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Identification of potato genes induced during colonization by *Phytophthora infestans*

KATINKA BEYER[1], ANDRES BINDER[2], THOMAS BOLLER[1] AND MARGARET COLLINGE[1,3]

[1]*Friedrich Miescher Institute, PO Box 2543, CH-4002 Basel, Switzerland,*
[2]*Syngenta AG, 4002 Basel, Switzerland,*
[3]*Current address: Institute of Plant Biology, University of Zürich, Zollikerstrasse 107, CH-8008 Zürich, Switzerland*

SUMMARY

Suppression Subtractive Hybridization (SSH) was applied in a search for genes induced during the compatible interaction between *Phytophthora infestans* and potato. Using potato leaves that had been treated with benzo(1,2,3)thiadiazole-7-carbothioic acid S-methylester (BTH) as the control tissue, a low redundancy library with a relatively low frequency of the classic plant Pathogenesis-Related (PR) genes was generated. 288 of the clones were screened for induced sequences using Inverse Northern analysis (hybridizing the arrayed clones with radiolabelled cDNA populations). Of the 75 clones that were detectable by this method, 43 appeared to be induced. Eleven of these clones were then analysed by total RNA blot analysis, and elevation of transcript levels during *P. infestans* infection was confirmed for 10 of them. Some of the cDNAs analysed by RNA blot analysis have homology to genes already known to be induced during infection, e.g. to β-1,3-glucanase. Another group of cDNAs have homology to enzymes involved in detoxification: gamma-glutamylcysteine synthetase, cytochrome P450, glutathione S-transferase and an MRP-type ABC transporter. Other infection induced cDNAs encode putative proteins that have not previously been reported to be induced by infection: e.g. the ER-located chaperone BiP, and a homologue of *Aspergillus nidulans* SudD, which was isolated as a suppressor of a mutation in chromosome disjunction. The differential library therefore presents the opportunity to analyse the metabolic changes occurring during infection, and the disease process itself in more detail.

Abbreviations: HR, hypersensitive response; put., putative; hyp., hypothetical; p.i., post-inoculation; BTH, benzo(1,2,3)thiadiazole-7-carbothioic acid S-methyl ester.
Database accession numbers: The new cDNA sequences reported here have been deposited in the EMBL database with the accession numbers AJ302109 to AJ302141.
Correspondence: Dr Margaret Collinge, Friedrich Miescher Institute, PO Box 2543, CH-4002 Basel, Switzerland, E-mail: collinge@fmi.ch

INTRODUCTION

The oomycete *Phytophthora infestans* causes late blight of potato and tomato. Throughout the world, this devastating disease causes severe losses in potato fields every year. Despite intense research on the genetics and physiology of *P. infestans* (e.g. Erwin *et al.*, 1983; Ingram and Williams, 1991) and its interaction with potato (Cuypers and Hahlbrock, 1988; Freytag *et al.*, 1994; Gees and Hohl, 1988; Kamoun *et al.*, 1999), progress in understanding the molecular processes involved in infection and resistance is still limited.

P. infestans is able to penetrate epidermal cells of many different plant species, including nonhost plants, which indicates that the defence responses mainly occur post-penetration (Vleeshouwers *et al.*, 2000). In the early phases of interaction there are only small differences between the compatible and incompatible reactions, if any (Cuypers and Hahlbrock, 1988; Freytag *et al.*, 1994; Gees and Hohl, 1988). Early reactions at the penetration site include rearrangement of the cytoplasm, rapid apposition of callose and hypersensitive response (HR) in the penetrated cell. Differences in the plant's reaction are discernible once the hyphae reach the mesophyll. In the compatible interaction, some cells undergo HR, but the hyphae can escape and continue to colonize the tissue. In the incompatible interaction, a larger number of cells undergo HR and the pathogen is contained and dies.

As part of these defence mechanisms, specific genes have to be temporally and spatially regulated. Several studies demonstrated that the attack of *P. infestans* leads to transcriptional activation of various genes in potato (Avrova *et al.*, 1999; Birch *et al.*, 1999; Choi *et al.*, 1992; Fritzemeier *et al.*, 1987; Schröder *et al.*, 1992; Taylor *et al.*, 1990; Zhu *et al.*, 1995). A comprehensive analysis of induced genes might lead to a better understanding of the molecular processes involved in infection and resistance, as well as potentially contributing to the development of biotechnological strategies for the fight against this disease.

The majority of screens for pathogen-induced genes have targeted the resistant reaction of the host, with the aim of finding factors involved in resistance (e.g. Birch *et al.*, 1999; Jung and Hwang, 2000; Kim *et al.*, 2000). In contrast, we are analysing the

compatible interaction. This analysis of the sensitive reaction complements the existing studies of the resistant reaction, but, more importantly, it allows us to look at gene expression in both pathogen and host and to find out more about the disease process.

Here, we present a screen for genes induced during the compatible interaction between potato and *P. infestans* using Suppression Subtractive Hybridization (Diatchenko *et al.*, 1996). We identified a number of genes that were previously shown to be activated during resistance responses, as well as genes that had not been shown to be induced by pathogens before.

