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Potato oxysterol binding protein and cathepsin B are rapidly up-regulated in independent defence pathways that distinguish *R* gene-mediated and field resistances to *Phytophthora infestans*

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SUMMARY

Suppression subtractive hybridization was used to isolate the genes which are specifically up-regulated in the biotrophic phase of the incompatible interaction between a potato genotype, 1512 c(16), containing the resistance gene *R2*, and a *Phytophthora infestans* isolate containing the avirulence gene *Avr2*. Eight cDNAs were up-regulated in the biotrophic phase of the incompatible interaction. Seven of these were also up-regulated in the compatible interaction, but not until late in the necrotrophic phase. Amongst the sequences to be isolated were genes encoding the cysteine protease cathepsin B, *StCathB*, and an oxysterol binding protein, *StOBP1*; equivalent genes are involved in programmed cell death (PCD) processes in animals, but have yet to be implicated in such processes in plants. Whereas *StOBP1* was up-regulated early in potato plants containing either *R* gene-mediated or moderate to high levels of field resistance, the highest levels of up-regulation of *StCathB* were observed early in *R* gene-mediated resistance but gradually increased from the early to late stages of field resistance, revealing these genes to be components of independent defence pathways and providing a means of distinguishing between these forms of resistance. *StOBP1* was up-regulated by oligogalacturonides (plant cell wall breakdown products generated by pectinase activities), indicating that it is also a component of a general, non-specific defence pathway and is unlikely to play a role in PCD. In contrast, the expression of *StCathB* was unaffected by oligogalacturonide treatment, further associating its up-regulation specifically with the gene-for-gene interaction.

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INTRODUCTION

Phytophthora infestans (Mont.) de Bary, the cause of late blight, is the most serious disease of potato, the world's fourth most important food crop. Although chemicals targeted against *P. infestans* provide some level of disease control, world-wide losses due to late blight and measures for its control are estimated to exceed US$5 billion annually. *P. infestans* is thus regarded as a threat to global food security (Duncan, 1999).

Genetic resistance to *P. infestans* in both wild and cultivated species of potato is either race- or non-race-specific (quantitative, field or partial resistance). Race-specific resistance is characterized by interactions between the products of dominant resistance (*R*) gene alleles and corresponding avirulence (*Avr*) alleles to trigger a rapid, localized programmed cell death (PCD) called the hypersensitive response (HR) that prevents further spread of the pathogen. Non-race-specific resistance has been mapped as quantitative trait loci (QTL) in a number of studies, but the molecular basis of this response is poorly understood. The genetics of late blight resistance in potato are reviewed in Gebhardt and Valkonen (2001). Recently, the first potato late blight resistance gene, *R1*, was cloned (Ballvora *et al.*, 2002) but no *Avr* genes have yet been isolated from *P. infestans*, although several have been genetically mapped (reviewed in Birch and Whisson, 2001).

By the 1960s, in potato breeding programmes around the world, it was becoming apparent that *R* genes do not provide durable resistance to late blight. Indeed, when the prevalent race of *P. infestans* in the UK was race 4 (compatible with the *R4* gene), cv. Pentland Dell was released, possessing resistance genes *R1*, *R2* and *R3*. It rapidly succumbed to a new race of the pathogen, and did so in the days when the *P. infestans* population was confined to the A1 mating type, and thus restricted to asexual reproduction

(reviewed in Bradshaw *et al.*, 1995). Since then, breeding programmes have sought to exploit the more durable non-race-specific resistance to this pathogen. Nevertheless, observations suggest that in all forms of resistance, whether race-specific, non-race-specific or non-host, the HR plays a crucial role (Kamoun *et al.*, 1999; Vleeshouwers *et al.*, 2000). Thus, an effective strategy for overcoming late blight might involve the identification and biotechnological exploitation of genes encoding HR effector proteins. Few such genes have been identified to date, possibly due to the complex nature of the HR, which may vary from pathosystem to pathosystem (Heath, 2000).

The potato cultivar Stirling contains both *R* gene-mediated and high-level field resistances to *P. infestans*. In a genotype-by-environment experiment instituted by the Global Initiative on Late Blight (GILB) designed to provide information on the stability of field resistance to *P. infestans*, 14 potato varieties were tested in nine different countries in 1997 and 1998. Cv. Stirling was one of the most resistant varieties at all sites and on all occasions that it was tested (Forbes, 1999). Stirling's *R* gene has been mapped to linkage group (LG) XI (Pande, 2002) and a major QTL for blight resistance to the 76 cM on LG IV, the region of the potato genome which also contains *R2* (Meyer *et al.*, 1998; Pande, 2002).

The induction of host defence responses following challenge with *P. infestans* involves the up- or down-regulation of countless genes, and an initial understanding of many of the molecular events underpinning resistance can thus be gained by studying differential gene transcription during the plant–pathogen interaction (Birch and Kamoun, 2000). In 1999, a powerful PCR-based method, suppression subtractive hybridization (SSH), was used to generate a cDNA library enriched for genes which were up-regulated in the interaction between cv. Stirling and *P. infestans*, but which were not induced in the susceptible interaction with cv. Bintje (Birch *et al.*, 1999). However, the pathogen isolate used triggered both *R* gene-mediated as well as field resistances in cv. Stirling. The SSH may thus have enriched for genes involved in both forms of resistance, as well as the differences in constitutive gene expression that are independent of defence responses between the cultivars Stirling and Bintje.

To identify genes involved specifically in *R* gene-mediated resistance, including candidate genes encoding HR effector proteins, we aimed to make a cDNA library enriched for genes up-regulated in the biotrophic phase of the incompatible interaction between a potato genotype from Black's *R* gene differential set (Black *et al.*, 1953), 1512 c(16), containing the resistance gene *R2*, and a *P. infestans* isolate containing the avirulence gene *Avr2*. In this SSH, driver cDNA used to subtract common gene sequences was derived from the same potato genotype, 1512 c(16), challenged with a compatible isolate of the pathogen. We investigated the expression of selected genes with a putative role in animal PCD processes in other resistant (containing *R* genes or moderate to high levels of field resistance) or susceptible potato plants across a range of times post-inoculation with avirulent and virulent *P. infestans* isolates, to determine whether they had an implied role in both forms of resistance to late blight. We also investigated whether genes up-regulated early only in resistant interactions were also up-regulated by the soft-rot pathogen *Erwinia carotovora* ssp. *atroseptica* (*Eca*). *Eca* lacks gene-for-gene interactions with the cultivated potato, but is a prolific producer of plant cell wall degrading enzymes, particularly pectinases (e.g. Hinton *et al.*, 1989; Palva *et al.*, 1993) that activate general, non-specific plant defences (Davis *et al.*, 1984). We subsequently investigated whether potato genes associated with resistance to *P. infestans* were also up-regulated by individual pectinase enzymes derived from *Eca*, and by oligogalacturonides, the products of pectinase activities, to eliminate such genes as candidate effectors of PCD in the HR.

