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Characterization of *Phytophthora infestans* genes regulated during the interaction with potato

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SUMMARY

Suppression Subtractive Hybridization (SSH) was used to search for genes of *Phytophthora infestans* that are induced during the infection of potato. To avoid having to distinguish the genes of the pathogen from the plant genes involved in defence responses and to isolate the genes involved in the early stages of interaction, mycelium of *P. infestans* was induced by contact with the host plant and then separated from the plant tissue. A differential cDNA library was generated by SSH that compared such induced mycelium with mycelium incubated in water. The expression of about 100 cDNA fragments from this differential cDNA library was analysed by hybridization of the arrayed PCR products with mRNA from control and induced mycelium. Twenty per cent of them showed increased transcript levels in mycelium within the first 24 h after exposure to a potato leaf. For six of these cDNA clones the elevated expression in response to the potato leaf could be proven by RNA gel blot analysis. Five of these cDNA clones have predicted amino acid sequence homologies to entries in the databases, including an amino acid transporter, a sucrose transporter, a spliceosome-associated factor, an ABC transporter, and a cell division control protein. We showed that the genes corresponding to these six cDNA clones are differentially regulated during their life. Reliable gene expression analysis of *Phytophthora* in infected leaf tissue is not possible until *c.* 48 h post-infection, but for two of the genes we identified, induction during *in planta* growth was detectable by RNA gel blot analysis. Therefore the SSH library that we have created provides a basis for the identification of *P. infestans* genes that are up-regulated during the interaction with the plant, which could be important for pathogenicity.

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INTRODUCTION

The oomycete *Phytophthora infestans* causes the devastating late blight disease of potato and tomato. Historically it is notorious as the causal agent of the Irish potato famine in 1845 (Ristaino *et al.*, 2001) that led to the death of over a million people and the emigration of many more (Duncan, 1999). Today *P. infestans* remains a destructive and economically important pathogen. Throughout the world the costs of fighting this pathogen and the losses in yield due to late blight are enormous (Duncan, 1999). Since *P. infestans* was first described as the cause of late blight disease, intense research was devoted to an understanding of its genetics and physiology (e.g. Erwin *et al.*, 1983; Ingram and Williams, 1991), as well as its interaction with potato (Freytag *et al.*, 1994; Kamoun *et al.*, 1999b). However, knowledge of the molecular processes involved in pathogenicity is still limited. A better understanding of the molecular mechanisms underlying this plant–microbe interaction could lead to the development of new strategies to reduce crop losses and thereby benefit agricultural practices.

One approach to identifying important factors in the establishment of an infection lies in defining the transcriptional changes occurring therein. Genes differentially expressed during contact with the plant would be of special interest, since such genes might contribute to pathogenicity or virulence. Components essential for pathogenesis could be involved in different phases of plant colonization, e.g. attachment, germination, appressorium formation, invasive growth, nutrient uptake and sporulation. In addition, these factors could also play a role in protection against plant defence responses.

Several previous studies focusing on potato genes regulated during colonization by *P. infestans* identified a broad range of induced genes (e.g. Avrova *et al.*, 1999; Beyer *et al.*, 2001; Collinge and Boller, 2001; Zhu *et al.*, 1995). From *P. infestans*, however, only a few genes could be identified that were differentially expressed during infection of its host (Görnhardt *et al.*, 2000; Kamoun *et al.*, 1997; Pieterse *et al.*, 1991; Pieterse *et al.*, 1992, 1993a,b, 1994).

In this study, we performed a PCR-based differential screen using Suppression Subtractive Hybridization (SSH) to identify the *P. infestans* genes involved in the infection of potato. Previously, we reported the successful application of this method to create a differential library from infected leaf tissue and the identification of several potato genes highly induced in response to *P. infestans* (Beyer *et al.*, 2001). The clear under-representation of *Phytophthora* genes in this library made it inefficient for isolating regulated genes of the pathogen, and thus a more pathogen orientated experimental set-up had to be sought. Görnhardt *et al.* (2000) generated an SSH library from *in vitro* germinated cysts of *P. infestans* in order to identify genes important for the initial step of the infection process. It is, however, not yet known if the genes identified in this approach also play a role in the later stages of the potato–*Phytophthora* interaction.

In the screen described here, the differentiation of *in planta*-induced genes of *P. infestans* from the high background of pathogen-activated plant genes was circumvented by exposing mycelium to potato leaves and then separating it from the plant tissue. Several *Phytophthora* genes were identified that are induced in response to potato, including genes with predicted homologies to two nutrient transporters, an ATP binding cassette-(ABC) transporter, a protein involved in cell division, a spliceosome associated factor, as well as an unknown protein. Thus, the screening system described enables the analysis of genes from a hemibiotrophic pathogen that might play a role in the interaction with its plant host.

EXPERIMENTAL PROCEDURES

Growth of potato plants and *P. infestans*

Phytophthora infestans strain 88069 was kindly provided by Francine Govers (University of Wageningen, Netherlands). It was grown on rye agar (Caten and Jinks, 1968) at 18 °C in the dark. Virulence was maintained by infecting leaves and re-isolating every 3–4 months. Sporangia, zoospores and cysts were obtained as described by van West *et al.* (1998). Germinated cysts were obtained by incubation of cysts in water at 18 °C in the dark for 4–6 h. To obtain mycelium, zoospores were germinated and grown in liquid rye medium at 18 °C in the dark for 2–6 days. Isolation and purity of the different developmental stages was confirmed microscopically.

Axenic potato plants, cultivar Bintje, were kindly provided by Patrick Schweizer (University of Fribourg, Switzerland). The plants were grown and maintained as described in Beyer *et al.* (2001).

Media preparation

Defined medium was prepared according to Xu *et al.* (1982). For solution A, 20 g glucose, 1.32 g $(NH_4)_2SO_4$, 1.32 g $CaCl_2 \times H_2O$, 0.5 g $MgSO_4 \times 7H_2O$, 0.7 g KH_2PO_4, 0.3 g K_2HPO_4, 0.01 g $Fe(SO4)_3$ were mixed with 500 mL H_2O and the pH was adjusted to 4.6. For solution B, 50 mg each of $MnSO_4 \times H_2O$, $ZnSO_4$ and thiamine were dissolved in 100 mL H_2O. For solution C, 2.32 g fumaric acid was dissolved in 300 mL H_2O and the pH was adjusted to 4.6. Solutions A and C were mixed together with 1 mL of solution B and the pH was adjusted to 4.6. For the N-starvation medium the defined medium was prepared without $(NH_4)_2SO_4$, while the C-starvation medium was prepared with only 2 g glucose.

