25.29	1639.08 ± 499.62	3397.68 ± 702.11
Tyr	91.83 ± 2.80	73.40 ± 21.55	52.88 ± 6.02	430.61 ± 125.30	28.33 ± 14.52	131.58 ± 59.77	599.40 ± 56.38
Val	249.64 ± 3.71	144.42 ± 12.68	75.01 ± 12.14	524.80 ± 112.90	74.00 ± 37.04	170.48 ± 37.96	956.08 ± 119.93
Met	43.11 ± 5.12	41.90 ± 13.75	224.92 ± 113.16	468.15 ± 219.66	88.67 ± 46.45	21.25 ± 24.54	—[a]
Cys	453.55 ± 139.11	—[a]	279.01 ± 46.41	96.91 ± 61.06	—[a]	—[a]	—[a]
ile	27.76 ± 14.0	50.68 ± 9.89	79.82 ± 45.62	273.30 ± 62.90	50.67 ± 39.73	63.00 ± 43.15	201.85 ± 20.77
Leu	91.55 ± 4.60	125.57 ± 23.12	61.90 ± 19.92	467.65 ± 124.08	81.67 ± 40.84	215.13 ± 59.30	722.73 ± 89.82
Phe	120.03 ± 10.44	81.57 ± 18.94	25.82 ± 2.91	1371.29 ± 1246.93	37.67 ± 19.01	113.78 ± 39.50	125.00 ± 16.18
Orn	602.74 ± 20.91	123.00 ± 4.62	44.31 ± 3.62	nd	59.00 ± 3.27	708.50 ± 178.40	731.00[b]
Trp	164.86 ± 8.40	349.12 ± 202.17	130.92 ± 21.10	566.38 ± 454.97	234.03 ± 23.33	120.18 ± 81.38	199.30 ± 75.19
Lys	1295.62 ± 48.90	116.18 ± 20.12	49.20 ± 3.02	147.10 ± 19.06	38.60 ± 2.39	128.25 ± 14.39	289.85 ± 42.06

Results presented are mean values from three experiments each containing three replicates ± standard error of the means.
It was not possible to determine ornithine levels in sporangial samples.
[a] Below detection limit.
[b] Data obtained from one measurement.

Table 4
Free amino acids present in uninfected and infected potato

Amino acid	Uninfected Bintje	Bintje 12 hpi	Bintje 24 hpi	Bintje 33 hpi	Bintje 48 hpi	Bintje 72 hpi
Asp	1598.93 ± 117.26	3992.70 ± 160.53	4282.23 ± 232.44	2936.67 ± 467.98	2602.37 ± 188.80	1714.89 ± 470.00
Hyp	—[a]	—[a]	—[a]	—[a]	—[a]	—[a]
Glu	2014.87 ± 149.95	2314.77 ± 65.47	2841.37 ± 130.77	3066.53 ± 495.09	2604.97 ± 100.22	1997.15 ± 509.56
Asn	567.63 ± 49.05	2438.97 ± 59.00	4308.77 ± 187.84	3288.87 ± 531.53	2632.00 ± 83.59	1651.87 ± 270.21
Ser	156.30 ± 6.38	225.97 ± 44.28	352.90 ± 16.69	1457.87 ± 1196.42	277.17 ± 23.32	618.78 ± 20.96
Gln	122.67 ± 6.85	2221.83 ± 74.07	3756.67 ± 238.57	2266.87 ± 370.67	1202.40 ± 81.73	898.25 ± 85.90
Gly	15.03 ± 0.66	53.97 ± 7.30	108.43 ± 6.17	83.30 ± 12.40	68.30 ± 1.36	40.31 ± 5.15
His	10.90 ± 5.47	19.83 ± 10.23	51.37 ± 3.52	46.37 ± 9.47	56.90 ± 3.22	30.72 ± 12.73
Tau	—[a]	7.17 ± 7.17	37.03 ± 4.85	33.77 ± 7.06	—[a]	8.17[b]
Arg	5.90 ± 5.90	66.47 ± 2.52	294.97 ± 19.54	171.00 ± 32.83	85.17 ± 5.43	65.55 ± 11.48
Thr	48.30 ± 3.75	80.70 ± 2.69	136.43 ± 7.59	111.27 ± 20.01	105.40 ± 5.23	63.11 ± 21.24
Ala	73.20 ± 2.19	176.17 ± 6.04	224.03 ± 12.02	204.27 ± 33.56	276.57 ± 13.94	130.53 ± 47.13
Pro	89.97 ± 3.18	132.67 ± 2.14	101.10 ± 3.38	76.37 ± 11.37	85.47 ± 11.44	54.25 ± 18.59
Tyr	15.60 ± 7.86	73.10 ± 2.18	215.50 ± 10.84	191.37 ± 34.03	193.07 ± 3.79	95.14 ± 30.72
Val	26.00 ± 1.25	161.67 ± 3.77	326.73 ± 14.90	210.003 ± 33.44	153.37 ± 7.13	89.09 ± 14.20
Met	—[a]	—[a]	—[a]		—[a]	—[a]
Cys	—[a]	—[a]	—[a]		—[a]	—[a]
ile	25.90 ± 0.57	90.83 ± 4.46	192.67 ± 13.03	156.97 ± 27.13	127.20 ± 5.31	75.05 ± 16.92
Leu	76.30 ± 2.61	161.70 ± 5.80	271.47 ± 8.29	172.30 ± 26.48	154.00 ± 6.27	92.52 ± 28.68
Hcys	79.93 ± 6.55	—[a]		54.77 ± 54.77	—[a]	23.52 ± 52.19
Phe	124.93 ± 14.73	211.20 ± 9.53	398.07 ± 21.08	258.83 ± 73.79	283.27 ± 4.90	173.01 ± 50.41
Trp	388.97 ± 18.53	450.30 ± 6.73	392.27 ± 6.95	369.33 ± 53.29	395.33 ± 18.83	224.58 ± 111.90
Lys	30.30 ± 3.40	70.10 ± 2.90	183.17 ± 11.84	83.17 ± 12.63	74.77 ± 5.04	41.12 ± 9.59