RESULTS

SSH enrichment for genes up-regulated in *R2*-mediated resistance to *P. infestans*

Suppression subtractive hybridization (SSH) was used to generate a cDNA library enriched for sequences up-regulated in potato *R* gene differential 1512 c(16) (*R2*) inoculated with an incompatible isolate (*Avr2*) of *P. infestans*. The first 24 h post-inoculation (hpi) of a compatible interaction are regarded as the biotrophic phase, as haustoria are readily observed (Vleeshouwers *et al.*, 2000). It can be argued that the pathogen is most vulnerable to the HR in this stage of infection, due to the requirement for living host cells. Therefore, test material comprised double stranded cDNA (dscDNA) synthesized from RNA extracted at 15 h p.i. DscDNA derived from 1512 c(16) 15 h p.i., with a compatible isolate of *P. infestans*, was used as the driver to subtract common sequences.

To test the efficiency of subtraction within the SSH, replicate Southern blots were made with PCR-amplified subtracted cDNAs and unsubtracted cDNAs from both tester and driver. The blots were hybridized with either subtracted cDNAs or unsubtracted driver cDNAs (results not shown). The subtracted probe only hybridized strongly to subtracted material on the filter. Unsubtracted driver cDNAs hybridized strongly to both unsubtracted tester and driver material, indicating that there were cDNAs in common between them. In contrast, this probe hybridized only weakly to subtracted cDNA, indicating the removal of most of these common sequences in the subtraction process, and thus implying enrichment for tester-specific cDNAs.

Only 10 classes of sequence (from 119 successfully sequenced clones) were obtained from the subtracted cDNA library (Table 1), indicating a considerable sequence redundancy and thus enrichment for tester-specific cDNAs. These were termed plbr1 to plbr10 (potato late blight resistance, accession numbers AY450630 to

Table 1 Similarities at the DNA or protein level between cloned cDNA sequences from infected potato cv. 1512 c(16) and sequences in databases.

Sequence name	Size (bp)	Number of clones	Similar sequence from database	Origin of similar sequence and accession no.	Score		DBEST	
					BlastN	BlastX	Origin of matching sequence and accession no.	Score
plbr1	313	5	Catalase (cat1)	*Lycopersicon esculentum* M93719.1	1e-168	1e-58	*Solanum tuberosum* BI432949.1	1e-176
plbr2	655	9	Cathepsin B-like cysteine proteinase	*Nicotiana rustica* X81995.1	1e-102	1e-100	*Solanum tuberosum* BG590588.1	0.0
plbr3	656	29	Putative oxysterol-binding protein	*Arabidopsis thaliana* AY062703.1	8e-13	1e-106	*Lycopersicon esculentum* AW030843.1	0.0
plbr4	219	3	ABC1 protein	*Nicotiana plumbaginifolia* AJ404328.1	2e-32	7e-32		
plbr5	429	16	Cold stress inducible protein (C17)	*Solanum tuberosum* U69633.1	0.0	8e-31	*Solanum tuberosum* BM113608.1	0.0
plbr6	277	8	Shaggy like protein kinase (NtK-1)	*Nicotiana tabacum* X77763.1	3e-87	3e-48	*Solanum tuberosum* BG59833.1	1e-146
plbr7	320	7					*Solanum tuberosum* BI434154.1	3e-39
plbr8	669	7	ATP-dependent protease (CD4B)	*Lycopersicon esculentum* M32604.1	0.0	1e-114	*Solanum tuberosum* BG888730.1	0.0
plbr9	614	33	Putative protein kinase	*Arabidopsis thaliana* AAC06160.1		6e-37		
plbr10	241	2	Unknown protein	*Arabidopsis thaliana* AAF14829.1	6e-05	3e-26	*Lycopersicon esculentum* BG130989.1	1e-121

AY450639). All but two of the sequences were significantly similar to potato or tomato ESTs and were therefore assumed to be from the host and not the pathogen.

Genes up-regulated at 15 h p.i. in the incompatible interaction are not up-regulated until 72 h p.i. in the compatible interaction

Probes derived from clones representative of each of the sequences in Table 1 were hybridized to RNAs extracted from uninoculated 1512 c(16) and from 1512 c(16) 15, 48 and 72 h p.i. with an avirulent isolate (*Avr2*) and 15, 48 and 72 h p.i. with a virulent isolate of *P. infestans* (Fig. 1). For two of the sequences, plbr9 and plbr10, no hybridization to corresponding gene transcripts was detected. The remaining eight probes revealed an up-regulation of corresponding genes at 15 h p.i. only in the incompatible interaction. In contrast, seven of the genes were only strongly up-regulated by 72 h p.i. in the compatible interaction (Fig. 1).

One sequence, plbr2, showed a strong similarity to cathepsin B, which has been implicated in programmed cell death (PCD)

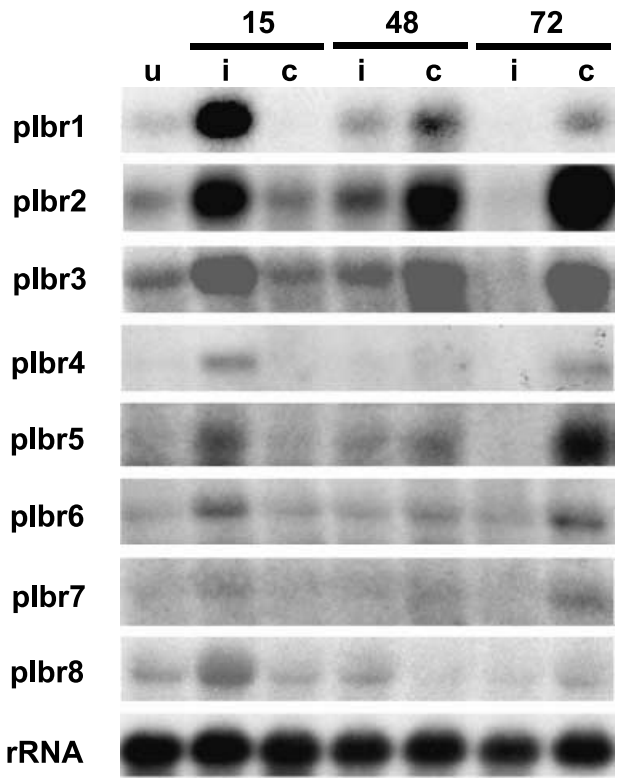

Fig. 1 Northern (RNA) hybridization of the plbr1 to plbr8 cDNA probes, and, as a control ribosomal DNA, to 10 μg of total RNA (each lane) extracted from control uninfected plants of potato clone 1512 c(16), from plants 15, 48 and 72 h post-inoculation with an incompatible race of *P. infestans*, and 15, 48 and 72 h after inoculation with a compatible race of *P. infestans*.

processes in animals (e.g. Foghsgaard *et al.*, 2001). A second sequence, plbr3, showed a strong similarity to oxysterol binding proteins (OBPs), which have also been implicated in PCD processes in animals (e.g. Bakos *et al.*, 1993). To study these sequences in more detail, the 5′ and 3′ ends of the cDNAs from which they were derived were obtained by rapid amplification of cDNA ends (RACE).