Infection of potato

Leaves were excised from 6-week-old plants, placed with the abaxial side up on moist filter paper in Petri dishes and inoculated with *P. infestans* zoospores. Droplets (about 10 μL) containing approximately 1000 zoospores were spotted on the abaxial side of the leaf. To maintain the high humidity required for infection, the Petri dishes were closed with Parafilm. They were placed in the dark at 18 °C for 16 h and then transferred to a growth chamber (16–20 °C).

Differential screen

Mycelium was grown in liquid rye medium at 18 °C in the dark for 6 days. The mycelium was washed with water and small portions were placed into a vessel containing 50 mL water (control) or on the abaxial side of a potato leaf floating in 50 mL water (tester). The mycelium was incubated for 4, 8 or 24 h and the samples were pooled to create one control and one tester sample. The tester samples were harvested by removing the mycelium from the potato leaf using forceps, and the control sample was handled in a similar manner. The mycelium was dried quickly on Whatman paper and frozen in liquid nitrogen. Poly(A) RNA was isolated from the mycelium using a Poly(A) Pure Kit (Ambion, Witney, Oxon). Suppression Subtractive Hybridization (SSH) was performed as described in Beyer *et al.* (2001).

Dot blot assay

The cDNA inserts of the clones from the SSH library were amplified by PCR using primers N1 and N2R (Clontech PCR-Select Kit) that are specific for the adaptors ligated on to the cDNA fragments during the SSH procedure. Then NaOH and EDTA pH 8 were added to each PCR reaction to a final concentration of 0.4 M NaOH and 10 mM EDTA and the PCR products were denatured at 95 °C for 10 min. A 30 μL aliquot of the PCR products was then loaded in duplicate on two Zeta-Probe Blotting Membranes (Bio-Rad) using a dot blot manifold. The slots of the dot blot manifold were each pre-washed with 500 μL H_2O, then the PCR samples were applied, and each slot was rinsed again with 0.4 M

NaOH. The membranes were then quickly rinsed in 2 × SSC, UV crosslinked and hybridized with the radioactively labelled cDNA probes. For this, poly(A) RNA was prepared from a pool of mycelium incubated in water for 4, 8 and 24 h and mycelium co-incubated with a potato leaf for the same time periods. To create radioactively labelled cDNA probes, first strand cDNA synthesis was carried out in the presence of α-^{32}P dCTP, as described in Beyer *et al.* (2001). The blots were hybridized and washed at 65 °C as described by Church and Gilbert (1984).

DNA sequence analysis

Sequencing was performed using Dye Terminators from PE Applied Biosystems using a Perkin-Elmer GeneAmp PCR system thermocycler and analysed using an ABI PRISM 377 DNA sequencer. Sequence data were analysed using the GCG package (Wisconsin Package ver. 10.1, Genetics Computer Group, Madison, WI). Similarity searches were made with BLAST programs at the National Center for Biotechnology Information <http://www.ncbi.nlm.nih.gov>.

RNA and DNA gel blot analysis

A series of duplicate RNA blots was made with total RNA extracted from different stages of *P. infestans* and necrotic parts of infected potato leaves. RNA of mycelium, sporangia, zoospores and cysts was extracted using the Trizol reagent (Life Technologies), RNA of germinated cysts was isolated using the RNAwiz Reagent (Ambion) and RNA of infected leaves was extracted as described by Chomczynski and Sacchi (1987). Total RNA samples (10–20 μg) were separated on formaldehyde-agarose gels and transferred to positively charged nylon membrane (Zeta-probe, Bio-Rad) (Sambrook *et al.*, 1989). DNA probes were labelled using the Prime It II random primed labelling kit (Stratagene), and hybridization and washing were carried out for RNA gel blots at 65 °C and for DNA gel blots at 50 °C, as described in Church and Gilbert (1984). For low-level transcripts, RNA gel blots were hybridized at 42 °C using the UltraHyb Buffer (Ambion) for greater sensitivity. The resulting radioactive signals were compared and quantified using a Phosphor imager (Storm 860, Molecular Dynamics).

RESULTS

Creation of a subtracted library

We screened for genes that are induced in *P. infestans* during the interaction with potato using the PCR-based method Suppression Subtractive Hybridization (SSH) (Diatchenko *et al.*, 1996). To specifically identify *P. infestans* genes involved in the interaction with potato, we used mycelium incubated in water as the control sample and mycelium that had been stimulated by the presence of a potato leaf as the tester. Importantly, this creates a subtracted library that contains only sequences of *P. infestans*. This approach is similar to the one used by Munoz and Bailey (1998) and enables the mechanical separation of the two interacting organisms before the applying the SSH procedure. To test the suitability of this *in vitro* system, we analysed the incubated leaves microscopically after staining for fungal infection structures (Wilson and Coffey, 1980). In leaves that had been exposed to the mycelium for 3 days, colonization of the leaf by *P. infestans* could be detected, and infection structures were indistinguishable from those seen after infection of leaves by zoospores (Fig. 1A). Furthermore, the recognition of the potato leaf as a potential host must take place very quickly, since the mycelium becomes firmly attached to the leaf after only 30 min of incubation, and has to be removed from the leaf using forceps. To determine if the regulation of genes occurs upon co-incubation with a potato leaf similar to that seen in infection of leaves with zoospores, the expression of the known *in planta* induced *Phytophthora* gene, *ipiO* (Pieterse *et al.*, 1993a, 1994) was analysed and a clear up-regulation was detected (Fig. 1B). Thus, our *in vitro* infection conditions closely mirror the infection process caused by inoculation with zoospores, and we used this system to generate a differential library using the 'induced mycelium' (IM) as the tester and the water-incubated mycelium as control.

Differential screening of the library clones

A dot blot assay was performed to determine what proportion of the clones in the SSH library represent transcripts that accumulate in the presence of a potato leaf. Ninety-six PCR-amplified cDNA inserts of about 200–800 bp were blotted on to two replica membranes using a dot blot manifold. Two genes that had been identified in a previous SSH screen and shown to be constitutively expressed in *in vitro* grown mycelium and *in planta* (data not shown) were loaded as controls on the membranes. The DNA blots were hybridized with the cDNA populations derived from the original control and tester samples that had been used to generate the SSH library. Level of induction for each clone was calculated by dividing the signal on the blot hybridized with tester cDNA by the signal obtained on the blot hybridized with control cDNA. The two control genes, as well as 80% of the tested clones, showed induction values in the range of 1–3-fold. Twenty clones, corresponding to 20% of clones from the SSH library, showed an induction of at least fourfold (data not shown).