Results presented are mean values from three experiments each containing three replicates ± standard error of the means.
[a] Below detection limit.
[b] Data obtained from one measurement.

the amino acids tested increased at 12 hpi, the onset of infection, compared to the uninfected control. The exception was homocysteine, which decreased and was detectable only at very low levels, or not at all in the stages tested. Similarly, the levels of most amino acids peaked at 24 hpi, corresponding to the mid-biotrophic phase when haustoria are abundant. There was a decrease in the amount of free amino acids at the onset of and during the necrotrophic phase of the infection (48–72 hpi), with the levels of some free amino acids (Ser, Tau, and Hcys) starting to increase again at the 72 hpi time point.

4. Discussion

Phytophthora infestans is a hemi-biotrophic pathogen and, as such, provides an opportunity to study both biotrophic and necrotrophic interactions within the same pathosystem. Approximately the first 36 h of the infection are biotrophic, following which it progresses to a necrotrophic phase (van West et al., 1998; Vleeshouwers et al., 2000). A number of cell types are formed prior to penetration of a host cell, including zoospores, cysts, and germinated cysts with appressoria, all of which can be generated in the absence of the plant, facilitating the study of transcriptional and translational changes required to establish infection. We used a combination of proteomics and SSH to identify five proteins and/or transcripts, that are up-regulated during these pre-infection stages. They showed significant database matches to enzymes involved in amino acid biosynthesis. Real-time RT-PCR expression analysis of identified genes, *Pi-met1*, *Pi-trp1*, *Pi-ts1*, *Pi-kari1*, and *Pi-als1*, demonstrated that they were all up-regulated in germinating cysts with appressoria when compared to non-sporulating mycelium. It would therefore appear that the biosynthesis of these amino acids is important during the establishment of potato infection by *P. infestans*.

Levels of free amino acids were investigated in several pre-infection stages. Our findings show that, in most cases, there was a decrease in free amino acids in zoospores and germinating cysts, relative to both sporangia cultured in vitro and in planta, followed by a sharp increase in germinating cysts with appressoria. The increases observed in germinated cysts with appressoria could be due either to amino acid biosynthesis and/or the result of protein degradation. Evidence is provided, at least in the cases of threonine and the branched chain amino acids valine and isoleucine, that elevated amino acid levels in germinating cysts with appressoria are accompanied by elevated transcription of the corresponding biosynthesis genes, *Pi-ts1*, *Pi-kari1*, and *Pi-als1*. Although levels of these amino acids are supported by up-regulation of the respective biosynthesis genes in germinating cysts with appressoria, this is not the case for sporangia harvested from in vitro grown mycelium, where the highest levels of many amino acids were observed. Interestingly, sporangia harvested from infected leaves showed reduced amounts of amino acids in almost all cases, suggesting that during sporangia formation in planta, lower amounts of amino acids are synthesized by *P. infestans* or taken up from the plant, or less protein degradation takes place. The observed lack of amino acid biosynthesis gene up-regulation in sporangia that are cultured in vitro implies that protein degradation is responsible for the high levels of free amino acids in sporangia, or that free amino acids are actively transferred from the mycelium into the sporangia during sporangial development. In contrast, despite up-regulation of the *Pi-met1* gene in germinating cysts and in germinating cysts with appressoria, free methionine decreases through the pre-infection stages until it is below the detection level in germinating cysts with appressoria (Table 3), suggesting that it is rapidly incorporated into new proteins or other compounds. Similarly, although *Pi-trp1* is highly up-regulated in zoospores, germinating cysts, and germinating cysts with appressoria, the levels of free tryptophan decrease 2-fold in zoospores in comparison to sporangia produced in vitro, suggesting that it is also rapidly incorporated into new proteins or used for the synthesis of other amino acids.