RESULTS

Creation of a subtracted library

We screened for genes that are induced during the compatible interaction between potato and *P. infestans* using the PCR-based method Suppression Subtractive Hybridization (SSH) (Diatchenko *et al.*, 1996). SSH is a powerful technique that produces a library of cDNA clones that are (putatively) differentially expressed in one, tester, mRNA-population compared to a second, control, population. Forty-eight hours post-inoculation (p.i.) was chosen for the screen because it is still relatively early in the disease process, but *P. infestans* is already colonizing the leaf tissue.

Although SSH produces a normalized library, preliminary experiments showed that the pathogenesis-related (PR) gene transcripts that accumulate to very high levels during infection can predominate in the resulting differential library. To prevent this, and to enrich for less abundant transcripts, we used control plant tissue in which PR genes were induced. The control leaves were treated with benzo(1,2,3)thiadiazole-7-carbothioic acid S-methylester (BTH), which induces the same set of PR genes as salicylic acid (Görlach *et al.*, 1996; Ryals *et al.*, 1996). To subtract constitutively expressed *P. infestans* sequences, RNA from the control plant tissue was mixed with RNA from *P. infestans* zoospores to create the control RNA population for the SSH screen.

Analysis of the library content

To determine what proportion of clones in the SSH library represent transcripts that accumulate during infection, we used a similar method to that described by Von Stein *et al.* (1997). In this 'Inverse Northern' procedure, DNA gel blots were made of the PCR amplified cDNA inserts from 288 clones. These DNA blots were hybridized with the ds-cDNA populations derived from the original control and tester samples that were used in the SSH screen. The cDNA was labelled by random-primed labelling, and the resulting radioactive signals were compared. Seventy-five of the 288 analysed clones were detectable on the blots and 43 of these showed a clear induction. Since the sensitivity of this Inverse Northern assay was relatively low, hybridization of the Inverse Northern blots was repeated using another labelling method. For this assay, the cDNA probes were derived from mRNA of leaf tissue taken from intact plants sprayed either with *P. infestans* spores or with water (control) and incubated for 48 h. The probes were radiolabelled by carrying out first strand cDNA synthesis in the presence of labelled dCTP. Using this technique, almost all the clones could be detected, and for most of the clones that showed an induced expression in the first experiment, the induction was confirmed and rate of increase was similar (data not shown).

The cDNA inserts of the apparently induced clones were sequenced and BLAST searches were performed. The results are shown in Table 1. If BLAST analyses did not indicate very strong homologies with plant genes, the origin of the sequences, potato or *P. infestans*, was determined by genomic DNA blot analysis. All the induced clones identified in the Inverse Northern assay originate from potato.

The genes in Table 1 have homology to diverse classes of genes, and can thus be grouped according to their putative function, including: defence and stress, e.g. β-1,3-glucanase and cytochrome P450 monooxygenase (cytochrome P450); transcription, e.g. WRKY DNA binding protein and poly(A) binding protein; signal transduction, e.g. calmodulin; transport, e.g. ABC-transporter and porin; metabolism, e.g. isopropylmalate dehydrogenase; energy, e.g. ubiquinol-cytochrome C reductase complex; and unclear function, e.g. SudD-like protein.

The level of redundancy in the library was low. Only two clones for a β-1,3-glucanase and a cytochrome P450 occurred more than twice among the clones that were positive in the Inverse Northern screen, and a few other clones occurred twice. Five clones, represented by clone S3-12E, were from a basic, class III β-1,3-glucanase, that is homologous to TomQ'b from tomato (Domingo *et al.*, 1994). Six genes derived from two different genes for cytochrome P450s, represented in Table 1 by clones S1-8D and S2-12B.

Expression analysis of selected genes

Expression in response to infection with P. infestans

To test the results of the Inverse Northern assay, the expression of several potentially induced genes was analysed using RNA gel blots. We chose some clones with homology to genes known to be pathogen or stress induced, and other clones with similarity to genes that had not previously been reported to be induced by infection. A series of duplicate RNA blots was made with total RNA extracted from control and infected plants and hybridized with the cDNA clones.

In total we analysed 11 clones on RNA blots, and for 10 of them a clear increase in transcript levels during infection was observed (Fig. 1). One clone (S2-5C) with homology to 2-oxoglutarate dehydrogenase, gave no signal on total RNA blots (not shown).

