Molecular Plant Pathology

MOLECULAR PLANT PATHOLOGY (2010) **11**(4), 513–530 DOI: 10.1111/J.1364-3703.2010.00622.X

Comparison of transcript profiles in late blight-challenged *Solanum cajamarquense* and B3C1 potato clones

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SUMMARY

Two *Solanum* genotypes, a wild relative of cultivated potato *S. cajamarquense* (Cjm) and an advanced tetraploid clone B3C1 (B3), were inoculated with two *Phytophthora infestans* isolates and leaves were sampled at 72 and 96 h after inoculation. Gene expression in the inoculated versus noninoculated samples was monitored using the Institute of Genomic Research (TIGR) 10K potato array and real-time reverse transcriptase-polymerase chain reaction (RT-PCR). The current experiment is study number 83 of the TIGR expression profiling service project, and all data are publicly available in the Solanaceae Gene Expression Database (SGED) at ftp://ftp.tigr.org/pub/data/s_tuberosum/SGED. Differentially regulated cDNA clones were selected separately for each isolate–time point interaction by significant analysis of microarray (SAM), and differentially regulated clones were classified into functional categories by MapMan. The results show that the genes activated in B3 and Cjm have largely the same biological functions and are commonly activated when plants respond to pathogen attack. The genes activated within biological function categories were considerably different between the genotypes studied, suggesting that the defence pathways activated in B3 and Cjm during the tested conditions may involve unique genes. However, as indicated by real-time RT-PCR, some of the genes thought to be genotype specific may be activated across genotypes at other time points during disease development.

INTRODUCTION

Late blight is one of the most devastating diseases of cultivated potato worldwide. Despite a long history of breeding, durable resistance has not been achieved and the disease is mainly controlled by pesticide applications. Previously incorporated resistance originating from *Solanum demissum* (Black *et al*., 1953; Malcolmson and Black, 1966) is based on resistance (*R*) genes that each recognize a specific avirulence (AVR) factor of the pathogen, and changes in pathogen population have led to a loss of resistance. In searches for durable resistance, focus has turned to the close wild relatives of potato and, indeed, many *Solanum* species have been proven to contain promising levels of late blight resistance (Colon and Budding, 1988; Pérez *et al*. 2000), and new *R* genes have been discovered in *S. berthaultii* (Ewing *et al*., 2000), *S. bulbocastanum* (Naess *et al*., 2000; Park *et al*., 2005; Song *et al*., 2003; Van der Vossen *et al*., 2003, 2005), *S. venturii* (Foster *et al*., 2009; Pel *et al*., 2009), *S. pinnatisectum* (Kuhl *et al*., 2001), *S. phureja* (Sliwka *et al*., 2006), *S. mochiquense* (Smilde *et al*., 2005;), *S. stoloniferum* and *S. papita* (Vleeshouwers *et al*., 2008). In addition, quantitative trait loci (QTLs) for late blight resistance have been found in *S. tuberosum* (Gebhardt and Valkonen, 2001), *S. phureja* (Ghislain *et al*., 2001), *S. microdontum* (Sandbrink *et al*., 2000; Tan *et al*., 2008) and *S. paucissectum* (Villamon *et al*., 2005). Since the early 1990s, some breeding strategies have sought to exclude *R* genes from breeding populations to facilitate the combination of minor genes expected to govern partial resistance (Colon *et al*., 1995; Landeo *et al*., 1995; Turkensteen, 1993), as the strong resistance they provide can mask the expression of other types or components of resistance. Partial resistance is quantitative by nature, effective against many isolates (nonrace specific) and thought to be based on multiple minor genes. However, it has been shown that the presence of defeated *R* genes can also contribute to field resistance (Stewart *et al*., 2003), suggesting that the elimination of *R* genes from breeding populations may reduce the level of resistance attainable. Defeated *R* genes are genes that were once effective against a certain pathogen race, but no longer come into contact with matching *avr* factors because of changes in the pathogen population structure. It is not known whether it is the actual defeated *R* gene or genes linked to it that contribute to the partial resistance phenotype. Furthermore, genetic mapping has shown that *R* genes locate to the same regions as late blight QTLs, making them candidates for partial resistance

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(Gebhardt and Valkonen, 2001; Sandbrink *et al*., 2000; Tan *et al*., 2008). Functional *R* gene-based resistance is characterized by a rapid hypersensitive response (HR) and, consequently, stops pathogen invasion. Similar HR is noted in partially resistant phenotypes, except that the reaction is delayed and slowly growing necrotic lesions appear (Vleeshouwers *et al*., 2000a). Partial resistance is measured as variable levels of susceptibility (Bradshaw *et al*., 2004; Yuen and Forbes, 2009), and an estimate of the resistance level can be obtained by comparing disease progress with that of a known susceptible control (Yuen and Forbes, 2009).

Although gene expression studies have uncovered molecular events in *S. tuberosum* during late blight attack and have identified several candidate genes involved in resistance (Beyer *et al*., 2001; Birch *et al*., 1999; Restrepo *et al*., 2005; Ros *et al*., 2004), the global gene networks activated in closely related species, and potentially useful in breeding, remain largely unknown. The genetic base of the modern cultivated potato is narrow, and the case for broadening it specifically to include new sources or genetic components of resistance is compelling.

Comprehensive genetic studies in the model plant *Arabidopsis thaliana* have enabled the identification of complex networks of pathways involved in plant defence responses (reviewed by Thatcher *et al*., 2005) that are at least partly conserved between plant species. Comparison of global gene expression triggered by three different *R* gene-dependent defence pathways has shown that the pathways can converge, leading to the up-regulation of common sets of target genes (Eulgem *et al*., 2004). Furthermore, potato homologues of at least some of the genes required for the function of *R* genes in *A. thaliana* locate to late blight resistance QTLs in potato maps (Pajerowska *et al*., 2005), and QTL studies have shown that different species appear to carry both overlapping and unique QTLs (Leonards-Schippers *et al*., 1994; Sandbrink *et al*., 2000). The expression of pathogenesis-related (PR) genes *PR1*, *PR2* and *PR5* has been studied in several *Solanum* species, and has been shown to differ between species (Vleeshouwers *et al*., 2000b). Therefore, there is every reason to expect that similar gene networks for general defence will be activated in all species, but a comprehensive analysis of pathogen-induced genes in different germplasm sources may lead to the discovery of new genetic components of resistance traits.

The current study focuses on two genotypes of different origin, displaying partial resistance to late blight. Genotype 762 619.244 of *S. cajamarquense*, endemic to the Department of Cajamarca in the northern Peruvian Andes, belongs to one of the accessions previously identified as late blight resistant in field and glasshouse conditions at the International Potato Center (CIP) (Pérez *et al*. 2000). The species displays very high levels of late blight resistance, but has not yet been utilized in potato breeding. Taxonomically, *S. cajamarquense* belongs to the series Tuberosa (Hawkes, 1990).

Genotype 391 011.17 is a resistant selection from the tetraploid population B3C1, derived from CIPs first improved potato breeding population A developed between 1980 and 1990. Late blight resistance in population A originates from *S. demissum*-derived advanced sources of resistance introduced into *S. tuberosum* ssp. *tuberosum*, *Neotuberosum* and *S. tuberosum* ssp. *andigena* germplasm, and four-way hybrids between *S. acaule*, *S. bulbocastanum*, *S. phureja* and *S. tuberosum* (ABPTs). Quantitative resistance in population B3 has been improved whilst selecting against known *R* genes, and it has been shown to withstand high epidemic pressure in a wide range of tropical and subtropical agroecologies of Latin America, Africa and Asia (Landeo *et al*., 1997). Both plant genotypes were tested in a prestudy for their level of resistance to late blight, and were shown to display partial resistance to the same isolates that are used in the current study (results not shown). When whole plants were inoculated, both genotypes reacted first with HR, which later developed into growing lesions. In addition, in a previous glasshouse assay by Pérez *et al*. (2000), the Cjm genotype used here was shown to display partial resistance. The B3 genotype has shown partial resistance in field conditions in various field experiments from 1998 to 2006 in Peru. The average area under the disease progress curve (AUDPC) for this genotype is 835, which is low compared with the control Yungay with an AUDPC of 2707 (J. Landeo, International Potato Center (CIP), Lima, Peru, personal communication).

A major objective of this study was to test a hypothesis for unique components of resistance in *S. cajamarquense* with respect to B3C1, as a means to guide the use of wild species in efforts to broaden the base of resistance in advanced populations and future varieties.

RESULTS

Resistance phenotype

Whole-plant assay

Plants of a wild genotype of *S. cajamarquense*, 762 619.244, hereafter referred to as Cjm, and a genotype of a bred line of B3C1, 391 011.17, hereafter referred to as B3, as well as controls for quantitative resistance, were challenged with two *P. infestans* isolates: PE84006, hereafter referred to as PE, and POX067, hereafter referred to as POX (see further details in Experimental procedures). Late blight evaluation started at 3 days after inoculation. Each plant was evaluated for disease status (percentage of leaf area affected) each day for 5–7 days after inoculation. The estimation of resistance components was performed on the basis of the recorded percentage of leaf area affected in all the inoculated plants. The results are summarized in Tables 1 and 2, which show the number of plants that reacted with HR, the number of infected plants and the average leaf area

Table 1 Resistance components in microarray experiments obtained at 3, 4, 5 and 7 days after inoculation (dai), measured as the number of plants with a hypersensitive response (HR), number of plants infected and average leaf area affected in infected plants.