The entire cDNA for plbr2 contained an open reading frame of 1062 bp followed by a 3′ untranslated region (UTR) of 188 bp. The deduced polypeptide of 354 amino acids (Fig. 2A) is predicted to have a signal peptide (probability 0.966) with a possible cleavage site between positions 26 and 27 (probability 0.790) (Nielsen *et al.*, 1997). A BLASTX search using the full-length cDNA confirmed its strong protein similarity (expect value e-170) to a cathepsin B-like cysteine proteinase from *Nicotiana rustica.* The potato sequence was thus called *StCathB* (accession number AY450641).

The entire cDNA for plbr3 contained an open reading frame of 1377 bp flanked by 5′- and 3′-UTRs of 8 bp and 154 bp, respectively. The deduced polypeptide is a non-secretory, soluble protein of 459 amino acids (Fig. 2B). A BLASTX search using the full-length cDNA confirmed a strong protein similarity (expect value 0.0) to oxysterol binding proteins from *Arabidopsis* and revealed the OBP amino acid signature motif (Fig. 2B). The potato sequence was thus called *StOBP1* (accession number AY450640).

Expression of *StCathB* and *StOBP1* in potato leaves containing *R* gene-mediated, or different levels of field resistance to *P. infestans*

As the HR has been associated with all forms of resistance to *P. infestans* (Kamoun *et al.*, 1999; Vleeshouwers *et al.*, 2000), expression of the two genes associated with PCD that were up-regulated early in *R2*-mediated resistance, *StCathB, StOBP1*, was also compared between plants containing different *R* gene-mediated resistance and/or high levels of field resistance.

Potato cv. Stirling was crossed with cv. Maris Piper, which lacks *R* gene-mediated resistance and possesses low-level field resistance, to yield 58 F_1 clones with enough tubers for maintenance and blight testing. The clones were assessed for both types of resistance in glasshouse tests with incompatible (race 1, 4) and compatible (race 1, 2, 3, 4, 6, 7) isolates of *P. infestans*. Figure 3 summarizes the results. Thirty-three clones were found to possess cv. Stirling's *R* gene and 25 lacked it. Field resistance levels, assessed using the compatible isolate of the pathogen, followed a normal distribution, from 1 (susceptible) to 7 (highly resistant), across the clones, with cv. Maris Piper scoring 2.25 and cv. Stirling 7.25. One clone (no. 29) was selected as possessing *R* gene-mediated resistance but with low-level (2.0) field resistance. Two clones (nos. 53 and 61) were selected as lacking cv. Stirling's *R* gene but possessing moderate (4.5) and high-level (6.0) field resistance, respectively (indicated on Fig. 3). Field resistance was checked in a second glasshouse test the following year, giving scores of 3, 6.5 and 7, respectively, for clone nos. 29, 53 and 61 and scores of 3 and 8.5 for Maris Piper and Stirling, respectively. In this way, clones were identified which discriminated between the two forms of late blight resistance in cv. Stirling.

StCathB and *StOBP1* probes were hybridized to RNAs from uninoculated cv. Stirling, clone nos. 29 and 1512 c(16), and each of these plants 12, 24, 48 and 72 h p.i. with an incompatible isolate of *P. infestans* (activating *R* gene-mediated resistance); to RNAs from uninoculated clone nos. 53 and 61 and from each of these plants 12, 24, 48 and 72 h p.i. with the same *P. infestans* isolate (triggering field resistance); and to RNAs from uninoculated cv. Maris Piper and cv. Bintje and from each of these plants 12, 24, 48 and 72 h p.i., again with the same isolate of the pathogen (compatible interactions). To aid the visualization of expression patterns, Northerns were densitometrically scanned and relative hybridization intensities for each gene, normalized by comparison to ribosomal DNA hybridization, were plotted as histograms (shown above each Northern in Fig. 4). In *R* gene-mediated resistance, *StOBP1* was clearly up-regulated at 12 h p.i. and *StCathB* followed a cyclic pattern of expression, with maximum up-regulation at 12–24 h p.i. followed by further up-regulation at 72 h p.i. (Fig. 4A). In the earlier Northern using 1512 alone (Fig. 1), strong up-regulation of *StCathB* was only observed at 15 h p.i. in the incompatible interaction. However, it was noted, possibly due to different environmental conditions (day length due to time of the year, in particular, effecting the growth and response of the plants) that the infection was proceeding more rapidly in the earlier experiments used for Fig. 1. In the field resistant plants, maximum up-regulation of *StOBP1* was again occurring at 12 h p.i. (Fig. 4B). However, in these plants, *StCathB* showed a gradually increasing up-regulation from 12 to 72 h p.i. (Fig. 4B). Both sequences were not up-regulated until 48–72 h p.i. in the compatible interactions (Fig. 4C).

StOBP1 is up-regulated when potato leaves are challenged with *Erwinia carotovora* ssp. *atroseptica*, pectin degrading enzymes and oligogalacturonides

The bacterial potato pathogen *Erwinia carotovora* ssp. *atroseptica* (*Eca*) lacks gene-for-gene interactions with potato but is a prolific producer of cell wall degrading enzymes leading to the elicitation of general defence responses (Davis *et al.*, 1984). Thus, genes up-regulated by challenge with this pathogen are likely to be components of a general defence pathway. The expression of *StCathB*, *StOBP1* and, for comparison, plbr1 (similar to catalase) was investigated in cv. Stirling leaves at 3, 5 and 7 h p.i. with *Eca*. Expression was compared with that in control leaves treated with 10 mM $MgSO_4$ solution lacking *Eca*. To aid the visualization of expression patterns, Northerns were again densitometrically