In addition to the dot blot assay, about 600 random clones were sequenced and subsequently BLASTX searches were performed against GENBANK data. Hits were considered significant if the expectation value was less than 1e-5. Genes were grouped into different categories using the same functional classification

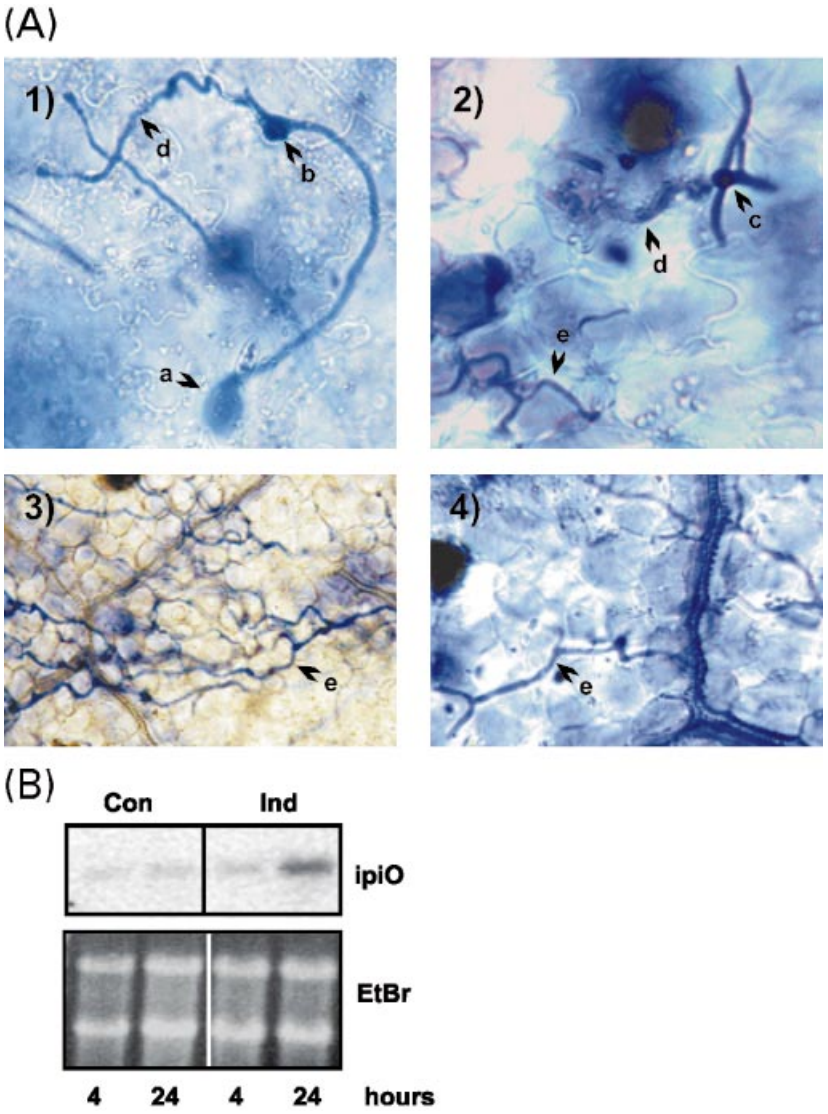

Fig. 1 (A) Infected potato leaves stained for infection structures. (1) and (3), potato leaf infected by spraying the leaf with a spore suspension of *P. infestans*, (1) 2 days after infection, (3) 3 days after infection. (2) and (4) potato leaf infected by co-incubation with P. infestans mycelium in water for 3 days. a = *infecting* spore, b = appressorium, c = appressorium or swollen hypha, d = infecting hypha, e = intercellular spreading hypha. Magnifications: (1) and (2) 320×, (3) 100×, (4) 200×. (B) Expression of *ipiO* in mycelium treated with water (Con) and incubated with a potato leaf (Ind). Top panel: RNA gel blot. Bottom panel: ethidium bromide stained gel before transfer.

as was employed for *S. cerevisiae* in MIPS (Munich information centre for protein sequences <http://www.mips.gsf.de> data not shown). Seventy per cent of the sequences could not be classified since they did not have significant hits in the databases or show homologies to proteins of unknown function. The sequences that could be assigned fell mainly into the functional classes of basic cellular processes, such as metabolism, energy, protein synthesis and fate and transport.

Transcriptional characterization of selected clones

Expression in mycelium induced by the plant

Twenty-five cDNA clones from the differential library were chosen for further analysis in RNA gel blot experiments. First, the 20 cDNA fragments that showed increased transcript levels in the dot blot experiment were chosen. Two of these, IM-002A and IM-003C, exhibit significant homology to an amino acid transporter and a sucrose transporter (accession nos AF159856 and AL445063), respectively. Furthermore, they show homology to similar transporters of the rust fungus *Uromyces fabae* that are up-regulated during the infection of pea (Hahn *et al.*, 1997; Voegele *et al.*, 2001). Since transporters are conceptually of clear importance for the interaction with the plant, we took advantage of our sequence collection from the subtracted library and searched for additional clones showing homologies to different kinds of transporters. Five such clones were found and included in our RNA gel blot analysis. They have homologies to a phosphate transporter, two different ABC transporters, a tSNARE and a putative vesicular transport protein in yeast (SRO9). The expression of the 25 selected cDNA clones was analysed on RNA gel

Table 1 Genes of *P. infestans* induced upon co-incubation with a potato leaf.

Clone name	Homology	Protein match % identity/ probability	Length of fragment*	Fold induction†
IM-002A	Amino acid transporter, *Mus musculus*, AF159856	35%/5e-11	500 bp	1.5
IM-003C‡	D-Xylose-proton symporter, *Thermoplasma acidophilum*, AL445063	48%/7e-5	300 bp	3.1
IM-004 A	No clear hits		370 bp§	2.6
IM-007F	No clear hits		360 bp	1.6
IM-008B	Spliceosome-associated protein, *Schizosaccharomyces pombe,* AL023589	30%/1e-12	690 bp§	2.5
IM-008D‡	Retinoblastoma protein, *Notophthalamus viridescens*, Y09226	34%/3e-06	740 bp	1.4
IM-01F1‡	ATP-binding cassette 2, subfamily 2, member 2, *Mus musculus*, X75927	56%/2e-59	700 bp§	2.6
IM-06D11	Cell division control protein 6, *Xenopus laevis*, U66558	30%/2e-7	480 bp	6.4
IM-06E10	Phosphate transporter Pho91, *Saccharomyces cerevisiae*, Z71628	33%/8e-24	590 bp	1.5
IM-09E10	Similarity to SRO9, *Schizosaccharomyces pombe*, AL355653	38%/2e-9	530 bp	1.5

*Length is indicated in base pairs.