		3 dai			4 dai			5 dai			7 dai		
Genotype	*n*	HR	Infected plants	Avg area affected (%)	HR	Infected plants	Avg area affected (%)	HR	Infected plants	Avg area affected (%)	HR	Infected plants	Avg area affected (%)
PE isolate													
B3	36	2	0	0	4	0	0	4	0	0	4	0	0
Cjm	36	0	0	0	3	0	0	3	0	0	4	0	0
Chata Blanca	12	1	0	0	2	0	0	5	2	10	5	6	15
Yungay	12	3	0	0	6	0	0	7	3	10	0	12	30
Monserrate	12	0	0	0	2	0	0	7	0	0	0	12	20
LBr-40	12	0	0	0	6	0	0	9	0	0	1	10	15
POX isolate													
B3	36	18	14	10	1	35	15	0	36	17	0	36	26
Cjm	36	4	0	0	20	0	0	20	0	0	21	9	11
Chata Blanca	12	9	0	0	4	8	11	1	11	19	0	12	43
Yungay	12	8	3	10	1	11	23	0	12	33	0	12	68
Monserrate	12	8	3	10	0	12	21	0	12	33	0	12	72
LBr-40	12	4	8	11	1	11	15	0	12	14	0	12	17

Table 2 Resistance components in real-time reverse transcriptase-polymerase chain reaction (RT-PCR) experiments obtained at 3, 4 and 5 days after inoculation (dai), measured as the number of plants with a hypersensitive response (HR), number of plants infected and average leaf area affected in infected plants.

		3 dai			4 dai			5 dai		
Genotype	*n*	HR	Infected plants	Area affected (%)	HR	Infected plants	Area affected (%)	HR	Infected plants	Area affected (%)
PE isolate										
B3	4	4	0	0	4	0	0	2	2	10
Cjm	4	3	0	0	4	0	0	2	2	10
Chata Blanca	4	3	1	10	0	4	86	0	4	100
Yungay	4	4	0	0	0	4	60	0	4	66
Monserrate	4	4	0	0	0	4	84	0	4	98
LBr-40	4	4	0	0	0	4	16	0	4	22
POX isolate										
B3	4	0	4	11	0	4	34	0	4	41
Cjm	4	4	0	0	4	0	0	4	0	0
Chata Blanca	4	0	4	33	0	4	99	0	4	100
Yungay	4	0	4	39	0	4	99	0	4	100
Monserrate	4	0	4	40	0	4	96	0	4	100
LBr-40	4	4	0	0	3	1	20	2	4	20

affected in the infected plants in microarray and real-time reverse transcriptase-polymerase chain reaction (RT-PCR) experiments, respectively.

Interaction with the PE84006 isolate. No successful infection was established in the interaction of B3 or Cjm genotypes with PE isolate during the microarray experiment. By the last evaluation date (day 7), four plants each of B3 and Cjm responded with HR, whereas the rest of the inoculated plants (total of 64) showed no visible reaction. The disease developed slowly in control clones, which at first reacted with HR; only from the fifth day after inoculation did the initial HRs develop into expanding lesions. The control genotype LBr40 was the most resistant, followed by Chata Blanca, Monserrate and Yungay (Table 1). In contrast, in the real-time RT-PCR experiment, successful infection was established in both B3 and Cjm, resulting in approximately 10% area affected in two of the inoculated plants by 5 days after inoculation. In this experiment, disease development was faster in control plants in comparison with the microarray experiment, as all controls became infected by 4 days after inoculation. At 5 days after inoculation, the two most susceptible control plants, Chata Blanca and Monserrate, were completely or almost completely destroyed by late blight (Table 2).

Interaction with the POX067 isolate. Genotype B3 reacted with HR faster than Cjm, and all of the HRs in B3 plants changed into developing lesions during the microarray experiment (Table 1). Approximately one-half of the genotype Cjm inoculated plants

Table 3 Resistance components in detached leaf assay.

Genotype	Successful infections (total)	IE (%)	LGR (mm/day)		Sporangia/mm^2
			Avg	Std	
PE isolate					
B3	3 (15)	20	4.28	1.81	0.00
Cjm	16 (25)	64	4.01	1.37	96.00
Monserrate	15 (15)	100	7.16	1.56	149.02
LBr40	12 (15)	80	6.36	1.34	59.34
Yungay	15 (15)	100	6.17	1.61	138.06
Chata Blanca	14 (15)	93	7.01	1.48	198.33
POX isolate					
B3	12 (15)	80	7.59	2.74	2.78
Cjm	11 (25)	44	3.99	1.28	5.20
Monserrate	15 (15)	100	9.05	1.70	117.43
LBr40	15 (15)	100	9.09	1.84	21.28
Yungay	15 (15)	100	10.50	2.00	136.95
Chata Blanca	15 (15)	100	9.10	1.80	157.37

IE, infection efficiency; LGR, lesion growth rate.

developed HR by 4 days after inoculation, but only nine plants showed developing lesions by the end of the experiment. In both genotypes, the average area affected in the infected plants was smaller than that in the susceptible controls. All of the inoculated plants in control genotypes showed developing lesions, but differed in the average leaf area affected (Table 1). In the real-time RT-PCR experiment, all inoculated plants of the B3 genotype already showed growing lesions 3 days after inoculation. The area affected grew more rapidly (41% by 5 days after inoculation) than in the microarray experiment. In contrast, Cjm showed no growing lesions during the experiment.

Detached leaf assay

In the detached leaf test, both genotypes were compatible with both isolates tested: the first reaction was hypersensitivity (HR) and, later, slowly expanding lesions appeared. In the interaction with the PE isolate, only 20% (three of 15) of the B3 leaflets became infected, whereas 64% (16 of 25) of the Cjm leaflets showed successful infection by 5 days after inoculation. In the control plants, all inoculated leaflets became infected. The difference in resistance was measured as the average length of the radius of the lesions established by 5 days after inoculation and the number of sporangia/mm^2 (Table 3). Lesion formation was reduced in B3 and the few lesions that were formed displayed no or very little sporulation, depending on the isolate used. In Cjm plants, successful infection occurred more frequently, but the lesions were small. Sporulation of the POX isolate in these plants was very low, whereas, surprisingly, many spores of the PE isolate were formed. The reaction of the potato genotypes to the PE isolate was different in detached leaf tests in comparison with the whole-plant test. Although successful infections were established in the detached leaf assay and real-time RT-PCR experiment, both genotypes remained uninfected in the microarray experiment.

General patterns of gene expression

Leaves were taken from the middle part of four individual plants of each genotype and combined into a single sample for RNA extraction. In total, three samples per isolate and genotype were collected at 72 h after inoculation and another three samples from the same plants at 96 h after inoculation. The three samples from a given time point, genotype and isolate were considered as independent biological replicates. Leaves of uninoculated plants were collected as control samples. The current experiment is study number 83 of the Institute of Genomic Research (TIGR) expression profiling service project, where all steps after RNA extraction were performed by TIGR according to their standardized protocols. The data of the experiment are publicly available in the Solanaceae Gene Expression Database (SGED) at ftp://ftp.tigr.org/pub/data/s_tuberosum/SGED.

A detailed description of the hybridization design is given in Experimental procedures.

Because of the difference in their response pattern in transcript accumulation, B3 and Cjm were analysed separately. For each genotype, all cDNA clones that showed more than two flagged spots throughout the experiment were discarded. Of the total of 11 412 verified cDNA clones present on the TIGR array, 11 001 for Cjm and 10 712 for B3 passed the quality spot criteria, and thus were used in the analysis. Differentially regulated clones were selected separately for each isolate–time point interaction by significant analysis of microarray (SAM), with a two-class unpaired design. Table 4 provides the SAM statistics and the number of differentially regulated clones at each genotype–isolate–time point interaction. The number of cDNA clones that were differentially regulated was higher in the POX interaction relative to the PE interaction in both potato genotypes, and up-regulation was more common than down-regulation. However, in the Cjm–POX interaction at 72 h after

Table 4 Statistical summary of significant analysis of microarray (SAM) and numbers of up- and down-regulated clones for each genotype–isolate–time point interaction.

	Delta	FDR	FDN	Up-regulated	Down-regulated	Total
Cjm PE 72 h	6.70	6.76	2	23	1	24
Cjm PE 96 h	45.04	20.40	4	18	3	21
Cjm POX 72 h	3.46	4.64	8	113	53	166
Cjm POX 96 h	4.26	4.64	7	147	9	156
B3 PE 72 h	3.62	50.00	2	4	0	4
B3 PE 96 h	6.29	2.60	2	46	15	61
B3 POX 72 h	5.50	1.35	2	148	0	148
B3 POX 96 h	7.52	0.66	1	88	6	94

FDN, false discovery number; FDR, false discovery rate.