Table 1 Genes showing an increased expression in potato leaves infected with *Phytophthora infestans*

Clone name	Homology*†	Protein match % Identity/ Probability	Fold induction‡
	Cell rescue/Defence/Cell death and ageing		
S2-12B	Cytochrome p450, *L. japonicus*, AF000403	45%/3E-13	3.4
S1-8D‖	Cytochrome p450, *N. tabaccum*, X95342	85%/4e-65	2.5
S1-2F	Endo-β-1,3-glucanase, *S. tuberosum,* O65787	35%/0.52	1.8
S3-12C	Endochitinase, *S. tuberosum*, X14133	94%/1e-86	3.3
S3-12E**	β-1,3-glucanase, *L. esculentum*, X74906	82%/5e-92	3.6
S2-3G	γ-glutamylcysteine synthase, *L. esculentum*, AF017983	68%/9e-26	4.2
S1-11B	Glutathione-S-transferase, *N. tabaccum*, X56266	92%/4e-94	2.7
S3-6F	Pathogenesis related protein, *H. vulgare*, X16648	43%/1e-30	5.0
S3-7G¶	Pathogenesis related protein, *O. sativa* AC007789	58%/1e-56	4.7
S1-10G	Peroxidase, *L. esculentum*, X71593	84%/2e-38	2.6
	Transcription		
S3-4A	Elongation factor 1-alpha, *L. esculentum*, P17786§	75%/5e-60	2.8
S2-5C	Poly(A) binding protein, *A. thaliana*, L19418	62%/3e-57	3.4
S1-9D	Putative DNA binding protein (WRKY DNA binding protein), *A. thaliana*, AF076243	38%/1e-37	1.4
	Cellular communication/Signal transduction		
S2-7G	Calmodulin 3 protein, *C. annuum*, AJ010645	99%/1e-77	1.3
S1-10C	Lipoxygenase, *S. tuberosum*, U60202	99%/8e/69	1.9
	Transport facilitation		
S1-12A	MRP-like ABC transporter, *A. thaliana*, U92650	60%/4e-47	3.3
	Cellular transport and transport mechanisms		
S2-3A	Mitochondrial porin, *S. tuberosum*, X80386	82%/1e-33	3.9
	Metabolism		
S3-9H	Allantoinase, *R. catesbeiana*, UO3471	41%/2e-18	2.2
S1-7H	Arginine decarboxylase, *L. esculentum*, L16582	84%/1e-72	2.7
S2-5B	3-deoxy-D-arabino-heptulosonate 7-phosphate synthase, *S. tuberosum*, J05191	94%/8e-99	4.1
S3-2B	Geranylgeranyl diphosphate synthase, *D. carota*, AB027706	70%/1e-60	3.6
S2-8D	3-isopropylmalate dehydrogenase, *B. napus*, X59970	87%/3e-70	2.8
S2-2G	2-oxoglutarate dehydrogenase, *A. thaliana*, AJ223802	83%/8e-67	3.7
S3-5H	12-oxophytodienoate reductase, *L. esculentum*, AJ242551	80%/7e-78	2.7
S3-6C	PHZF, *A. thaliana*, AC004044, similar to 3-deoxy-D-arabino-heptulosonate	55%/3e-46	2.8
	7-phosphate synthase, *S. tuberosum*, J05191		
S1-12D	Putative GDP-mannose pyrophosphorylase, *A. thaliana*, AAF21085	87%/2e-89	3.8
	Energy		
S2-6E	Ubiquinol-cytochrome C red. complex, *S. tuberosum*, X79273	94%/1e-35	2.5
	Unclear function		
S2-2H	Cockayne syndrome WD-repeat protein, *H. sapiens*, Q13216§	26%/2e-11	6.8
S3-9B	F12K11.12, *A. thaliana*, AC007592	68%/2e-51	4.7
S2-7H	Luminal binding protein, *N. tabaccum*, X60058	87%/2e-81	4.6
S3-2G	no clear hits		2.7
S1-4D	Putative protein, *A. thaliana*, AL049658	51%/1e-40	2.6
S1-12G	SudD-like protein, *A. thaliana*, AA23014	87%/1e-63	3.2

*Accession numbers referring to the DNA sequence are given, where available.

†Genes were grouped using the same functional classification used for *A.thaliana* MIPS (http://www.mips.biochem.mpg.de).

‡Fold induction calculated by normalizing the expression level in the control against the expression level in the tester cDNA population.

§Accession number refers to protein sequence.

¶Found twice among analysed clones.

‖Found five times among analysed clones.