†Fold induction calculated by normalizing the expression level in the control against the expression level in the induced sample.

‡Found twice among the analysed clones.

§Longer cDNA fragments have been isolated additionally and are available at the database.

blots containing either total or poly(A) RNA from pooled samples of mycelium collected at different time points after incubation in water only, or with a potato leaf. Of the 25 clones that were analysed on RNA gel blots, 15 were detectable and 10 of these showed higher transcript levels in the induced mycelium (Table 1 and Fig. 2).

The differential expression in response to the potato leaf vs. the water control observed in the pooled samples was then analysed at different time points of incubation, namely after 4 h, 8 h and 24 h. The five cDNAs showing at least twofold induction were selected for this analysis. These clones have homologies to a sucrose transporter (IM-003C), a spliceosome-associated factor (IM-008B), an ABC transporter (IM-01F1), a cell division control (CDC) protein (cdc6) (IM-06D11) and one clone without any clear homology in the database (IM-004 A). In addition, we included IM-002A, which has a lower level of induction, but shows significant homology to a known *in planta* induced gene of *Uromyces fabae* (Hahn *et al.*, 1997). For all of the six selected clones, the analysis was performed at least twice in independent experiments and the differential expression was confirmed for all of them (Fig. 3). In these time-course experiments, IM-004A showed the weakest induction, which is in contrast to the level of induction detected on the pooled RNA gel blots. Some of the genes, IM-003C, IM-008B and IM-06D11, showed a decrease in differential expression after 24 h (data not shown). This could be due to a general decrease in fitness caused by the long incubation in water. Before beginning the SSH screen, when testing the expression of different genes, we noted that actin transcript levels decreased after 24 h. Therefore, we suggest that the induced mycelium system only be used for the identification of early induced genes.

Expression at different stages of the life cycle

Expression of the genes represented by the six selected cDNA clones at different stages of the life cycle was analysed on RNA gel blots of total RNA from sporangia, zoospores, cysts, germinated cysts, mycelium grown in liquid rye medium and infected plant material (Fig. 4). Ten μg of RNA were loaded on the gel, except for germinated cysts, from which less RNA of lower quality was loaded because of consistent problems in RNA isolation from this stage. The RNA of infected plant tissue was a mixture of RNA from potato leaves 3, 4 and 5 days after infection. To assay the relative amount of *P. infestans* RNA loaded, the blot was subsequently hybridized with a fragment of the actin gene (actA) of *P. infestans*, a gene that is expressed at similar levels in all developmental stages tested (Unkles *et al.*, 1991). The strength of the actin signal in Fig. 4 demonstrates the lower amount of RNA loaded for the germinated cysts, as well as the dilution with plant RNA in the sample from infected potato leaves.

Three of the analysed genes, IM-002A (homologous to an amino acid transporter), IM-003C (homologous to a sugar transporter), and IM-008B (homologous to a spliceosome-associated factor) show a similar expression pattern. They were expressed mainly in sporangia, in mycelium and in the infected plant tissue. IM-002A was also expressed in zoospores and cysts, but IM-003C and IM-008B were not. Relative to the expression of actin, IM-008B also showed a relatively high expression in germinated cysts. IM-004A (no clear homology in the database) showed a similar expression pattern to that of actin. IM-01F1 (homologous to ABC transporter) was expressed in all stages but was clearly upregulated in mycelium and in the infected plant material. IM-06D11 (homologous to cdc6) was mainly expressed in zoospores and cysts, the main infectious agents, but also in mycelium and the

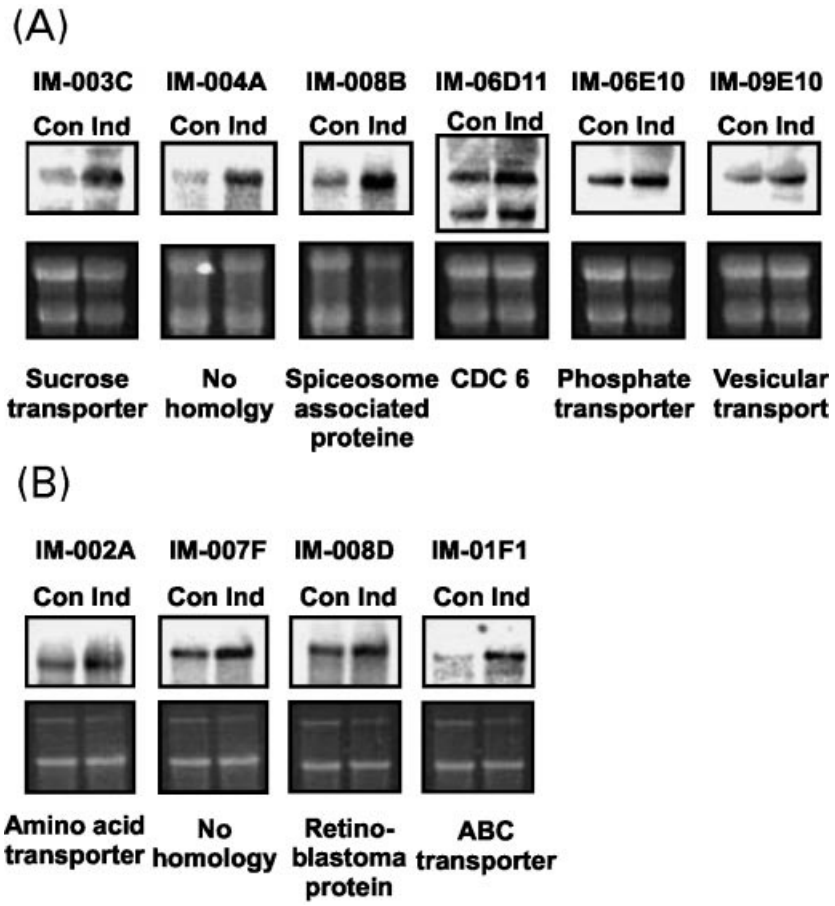

Fig. 2 Differential expression of selected library clones in response to co-incubation with a potato leaf. Bottom panel: ethidium bromide-stained agarose gel before transfer. Top panel: autoradiograph with signals after hybridization with radioactive probe. Mycelium was incubated in water (Con) or in water together with a potato leaf (Ind) for 2, 4, 8 and 24 h. The RNA was isolated and equal amounts from each time-point were pooled to create one control sample and one tester sample. (A) Fifteen μg of total RNA was loaded in each lane. (B) Two and a half μg of poly(A)RNA were loaded in each lane.

infected plant tissue. This cDNA clone detected three hybridizing bands of different sizes in all the *P. infestans in vitro* stages, but only the largest band of 3.6 kb was detected in the infected plant tissue.