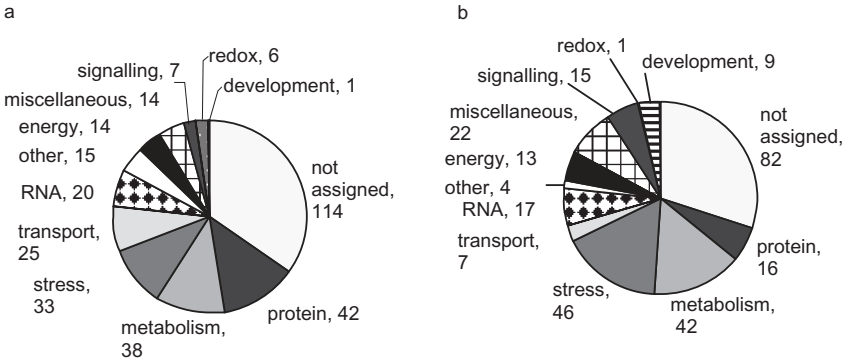

Fig. 1 Pie charts showing the proportions of differentially regulated clones in Cjm (a) and B3 (b) in each of the functional categories formed from functional bins of MapMan. The 'miscellaneous' category includes clones annotated as various short-chain dehydrogenases, cytochrome P450s, oxidases and proteinase inhibitors. The 'other' category includes functional categories DNA, cell, cell wall and tetrapyrrole synthesis. The classification is shown in Tables S1 and S2 (see Supporting Information).

inoculation, there were several down-regulated cDNA clones, which correspond to sequences that have no assigned function (Table S1, see Supporting Information). The response to PE inoculation was faster in Cjm than in B3, but, finally, the total number of affected cDNA clones was fairly similar in these genotypes (Table 4). In total, 367 (Cjm) and 307 (B3) cDNA clones were differentially regulated in at least one of the isolate–time point interactions. As some of these clones appeared to be differentially regulated at more than one of the interactions, the final numbers of differentially regulated clones were 329 (2.9%) and 274 (2.6%) for Cjm and B3, respectively. These differentially regulated clones were annotated and classified into functional bins by MapMan ontology (Thimm *et al*., 2004) (Tables S1 and S2, see Supporting Information). Functional bins were utilized to group clones into larger functional groups, shown in Fig. 1. A large proportion of the clones in both B3 (30%) and Cjm (35%) have no assigned function. The notable differences between B3 and Cjm are in the numbers of differentially regulated clones in the categories transport and protein, which are larger in Cjm than B3, and stress, signalling and development, which are larger in B3 than Cjm (Fig. 1).

Stress-related genes

MapMan ontology was utilized to select clones that were classified as having function or putative function in either biotic or abiotic stress for visualization as heat plots (Fig. 2a,b). In addition to clones selected by MapMan, clones similar to the storage protein patatin, alternative oxidase and RPM1-interacting protein 4 (RIN4) were included because of the recent evidence of their involvement in disease resistance. In addition, the ontology was adapted and some clones were transferred between functional groups. Tables S3 and S4 (see Supporting Information) show the detailed functional grouping of the cDNA clones that were differentially regulated in this study. The expression profiles of the stress-related clones (109 in B3 and 93 in Cjm), presented in Fig. 2, are organized according to gene function to allow for an easier comparison between genotypes. The stress-related clones are mostly genotype specific, as 94 (of 109) clones were unique for B3 and 78 (of 93) were unique for Cjm. However, the different clones correspond largely to the same defence pathways in both genotypes.

The induction and expression of defence pathways in plants in response to pathogens begin with recognition. Specific recognition is mediated by *R* genes that recognize pathogen signals. The first functional group of genes (group A, Fig. 2) includes *R* genes, proteins that interact with these genes and the genes essential for the function of some *R* genes. *R* genes (STMCL13, STMJC84, STMHZ79, STMIM79, STMCJ74) were only identified in the B3 genotype (Fig. 2b). These were mostly up-regulated, but, interestingly, clone STMCJ74, corresponding to bacterial spot resistance protein (BS2) of *Capsicum annuum*, was down-regulated by the PE isolate. *RIN4* (STMJI59, STMJH73) was induced by the POX isolate in B3. Signalling genes essential for the function of certain *R* genes were activated in both genotypes. *Enhanced disease susceptibility 1* (*EDS1*) (STMIX37), induced by the PE isolate in B3,

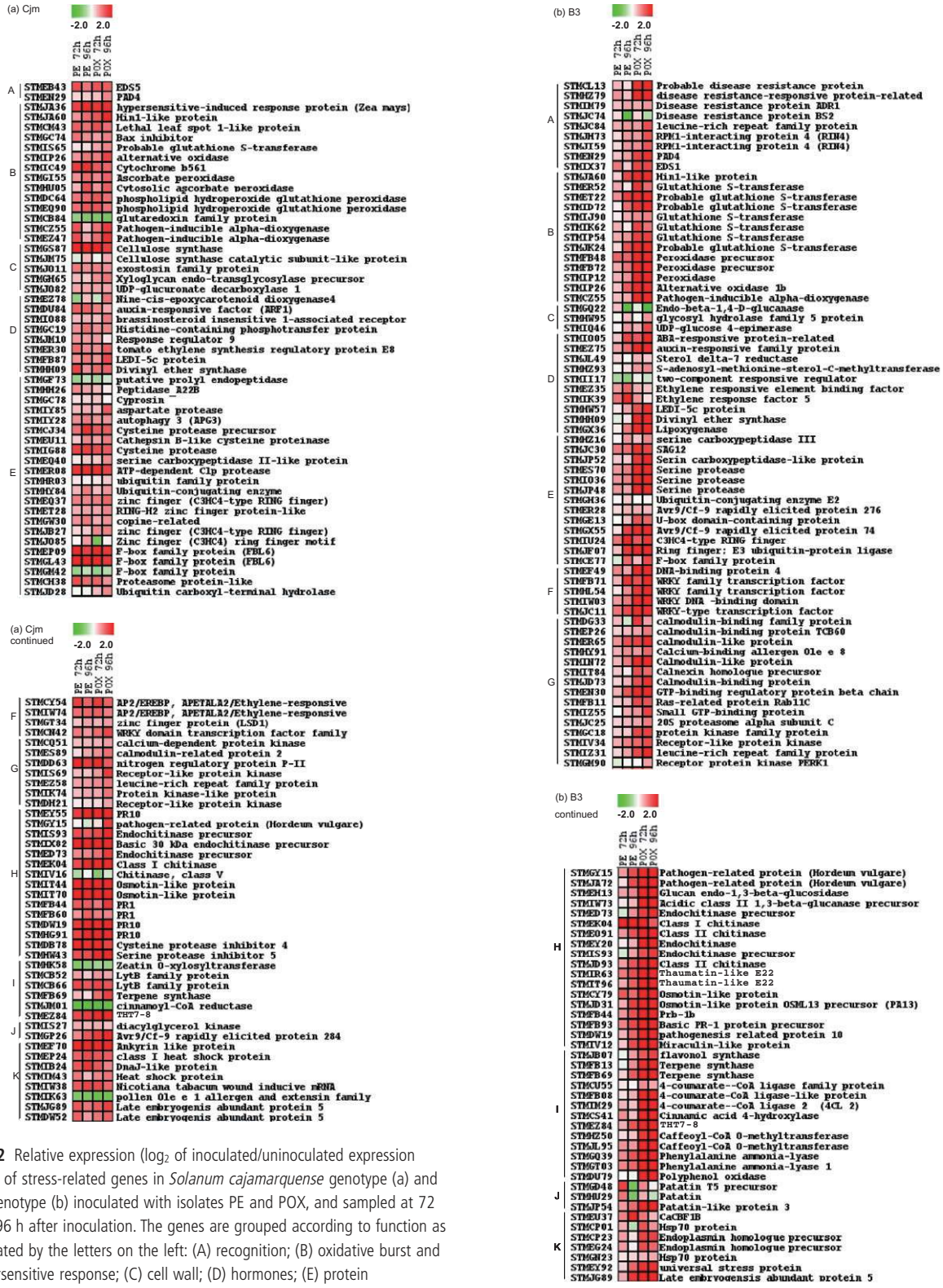

Fig. 2 Relative expression (log_2 of inoculated/uninoculated expression ratio) of stress-related genes in *Solanum cajamarquense* genotype (a) and B3 genotype (b) inoculated with isolates PE and POX, and sampled at 72 and 96 h after inoculation. The genes are grouped according to function as indicated by the letters on the left: (A) recognition; (B) oxidative burst and hypersensitive response; (C) cell wall; (D) hormones; (E) protein degradation; (F) transcription; (G) signalling; (H) pathogenesis-related (PR) proteins; (I) secondary metabolites; (J) other; (K) abiotic stress.

is an important component of the nucleotide-binding site-leucine-rich repeat (NBS-LRR) *R* gene-mediated response to specific pathogens in Arabidopsis and tomato. *EDS5* (STMEB43), induced in Cjm by the POX isolate, and *phytoalexin-deficient 4* (*PAD4*) (STMEN29), induced by the POX isolate in both B3 and Cjm, are components of salicylic acid (SA) signalling.