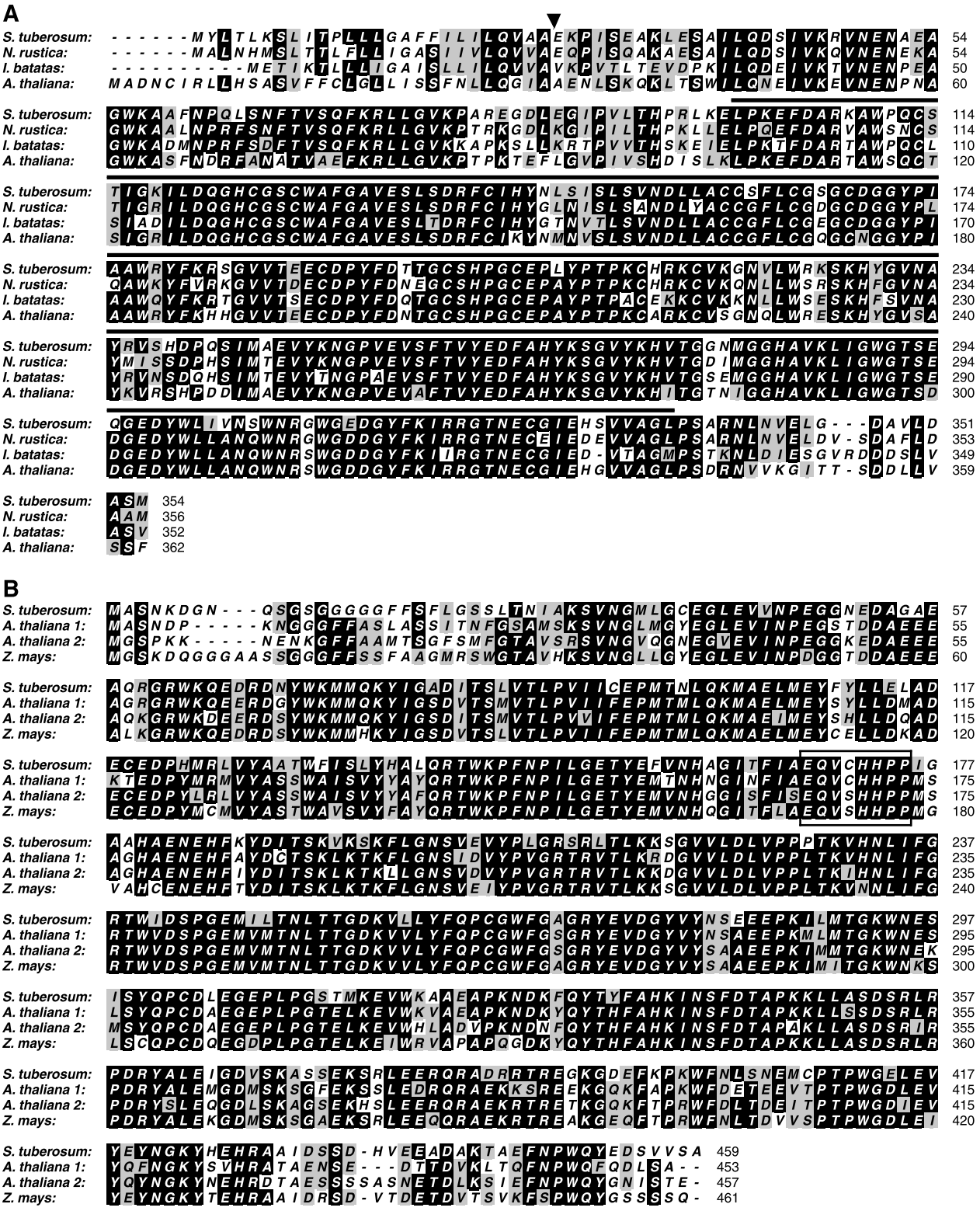

Fig. 2 Amino acid alignments, using ClustalW, of StCathB and StOBP1 with closely related proteins in databases. (A) StCathB (*S. tuberosum*) aligned with cathepsin B-like proteins from *Nicotiana rustica* (accession no. X1995), *Ipomoea batatas* (AF101239) and *Arabidopsis thaliana* (NM_100111), showing a likely signal peptide cleavage site (▼). The cysteine protease domain is over-lined. (B) StOBP1 (*S. tuberosum*) aligned with oxysterol binding protein-like proteins from *Arabidopsis thaliana* (1 and 2: accession nos., respectively, NM_120288 and NM_111764.3) and *Zea Mays* (AY103776) showing the OBP signature motif (boxed).

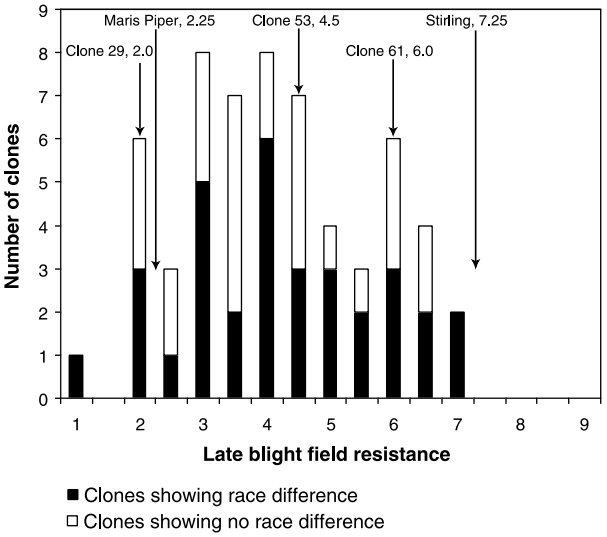

Fig. 3 Field resistance of the Stirling × Maris Piper progeny. The graph shows the number of progeny (*y*-axis) with field resistance levels (*x*-axis) from 1 to 8 on the 1 to 9 scale of increasing resistance (NIAB, 2003) following challenge with the *P. infestans* isolate 36.4.3, race 1, 2, 3, 4, 6, 7 (lacking a gene-for-gene interaction with cv. Stirling). The field resistances of Maris Piper (2.25), Stirling (7.25) and clone nos. 29 (2), 53 (4.5) and 61 (6.0) from the first year of field tests are indicated. Shaded portions represent the number of progeny showing an incompatible response to a *P. infestans* isolate that activates the *R* gene-mediated resistance in Stirling, and unshaded progeny lack any such gene-for-gene interactions.

scanned and relative hybridization intensities for each gene, normalized by comparison to rDNA hybridization, were plotted as histograms (shown above each Northern in Fig. 5). At each of the time points, a small but detectable increase in the expression of *StOBP1* was observed in leaves treated with *Eca* compared to control leaves (Fig. 5A). In contrast, at 5 and 7 h p.i., the plbr1 probe only detected up-regulation of a corresponding catalase gene in control leaves, suggesting that the salt solution alone induces expression, and that such induction is inhibited in the presence of *Eca* (Fig. 5A). The expression of *StCathB* was unaffected by either *Eca* challenge or salt treatment (results not shown).