To analyse the *in planta* expression in more detail, transcript levels were determined 1, 2, 3, 4 and 5 days after infection. RNA from mycelium grown in liquid rye medium and incubated for 8 h in water was used as a comparison. Expression of all the analysed genes was first detectable 3 days after infection (Fig. 5), and a continuous increase in transcript level was observed which was comparable to the expression of actin. The relative amount of actin mRNA, which specifically hybridizes to *P. infestans* actin, was taken as a measure of the *P. infestans* biomass in the infected leaf. Accordingly, the level of expression was determined by normalizing the signals for each gene against those for actin. This level of expression was then compared to the expression in *in vitro* grown mycelium (data not shown). Importantly, two genes, IM-01F1 and IM-06D11, showed a higher expression *in planta* than *in vitro*, which can also be seen in Fig. 5. IM-01F1 shows highest level of expression 5 days after infection, twofold higher than in the *in vitro* mycelium. IM-06D11 hybridizes only to a fragment of 3600 bp in RNA from infected leaves, whereas it hybridizes more strongly to smaller fragments in RNA from the *in vitro* grown mycelium. It is the only clone with a clearly induced expression at the first detectable time point after infection. Three days after infection its expression was fourfold greater than *in vitro*, and then subsequently its transcript level slightly decreased. This is consistent with the result obtained in the expression analysis of different stages (Fig. 4), where it was mainly expressed in zoospores and cysts, the main infectious agents. Therefore this gene might be important at early time points during the infection process. It is possible that the expression of the other genes is up-regulated before they are detectable in infected leaves, or perhaps later in the infection process, since their signals still seemed to be increasing 5 days after infection.

Expression analysis in response to different media

It has been reported that various genes of fungal pathogens expressed during infection are also regulated by nutrient starvation, which indicates that nutrient deprivation may be one of the conditions that biotrophic pathogens encounter during *in planta* growth (reviewed in Oliver and Osbourn, 1995). To determine if

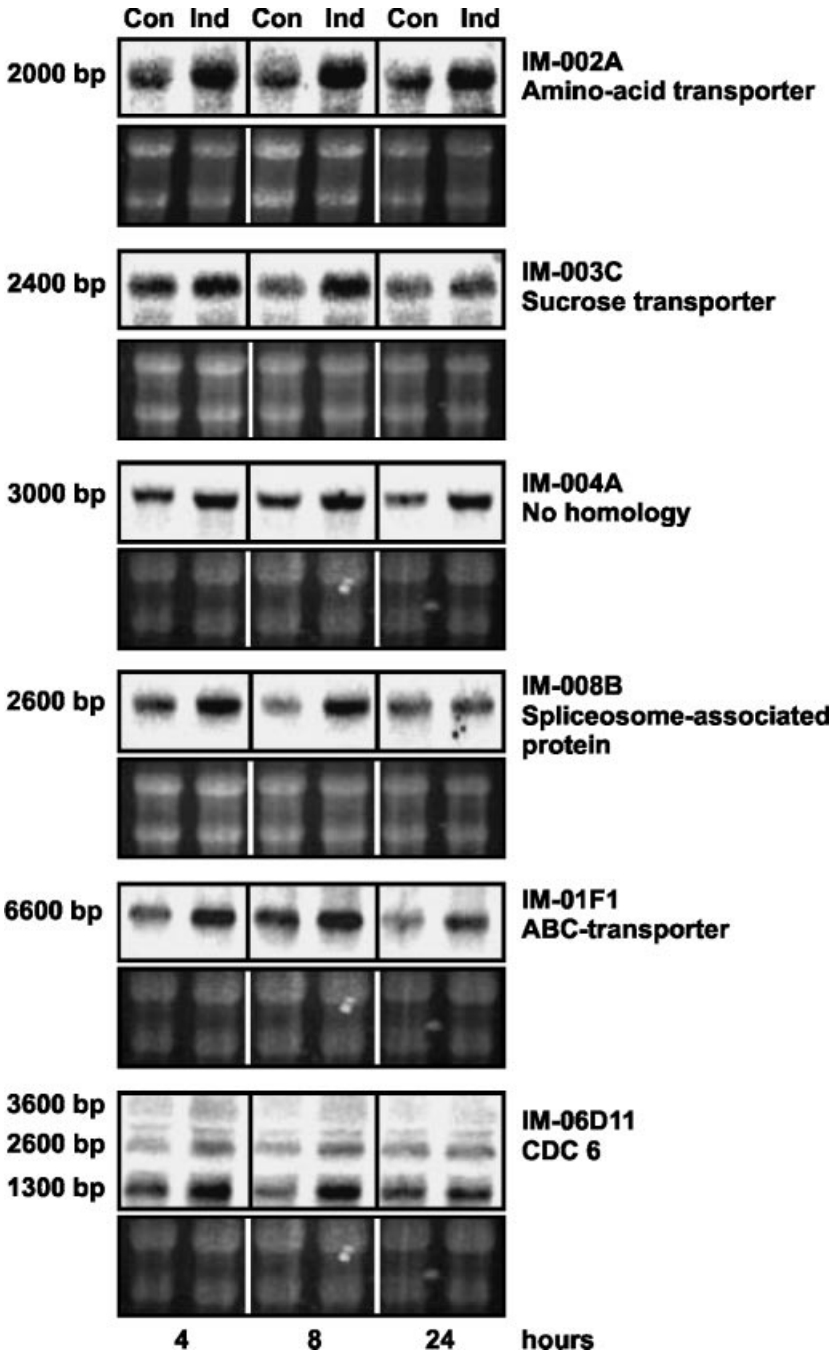

Fig. 3 Changes in transcript level over time of the differential clones in response to co-incubation with a potato leaf. Bottom panel: ethidium bromide-stained agarose gel before transfer. Top panel: autoradiograph with hybridization signals after radioactive detection. Mycelium was incubated in water (Con) or in water together with a potato leaf (Ind) for the indicated times. Fifteen μg of total RNA was loaded in each lane.