Recognition is followed by an oxidative burst characterized by the generation of extracellular reactive oxygen species (ROS) and the onset of HR (group B in Fig. 2). Miscellaneous glutathione *S*-transferases (GST) (STMER52, STMET22, STMID72, STMIJ90, STMIK62, STMIP54, STMJK24) and peroxidases (STMFB48, STMFB72, STMIP12) were activated in B3, but only one GST in Cjm (STMIS65). In Cjm, the oxidative stress-related clones correspond to ascorbate and glutathione peroxidases involved in the redox state (STMGI55, STMHU05, STMDC64, STMEQ90, STMCB84). Pathogen-induced α-dioxygenase (*PIOX1*) (STMEZ47, STMCZ55 in Cjm and STMCZ55 in B3) and alternative oxidase (*AOX*) (STMIP26 in both B3 and Cjm) were induced in both potato genotypes. HR-related Bax inhibitor (STMGC74), *lethal leaf spot 1* (STMCM43) and hypersensitive-induced response protein gene (STMJA36) were induced in Cjm by the POX isolate. Harpin-induced 1 (*HIN1*) (STMJA60) was induced in both genotypes by the POX isolate.

The cell wall acts as an efficient barrier against microbes (group C in Fig. 2). cDNA clones corresponding to cell wall degradation-related elements, such as glycosyl hydrolase family 5 protein (STMHW95) and cell wall precursor UDP-glucose 4-epimerase (STMIQ46), were found to be up-regulated in the B3 genotype, and *endo-β-1,4-D-glucanase* (STMGQ22), involved in cell wall degradation, was down-regulated. In Cjm, cellulose synthase (STMGS87) and other genes corresponding to cellulose (STMJM75) and hemicellulose (STMJO11) synthesis were up-regulated.

Plant hormones play important roles in biotic and abiotic stress responses (group D in Fig. 2). In both genotypes, transcripts involved in hormone signalling pathways of abscisic acid (ABA), auxin, brassinosteroids, ethylene (ET) and jasmonic acid (JA) were activated. Genes on the JA biosynthesis pathway, such as *divinyl ether synthase*, a CYP74D enzyme on the lipoxygenase pathway (STMHH09) and *LEDI-5c* (STMFB87 in Cjm and STMHW57 in B3), were up-regulated in both genotypes, and lipoxygenase *Lox1* (STMGX36) was up-regulated in the B3 genotype. The ET-related genes were entirely different between genotypes. In B3, two ET-responsive transcription factors (STMEZ35 and STMIK39) were up-regulated in response to inoculation with the PE isolate, whereas, in Cjm, a cDNA clone similar to ET synthesis regulatory protein E8 (STMER30) was up-regulated in response to the POX isolate. The other hormone-related genes either involved in the synthesis of ABA, auxin, brassinosteroids and cytokinins or responsive to these hormones were also different between genotypes (Fig. 2, group D).

Controlled protein degradation is part of successful defence, and genes in this category were affected in both genotypes (group E in Fig. 2). The proteolytic pathway involving the ubiquitination of target proteins for subsequent degradation by the 26S proteasome was activated in both genotypes, but the genes activated were genotype specific, as demonstrated by the differential regulation of cDNA clone corresponding to E2 ubiquitin conjugating enzyme (STMHY84 in Cjm and STMGH36 in B3), as well as SFX-type (STMEP09, STMGL43, STMGM42 in Cjm and STMCE77 in B3) and ring-type (STMEQ37, STMET28, STMGW30, STMJB27, STMJO85 in Cjm and STMER28, STMGE13, STMGX55, STMIU24, STMJF07 in B3) E3 ubiquitin ligases. Although cysteine and serine protease genes were up-regulated in both genotypes, the genes corresponding to these were genotype specific. Subtilases (STMES70, STMIO36 and STMJP48) were up-regulated only in the B3 genotype.

Different types of transcription factor are activated during defence (group F in Fig. 2). WRKY proteins are a family of transcription factors that, in plants, bind the W box sequence present in the promoters of various pathogen- and wounding-responsive genes. The activated WRKY-type transcription factors were unique to the genotype and more numerous in B3 (STMCN42 in Cjm and STMEF49, STMFB71, STMHL54, STMIW03, STMJC11 in B3). Two types of transcription factor relating to stress, DREB subfamily A-6 of the ERF/AP2 transcription factor family (RAP2.4) (STMCY54 and STMIW74) and zinc finger protein (LSD1) (STMGT34), were differentially expressed in Cjm only.

Various genes involved in calcium signalling (STMCQ51, STMES89 in Cjm and STMDG33, STMEP26, STMER65, STMHY91, STMIN72, STMIT84, STMJD73 in B3), as well as signalling receptor kinases (STMDD63, STMIS69, STMEZ58, STMIK74, STMDH21 in Cjm and STMJC25, STMGC18, STMIV34, STMIZ31, STMGM90 in B3), were activated in both genotypes, and all these were unique to the genotype. G-proteins involved in signalling (STMEN30, STMFB11, STMIZ55) were activated only in the B3 genotype. Signalling genes are found in group G in Fig. 2.

PR proteins are a diverse group of proteins affected by pathogens (group H in Fig. 2). In this group, there were some genes that were activated in both genotypes, such as PR-like (STMGY15), *PR1* (STMFB44), *PR10* (STMDW19) and chitinases (STMIS93, STMED73, STMEK04). However, each of these subgroups also showed genotype-specific genes. Osmotins and proteinase inhibitors were activated in both genotypes, but the genes were all genotype specific. *β1,3-Glucanase* (STMEH13, STMIW73) was up-regulated only in the B3 genotype. The only down-regulated gene in group H was class V chitinase (STMIV16) in genotype Cjm.

The secondary metabolite-related genes (group I in Fig. 2) were mostly genotype specific, as only two of the activated genes in this group, terpene synthase (STMFB69) and *THT7-8* (STMEZ84), were the same between genotypes. *THT7-8*

Table 5 Primers used in reverse transcriptase-polymerase chain reaction (RT-PCR) designed on six genes found to be differentially regulated by microarray experiment.

GenBank #	Annotation	Corresponding clone on TIGR array	Primer name	Primer sequences 5′–3′
P13046	Pathogenesis-related protein R major form precursor (thaumatin-like protein E22)	STMIR63 STMIT96	P8	Forward: ATGGGCCAGGGTCATGTA Reverse: GCCTTCAAGGGCAAAAGA
Q8S3U2	EDS1-like protein	STMIX37	P10	Forward: CTTCTGCACTGGGAATAGGA Reverse: TTCGGAACTCAGTTGAGAGG
Q9FZ08	Patatin-like protein 3	STMJP54	P13	Forward: TGGTATAGGCAAGCAGGAAG Reverse: TATCAAAGGAGTGGCACCAT
AAF09479	Phytoalexin-deficient 4 protein (PAD4)	STMEN29	P11	Forward: TTGCATTACCTTTGGCTCTC Reverse: CATGATGGGGAAAAGAACAG
Q84V46	Alternative oxidase 1b (AOX)	STMIP26	P14	Forward: GGTTGGATCAAAGCCCTACT Reverse: ATGCACGTTCGTACCATTTT
Q9AXU5	Pathogen-inducible α-dioxygenase (PIOX)	STMCZ55 STMEZ47	P15	Forward: ACAATGGGATGGAAGTGCTA Reverse: GTTCAAGGAGCCCATTTTCT

EDS1, enhanced disease susceptibility 1; TIGR, Institute of Genomic Research.

(*N-hydroxycinnamoyl-CoA:tyramine N-hydroxycinnamoyl transferase*), is involved in lignin biosynthesis, and many other lignin biosynthesis-related genes were affected in B3 (STMHZ50, STMJL95, STMGQ39, STMGT03), but not in Cjm. Flavonoid-related genes, zeatin *O*-xylosyltransferase in Cjm (STMHK58) and flavonol synthase in B3 (STMJB07), were genotype specific. Most of the genes involved in secondary metabolism were up-regulated and, in the B3 genotype, this up-regulation was especially strong in the POX interaction. There were two down-regulated secondary metabolism-related genes in the Cjm genotype, corresponding to cinnamoyl-CoA reductase (STMJM01) on the phenylpropanoid pathway and flavonoid zeatin *O*-xylosyltransferase (STMHK58).

Group J (Fig. 2) consists of genes that do not belong to any other functional group. In B3, this group includes only different patatin genes, whereas, in Cjm, the genes are involved in lipid metabolism (STMIS27) and protein post-translational modification (STMGP26).

Finally, group K (Fig. 2) consists of genes having function in abiotic stress. Only one of the genes in this group, corresponding to late embryogenesis abundant protein 5 (STMJG89), is shared by B3 and Cjm.

