Research Note

Isolation of Potato Genes That Are Induced During an Early Stage of the Hypersensitive Response to *Phytophthora infestans*

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Suppression subtractive hybridization (SSH) was used to generate a cDNA library enriched for sequences induced in a late-blight-resistant potato cultivar undergoing the hypersensitive response (HR). Of 100 partial cDNA sequences submitted to international DNA and protein data bases, 42 showed similarity to 35 genes, of which 31 were from plants. Of these, 13 were previously characterized as either defense-, stress-, or senescence-associated. One sequence matched (75 to 81%) all known serine palmitoyl-transferases (SPTs) at the protein level. SPT catalyzes the first committed step in the synthesis of sphingolipids, important signaling molecules involved in cell differentiation and apoptosis. Putative products of other genes identified here may play a role in programmed cell death, including protein degradation, DNA degradation, metal ion chelation, and signal transduction. cDNA-amplified fragment length polymorphism was used to confirm differential expression of sequences isolated by SSH.

Additional keyword: EST.

In recent years the connection has been made between the pathogen-induced hypersensitive response (HR) in plants and the form of programmed cell death (PCD) in animals known as apoptosis. Characteristic symptoms of apoptosis, such as DNA fragmentation, membrane blebbing, and nuclear and cytoplasmic condensation, have been observed in various forms of plant PCD, suggesting common processes in plants and animals (reviewed in Greenberg 1996, 1997; Heath 1998). Recently, a motif shared by plant resistance (*R*) gene products and animal cell death regulators was reported (van der Biezen and Jones 1998). Nevertheless, molecular bases of signal transduction and the various cascades of responses induced by *R* gene activation are poorly understood (Greenberg 1997).

PCD in plants has also been widely associated with developmental processes, including reproduction, senescence, and

fruit ripening. At least in part, PCD in all three requires the action of the plant hormone ethylene (reviewed in Greenberg 1996). In the case of senescence, ethylene may induce cysteine proteases (Grbic and Bleecker 1995). Members of the cysteine protease family are strongly implicated in initiation of apoptosis in animals. Nevertheless, it remains to be determined whether senescence-associated cysteine protease activity is linked to cell death initiation and, if so, whether such mechanisms are common to other plant processes such as the HR.

Interactions between potato and *Phytophthora infestans*, the cause of late blight, offer opportunities to study the HR. Genetic resistance to *P. infestans* in both wild and cultivated potato species is of two forms, either race specific or race nonspecific (field resistance) (reviewed in Wastie 1991). Race-specific resistance is characterized by interactions between products of dominant *R* gene alleles in the host and corresponding avirulence (*avr*) gene alleles in the pathogen, resulting in a localized HR and complete inhibition of infection.

The aim of this work was to determine molecular events associated with the potato HR, by isolating cDNA sequences that are induced in a highly resistant cultivar after infection with *P. infestans* but not induced in a susceptible cultivar. For this, recently developed PCR (polymerase chain reaction)-based methods for isolating differentially expressed genes (suppression subtractive hybridization [SSH]; Diatchenko et al. 1996) and for profiling gene expression (cDNA-amplified fragment length polymorphism [cDNA-AFLP]; Bachem et al. 1996) were utilized.

To isolate differentially expressed genes associated with the potato HR, choices of cultivars and time point after inoculation with the pathogen are major considerations. Whereas cv. Bintje is susceptible to *P. infestans*, cv. Stirling has both high levels of field resistance and an uncharacterized *R* gene (Wastie 1991). In the incompatible interaction a visible HR is seen 3 days after inoculation. *P. infestans* isolate 89-AF1 elicits such a response. Freytag et al. (1994) have shown, using microscopy, that, at 24 h post inoculation, whereas only slightly larger numbers of epidermal cells are undergoing hypersensitive cell death in the incompatible (HR) than in the compatible (susceptible) response, considerably more hypersensitive-like responses occur in mesophyll cells in the incompatible interaction. A time point for RNA extraction of 24

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Nucleotide and/or amino acid sequence information for clones STL1 to STL36 are to be found at the EMBL data base as accession nos. AJ235735 to AJ235770.