**Found six times among analysed clones.

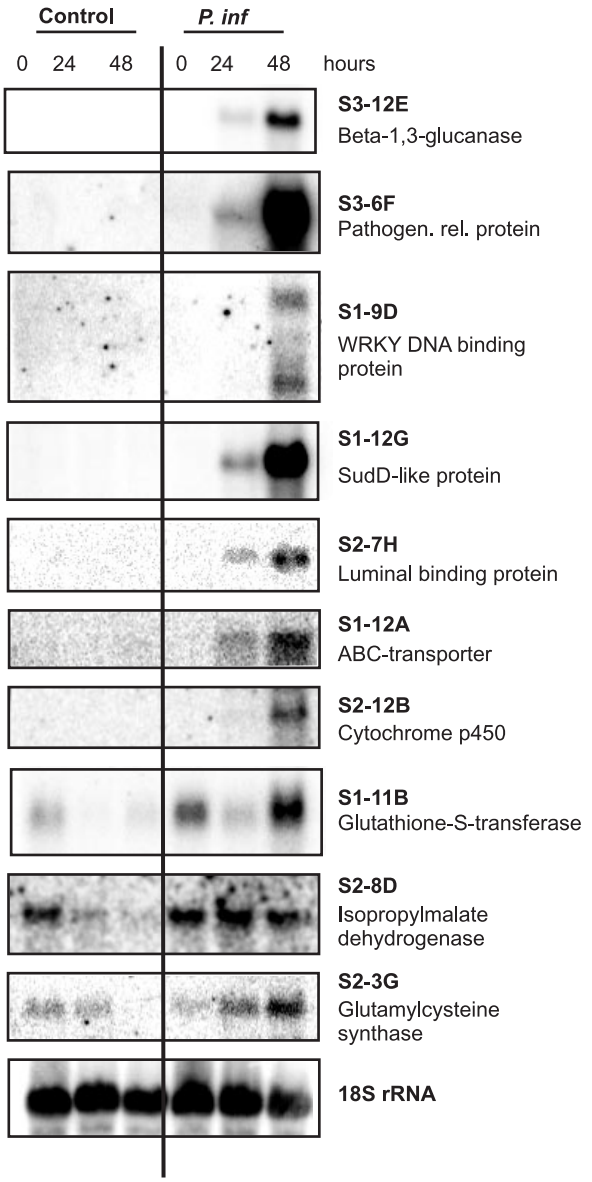

Fig. 1 Analysis of the transcript level of clones from the differential library on RNA gel blots. Intact plants were sprayed with water (control) or with *P. infestans* zoospores and leaves were harvested at the indicated times. Ten μg of total RNA from fully expanded leaves was loaded in each lane.

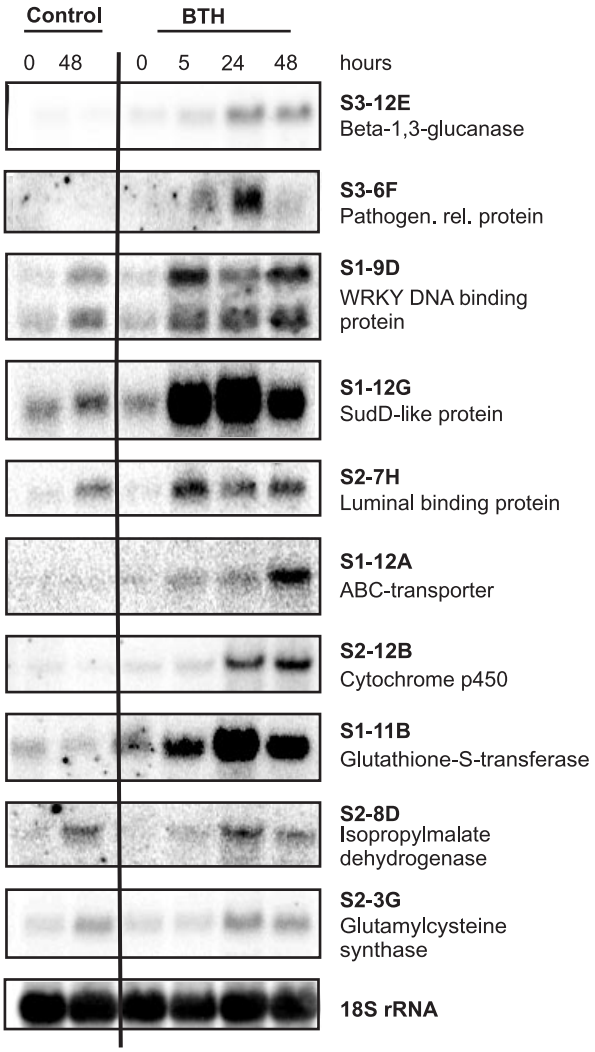

Fig. 2 Changes in transcript levels of the differential library clones in response to BTH. Detached leaves were sprayed with water (control) or BTH and incubated for the indicated times. Ten μg of total RNA was loaded in each lane.

The induced sequences can be assigned to two groups on the basis of their expression patterns. In the first group, S3-12E (β-1,3-glucanase), S3-6F (pathogenesis related protein from barley), S1-9D (WRKY-box transcription factor-like), S1-12G (*Arabidopsis* SudD-like protein), S2-7H (luminal binding protein, BiP), S1-12A (MRP-type ABC transporter), and S2-12B (cytochrome P450), there was no detectable message in controls, but the transcripts were induced one to two days after infection. The second group of clones, S1-11B (glutathione S-transferase, GST), S2-8D (isopropylmalate dehydrogenase), and S2-3G (γ-glutamylcysteine synthase), were expressed at $t = 0$ but the transcript level declined in the control plants over the 2-day period of the experiment. This could have been caused by covering both control and infected plants with Plexiglas boxes to maintain the high humidity required for infection, i.e. it could be due to reduced ventilation, high humidity, or the build-up of volatile substances. In infected leaves, transcript levels at $t = 0$ were as high as in the control but then the level increased (clone S2-3G), stayed high (clone S2-8D) or rose again after dropping (clone S1-11B). Thus, also in this second group, the expression level in infected plants was clearly higher at 48 h, compared to control plants.