Real-time RT-PCR results

Six genes that were differentially expressed in B3 at different time points were selected for confirmation by real-time RT-PCR utilizing RNA that originated from a separate biological experiment. Plants were inoculated with isolates POX and PE as described for the microarray experiment and leaves were sampled at 24, 72 and 96 h after inoculation. Disease symptoms were recorded at 3, 4 and 5 days after inoculation (Table 2). Instead of using single clone sequences for primer design, we utilized the best matching gene sequence that corresponded to the tentative consensus build, including the cDNA clone that was differentially expressed in the microarray (Table 5). In general, in B3 plants, the gene expression was similar in the microarray and real-time RT-PCR studies, but there was an interesting difference between time points as, at 72 h after inoculation, gene expression was always higher when measured by real-time RT-PCR but, at 96 h, the situation was reversed (Fig. 3). This is probably an indication that the time points between the experiments are not exactly the same. The differences in disease development between the microarray and real-time RT-PCR experiments (Tables 1 and 2) are not surprising, as partial resistance is affected by the environment. In Cjm plants, gene expression was quite different when measured by microarray and real-time RT-PCR, as many genes whose expression was not significant by microarray were found to be significantly up-regulated by real-time RT-PCR (Fig. 4). The *thaumatin* gene showed very inconsistent results between the experiments. In the microarray study, clones STMIR63 and STMIT96, similar to the *thaumatin*-like PR protein, were found to be up-regulated in the POX–B3 interaction. In Cjm plants, neither one of these clones was significantly different when compared with the control. In RT-PCR, however, this gene was found to be strongly up-regulated in both genotypes. A closer evaluation of the expression patterns in the microarray experiment revealed that, in B3, the expression of STMIR63 and STMIT96 clones was very similar, but, in Cjm, these two clones behaved very differently (Fig. 4), suggesting that the sequences present in the microarray hybridize to different genes in the Cjm transcriptome. The DNA sequences of STMIR63 and STMIT96 are highly similar, but contain several single nucleotide polymorphisms resulting in a change in amino acid composition (not shown). Thus, it is possible that Cjm contains several different *thaumatin*-like genes, and the gene(s) captured by real-time RT-PCR, for some reason, did not hybridize with the clones on the microarray. The expression of *EDS1*, *PAD4*, *PIOX*, *AOX* and *patatin* on the microarray was always

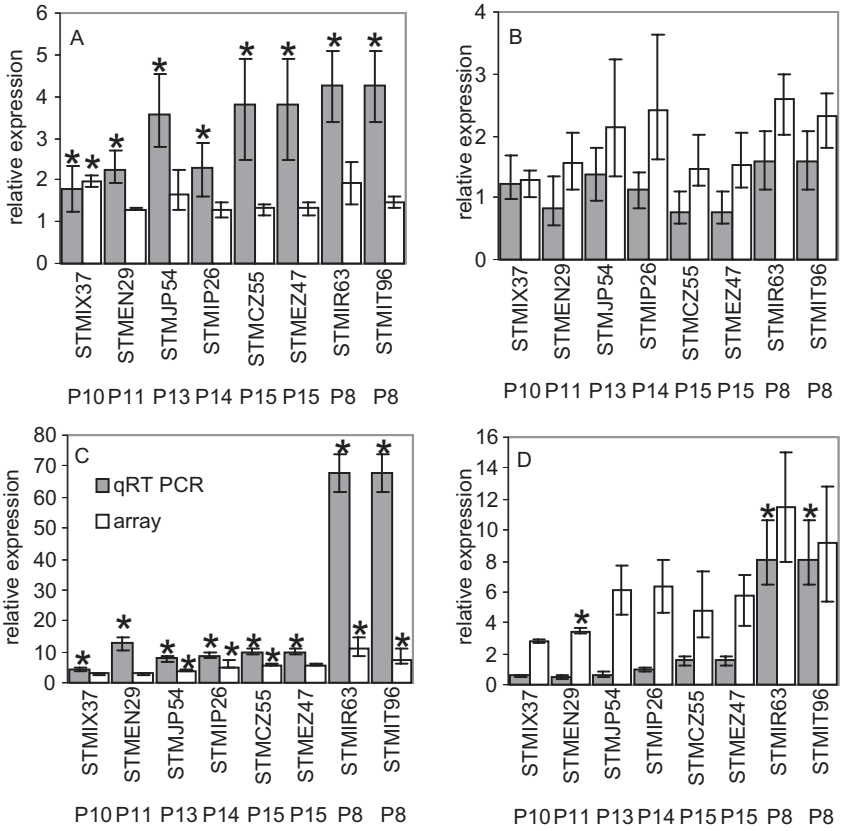

Fig. 3 Comparison of the relative expression of six genes using microarray and real-time reverse transcriptase-polymerase chain reaction (RT-PCR) in B3 plants inoculated with *Phytophthora infestans* PE isolate (A and B) and POX isolate (C and D). The samples were collected at 72 h (A and C) and 96 h (B and D) after inoculation. The names beginning with STM correspond to the cDNA clones present on the microarray, whereas P followed by a number corresponds to the genes analysed by real-time RT-PCR. Statistically significant values are indicated by asterisks. The gene annotations and GenBank numbers are shown in Table 5.

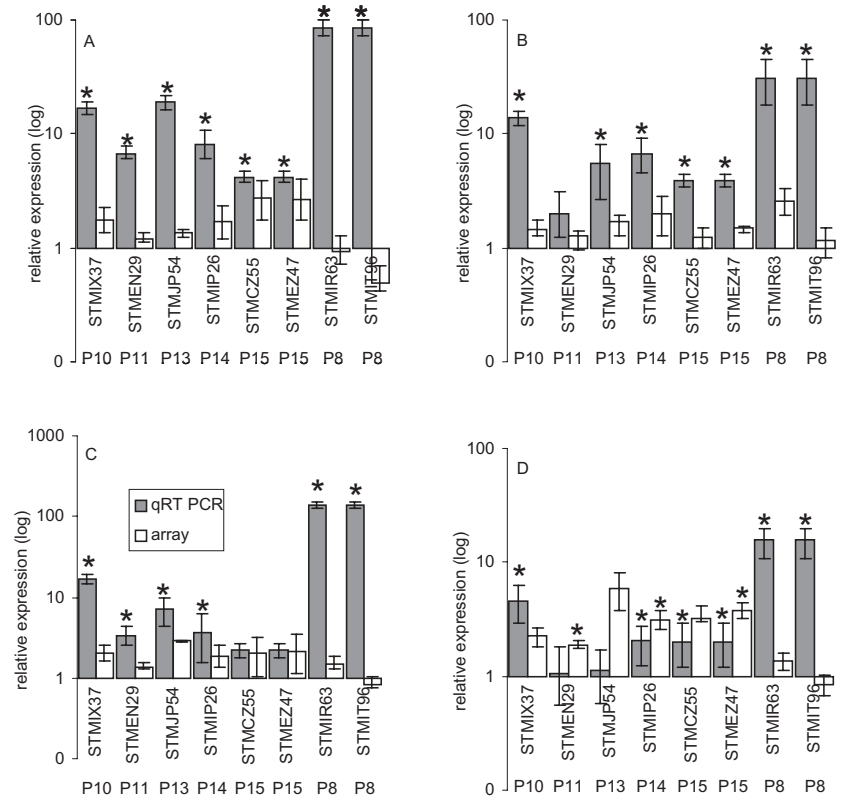

Fig. 4 Comparison of the relative expression of six genes using microarray and real-time reverse transcriptase-polymerase chain reaction (RT-PCR) in Cjm plants inoculated with *Phytophthora infestans* PE isolate (A and B) and POX isolate (C and D). The samples were collected at 72 h (A and C) and 96 h (B and D) after inoculation. The expression is shown on a logarithmic scale because of the large expression differences between the genes. Statistically significant values are indicated by asterisks. The names beginning with STM correspond to the cDNA clones present on the microarray, whereas P followed by a number corresponds to the genes analysed by real-time RT-PCR. The gene annotations and GenBank numbers are shown in Table 5.

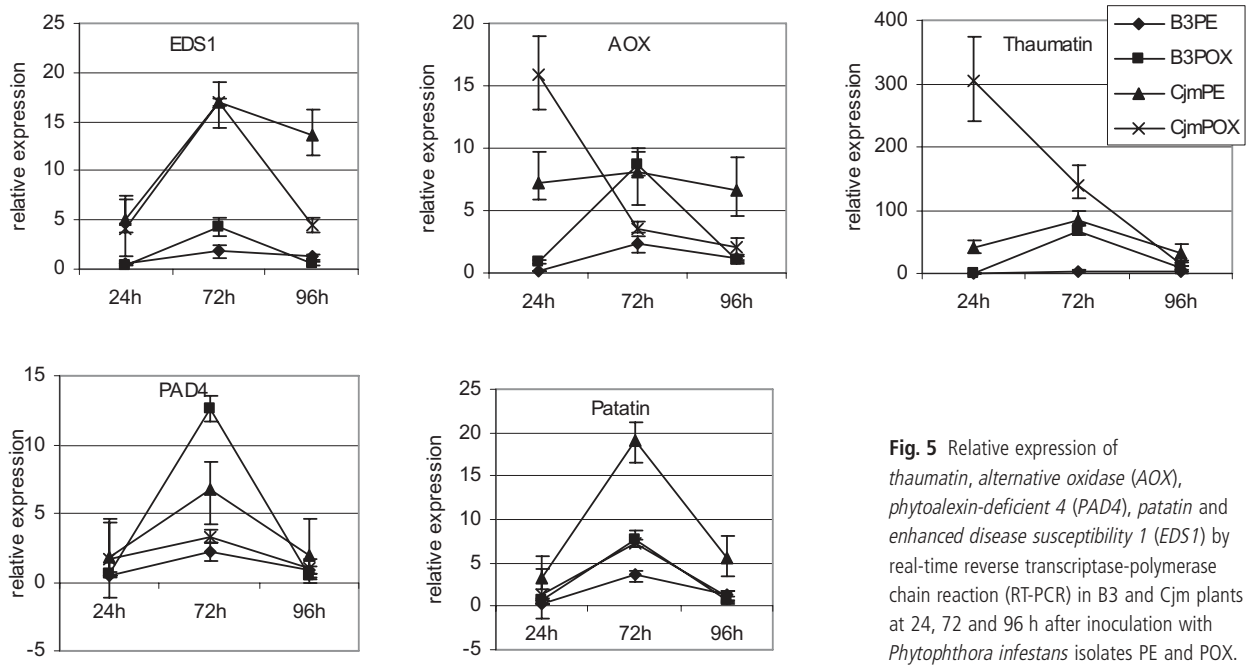

Fig. 5 Relative expression of *thaumatin*, *alternative oxidase* (*AOX*), *phytoalexin-deficient 4* (*PAD4*), *patatin* and *enhanced disease susceptibility 1* (*EDS1*) by real-time reverse transcriptase-polymerase chain reaction (RT-PCR) in B3 and Cjm plants at 24, 72 and 96 h after inoculation with *Phytophthora infestans* isolates PE and POX.

in the same direction as in real-time RT-PCR, but most of the time the expression in the microarray was not significant. This could be a result of too conservative a statistical method or differences in the biological response between experiments. It could also be caused by the divergence between *S. tuberosum* and *S. cajamarquense*, resulting in a poor hybridization of Cjm cDNA to the *S. tuberosum* probes on the TIGR array. However, the number of features on the arrays with good signal strength was fairly similar between Cjm and B3 (Fig. S1).