Amongst the cell wall degrading enzymes produced by *Eca*, pectinases in particular act not only as virulence determinants but also induce a variety of plant defence responses (Dellagi *et al.*, 2000a, 2000b; Hinton *et al.*, 1989; Palva *et al.*, 1993). Oligogalacturonides (OGAs) generated by pectin breakdown induce resistance to *Eca* in potato (Wegener *et al.*, 1996) and have been shown to up-regulate defence-associated genes (e.g. Montesano *et al.*, 2001). Given that *Eca* up-regulates *StOBP1*, we investigated whether individual cell wall degrading enzymes could also induce the expression of this gene. Cv. Stirling leaves were infiltrated with acellular extracts from recombinant *Escherichia coli* containing equivalent activity (0.5 A_{265} /min/mL) of pectate lyase

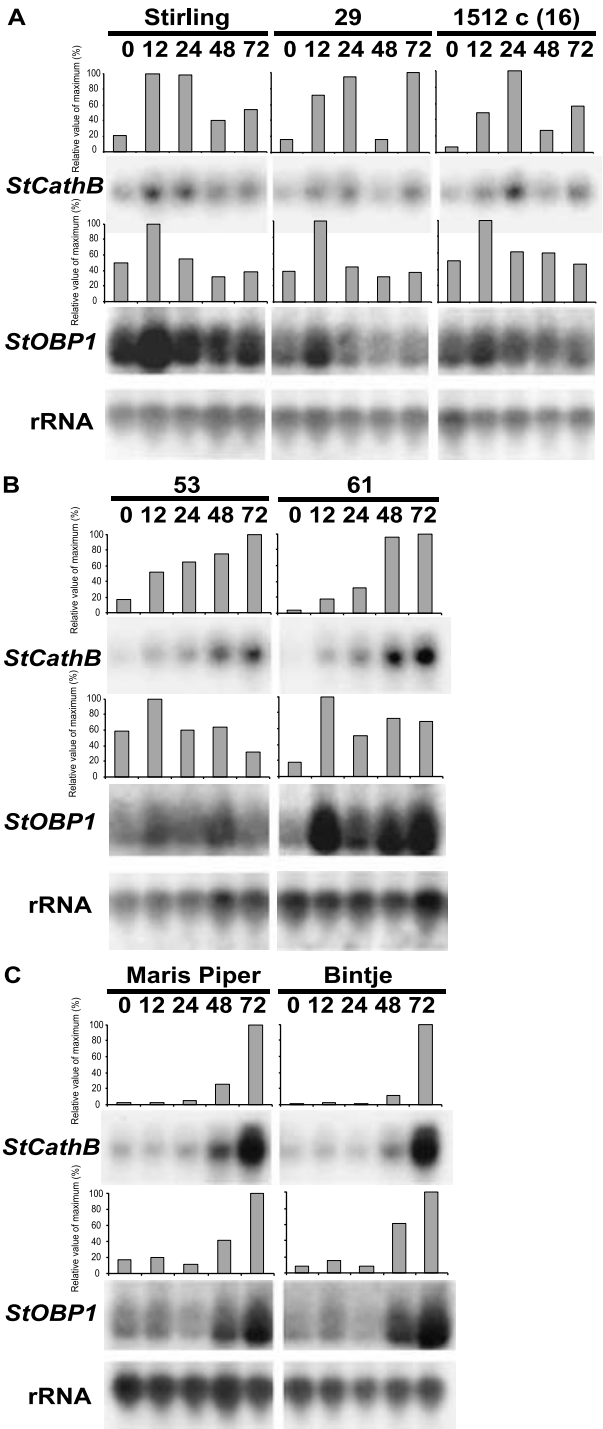

Fig. 4 Northern (RNA) hybridization of the *StCathB* and *StOBP1* probes and, as a control, ribosomal DNA, to 10 μg of total RNA (each lane) extracted from leaves of (A) *R* gene containing potato cv. Stirling, clone no. 29 and clone 1512 c(16). (B) field resistant clone no. 53 and clone no. 61, and (C) susceptible cv. Maris Piper and cv. Bintje, in each case uninfected (0) and 12, 24, 48 and 72 h post-inoculation with an incompatible race of *P. infestans*. The relative intensities of hybridizing signals for *StCathB* and *StOBP1*, normalized by comparison to ribosomal DNA hybridization after densitometric scanning and analysis using BioImage Intelligent Quantifier, are plotted as histograms above the relevant Northerns.