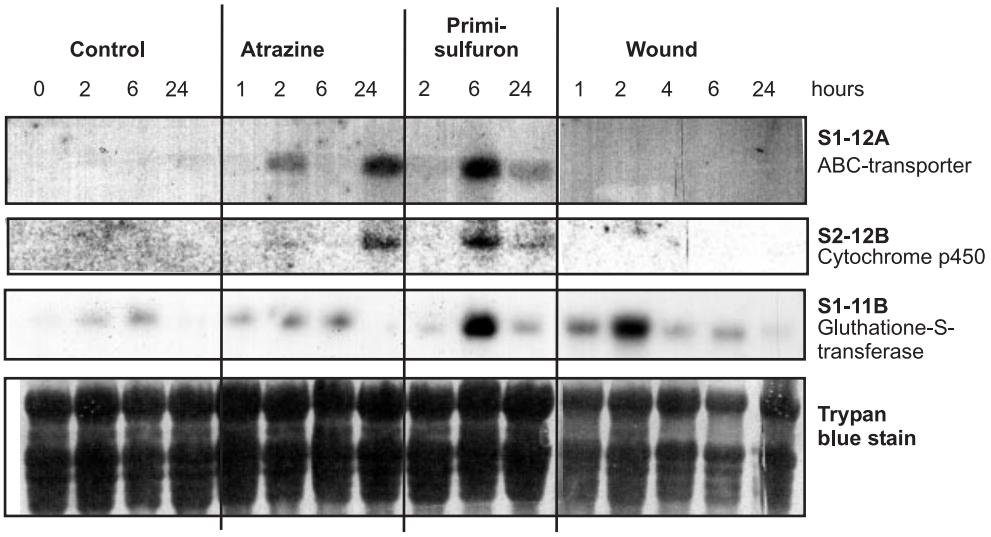

Fig. 3 Transcript levels of S1-12A, S2-12B and S1-11B in plants treated with 1% (v/v) DMSO (control), 500 mg/L atrazine, 20 mg/L primisulphuron, or mechanically wounded. Twenty μg of total RNA was loaded in each lane.

Expression in response to BTH

For the SSH screen, the control leaves were treated with BTH for 48 h and we therefore expected to identify genes that are induced by infection but not by BTH. To test this assumption, the expression of the 10 genes described above was analysed in detached leaves treated with BTH. As shown in Fig. 2, a number of the infection-induced genes, S3-12E, S3-6F, S1-12G, S1-12A, S2-12B and S1-11B, were induced by BTH treatment. However their transcript levels were again declining at 48 h after BTH treatment. In contrast, transcript levels in the infected tissue were high at 48 h (Figs 1 and 2). This probably explains the representation of these genes in the library. These findings are supported by a recently published study of the *A. thaliana* transcriptome during SAR (Maleck *et al.*, 2000). The expression pattern of plants inoculated with an oomycete pathogen resembles the expression pattern of early responses to BTH but not of responses after 48 h of BTH treatment.

Transcript levels of the other genes we analysed were similar at 48 h in both BTH-treated and control leaves, and were higher than in the infection experiment (Fig. 1) at $t = 0$. This increase could be due to detachment of the leaves or their incubation in Petri dishes during 48 h in the BTH experiment. Clone S2-7H was indeed shown to be induced 6 h after wounding (data not shown). This indicates that not all the genes are induced by BTH and it is possible that some of them are regulated by other signal transduction pathways or that they are more strongly induced by the kind of cell damage or stress occurring during a compatible interaction.

Analysis of cytotoxic stress related genes

The increase in transcript levels of genes encoding a γ-glutamylcysteine synthase (S2-3G), a GST (S1-11B), a cytochrome P450 (S2-12B) and an MRP-type ABC transporter (S1-12A) raised the possibility that these genes are involved in a common pathway that transports toxic molecules into the vacuole. The role of γ-glutamylcysteine synthetase is clearly defined, it catalyses the first step of glutathione biosynthesis (Cobbett *et al.*, 1998). However GSTs, cytochrome P450s and MRP-type ABC transporters are all members of gene families, and could also be involved in the biosynthesis of secondary metabolites and their deposition in the vacuole. To find out if these genes are coordinately regulated, and whether they respond to general cytotoxic stress or only to infection, we analysed their transcript levels in wounded plants and in plants that had been treated with herbicides (Fig. 3). Two herbicides with different cellular targets were applied: atrazine, which inhibits photosystem I and causes oxidative stress, and primisulphuron, which inhibits acetolactate synthesis, the first step in the synthesis of valine, leucine and isoleucine.

The ABC transporter (S1-12A) and the cytochrome P450 (S2-12B) do appear to have similar expression patterns: transcript levels increased in response to *P. infestans* (Fig. 1), BTH (Fig. 2), atrazine, and primisulphuron, but not after wounding (Fig. 3). The initial mRNA accumulation in response to atrazine was not apparent at 6 h but reappeared at 24 h. This pattern was not seen with constitutively expressed genes (not shown) or GST (S1-11B), and could reflect a diurnally occurring stress. Such a fluctuation in cytotoxic effects would be consistent with the mode of action of atrazine. It affects chloroplast function and relies on light for its action. The GST represented by clone S1-11B was induced by the same stresses, but the pattern was different, and it was also induced by wounding.