Kinetics of expression of defence genes

Of the genes tested by real-time RT-PCR, *EDS1*, *PAD4* and *patatin* showed similar kinetics of expression regardless of genotype, isolate or time point, with the highest expression at 72 h after inoculation. *AOX* and *thaumatin*-like genes showed a different pattern for the Cjm–POX interaction, with extremely high expression at 24 h when no macroscopic symptoms of HR or disease were seen in the inoculated plants, suggesting that these genes may have key functions for resistance in Cjm (Fig. 5).

DISCUSSION

The TIGR 10K potato array was used to monitor global gene expression in two *Solanum* genotypes, a wild relative of cultivated potato *S. cajamarquense* and an advanced tetraploid genotype B3C1, during late blight attack. The inoculated samples were compared with healthy noninoculated plants to filter out the genes expressed for reasons other than pathogen attack. To improve data quality, three biological replicates were collected, each consisting of a pooled sample of several plants.

The quality of the microarray results was validated for a set of six differentially expressed genes by real-time RT-PCR. The pattern of gene expression was comparable with that of the microarray in the B3 genotype but, in the Cjm plant, several genes that were not significant in the microarray were found to be highly up-regulated by real-time RT-PCR, indicating that the differences between genotypes may not be as pronounced as suggested by the microarray. The differences between the array and real-time RT-PCR results could be caused by differences between disease development in the experiments, and indicate that it is beneficial to investigate several time points in order to capture more of the genes involved. However, it is possible that the microarray containing cDNA clones from *S. tuberosum* is not optimal for use in more distantly related species such as *S. cajamarquense*. However, the number of usable elements on the arrays and the signal strength were comparable between Cjm and B3, indicating that the cDNA of both genotypes hybridized well on the array.

The proportion of differentially regulated cDNA clones, 3% in Cjm and 2.5% in B3, was relatively low considering that a large number of the clones present on the array originate from *P. infestans*-challenged cDNA libraries. Using the same TIGR array to monitor gene expression during compatible late blight interaction in potato cultivar Kennebec, Restrepo *et al*. (2005) found that 13% of the clones were differentially regulated. A large proportion of the differentially regulated cDNA clones corresponded to genes that had no previously assigned biological

function, implicating them as novel genes not yet identified, but involved in the defence response.

Disease progress was monitored up to 7 days after inoculation, and comparison with control genotypes allowed for the verification of the level of resistance in the B3 and Cjm genotypes during the experiment. Both genotypes appeared to be resistant to the PE isolate, as no successful infection was established during the experiment. The interaction with the PE isolate in the microarray study could actually be considered as an incompatible interaction indicative of the presence of *R* genes, but, when tested with a detached leaf assay with the same isolate, the reaction was a compatible interaction that began with HR, which later developed into slowly growing lesions (Table 2). The other whole-plant assay used for real-time RT-PCR resulted in successful infection in both B3 and Cjm, with slowly growing lesions (Table 2). Our other experiments with the PE isolate in whole-plant tests, using the same conditions as described in the present study, have also produced contradictory results, sometimes inducing a low level of infection and sometimes complete resistance. Therefore, we suggest that the resistance phenotype of Cjm–PE and B3–PE interaction is indicative of a high level of partial resistance, which, in some environmental conditions, results in complete resistance. It is to be expected that the phenotype of partial resistance is affected by the environment. Cjm appears to be more resistant than B3, but the B3 genotype is also more resistant than susceptible control plants, thus displaying partial resistance. Potato genotypes with high levels of partial resistance do not need as many fungicide applications as susceptible genotypes (Kromann *et al*., 2009), and this can mean significant savings for farmers.

During the interaction with the PE isolate, the expression of many fewer cDNA clones was affected than during interaction with the POX isolate (Table 4). This could be explained by the low aggressiveness of the PE isolate, perhaps relating to the slow germination of spores, and resulting in few contact sites between the plant and the pathogen. Genotype Cjm appeared to be more resistant than genotype B3 to the POX isolate, which also showed good resistance, as indicated by the slow development of lesions. Interestingly, the inoculation with POX affected the expression of more cDNA clones in Cjm relative to B3, suggesting that the higher level of resistance of Cjm could be caused by a wider array of responses deployed.

Recognition

With the hybridization design of the current study we were not able to identify *R* genes that were constitutively expressed in the absence of the pathogen, only induced or suppressed genes. Nevertheless, cDNAs annotated as putative disease resistance proteins were found to be differentially expressed in B3, indicating the potential usefulness of the identification of functional *R* genes by transcriptome profiling. So far, *R* genes are probable candidates responsible for the QTL effect only because of their colocalization on genetic maps. As suggested by Tan *et al*. (2008), it is probable that the quantitative resistance to late blight is determined by NBS-type *R* genes, but biological evidence for this is still needed. To date, we have preliminary indication of an expression of a major QTL in chr9 in a progeny of a cross in which the resistance originates from one of the genotypes of the advanced B3 population (J. Landeo, M. Gastelo, L. Portal, International Potato Center (CIP), Lima, Peru, personal communication); therefore we may begin testing the hypothesis of the colocalization of some candidate *R* genes in this genomic region. One of the *R* gene-like clones differentially regulated is similar to the *ADR1* gene. This gene in Arabidopsis is induced by pathogen challenge, and transient expression of this gene results in defence-related gene expression as well as the establishment of resistance (Grant *et al*., 2003).

Genes required for *R* gene function and their downstream signalling pathways were found to be induced in both genotypes studied. *RIN4* is an R protein-guarded host target, whose degradation or phosphorylation induced by pathogen effectors activates the cognate R proteins RPM1 and RPS2 in Arabidopsis (Mackey *et al*., 2002, 2003). *EDS1* is required for resistance conferred by a number of NBS-LRR-type *R* genes in Arabidopsis, tobacco and tomato (Feys *et al*., 2001; Hu *et al*., 2005; Venugopal *et al*., 2009). In tomato and Arabidopsis, it is also indispensable for basal resistance to virulent pathogens (Feys *et al*., 2001; Hu *et al*., 2005), but has also been shown to enhance disease caused by the necrotrophic pathogen *Botrytis cinerea* in tobacco (El Oirdi and Bouarab, 2007). *EDS1* is necessary for HR to develop (Feys *et al*., 2001) and, apparently, necrotrophic pathogens can use this to invade the host. *EDS1* was highly expressed in Cjm plants during the HR phase, but not in B3 plants. Detailed functional studies are necessary to determine whether the difference is caused by *R* gene-based versus basal resistance. *EDS1* has been reported to interact physically with *PAD4* in Arabidopsis (Feys *et al*., 2001). *PAD4* was found to be up-regulated in both B3 and Cjm, being highly expressed in the B3–POX interaction, where all plants were infected and had growing lesions.

Oxidative stress and HR

ROS production can be linked to both incompatible and compatible plant–pathogen interactions, but the timing and amplitude of the ROS burst in these interactions differ. In soybean and tobacco cells, ROS production during incompatible interaction occurs in two phases, the second of which is of much greater magnitude and lasts longer, whereas during the compatible interaction only the early burst of lower magnitude is seen. The weak first-phase burst is biologically nonspecific and does not

lead to HR, whereas the second burst requires specific recognition and leads to HR (Lamb and Dixon, 1997). The induction of the expression of ROS-related enzymes and the clearly visible HR in the POX interaction are indicative of successful pathogen recognition. However, although ROS production is usually linked to successful resistance responses and defence signalling, some necrotrophic pathogens may induce ROS production to accelerate cell death that facilitates subsequent infection (reviewed by Torres *et al*., 2006). A considerably greater induced expression of peroxidases and GSTs has been reported in susceptible potato than in moderately resistant potato as a result of late blight attack (Ros *et al*., 2004). In the current study, *P. infestans* had at least partly established a necrotrophic phase in B3 plants infected with POX by 96 h after inoculation, as indicated by the presence of necrotic lesions (Table 1). Thus, it is not clear whether the induction of peroxidases and GSTs acts as a defence or susceptibility mechanism. However, a gene *prp1* that encodes a GST (Hahn and Strittmatter, 1994) has been mapped to a QTL against late blight (Oberhagemann *et al*., 1999), and this same gene was also affected in Cjm, in which no infection was established (Table 1), suggesting a successful role in defence. AOX of plant mitochondria functions to ameliorate ROS production when the cytochrome pathway is chemically inhibited, and may be involved in acclimation to oxidative stresses (Umbach *et al*., 2005). When transgenic tobacco plants overexpressing AOX were challenged with *Tobacco mosaic virus* (TMV), they developed smaller HR lesions, indicating reduced programmed cell death (Ordog *et al*., 2002), perhaps by reduced ROS. Comparing the kinetics of expression of AOX by real-time RT-PCR showed significant isolate and time point-dependent up-regulation in both genotypes (Fig. 5), illustrating that the genes involved in ROS production play a significant role in late blight resistance. The early high level of AOX expression seems to be associated with resistance in the incompatible Cjm–POX interaction, but functional studies are required to prove this.