Recently, using differential hybridization of a cDNA library, Shan et al. (2004) also identified several amino acid biosynthesis genes, including a methionine synthase, a glutamine synthase, and a cysteine synthase, up-regulated in *P. nicotianae* germinated cysts. Interestingly a large proportion of 300 clones up-regulated in germinating cysts consisted of sequences similar to genes involved in protein biosynthesis and energy production, suggesting that *Phytophthora* species require elevated amino acid production and metabolism during germination.

Although enzymes involved in the biosynthesis of proline were not detected in the proteomics or SSH screens in germinating cysts with appressoria, there is 4-fold more free proline in germinating cysts with appressoria than in non-sporulating mycelium. There is also approximately 5- to 7-fold more proline in sporangia than in non-sporulating mycelium. It is likely that both of these stages of the infection cycle require accumulation of osmolytes. In sporangia, a high pressure is required for release of zoospores, a process termed sporangial cleavage. In appressoria it is likely that an equal or greater pressure is required to penetrate the host leaf cuticle. Proline has already been implicated in osmoregulation of zoospore release in *P. nicotianae* (Ambikapathy et al., 2002). Therefore we are currently investigating the role of proline in the osmoregulation of appressorium formation in *P. infestans*.

In the present study, we have shown that transcripts of several *P. infestans* amino acid biosynthesis genes are

only slightly up-regulated during the biotrophic phase of infection, and that expression increases at the transition to the necrotrophic phase. We have shown that during the biotrophic phase of the interaction the free amino acid pool within the potato leaf increases, thereby precluding the need for the pathogen to synthesize amino acids during this part of the infection process. Moreover, three of the corresponding host amino acid biosynthesis genes (*St-als1*, *St-kari1*, and *St-ts1*) are up-regulated during the biotrophic phase, with the corresponding amino acids levels also increasing, raising speculation that the pathogen may also obtain these amino acids from the host. As infection proceeds to the necrotrophic phase of the interaction, there is a drop in the levels of free amino acids within the plant tissue and a corresponding increase in the expression of both the host and pathogen amino acid biosynthesis genes, revealing increased metabolic activity in each and potentially greater competition for resources.

It has been suggested that plant pathogenic fungi are starved of nitrogen within host tissues (Talbot et al., 1997). Solomon and Oliver (2001) have used the biotrophic *Cladosporium fulvum*–tomato pathosystem to study nutritional requirements of phytopathogenic fungi. Contrary to previously held views, that plant pathogens deplete the nutrient supply of the host, they showed that the amino acid and total nitrogen content of the apoplast increased during infection. Our results also show that during the biotrophic phase of the *P. infestans*–potato interaction, the free amino acid pool increases. Solomon and Oliver (2001) noted that all 20 amino acids were present in apoplastic wash fluids from *C. fulvum* infected tomato plants, except for tryptophan and cysteine. Methionine, arginine, histidine, and proline were only detected at very low levels (Solomon and Oliver, 2001). During our study, we found that methionine, cysteine, and hydroxyproline were undetectable within the plant and that histidine, homocysteine, and lysine were present at relatively low levels. Overall, these results suggest that the synthesis of many amino acids is up-regulated in plants by fungi and oomycetes during biotrophic growth and support our findings that genes involved in the biosynthesis of methionine, tryptophan, threonine, and branched chain amino acids are up-regulated in planta. Furthermore, we present real-time RT-PCR evidence that also shows up-regulation of host biosynthetic genes, especially during the necrotrophic phase of the *P. infestans* infection cycle.

Recent studies have also shown that a *C. fulvum* methionine synthase gene is up-regulated during infection (Solomon et al., 2000). Furthermore, disruption of a methionine synthase gene using restriction enzyme-mediated DNA integration (REMI) in the rice pathogen *Magnaporthe grisea* caused reduced pathogenicity. Conidiogenesis was greatly reduced and significantly less appressoria were formed on rice epidermal cells compared to the wild type strain (Balhadere et al., 1999). A histidine auxotroph has also been identified in *M. grisea* that is significantly less pathogenic than the wild type (Sweigard et al., 1998). These data indicate that there is a requirement for plant pathogenic fungi to be able to synthesize amino acids such as methionine and histidine during infection and our data suggest that this might also be the case for the oomycete *P. infestans*.

Essential biosynthetic pathways, including the biosynthesis of tryptophan, methionine, threonine, and branched chain amino acids, may therefore be exploited as potential fungus or oomycete chemical control targets; methionine biosynthesis has already been targeted in fungi. For example *Botrytis cinerea* grown in the presence of the commercial fungicide pyrimethanil becomes a leaky methionine auxotroph (Daniels and Lucas, 1995). It is thought that the target for pyrimethanil is cystathionine β-lyase, which converts cystathionine to homocysteine, which can then be converted to methionine by methionine synthase (Daniels and Lucas, 1995). Methionine can also be produced from homoserine, hence this type of fungicide will not completely prevent methionine biosynthesis. Based on our findings, it is possible that oomycetes may also respond to this type of chemical control.

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