DISCUSSION

In this report we describe a screen designed to isolate a large number of sequences induced during the compatible interaction between potato and the late blight agent, *P. infestans*. By a judicious choice of the control treatment and use of the SSH technique, we have generated a very low redundancy library with a high frequency of positive clones. A special advantage of this library is that the frequency of clones derived from classic PR genes was low. This indicates that the induction of these genes by BTH in the control tissue effectively reduced the proportion of these sequences in the library, allowing the detection of transcripts of lower abundance. Similar approaches have been used before to minimize the representation of PR genes in libraries and to isolate pathogen sequences (e.g. Benito *et al*., 1996), but not in combination with SSH.

The majority of the clones we sequenced have homologies to known genes, at least at the protein level. Those which did not, e.g. S3-2G, contain poly(A) stretches and probably contain little or no coding sequence. A number of clones, e.g. S1-4D and S3-9B, have a strong homology to *Arabidopsis* sequences of no known function. Our observation that these genes are induced by infection may shed light on the function of these probable plant-specific proteins. According to their homology, the majority of the clones could be assigned to two main functional classes: defence and stress, and metabolism. Defence and stress responses are known to be induced when the plant is colonized by a pathogen but also many changes in metabolism are likely to occur, especially in a compatible interaction.

A number of the clones we identified are not only homologues of genes known to be induced during disease resistance responses but are also inducible by BTH, which indicates that they are most likely also induced in an incompatible interaction. Similar results were obtained in a study by Maleck *et al*. (2000), describing that gene expression changes are similar in plants inoculated with either incompatible or compatible isolates of the oomycete *Peronospora parasitica*. This is consistent with the numerous observations that similar responses are involved in both compatible and incompatible interactions, but that their timing and extent can differ (e.g. Birch and Kamoun, 2000; Freytag *et al*., 1994). Furthermore, some pathogenesis-related genes are not only up-regulated in both susceptible and resistant responses but also in some symbiotic relationships (Salzer *et al*., 2000).

There is genetic evidence for the existence of susceptibility factors required in compatible interactions (Vogel and Somerville, 1999). Such genes might be expressed only at low levels or undergo only minor or no changes in transcript levels and would therefore be difficult to detect by differential expression analysis.

Surprisingly, we did not identify any *P. infestans* genes among the clones analysed. The Inverse Northern screen may not be sensitive enough to detect the *P. infestans* sequences in the background of plant transcripts. It is more likely that the *P. infestans* genes are underrepresented in the library, either because the *P. infestans* biomass was still too low compared to the plant biomass in the infected tissue, or because there is a difference in GC content between the genomes. Qutob *et al*. (2000) showed that the GC content of *Phytophthora sojae* DNA is higher than that of its host soybean, and AFLP DNA fingerprinting protocols had to be modified on account of the relatively high GC content of *P. infestans* genomic DNA (Van der Lee *et al*., 1997). This difference in GC content could bias the SSH procedure towards recovery of potato sequences at any or all of the following steps: restriction enzyme digestion of ds-cDNA, differential hybridization, and PCR amplification.

Some of the clones that were chosen to be analysed in more detail in RNA gel blot assays have putative functions that are clearly related to pathogen infection, e.g. defence and stress, and transcription. Others are homologous to genes which have not previously been connected with the response to infection. For almost all the clones that have been analysed in more detail, the induced expression shown by Inverse Northern assay was confirmed by RNA gel blot analysis.

The only classic PR gene whose expression was further analysed is a putative glucanase (S3-12E) that has highest homology with the basic, class III β-1,3-glucanase, TomQ'b, from tomato, which is induced by viroid infection (Domingo *et al*., 1994). Another pathogenesis related gene is represented by clone S3-6F. It is homologous to the PRPX HORVU gene from barley (Jutidamrongphan *et al*., 1989) that was shown to be induced upon infection with *Erysiphe graminis* (Davidson *et al*., 1987).

Clone S1-9D has homology to genes that are involved in the regulation of PR protein genes. It has a strong similarity to WRKY box transcription factors. WRKY1 has been shown to mediate elicitor induced gene expression (Eulgem *et al*., 1999). Recently, it was demonstrated that a putative WRKY protein, *St-WRKY*, is up-regulated in potato in a compatible interaction with *P. infestans* (Dellagi *et al*., 2000).

One of the cytochrome P450 genes identified in the screen (S2-12B) was shown to be induced during *P. infestans* infection. Cytochrome P450s form the largest class of plant enzymes (Werck-Reichhart *et al*., 2000) and are involved in a multitude of reactions in secondary metabolism and in the metabolism of xenobiotics (Schuler, 1996). A number of cytochrome P450s have been shown to be induced by pathogens (e.g. Hull *et al*., 2000; Schopfer and Ebel, 1998), and are involved in the synthesis of secondary metabolites, e.g. phytoalexins. However, cytochrome P450s also play a role in the excretion of toxic substances into the vacuole. During a pathogen infection, many toxic compounds can be formed that have to be transported actively to the vacuole (reviewed in Rea, 1999). Toxic molecules are first activated by a cytochrome P450 and then conjugated to a hydrophilic molecule; often glutathione conjugates are formed by a glutathione S-transferase.