In accordance with the resistance phenotype, several genes relating to HR and the oxidative burst were induced in B3, but the up-regulation was statistically significant only in the interaction with the POX isolate. The induction of these genes is well correlated with the proportion of B3 plants reacting with HR (Table 1). In the interaction with the PE isolate, very few plants reacted with HR, whereas, at 72 h after inoculation with the POX isolate, 18 (50%) of the inoculated plants had HR. At 96 h after inoculation with POX, only one plant remained with HR, whereas all the rest had expanding lesions, and consequently the expression of most of the HR- and oxidative burst-related genes was no longer significant. *HIN1* has been found to be induced during late blight attack in potato (Wang *et al*., 2005). The map location of *StHIN1* in potato on chromosome X is not in the vicinity of any known late blight resistance QTL (Pajerowska *et al*., 2005).

JA, ET, ABA, auxin, brassinosteroid and cytokinin pathways are activated during late blight attack

JA is involved in late blight resistance in both genotypes studied, as indicated by the up-regulation of genes on the pathway leading to JA synthesis. One of the genes activated on the JA pathway was *divinyl ether synthase* (*DES*). *DES* is a CYP74D family enzyme involved in the synthesis of the cytotoxic oxylipin, named colneleic acid (Stumpe *et al*., 2001), which has antimicrobial function against *P. infestans* (Weber *et al*., 1999). *DES* transcripts have been shown to accumulate in potato leaves on infection with *P. infestans* (Stumpe *et al*., 2001). However, recently, it has been shown that, in spite of the high accumulation of *DES* transcripts, no correlation with late blight resistance level could be established (Fauconnier *et al*., 2008.) JA and ET signalling often operate synergistically to activate the expression of defence-related genes after pathogen inoculation (reviewed by Bari and Jones, 2009). Examples of this are ET response factors (ETRFs) that function in JA signalling and disease resistance in Arabidopsis (McGrath *et al*., 2005). Two of the genes found to be differentially regulated in B3 correspond to ETRFs. Interestingly, these genes were significantly up-regulated only in the PE interaction, in contrast with the JA pathway genes in B3 which were significantly up-regulated only in the POX interaction. In Cjm, both JA- and ET-related genes were up-regulated only in the POX interaction. JA and ET pathway activation is usually associated with necrotrophic pathogens (Bari and Jones, 2009). The activation of these pathways in the potato genotypes studied here is in agreement with the timing of the establishment of necrotic lesions in the POX interaction.

We also found an indication of the activation of ABA-, auxin-, brassinosteroid- and cytokinin-related genes in response to late blight attack. The role of cytokinins and brassinosteroids in defence is poorly understood, but there is emerging evidence of their involvement in the regulation of defences in some plant–pathogen interactions (reviewed by Bari and Jones, 2009).

We found indirect evidence of SA in the defence response to late blight in both genotypes studied, as indicated by the differential expression of *EDS1* and *PAD4* and several SA-induced PR proteins. SA accumulation induced in response to pathogen infection in Arabidopsis is controlled by *EDS1* and *PAD4* (Wiermer *et al*., 2005; Zhou *et al*., 1998), which are also required for the function of NBS-LRR-type *R* genes (Feys *et al*., 2001; Hu *et al*., 2005). Recently, it has been shown that potato *NahG* transgenic lines that exhibit reduced accumulation of SA allow for more *P. infestans* growth, suggesting that SA is important for basal defence in potato (Halim *et al*., 2007). However, we found no significant induction of genes considered as marker genes of the SA synthesis pathway as defined in MapMan.

Proteolysis

We found evidence of the involvement of proteolysis in late blight resistance in both genotypes studied. Of particular interest among the proteolysis-related genes activated are the components of the ubiquitination pathway, which has recently been shown to contribute to disease resistance (reviewed by Birch *et al*. 2009 and Delaure *et al*., 2008). The U-box proteins ACRE74 and ACRE276, are active as single subunit E3 ubiquitin ligases and are required for *Cf-9* resistance in Solanaceae (reviewed by Delaure *et al*., 2008). Furthermore, both proteins are required for HR in *P. infestans*-secreted INF1-elicitor and tobacco interaction.

PR proteins, phenylalanine ammonia-lyase (PAL) and secondary metabolism

As expected, several PR proteins, as well as PAL, indicating the activation of systemic acquired resistance, were induced in both genotypes. Chitinases (Beyer *et al*., 2001; Birch *et al*., 1999; Wang *et al*., 2005) and osmotin-like genes (Castillo Ruiz *et al*., 2005; Liu *et al*., 1994; Trognitz *et al*., 2002; Vleeshouwers *et al*. 2000b, Zhu *et al*., 1996) have been reported to be associated with late blight resistance in potato. A high level of *thaumatin*-like PR-5 expression was discovered in both genotypes by real-time RT-PCR. The extremely high early expression of *thaumatin* in Cjm is an interesting result, and further studies are required to elucidate whether it is caused by a higher copy number of the genes or other regulatory-related reasons. Thaumatin-like proteins are acidic PR-5 proteins that also show sequence similarity to PR-5 osmotins (Castillo Ruiz *et al*., 2005).

Other interesting genes

Three different patatin genes were found to be differentially expressed in the B3 genotype. Patatin is a nonspecific lipid acyl hydrolase and a major potato tuber storage protein that has recently been shown to have antimicrobial function against *P. infestans* (Sharma *et al*., 2004). In Arabidopsis, patatin-like phospholipases activated by plant–pathogen interaction have been implicated in signal transduction, leading to systemic acquired resistance (Holk *et al*., 2002), and those of tobacco are rapidly activated during HR associated with TMV (Dhont *et al*., 2000). Recent evidence suggests that patatins may act as precursors for oxylipin synthesis (Dhont *et al*., 2000). In our real-time RT-PCR study, we found a strong up-regulation of patatin in Cjm at 72 h after PE inoculation.

CONCLUSIONS

Although gene expression studies have uncovered molecular events in *S. tuberosum* during late blight attack and have identified several candidate genes involved in resistance (Restrepo *et al*., 2005; Ros *et al*., 2004), the gene networks activated in closely related species, and potentially useful in breeding, remain largely unknown. The main objective of this study was to test a hypothesis for unique components of resistance in *S. cajamarquense* with respect to B3C1, as a means to guide the use of wild species in base-broadening efforts. Our results show that the genes activated in B3 and Cjm have largely the same biological functions that are commonly activated when plants respond to pathogen attack. This was to be expected, as gene expression changes caused by pathogen attack or stress generally share similarities between species and different resistance mechanisms. However, the genes activated within biological function categories were mostly different between the genotypes studied, suggesting that the defence pathways activated in B3 and Cjm by the *P. infestans* isolates tested seem to involve mostly unique genes. However, as indicated by the real-time RT-PCR study, the differences observed in microarray experiments may not be as pronounced, as several genes that were not significant in the microarray experiment were found to be highly significant when tested by real-time RT-PCR. The differences between the two methods are probably caused by different kinetics of expression between biological experiments, and indicate that more time points need to be analysed in order to capture all genes activated during resistance.

EXPERIMENTAL PROCEDURES

Plant material

Rooted *in vitro* plantlets of a wild genotype of *S. cajamarquense*, 762 619.244, referred to as Cjm, and a genotype of a bred line of B3C1, 391 011.17, referred to as B3, as well as controls for quantitative resistance, LBr-40 (highly resistant), Monserrate and Chata Blanca (moderately resistant) and Yungay (susceptible), were transplanted to Jiffystrips®, Product 300522 [Jiffy Products (N.B.) Ltd., Shippagan, NB, Canada] and placed in a screen house at CIPs experiment station in the Peruvian highlands, Huancayo (latitude 12°38′S, longitude 75°13′W, altitude 3289 m) in March 2005. After 1 week of rooting, the plants were transferred into pots with a sterile soil mix in the same screen house and grown in natural short day length until inoculation. The average temperature during the experiment was 15 °C, ranging from the night-time minimum temperature of 0.5 °C to a daytime maximum of 34.5 °C.

The real-time RT-PCR experiment was conducted in La Molina, Lima (latitude 12°00′07″S, longitude 76°51′00″, altitude 240 m) in December 2006, under natural short-day conditions. The average temperature during the experiment was 19.5 °C, ranging from the night-time minimum temperature of 11 °C to a daytime maximum of 35 °C.