the genes that were identified in our screen are regulated in response to nutrient starvation, their expression was analysed in mycelium incubated in different media. Northern blots were prepared with 15 μg of total RNA from mycelium grown for 6 days in rye medium followed by incubation for 8 h in water, in defined medium (Xu *et al.*, 1982), in defined medium without either nitrogen or carbon source, or in rye medium (Fig. 6). Three of the analysed genes—IM-003C, IM-004A and IM-008B—showed similar expression in all the different media. In contrast, transcript levels of IM-002A increased slightly in response to the N-starvation medium and strongly in response to the C-starvation medium, as well as to the rye medium. Moreover, IM-01F1 and IM-06D11 were both induced in response to the complete defined medium, and they showed a slight induction in response to the N-starvation medium and a strong induction to the C-starvation medium, compared to their expression in water and

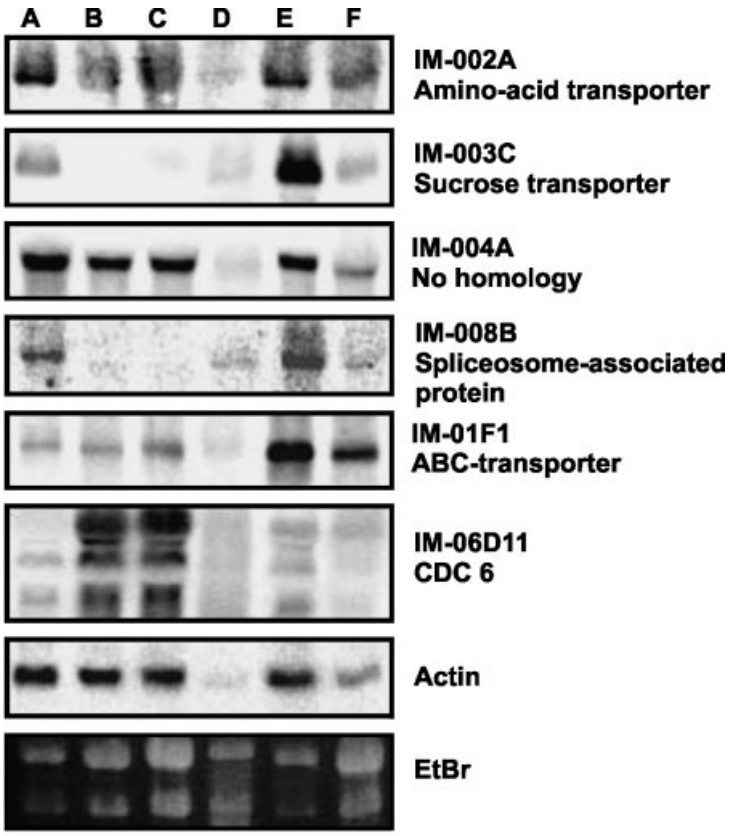

Fig. 4 Transcript levels of selected clones at different stages of the *P. infestans* life cycle. Bottom panel: ethidium bromide-stained agarose gel before transfer. Top panel: autoradiograph with signals after hybridization with radioactive probe. Ten μg of total RNA were loaded in lanes A, B, C, E and F, and 5 μg total RNA was loaded in lane D. (A) sporangia, (B) zoospores, (C) cysts, (D) germinated cysts, (E) mycelium and (F) infected potato leaves.

rye medium. It is possible that their expression is induced in response to a nitrogen source.

Database accession numbers

The new cDNA sequences reported here have been deposited in the EMBL Nucleotide Sequence Database with the accession numbers AJ487842 to AJ487851.

DISCUSSION

The destructiveness of late blight disease makes it very important to investigate the causal organism in more detail. The infection process involves specific morphological changes and many metabolic processes, which require the regulation of a wide variety of genes.

Genes regulated during infection have been described for a number of phytopathogenic fungi and oomycetes. For example, from *Uromyces fabae* a total of 31 *in planta* induced genes (PIGs) were isolated from a haustorium specific library (Hahn and Mendgen, 1997). Two proteins of *Cladosporium fulvum* that are secreted into the intercellular space of infected tomato leaves (Wubben *et al.*, 1994), as well as the race specific elicitors *AVR4* and *AVR9*, were shown to be transcriptionally induced during plant infection (Laugé and de Wit, 1998). From *Magnaporthe grisea* a putative hydrophobin gene, *MPG1*, that is induced during infectious growth, was identified by differential cDNA cloning (Talbot *et al.*, 1993), and several differentially expressed genes were isolated by screening cDNA libraries of infected rice plants (Kim *et al.*, 2001; Rauyaree *et al.*, 2001). In *Phytophthora capsici*, a cutinase-encoding gene is induced in the interaction with pepper (Munoz and Bailey, 1998). From *P. infestans*, nine *in planta* induced genes have previously been isolated (Pieterse *et al.*, 1993a). In addition, several genes have been reported to be expressed during specific stages of the life cycle which are important for infection (Görnhardt *et al.*, 2000; Judelson and Michelmore, 1990). *MPG1* of *M. grisea* (Talbot *et al.*, 1993) was shown to be a determinant of pathogenicity, which demonstrates the success of differential screening approaches.

Here we describe an efficient system for isolating *P. infestans* genes that may play a role in the interaction with potato. This *in vitro* experimental system allows the physical separation of the induced mycelium from the plant tissue after the initiation of infection. It has the advantage that genes of the pathogen do not have to be discriminated from the host genes, a process that is very difficult in the analysis of infected plant tissue due to the overwhelming representation of plant sequences (Beyer *et al.*, 2001).