The conjugated form is then actively transported into the vacuole by an ABC-transporter.

Up-regulation of excretion to the vacuole would explain the induction of other genes identified in this screen that are similar to other members of this detoxification pathway. Clone S2-3G is from a γ-glutamylcysteine synthetase, which is involved in glutathione synthesis (Cobbett *et al.*, 1998). The genes responsible for glutathione synthesis are induced by certain heavy metals, as well as by jasmonic acid (Xiang and Oliver, 1998). Here we show, that γ-glutamylcysteine synthetase is induced by *P. infestans,* which could possibly occur via the jasmonate signalling pathway. Clone S1-11B encodes a GST. Clone S1-12A encodes a polypeptide with similarity to MRP type ABC transporters. It has similarity to AtMRP3 (Tommasini *et al.*, 1998), which has been shown to have glutathione-conjugate and chlorophyll-catabolite transport activity.

The precise functions of GSTs, cytochrome P450s and ABC-transporters are difficult to determine, as they are members of gene families. We analysed the expression of the genes we identified after wounding and exposure to two herbicides with different modes of action, atrazine and primisulphuron. The patterns of expression of the cytochrome P450 (S2-12B) and the ABC transporter (S1-12A) were similar, and there was no detectable response to wounding. This would be consistent with their involvement in the same pathway. In comparison, the mRNA level of the GST represented by clone 11B-S1, increased after infection, BTH and xenobiotic stress, but also after wounding. It may therefore have a different or additional role to the other two genes, for instance in the reaction to oxidative stress.

Another gene that may be up-regulated to cope with stress and toxic effects is BiP (clone S2-7H). BiP, also known as luminal binding protein, is a highly conserved protein located in the endoplasmic reticulum (Gething, 1999). It binds transiently to newly synthesized proteins as they enter the ER through the translocon. BiP binds more permanently to misfolded, underglycosylated or unassembled proteins and mediates their transport back out of the ER for degradation by the proteosome, in the Unfolded Protein Response. As such, BiP is a manager of stress in the cell, and its synthesis is induced under stress conditions that lead to the accumulation of misfolded proteins in the ER. BiP of soybean has been reported to be induced by wounding (Kalinski *et al.*, 1995). Here we show that its transcript level also accumulates during pathogen infection, which is consistent with the recently published results of Sticher and Metraux (2000) demonstrating that levels of BiP protein increased in cucumber seedlings infected with a bacterial pathogen *Pseudomonas syringae* pv. *lachrymans.*

One clone that we analysed, S2-8D, is involved in primary metabolism. It has very high similarity both at the DNA and amino acid level to 3-isopropylmalate dehydrogenase, which catalyses the penultimate step in leucine biosynthesis. Here we show that its transcript level is up-regulated by infection and in detached leaves.

Clone S1-12G, which encodes a protein showing homology to SUDD of *Aspergillus nidulans*, is the most enigmatic clone that we analysed. The *sudD* gene was isolated as an extragenic suppressor of the *bimD6* mutation in *A. nidulans* (Anaya *et al.*, 1998). It is evolutionarily conserved and seems to have a role in chromatin regulation. One possible function for the induction of the *sudD* homologue by pathogen infection is the change in chromatin organization required for the dramatic changes in gene expression upon infection. Histone acetyltransferase is the putative target of the HC-toxin of *Cochliobolus carbonum* race 1 in its host maize (Brosch *et al.*, 1995). By inhibiting histone acetyltransferase, the toxin has been proposed to affect chromatin dynamics and thus inhibit the changes in gene expression connected with induced plant defence responses, preventing the plant from mounting an effective response to infection.

In conclusion, the analysis presented here identified novel pathogen and stress induced potato genes that may be important in the response to parasitic threat. Studies of the pathways that these genes are involved in will give more information about the physiology of late blight disease and may elucidate the mechanisms of tolerance. In any case, the analysis of these genes will contribute to a more comprehensive view of the potato–*P. infestans* interaction.

EXPERIMENTAL PROCEDURES

Growth of potato plants and *P. infestans*

P. infestans strains 96F10 and RDA49 were kindly provided by Mrs M.-C. Wybrecht of Novartis Crop Protection, Switzerland and by Dr David Johnson, then at Changins Research Station, Nyon, Switzerland, respectively. They were grown on rye agar at 18 °C. Virulence was maintained by infecting leaves and re-isolating every 3–4 months. Zoospores were released by flooding 10–14-day-old cultures with 10 mL cold distilled water and incubating at 4 °C for 1–3 h.