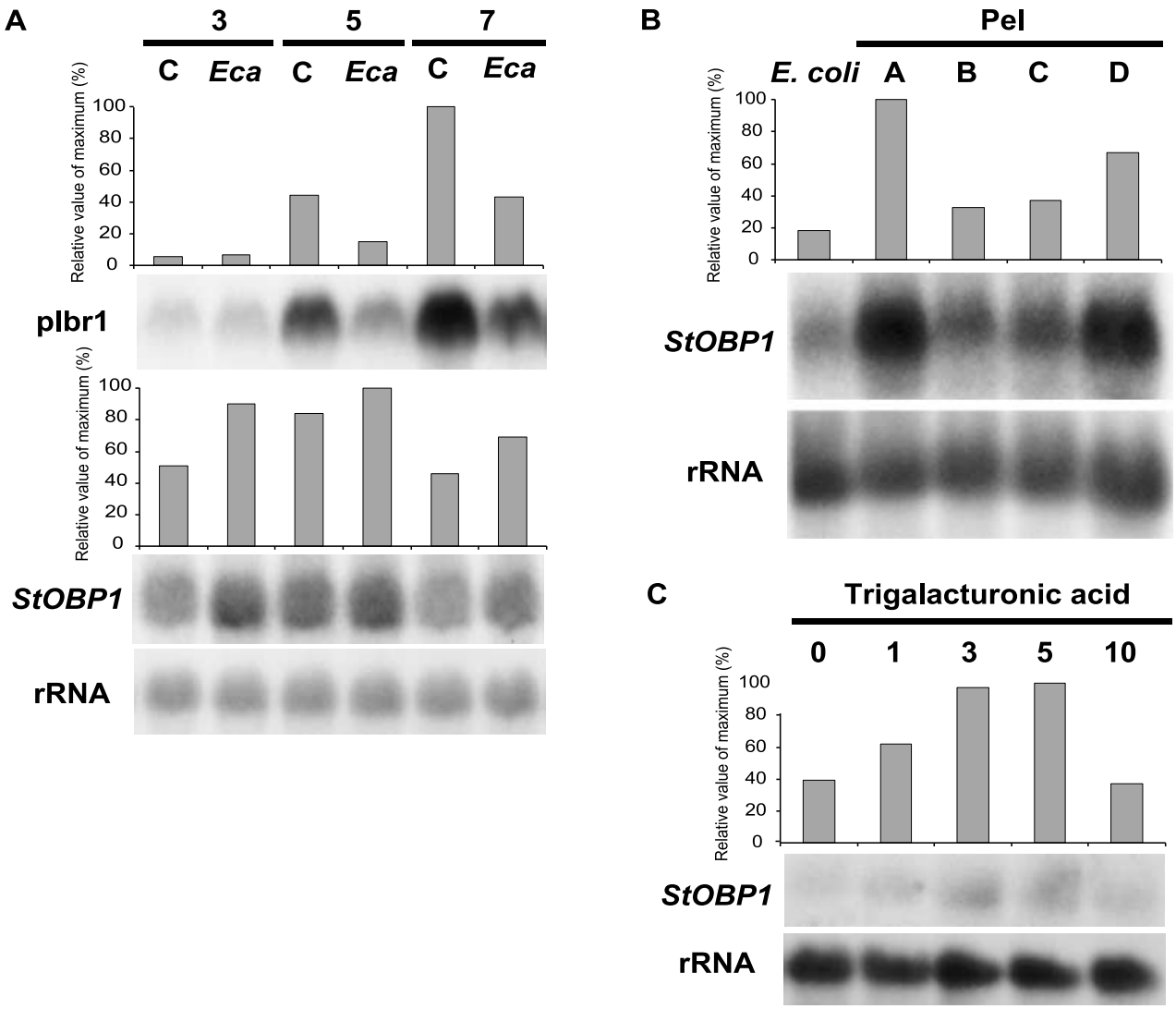

Fig. 5 (A) Northern (RNA) hybridization of the plbr1, *StOBP1*, and, as a control, ribosomal DNA, to 10 μg of total RNA (each lane) extracted from (A) leaves of potato cv. Stirling 3, 5 and 7 h after vacuum infiltrated with $MgSO_4$ (C), and *Erwinia carotovora* ssp. *atroseptica* (*Eca*). (B) Northern hybridization with *StOBP1* and ribosomal DNA to RNA prepared from leaves vacuum infiltrated with culture filtrates from non-recombinant *Escherichia coli* and recombinant *E. coli* expressing the *Eca* genes *PelA*, *PelB*, *PelC*, *PelD*. (C) Northern hybridization with *StOBP1* and ribosomal DNA to RNA prepared from leaves 0, 1, 3, 5 and 10 h post-treatment (vacuum infiltration) with 0.1 mM trigalacturonic acid. The relative intensities of hybridizing signals for plbr1 and *StOBP1*, normalized by comparison to ribosomal DNA hybridization after densitometric scanning and analysis using BioImage Intelligent Quantifier, are plotted as histograms above the relevant Northerns.

(Pel) enzyme produced by *PelA*, *PelB*, *PelC* or *PelD* genes cloned into high-copy-number plasmids. Northern analysis 1 h after infiltration revealed a strong up-regulation of *StOBP1* by PelA and PelD, whereas PelB and PelC only weakly up-regulated *StOBP1* relative to the supernatant from non-recombinant *E. coli* (Fig. 5B). Although a range of enzyme dilutions and time points after infiltration would be required to fully characterize the Pel-responsive induction of *StOBP1*, this preliminary result clearly indicates that either specific recognition of PelA and PelD, or the classes of OGA molecules they generate 1 h after infiltration, contributes to triggering *StOBP1* expression.

Finally, expression of *StOBP1* was investigated in response to OGA treatment. RNA was extracted from cv. Stirling leaves untreated and 1, 3, 5 and 10 h post-treatment with trigalacturonic acid. Hybridization with the *StOBP1* probe revealed a gradual up-regulation of this gene across the first 5 h post-treatment (Fig. 5C).

DISCUSSION

Suppression subtractive hybridization was used to isolate cDNAs specifically up-regulated at 15 h p.i. in the incompatible interaction between potato and *P. infestans* following activation of *R2*-

mediated resistance. From 119 clones in the subtracted library, only 10 independent cDNA sequences were observed, implying a considerable enrichment in the subtraction process. Northern hybridizations with eight of these cDNAs confirmed the up-regulation of corresponding genes by 15 h p.i. in the incompatible interaction. Seven of the sequences were also up-regulated in the compatible interaction, but not until 48–72 h p.i. Two of the clones (plbr9 and plbr10) were not detected in Northern experiments, possibly due to the very low levels of expression. One of these, plbr9, was the most abundantly enriched sequence in the SSH. Nevertheless, it has been noted before that SSH, which uses PCR to enrich target cDNA fragments, is more than 1000-fold more sensitive than Northern blots (Diatchenko *et al.*, 1996). Real-time RT-PCR may be needed to assess the expression of these genes.

P. infestans is a haemibiotrophic pathogen. During the first 24 h p.i. of a compatible interaction, haustoria indicative of biotrophy are readily observed (Vleeshouwers *et al.*, 2000). From this period onwards, the pathogen enters a highly destructive necrotrophic phase of infection. It has been argued that biotrophic pathogens, requiring living plant cells for their survival, must not only suppress host cell death but also prevent the defence responses that living cells readily induce (Heath, 2000). Indeed, Doke *et al.* (1998) have proposed the production of a death-suppressing glucan by *P. infestans*. It is therefore interesting that the genes in this study that are rapidly activated in *R* gene-mediated resistance are also up-regulated during the necrotrophic phase of the compatible interaction, when there is possibly no longer a requirement for suppressing or avoiding host cell death. The question is: does *R* gene-mediated resistance induce genes that are being actively suppressed by the pathogen during biotrophy, or does it simply cause the earlier induction of genes that are actively up-regulated by the pathogen during the necrotrophic phase, when there may be a 'desire' for host cell death? The Guard hypothesis would support an active role for the pathogen in the biotrophic phase, producing virulence proteins that, through direct interaction with virulence targets in the host, suppress host defences (Dangl and Jones, 2001; Mackey *et al.*, 2003).

In 1999, SSH was used to generate a cDNA library enriched for genes up-regulated in the interaction between cv. Stirling and *P. infestans*, but which were not induced in the susceptible interaction with cv. Bintje (Birch *et al.*, 1999). None of the 10 cDNAs identified in the current study were obtained by this previous SSH. However, not only were both field and *R* gene-mediated resistances activated in Stirling in the earlier study, in contrast to only *R* gene-mediated resistance in this study, but also a different time-point after inoculation was selected. Moreover, in the current study a highly stringent subtraction was performed, using a ratio of driver : tester cDNA of 600 : 1, compared to the ratio of only 40 : 1 used in the previous study. More sequencing of clones from the SSH library prepared in 1999 (Birch *et al.*, 1999) may reveal some of the sequences identified in this study.

R2-mediated resistance to *P. infestans* is characterized by a rapid HR, implying the activation of genes involved in programmed cell death (PCD). One sequence with a role in PCD in animals is the lysosomal cysteine protease, cathepsin B (Castino *et al.*, 2002; Foghsgaard *et al.*, 2001; Guicciardi *et al.*, 2001; Nakayama *et al.*, 2002) and a potato gene encoding cathepsin B, *StCathB*, was isolated in this study. Cysteine protease involvement in the HR in plants has been reported (D'Silva *et al.*, 1998; Del Pozo and Lam, 1998; Solomon *et al.*, 1999) and up-regulation of a cathepsin K-like gene in the incompatible potato—*P. infestans* interaction was described by this group (Avrova *et al.*, 1999). D'Silva *et al.* (1998) demonstrated the cleavage of poly (ADP-ribose) polymerase (PARP) by cysteine proteases present in the cowpea HR. Interestingly, cathepsin B has been shown to cleave PARP during animal PCD (Gobeil *et al.*, 2001).