h post inoculation was chosen to see if early induced gene expression differences could be detected between the infected cultivars. To allow for variation in response, three plants from each cultivar were point inoculated with zoospore droplets (10^5 per ml) and leaf material pooled prior to RNA extraction (with an RNeasy Plant minikit; Qiagen, Hilden, Germany), DNase I treatment, and mRNA purification (with the mRNA Separator kit; Clontech, Palo Alto, CA). From mRNA, double-stranded cDNA was synthesized (with the Riboclone cDNA Synthesis System; Promega, Madison, WI) and used for both cDNA-AFLP and SSH (see below). To test the phenotype of the interactions, a fourth plant of each cultivar was simultaneously inoculated and infection allowed to proceed for 5 days. Typical leaf symptoms of each infected cultivar after this period were observed (results not shown).

SSH (with the Clontech PCR-Select cDNA subtraction kit, protocol PT1117-1) was used to generate a cDNA library enriched for sequences expressed specifically in infected leaves of cv. Stirling, with cDNA from infected leaves of susceptible cv. Bintje as a driver to remove common sequences. Following SSH, amplification products from 100 bp to over 1 kb were generated, purified with the Promega Wizard PCR Preps kit, directly cloned with the Promega pGEM-T vector cloning kit, and transformed into Ultracompetent *Escherichia coli* XL2-blue MRF' cells (Stratagene, La Jolla, CA). Seven hundred sixty-eight of the approximately 4,000 recombinant clones were screened by hybridization to identify abundant cDNA sequences. Two complex probes were used. First, an *Rsa*I-digested portion of unsubtracted driver cDNA was ligated to adapters 1 and 2 (Clontech PCR-Select kit), amplified, and used as a probe following removal of the adapter sequences. A probe from subtracted target cDNA (material used for cloning) was prepared in the same way. As expected, the subtracted cDNA probe hybridized reproducibly to all clones to varying degrees, strength of signal presumably reflecting copy number of each sequence in the probe. Driver cDNA hybridized reproducibly to approximately 25% of clones, most of which also hybridized strongly to the subtracted cDNA probe (results not shown). Sequences from 15 of these were all identified as rRNA, demonstrating that significant amounts of this had been converted to cDNA and come through the subtraction process.

Plasmid preparations, with the Qiagen Plasmid Miniprep kit, from 100 clones hybridizing only to subtracted cDNA, were sequenced (Perkin Elmer ABI PRISM Dye Terminator cycle sequencing kit and ABI Model 377 DNA sequencer) in both directions (with Promega M13 forward and reverse primers). Insert sizes varied from 40 to over 600 bp and the average was 162 bp (excluding primer sequences). Eighteen clones were redundant, with nine containing the same short (61 bp) sequence.

In all, 42 clones, with an average insert size of 175 bp, showed significant similarity to data base sequences, 29 with FASTA, 11 with BLASTP, and 2 with BLASTX. All FASTA matches were also submitted to BLASTX searches. Sequences from 19 clones that revealed significant homologies by FASTA failed to make a significant match with BLASTX. In each case, the cloned region matched the 3' untranslated region (UTR) of a gene. BLAST scores fell into the three classes described by Nelson et al. (1997) for *Neurospora crassa* expressed sequence tags (ESTs): 8 highly significant ($P < 10^{-19}$); 15 moderately significant ($P = 10^{-5}$ to 10^{-19}); and 4 weakly significant ($P = 10^{-2}$ to 10^{-4}). From the 42 clones, sig-

nificant similarities to 35 gene sequences (Table 1) were detected, with 11 clones containing redundant inserts and two independent clones, STL2 and STL3, revealing homology to contiguous regions of a cysteine protease from tomato. Several BLASTP matches were made with reading frames containing a translation stop codon. In these cases, matches were to C termini of proteins. Indeed, the majority of matches with FASTA were to 3' ends of genes.