Axenic potato plants, cultivar Bintje, were kindly provided by Dr Patrick Schweizer (then at the University of Fribourg, Switzerland). The plants were grown from internode cuttings in Murashige and Skoog medium containing 2% (w/v) sucrose, without hormones, for up to 8 weeks. Soil-grown plants were raised from 2-week-old axenic cuttings in a growth chamber with a 16-h light period and temperatures of 17 °C in the night and 20 °C in the day.

Infection of potato plants

Potato plants were infected with *P. infestans* strain RDA49 by spraying until run-off with freshly isolated spores at 1×10^6/mL and maintained in the conditions described above. To maintain the high humidity required for infection, plants were covered with Plexiglas boxes, of which the inner surfaces had been sprayed

with water. Control plants were sprayed with water and covered in the same way. A number of treated plants were kept for 4–5 days to confirm that the infection had been successful.

Treatment with xenobiotics

Atrazine and primisulphuron were obtained from Dr K. Kreuz of Novartis Crop Protection AG. Atrazine was suspended in water at 500 mg/L. Primisulphuron was first dissolved in dimethylsulphoxide (DMSO) at 2 g/L and diluted 100-fold in water to 20 mg/L. Control plants were treated with 1% (v/v) DMSO. The uppermost fully expanded leaves were sprayed until run-off with the appropriate solution in the morning, since atrazine requires light for its herbicidal effects. Leaves from two plants were removed and pooled for each sample. Symptoms were seen after 2 days in the case of atrazine and after ≈ 4 days with primisulphuron.

Differential screen

Suppression Subtractive Hybridization (SSH) was used to create a differential library of clones whose expression may be increased during infection of potato by *P. infestans*. Leaves were excised from 6-week-old plants and placed on moist filter paper in Petri dishes. Tester leaves were inoculated by spraying until run-off with a zoospore suspension of strain 96F10 (≈ 10^6 spores/mL). Control leaves were sprayed with 300 μM benzo(1,2,3)thiadiazole-7-carbothioic acid S-methyl ester (BTH). The leaves were incubated in the growth chamber and harvested after 48 h by freezing in liquid nitrogen. Poly(A) RNA was isolated from the leaves and from the zoospores used for infection using the Poly(A) Pure Kit (Ambion, Witney, Oxon). Poly(A) RNA of the control, BTH treated leaves was mixed with the poly(A) RNA of the *P. infestans* spores in a ratio of 4 : 1 to form the control RNA population for the differential screen. SSH was performed using Clontech's PCR Select cDNA Subtraction Kit with 2 μg of control and tester mRNA, according to the instructions supplied by the manufacturer. The resulting PCR products were cloned into the pGEM-T vector (Promega pGEM-T cloning Kit) and transformed into Ultracompetent SL2-blue MRF′ cells (Stratagene).

Inverse Northerns

The cDNA inserts in the clones 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. The PCR products were then loaded in duplicate on two 2% agarose gels (96 clones per gel) and after separation by gel electrophoresis they were transferred to a HybondN Membrane (Amersham). Two procedures were used to make radioactively labelled cDNA probes. First, Inverse Northerns were performed with radioactive probes, generated by labelling 100 ng each of the control and tester cDNA generated in the SSH procedure using the Prime It II random-primed labelling kit (Stratagene). For a second experiment with the same blots, first strand cDNA synthesis was carried out in the presence of labelled dCTP. Poly(A) RNA was prepared from intact plants 48 h after they had been sprayed with water (control) or *P. infestans* RDA49 spores (infected). The Poly(A) RNA was treated with DNase, and then 1 μg was incubated with 2 μg of random primer (Roche) in 9.5 μL at 70 °C for 10 min. The mixture was cooled on ice, 5 μL 5 × Superscript II buffer, 2.5 μL 100 mM DTT, and 1 μL of dNTP mix (10 mM each dATP, dTTP and dGTP, and 0.1 mM dCTP) were added, and the mixture was incubated at 25 °C for 10 min 5 μL ^{32}P-dCTP (30 TBq/mmol, Amersham Pharmacia Biotech), 1 μL RNasin (Promega) and 1 μL Superscript II Reverse Transcriptase (Gibco BRL) were added and the mixture incubated at 42 °C for 60 min. RNA was removed (Bernard *et al.*, 1996) by adding 1 μL 500 mM EDTA, 1 μL 10% (w/v) SDS and 1 μL 10 N NaOH and incubating at 68 °C for 30 min. After neutralization by addition of 3 μL 2 N HCl, the radiolabelled cDNA was purified using a NICK™ column (Amersham Pharmacia Biotech).

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 DNASIS (Hitachi Software) and the GCG package (Wisconsin Package Version 10, Genetics Computer Group, Madison, WI). Similarity searches were made with BLAST programs at the National Centre for Biotechnology Information (http://www.ncbi.nlm.nih.gov) or at EMBL (http://www.ch.embnet.org/software/aBLAST.html).

RNA and DNA gel blot analysis

A series of duplicate RNA blots was made with total RNA extracted from control and infected intact plants. RNA was extracted as described by Chomczynski and Sacchi (1987). Total RNA samples (10 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 at 65 °C as described in Church and Gilbert (1984).

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