Phytophthora infestans isolates, maintenance and inoculum preparation

Two *P. infestans* isolates with different virulence and aggressiveness were used in this study. The PE84006 isolate, referred to as PE, is a race 0, which is incompatible with all known *S. demissum R* genes. This isolate, collected in Junin, Peru, in 1984, belongs to the US-1 lineage and is of the A1 mating type (Pérez *et al*. 2001). It is not very aggressive and usually causes slow disease progress during compatible interaction. The POX067 isolate, referred to as POX, is avirulent (i.e. incompatible) with the *S. demissum* genes *R8* and *R9*. It is a highly aggressive isolate originating from Oxapampa, Peru, and belongs to the EC-1 lineage (Villamon *et al*., 2005). The US-1 isolates have been displaced by isolates belonging to the EC-1 lineage, which is currently the predominant type of *P. infestans* found in Peru (Pérez *et al*. 2001). Aliquots of sporangia suspension were preserved in liquid nitrogen. For the experiments, a fresh sample of sporangia was placed on tuber slices of the susceptible potato cultivar Huayro (native potato without known *R* genes) and incubated for 6–7 days at 18 °C and photoperiods of 12 h in a moist chamber to promote sporulation. Sporangia were collected on 10 μm filter paper and rinsed with distilled water.

Inoculation and estimation of resistance components

Whole-plant tests

After 60 days, all plant material was moved to an inoculation glasshouse in the same experimental station. Before flowering, whole plants were inoculated with *P. infestans* by spraying each plant until run-off with a hand-held sprayer. In total, 36 plants/genotype/isolate were inoculated with *P. infestans* sporangial suspension (approximately 5×10^3 sporangia/mL) in the microarray experiment. In real-time RT-PCR, four plants/genotype/isolate were inoculated. Water-treated plants were included as a control. After inoculation, conditions were maintained conducive to late blight by the delivery of cool mist through an overhead sprinkler system operating on a thermostat. Shade cloth was used to maintain the temperature in the range of 15–20 °C and plastic film was used during the night over the plants to maintain appropriate humidity for disease development. Late blight evaluation started 3 days after inoculation. Each plant was evaluated for disease status (percentage of leaf area affected), as recommended internationally (Bonierbale *et al*., 2007; Forbes and Korva, 1994), and representative individuals were marked for leaf sampling. The percentage of leaf area infected was recorded each day for 7 days after inoculation. The evaluation was performed with the scale 0–100% at five-unit intervals, where 0 = no visible reaction, 5 = HR and 10–100 = successful infection. Resistance components consisting of the number of plants with HR, number of infected plants and average leaf area affected at 3, 4, 5 and 7 days after inoculation are shown in Tables 1 and 2.

Detached leaf assay

Leaves from the middle part of the plants, grown as described above, were detached and placed on inverted Petri dishes lined with 1.5% water agar, such that the leaflets were positioned in the lids below the agar layer. A 20-μL drop of sporangia (approximately 5×10^3 sporangia/mL) was placed on the abaxial surface of each leaflet. For clone B3 and the control clones, three leaflets per leaf were inoculated, whereas, for clone Cjm, five leaflets were inoculated. The plates were sealed with Parafilm and covered with black plastic overnight. On the next day, the black plastic was removed and the plates were exposed to natural daylight. The test was performed at room temperature. The largest length and width of each lesion were measured at 3, 4 and 5 days after inoculation, and the ellipse area was calculated as described by Vleeshouwers *et al*. (1999). The lesions that remained the size of the inoculum droplet or smaller were considered as HR, and were not included in the estimation of the lesion growth rate. Thus, only growing lesions were used to calculate the estimate of the lesion growth rate (Vleeshouwers *et al*. 1999). The infection efficiency was calculated as the percentage of successful infections relative to the number of inoculations per genotype. To obtain an estimate of the additional components of resistance, we counted the number of sporangia per leaflet of five inoculated leaves per genotype at 5 days after inoculation. An estimation of the degree of sporulation was obtained by dividing the number of sporangia by the size of the lesion.

Sampling and RNA extraction

To obtain a representative sample of each genotype, inoculated with each isolate, individual plants were selected to include those with visible HR and those without any visible reaction. Leaves were taken from the middle part of four individual plants of each genotype and combined for a single sample. In total, three samples per isolate and genotype were collected at 72 h after inoculation, and another three samples from the same plants at 96 h after inoculation. The three samples from a given time point, genotype and isolate were considered as independent biological replicates. Leaves of uninoculated plants were collected as control samples. After excision, the leaves were immediately placed in liquid nitrogen and kept frozen until RNA extraction. RNA was extracted from the leaves using the Trizol method indicated by TIGR, available at: http://jcvi.org/potato/images/SGED_SOP_3.1.1.pdf.

Hybridization design

In total, 24 hybridizations were performed. Hybridizations consisted of a reference sample (uninoculated sample) labelled with

Cy5 and a test sample (inoculated sample) labelled with Cy3 at each time point. There were two genotypes (Cjm and B3), two isolates (PE and POX) and two time points (72 and 96 h after inoculation), and each combination was present in three biological replicates, yielding a total of 24 arrays or hybridizations. Details concerning the potato cDNA array, expressed sequence tag (EST) sequences, the clones on the array, annotation of the clones and all methods are also available at the TIGR website: http://www.jcvi.org/potato/sol_ma_microarrays.shtml.

Data analysis

For both genotypes, all cDNA clones that showed more than two flagged spots throughout the experiment were discarded. Differentially regulated clones were selected separately for each genotype–isolate–time point interaction by SAM, with a two-class unpaired design. The clones were considered to be differentially regulated if the mean expression of the inoculated samples was significantly higher or lower than that of the uninoculated samples, as measured by the Tusher method (Tusher *et al*., 2001), and with the lowest possible false discovery rate of the 90th percentile. The missing values were imputed by the *K*-nearest-neighbour method and 100 permutations were performed. The analysis was performed with the TIGR Multiexperiment Viewer (MeV version 4.2) (http://www.tm4.org/mev.html). MapMan software (https://www.gabipd.org/projects/MapMan/), using ontology adapted to solanaceous species, was used to obtain an overview of the late blight-affected genes belonging to various metabolic pathways in Cjm and B3.

Real-time RT-PCR

Six genes that were differentially expressed in B3 at different time points were selected for confirmation by real-time RT-PCR (Table 3). Instead of using the single clone sequences for primer design, we utilized the best matching gene sequence that corresponded to the tentative consensus build including the cDNA clone that was differentially expressed in the microarray. The DNA sequences of these genes were obtained from the National Center for Biotechnology Information database, and primers for quantitative detection were designed using Primer3 (http://frodo.wi.mit.edu/) (Table 3). All primer combinations yielded a single PCR product of the expected length as verified by PCR and gel electrophoresis (not shown). Optimal melting temperatures were determined for each primer combination, and the efficiency of PCR was determined by a dilution series of cDNA.

RNA was collected from B3 plants inoculated with the POX isolate, as described for the microarray experiment. cDNA was synthesized from 3 μg of RNA using the SuperScript® III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA). PCR was run in a final volume of 10 μL using 25 μM of each primer on a PTC 200 Peltier Thermal Cycler, equipped with a Chromo4 Continuous Fluorescence Detector and DNA Opticon 2 software (MJ Research, Alameda, CA, USA), using iQ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA), following the manufacturer's instructions. The gene expression levels obtained were normalized to the abundant constitutive cytochrome oxidase (*Cox*) gene (Weller *et al*., 2000), whose expression, as determined by expression of the corresponding cDNA clone STMIL60 on the microarray, was not affected by late blight in the conditions tested. The calculations were made with REST2008 (Relative Expression Software Tool) (Pfaffl *et al*., 2002).

ACKNOWLEDGEMENTS

Funding from the Austrian Government for the CIP-ARC collaborative research project, 'Genomic analysis of the effect of photoperiod on late blight resistance in potato' (2003–07), is gratefully acknowledged. We would also like to thank the following people from CIP: Juan Landeo for recommending the B3 clone used in this study, Elvin De la Torre for technical support, and Jan Kreuze for critical reading of the manuscript.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Fig. S1 Average signal strength of cDNA clones that were accepted as good spots for Cjm and B3 in each isolate–time point interaction and for the two dyes (Cy3 and Cy5). Error bars show the standard deviations of the biological replicates.

Table S1 Log_2 expression values (inoculated vs. uninoculated sample) of all cDNA clones that were found differentially expressed in *Solanum cajamarquense* (Cjm) at each isolate time point interaction. Values in bold indicate statistically significant up- or down-regulation. Functional categories (FunCat), MAPMAN descriptions and bin names are given for each clone.

Table S2 Log_2 expression values (inoculated vs. uninoculated sample) of all cDNA clones that were found differentially expressed in B3 genotype at each isolate time point interaction. Values in bold indicate statistically significant up- or down-regulation. Functional categories (FunCat), MAPMAN descriptions and bin names are given for each clone.

Table S3 Functional grouping of the stress-related cDNA clones in Cjm. Stress function, group name and MeV name columns are used in heat plot (Fig. 2a). Functional category (FunCat), Bin name, MAPMAN description, and log_2 expression values are the same as in Table S1.

Table S4 Functional grouping of the stress-related cDNA clones in B3. Stress function, group name and MeV name columns are used in heat plot (Fig. 2b). Functional category (FunCat), Bin name, MAPMAN description, and log_2 expression values are the same as in Table S2.

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