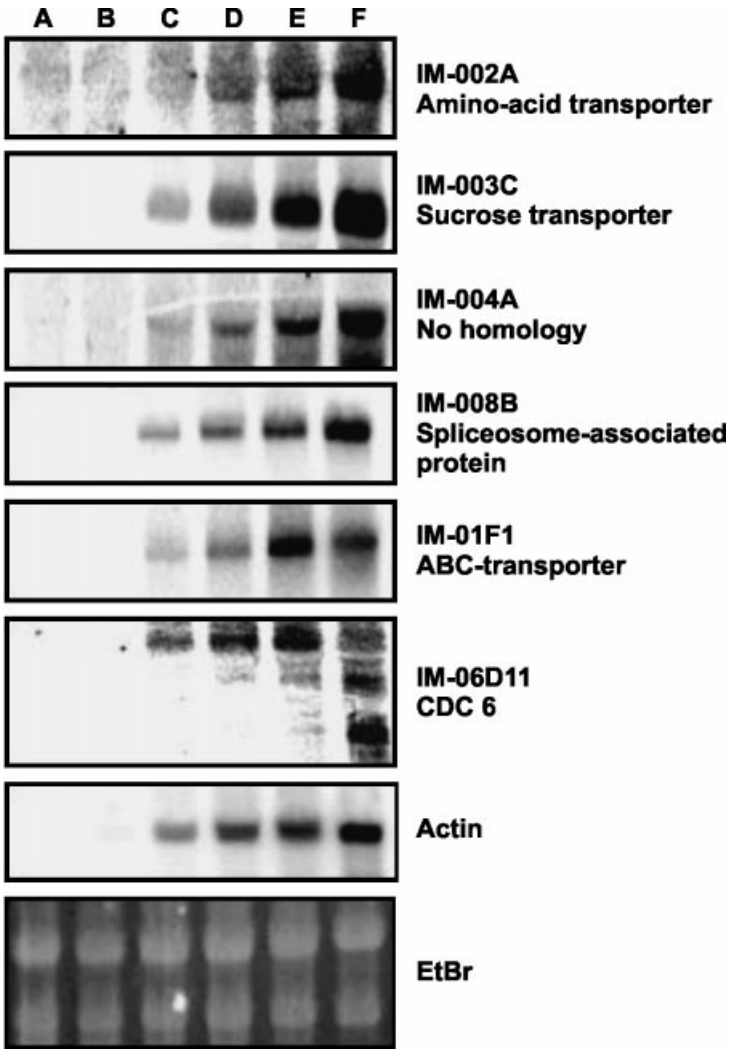

Fig. 5 Transcript levels during *in planta* growth and in *in vitro* grown mycelium. Bottom panel: ethidium bromide-stained agarose gel before transfer. Top panel: autoradiograph with hybridization signals after radioactive detection. Fifteen μg of total RNA was loaded in each lane. (A–E) Infected plant tissue at: (A) 1, (B) 2, (C) 3, (D) 4 and (E) 5 days after infection. (F) mycelium incubated in H_2O for 8 h.

About 600 clones from the subtracted library were sequenced and grouped into different classes according to their predicted sequence homologies. This classification suggested that growth and the maintenance of metabolism are important processes in mycelial growth stage. A similar grouping was described for ESTs of a mycelial library of *P. infestans* (Kamoun *et al.*, 1999a) and for other host-pathogens. Most of the expressed genes identified in *Magnaporthe grisea* during the colonization of rice were grouped according to gene/protein expression and metabolism (Kim *et al.*, 2001). Furthermore, the most abundant *P. sojae*-derived ESTs of an interaction library are involved in intermediary metabolism, indicating that rapid growth and invasion of the host tissue make a massive demand on central metabolic processes (Qutob *et al.*, 2000).

One hundred of the clones from the subtracted library were screened by dot blot analysis. Twenty-five clones were selected for further analysis on RNA gel blots comparing mycelium incubated with a potato leaf with mycelium incubated only in water. Ten of the cDNAs that showed induction on the dot blot also showed induction in this assay (Figs 2 and 3). The increases in transcript level observed in these experiment were moderate, and level of induction ranged from 1.5- to 3-fold. This may be because in this system only a few hyphae of the induced mycelium have direct contact with the plant surface and therefore only a part of the 'induced' mycelium is really induced. Another reason could be that we searched for expression changes triggered by the presence of the host plant in the mycelium of a hemibiotrophic pathogen that does not normally exist in an independent mycelial form in the field (Hohl, 1991). This developmental stage probably does not undergo major changes in terms of adaptation to new conditions, and therefore even small changes in gene expression could have an important impact on *in planta* growth. Indeed, it was shown in other systems that genes showing a low level of induction following a certain stimulus can be very important

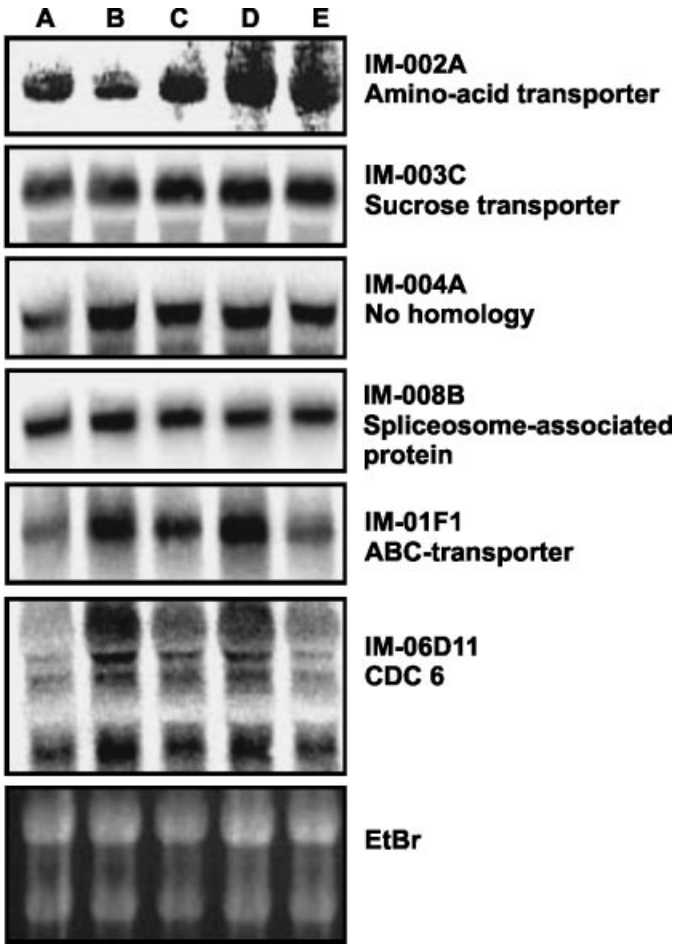

Fig. 6 Changes in transcript level in response to different media. Bottom panel: ethidium bromide-stained agarose gel before transfer. Top panel: autoradiograph with hybridization signals after radioactive detection. Fifteen μg of total RNA was loaded in each lane. Six-day-old mycelium was incubated for 8 h in different media. (A) H_2O, (B) defined medium, (C) N-starvation medium, (D) C-starvation medium, (E) Rye medium.

for the corresponding adaptation. In yeast, for example, a gene knock-out study revealed that a heat shock protein exhibiting a twofold induction following sorbic acid treatment conferred resistance to this treatment (de Nobel *et al.*, 2001).