A second family of genes with a role in PCD in animals are the oxysterol binding proteins (OBPs) and a potato gene encoding an OBP, *StOBP1*, was isolated in this study. Oxysterols are oxygenated derivatives of cholesterol that influence a variety of biological functions, including sterol metabolism, sphingolipid metabolism, lipid trafficking, apoptosis and necrosis in mammals (e.g. Anniss *et al.*, 2002; Panini and Sinensky, 2001; Schroepfer, 2000). Oxysterol-induced cell death in animals correlates with the activity of OBPs (Bakos *et al.*, 1993). Oxysterol binding proteins (OBPs) are nuclear receptors that also function as transcription factors, co-ordinately regulating sterol catabolism, storage, efflux and elimination (Repa *et al.*, 2002).

Although a role for OBPs in plant defence responses remains to be demonstrated, there is a connection between the sterol-binding capacity of elicitins, small cysteine-rich proteins secreted by *Phytophthora* spp., and their ability to activate defence responses, including the HR, in tobacco (Blein *et al.*, 2002; Osman *et al.*, 2001). Elicitins have been proposed to act as lipid transfer proteins (LTPs) and must form an elicitin–sterol complex before binding to a plant plasmalemma receptor and activating the HR (Osman *et al.*, 2001; Ponchet *et al.*, 1999). Recently, a role for plant LTPs in defence has been demonstrated (Maldonado *et al.*, 2002). The elicitin–tobacco interaction may offer an excellent model for study of the role of OBPs in plant defence, as they may either regulate levels of sterol available to LTPs/elicitins, or be activated downstream of sterol–elicitin/LTP complexes to, in turn, regulate defence processes.

Although a major feature of the HR is a rapid, localized cell death, many defence-related genes that are not involved in cell death are also activated and, indeed, may often play a more crucial role in preventing the further spread of a pathogen (Heath, 2000). It was thus interesting that one of the potato genes activated early in the *R2*-mediated resistance response showed a significant similarity to a gene encoding an ABC transporter in *Nicotiana plumbaginifolia*, *NpABC1*, which is involved in the secretion of an antifungal terpenoid (Jasinski *et al.*, 2001).

The expression patterns of the two cell death-associated genes, *StCathB* and *StOBP1*, demonstrated them to be components of independent defence pathways that allow discrimination between *R* gene-mediated resistance and the field resistance in cv. Stirling. Crucially, the highest levels of expression of *StCathB* were at 12–24 h p.i. in *R* gene-mediated resistance, whereas in field resistance, the gene was gradually up-regulated from 12 h p.i. to its highest level at 72 h p.i. If this gene is involved in the PCD process of the HR, its expression agrees with the phenotypic observations of a rapid, localized HR in *R* gene-mediated resistance and a slowly developing lesion and trailing HR in field resistance. Its strong up-regulation by only the necrotrophic phase (48–72 h p.i.) of the compatible interaction also correlates with the occurrence of PCD at this stage of infection. This has been observed in compatible interactions for many pathosystems (e.g. Heath, 2000). Thus, *StCathB* provides a gene expression marker for the presence of either *R* gene-mediated or field resistance in the progeny of Stirling crosses, a useful tool for the rapid assessment of late blight resistance in a breeding programme.

The *StOBP1* gene was shown to be up-regulated by the soft rot pathogen *Eca*, by pelA and pelD enzymes, and by trigalacturonic acid, a plant cell wall breakdown product derived from pel activity. It can therefore be assumed to be a component of a general, non-specific defence pathway in potato. The failure to detect an up-regulation of this gene until the later stages of the compatible interaction could be due to the active inhibition of this defence pathway by the pathogen during the biotrophic phase, as it is likely that non-specific elicitors, such as OGAs, will be generated during this phase of infection. As *Eca* lacks gene-for-gene interactions with potato, the up-regulation of *StOBP1* by *Eca* may indicate that it is unlikely to play a role in PCD in the HR. In contrast, *StCathB* was not up-regulated by *Eca* and remains a strong candidate effector in the HR.

Further analyses of the *StCathB* and *StOBP1* genes, including over-expression or silencing in model plants, or in potato, as technologies such as virus induced gene silencing improve, will reveal their roles in resistance to late blight and/or in general plant PCD processes. In conclusion, SSH has been successfully applied to identify the genes up-regulated early in *R* gene-mediated resistance to late blight. It could also be used to identify the genes which are specifically up-regulated in either *R* gene or field resistances by further subtractions between clones of the Stirling × Maris Piper cross that distinguish between these forms of defence.

EXPERIMENTAL PROCEDURES

Plants, *P. infestans* and *P. infestans* inoculation

To isolate the genes up-regulated by *R* gene-mediated resistance to *P. infestans*, a genotype from Black's *R* gene differential series (Black *et al.*, 1953), clone 1512 c(16) (*R2*), was inoculated with a race 1, 4 isolate (*Avr2*) or a race 1, 2, 3, 4, 6, 7 isolate (virulent) of *P. infestans*. The race 1, 4 isolate was also used for incompatible interactions with cv. Stirling, which has high levels of field resistance (rating 8 on the 1–9 scale of increasing resistance; NIAB, 2003) and an uncharacterized *R* gene (Meyer *et al.*, 1998; Pande, 2002), clone no. 29 (containing Stirling's *R* gene but with a field resistance of only 2, comparable to the susceptible cv. Bintje) and clone nos. 53 and 61 (lacking Stirling's *R* gene but with field resistances of 4.5 and 6.0 [moderate to high], respectively); all clones were from an F_1 population resulting from a cross between cv. Stirling (female parent) and cv. Maris Piper (male parent) made in 1994. The race 1, 4 isolate was also used in compatible interactions with cv. Maris Piper and cv. Bintje (both lacking *R* genes, and with NIAB field resistance scores of only 4 and 2, respectively). To allow for variation in response, three glasshouse grown plants of each clone or cultivar were sprayed with a suspension of zoospores as described by Stewart *et al.* (1983) and the leaf material was pooled prior to RNA extractions. In each case, RNAs from uninoculated leaves were prepared as controls. Additional plants (inoculated and uninoculated) were kept as controls for 7 days after inoculation and gave the expected interaction phenotypes.

The F_1 population resulting from the cross between Stirling and Maris Piper was screened for *R* gene resistance and for field resistance in a glasshouse test in the summer of 1999, and repeated in a second glasshouse test in 2000. *R* gene resistance was assessed using the race 1, 4 isolate that is incompatible on Stirling, and the race 1, 2, 3, 4, 6, 7 isolate that is compatible (does not trigger the *R* gene resistance). Glasshouse tests to assess *R* gene resistance and field resistance in Maris Piper, Stirling and the 58 F_1 clones involved the inoculation and testing of four plants each in a randomised complete block design with four replicates (i.e. one plant per replicate) as described in Stewart *et al.* (1983), using 5×10^4 zoospores/mL. The first two replicates were inoculated with race 1, 2, 3, 4, 6, 7 and the second two replicates with race 1, 4. The plants were placed in a 100% r.h. cabinet at an ambient temperature of about 18 °C at least 4 h prior to inoculation. The plants were sprayed with 150–200 mL of zoospore suspension using a hand sprayer and left at 100% r.h. for 24 h post-inoculation before being transferred to a north-facing glasshouse with cooling, where they were left at 15 °C for 7 days before scoring. These conditions were also used for glasshouse inoculations for RNA extractions for the SSH and Northern analyses (paragraph above).