Six genes whose transcript levels were at least twofold higher in 'induced mycelium' were identified in this small-scale screen of the subtracted library. These were an amino acid transporter, a sucrose transporter, an ABC transporter, a spliceosome associated protein, a cdc6 homologue and one with no homology to entries in the databases. The changes in transcript levels were already detectable after 4 h of exposure to a potato leaf. Transcript levels of these genes were also regulated in different developmental stages and by nutrient conditions.

The fact that we identified two genes similar to those induced *in planta* in another plant-pathogen indicates a convergent evolution of gene expression changes in distinct plant pathogens. One of these clones, IM-003C, exhibits homology to a sugar transporter (D-Xylose-proton symporter, *Thermoplasma acidophilum*, AL445063), and also shows significant homology to a hexose transporter of *Uromyces fabae* that is exclusively expressed in haustoria while colonizing pea, and is needed for sugar uptake (Voegele *et al.*, 2001). The other clone, IM-002A, shows homology to an amino acid transporter of *Mus musculus* (AF159856) and is also homologous to an amino acid transporter that is mainly expressed in haustoria of *Uromyces* (Hahn *et al.*, 1997). In *Uromyces* these two genes are expressed exclusively in haustoria and infected plants, but their expression has not been analysed in teliospores or in *in vitro* mycelium, since *Uromyces* is an obligate biotroph. The two transporters that we isolated are expressed in mycelium, *in planta* and in sporangia. There was no clear induction *in planta* compared to expression in *in vitro* grown mycelium. However, it has been observed that, unlike typical haustoria of many higher fungi, those of *P. infestans* often look like side branches or infection hyphae, and it was therefore suggested that the nutrient uptake of *P. infestans* may not be restricted to haustoria but include intercellular, intracellular and transcellular hyphae (reviewed in Hohl, 1991). Thus, these transporters may be important during *in planta* growth for nutrient uptake.

Since it is very likely that transporters are needed during infection for the uptake of nutrients and metabolites in the colonized host tissue, we selected other clones from the subtracted library

with homologies to transporters. Indeed, one of them, IM-01F1, which exhibits homology to an ABC transporter, showed a clear induction. ABC transporters are highly conserved traffic ATPases that occur ubiquitously, and transport a wide spectrum of compounds over various membranes (Senior *et al*., 1995). It has been postulated that the function of these transporters in plant pathogens could be the secretion of endogenous pathogenicity factors (e.g. toxins) or exogenous plant defence compounds. Indeed, several studies have shown that ABC transporters are involved in the secretion of antibiotics, toxins, plant defence compounds and fungicides (reviewed in Del Sorbo *et al*., 2000). However, all of these fungal ABC transporters belong to the class of multidrug or pleiotropic drug resistance (MDR or PDR) type transporters, whereas the ABC transporter that we identified in *P. infestans* displays a rather weak homology to this subgroup. A greater homology of IM-01F1 is found with a human ATP-binding cassette transporter (X75927), called ABCA2. This transporter was shown to be involved in drug resistance in the brain (Dean *et al*., 2001), but, interestingly, it was also shown to be transcriptionally up-regulated by sterols (Kaminski *et al*., 2001). IM-01F1 is clearly induced in response to a potato leaf in the *in vitro* system and it is also clearly *in planta*-induced (Fig. 5). During the life cycle it is highly expressed only in mycelium and infected plants, indicating that it plays a role during colonization of the host. Like other *in planta*-induced genes it is transcriptionally activated under starvation conditions, defined medium and carbon starvation. Based on its homology, it is possible that IM-01F1 is involved in sterol uptake from the host, which is thought to be necessary since *Phytophthora* species are unable to produce their own sterols (Hendrix, 1970). It is also possible that it plays a role in the secretion of exogenous plant toxins and fungicides, since the homolog in humans is also involved in drug resistance. The precise function of IM-01F1 remains to be elucidated; however, it represents a novel type of ABC transporter that may play a role in the interaction with potato.

Another interesting gene identified in the subtracted library, represented by clone IM-06D11, shows significant homology to cdc6 from *Xenopus laevis* (U66558), a protein that is crucial to the control of DNA replication during the cell cycle (Coleman *et al*., 1996, and references therein). *P. infestans* has to undergo morphological changes to infect the host plant and it has to adapt to the new environmental conditions as well as to grow rapidly to overcome the defence responses of the host. Therefore, cell division control could play a crucial role in the infection process. The gene that we identified is induced in response to the potato leaf and it is also clearly induced *in planta* at early time points of infection. During the life cycle it shows a high up-regulation in the zoospore and cyst stages. Therefore it could be important for the establishment of infection and colonization. Like IM-01F1, it shows an induction in defined medium as well as in response to carbon starvation. Interestingly, IM-06D11 hybridizes to three bands on RNA gel blots, and the largest band predominates in the *in planta* growing mycelium. This could be due to cross-hybridization to a potato gene induced by the infection with *P. infestans*, but this is rather unlikely since no hybridization occurred with potato genomic DNA. The possibility of cross-hybridization to a homologous gene product of *P. infestans* can be also excluded, since on the genomic DNA gel blot analysis IM-06D11 only recognizes one gene. The different RNA bands most likely correspond to different splicing products that may play different roles during the life cycle.

Interestingly, we also identified a clone, IM-008B, that shows homology to a spliceosome-associated factor (*Schizosaccharomyces pombe*, AL023589), which is possibly involved in the provision of specific proteins necessary for the diverse adaptations during colonization of the plant. Importantly, it is mainly expressed in the stages necessary for colonization of the host, such as germinated cysts, mycelium and *in planta* growth, and also in sporangia, suggesting an involvement during infection and colonization.

Of the six genes that were shown to be induced in the induced mycelium system used to create the subtracted library, two also showed a clear *in planta* induction in infected leaf tissue compared with *in vitro* grown mycelium (Figs 4 and 5). The others might be specifically induced by first contact with plant compounds or they might be induced later or, more likely, earlier in the course of infection when their expression in infected leaves was still undetectable. This highlights the value of the induced mycelium system. First, it allowed the production of a differential library containing only *P. infestans* sequences. Second, changes in expression patterns within hours of exposure to the host (or non-host) can be assayed: time points that cannot be accurately analysed in infected leaf tissue because of the small amount of *P. infestans* biomass.

In our screen we have identified a number of genes that are regulated following co-incubation of mycelium with the host leaf. Some of them are mainly expressed in mycelium and *in planta*, indicating a role during colonization. Future work will reveal what role they play in pathogenicity or virulence.

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