Bacterial strains, production of pectin degrading enzymes and treatments with oligogalacturonides

The strains used in this study were the wild-type *Eca* SCRI1039 (Hyman *et al.*, 1997) and the *Escherichia coli* TG1 described in Hinton *et al.* (1989) with plasmids pH5 (*pelA* cloned into pBR322), pB6 (*pelB* cloned into pUC8), pJS619 (*pelC* cloned into pUC19)

and pJS616 (*pelD* cloned into pUC19). Bacteria were grown in LB medium supplemented with ampicillin (100 μg/mL when necessary). Potato cv. Stirling was used for assessing the expression of genes in response to challenge with *Eca*. Detached leaves were vacuum infiltrated with a suspension of *Eca* at 10^8 cfu/mL in 10 mM $MgSO_4$ for 15 min, and incubated at 18 °C for 3, 5 or 7 h before freezing in liquid nitrogen and storing at −80 °C. Control RNA extractions were made at the same time-points following vacuum infiltration with 10 mM $MgSO_4$ alone. The acellular Pel enzyme extracts were prepared as described in Dellagi *et al.* (2000a). Stirling leaves were vacuum infiltrated with Pel extracts as described in Dellagi *et al.* (2000a). Stirling leaves were vacuum infiltrated with 0.1 mM trigalacturonic acid (Sigma-Aldrich) and incubated at 18 °C under strong light. For each of the experiments described in this section, RNA was extracted from two leaves pooled from four plants for each time-point (0, 1, 3, 5 and 10 h post-treatment). Reproducible Northern results were obtained (two independent replicates) in all cases.

Generation of a subtracted cDNA library using suppression subtractive hybridization (SSH)

Leaves from three clone 1512 c(16) plants 15 h post-inoculation (hpi) with avirulent *P. infestans* and three plants 15 h p.i. with virulent *P. infestans* were ground in liquid nitrogen and total RNA was extracted using an RNeasy Plant Mini Kit (Qiagen, Hilden, Germany) and subjected to DNase treatment (Ambio, Austin, TX) according to the manufacturer's instructions. First-strand cDNA was generated from total RNA using a First Strand cDNA Synthesis Kit (Amersham Pharmacia Biotech, Little Chalfont, UK). Second-strand cDNA synthesis was carried out in a total volume of 100 μL containing 31 μL first-strand cDNA, 1 × second-strand buffer (40 mM Tris-HCl, pH 7.2, 90 mM KCl, 3.0 mM $MgCl_2$, 3.0 mM DTT, 5.0 ng BSA), dNTP's Mix (200 μM), 25 U DNA Polymerase I (Promega) and 1 U RNase H (Promega). Samples were incubated at 14 °C for 16 h. Reactions were stopped by incubating at 70 °C for 10 min. T4 DNA Polymerase (10 U) (Promega) was added to the reaction and incubated for 20 min at 12 °C followed by heat-denaturation of the enzyme for 10 min at 75 °C.

After dscDNA synthesis, SSH was performed with cDNA from the compatible interaction ('driver') and the incompatible interaction ('tester') using the a PCR-Select™ cDNA subtraction kit (Clontech, Palo Alto, CA) according to the manufacturer's instructions, but with a ratio of 450 : 1 for the driver to tester material in the primary hybridization.

Following an assessment of the cDNA enrichment in the SSH process (see below) PCR amplified SSH products were purified and cloned into pGEM-T Easy vector (Promega) followed by electroporation into electromax DH10B cells (Invitrogen Life Technologies, Auckland, New Zealand). Recombinant transformants were transferred into 384-well plates (AB Gene) containing 80 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 22–24 h at 37 °C.

Assessment of the efficacy of subtraction in the SSH

Southern-based screening was performed by transferring 8 μL PCR amplified subtracted and unsubtracted material to Hybond-N^+ nylon transfer membrane (Amersham-Pharmacia) after electrophoretic separation on two replicate agarose gels. Each blot was hybridized, in turn, with ^{32}P-radiolabelled PCR amplified subtracted or driver cDNAs, following *Rsa*I restriction digestion to remove the adaptor sequences and probe recovery using QIAquick gel extraction (Qiagen). DNA ^{32}P radiolabelling was carried out using High Prime (-dCTP) (Roche Diagnostics GmbH, Mannheim, Germany). Unincorporated radiolabelled nucleotides were removed using MicroSpin™ G-50 columns (Amersham Pharmacia Biotech). Hybridizations were carried out under stringent conditions as described by Sambrook *et al.* (1989).

Northern analysis

Northern blot analyses were performed with 10 μg of total RNA separated on 1% formaldehyde agarose gels and transferred to a BrightStar-Plus nylon membrane (Ambion) according to the NorthernMax™ and Strip-EZ DNA™ protocol. Probes were generated from SSH clone insert DNA by *Eco*RI restriction digestion and gel purification. DNA was ^{32}P-radiolabelled according to the Strip-EZ DNA™ manual (Ambion) and purified using MicroSpin™ G-50 columns (Amersham Pharmacia Biotech). All Northerns were performed twice, each time using freshly prepared RNAs isolated from independently inoculated/treated plants and gave reproducible results.

Sequencing, 5′ and 3′ RACE and standard molecular biological methods

Plasmid preparations (Qiagen Plasmid Miniprep kit) were sequenced (ABI PRISM Dye terminator cycle sequencing kit and ABI Model 377 DNA sequencer, Perkin Elmer, Warrington, UK) with the Promega M13 forward primer. Sequences were compared to international database sequences using BLASTX (Altschul *et al.*, 1990). 5′ RACE was performed using the Clontech Smart RACE kit as described by the manufacturer, with primers OXYR1 (GATAGAAGTATTCCATCAACTCTGCC) and OXYR2 (AACTGGCAACGTCACCAGTGAGG) (nested) for *StOBP1*. For the 3′ RACE, primers CathBF1 (CCCACCCTGGTTGTGAACCGC) and CathBF2 (nested) (TACCCCACTCCAAAATGTCATAGG) for *StCathB* and OxyF1 (GATGATATTGACGAACTTGACG) and OxyF2 (nested) (GTTTGGTGCTGGTCGCTATG) were used in conjunction with an oligo dT primer as described by Dellagi *et al.* (2000a). Other molecular biological methods

(including gel electrophoresis and Southern hybridizations) were as described in Sambrook *et al*. (1989).

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