Thirty-one of the genes identified in data bases were from plants. Of these, 13 are either senescence-, stress-, or defense-related (Table 1: STL2s 2/3; 6, 7, 8, 9, 10, 12, 13, 14, 16, 17, 18, and 24). While the strongest match to STL8 was anther-specific APG from *Arabidopsis thaliana*, strong protein similarity was also seen to wound- and jasmonate-inducible myrosinase from *Brassicaceae* (Taipalensuu et al. 1996). Similarly, the *SAC51* gene from *Brassica napus* induced during pod development (which matches STL9), shows strong similarity, at the protein level (P e-30 to e-33), to related proline-rich proteins including extensins and a protein (TID23) involved in the formation of genetic tumors in tobacco (Fujita et al. 1994). Interestingly, another sequence (STL17) matched a second tumor-related protein (TID617) described by these authors. Such tumors are initiated by stress and may arise from the formation of interspecific or intraspecific hybrids.

The first question asked was whether matches were made to genes assumed to have a function in the HR. Two types of *PR-1*-like sequence, STL6 matching *prb-1b* from tobacco and STL7 matching *P14a* from tomato, encode ethylene-induced, basic forms of the *PR-1* family in tobacco (Sessa et al. 1995) and tomato (Tornerio et al. 1994), respectively. *P14a* has antifungal activity to *P. infestans* (Niderman et al. 1995) and is associated with the tomato HR (Tornerio et al. 1997). Intriguingly, both proteins show significant homology to a human *PR* protein, GliPR, which may suggest a functional link between the human immune system and a plant defense system (Szyperski et al. 1998).

In addition, a gene encoding a basic chitinase (*PR-3*) was isolated. Although there was a 90% protein match to three potato endochitinases, the strongest DNA match (81.8%) was to the *ChtB1* gene (accession no. U02607). Thus, STL18 is from a related but unique gene. Recently, Takemoto et al. (1997) showed that actin, involved in cytoplasmic aggregation during the early stages of the potato HR to *P. infestans*, is intimately associated with basic chitinase and osmotin. It is therefore interesting that another sequence isolated in this study (STL19) encodes actin.

An early event associated with the HR is the oxidative burst, leading to generation of reactive oxygen intermediates such as O_2^- , H_2O_2 , and $\cdot OH$ (Dangl et al. 1996). The oxidative burst is implicated in a direct defense response by stimulating oxidative cross-linking in the cell wall. However, it has been noted that extracellular H_2O_2 could not be detected when *P. infestans* invaded nonhost parsley cells (Naton et al. 1996). One sequence detected here (STL25) encoded a putative open reading frame similar to hyoscyamine 6-dioxygenase. This is a member of the FeII/ascorbate-dependent oxidase superfamily, which generate a number of radical species, including ascorbyl. A related protein is implicated in senescence in *A. thaliana* (Callard et al. 1996), a process associated with PCD (Greenberg 1996). Ascorbyl radical can directly induce apoptosis in animals (Sakagami and Satoh 1997).

Evidence gathered in the last few years suggests that many plant processes, including the HR, involve PCD similar to apoptosis in animals (Greenberg 1996; Heath 1998). The oxidative burst and temporally associated Ca^{2+} and H^+ influx early in HR may act as stimulators of further signaling mechanisms promoting cell death (Dangl et al. 1996; Somssich and Hahlbrock 1998). In animal cells the phosphoinositide and sphingolipid pathways are major lipid-based signaling systems involved in regulating cell growth, differentiation, and apoptosis (respectively, Carpenter and Cantley 1996; Peña et al. 1997). Much evidence exists for phosphoinositide signaling in plant stress responses (e.g., Kopka et al. 1998), but, as yet, little attention has been directed toward sphingolipids (Heath 1998; Gilchrist 1997). Two cDNA sequences described here, STL5 matching a phosphatidylinositol-4-phosphate 5-kinase (PI4P5 kinase) gene and STL1 matching a serine palmitoyltransferase (SPT) gene, encode central enzymes to each respective pathway. Until now, there has been no published information relating to either gene in plants.

In animals, PI4P5 kinase is implicated in the generation of all polyphosphoinositides (Zhang et al. 1997) and is associated, among other things, with massive actin polymerization

(Shibasaki et al. 1997). SPT catalyzes the first committed step in sphingolipid biosynthesis, by condensation of palmitoyl CoA and serine to form sphinganine. Sphinganine is converted to two potent second messengers, either sphinganine-1-phosphate (SPP), involved in Ca^{2+} mobilization, or ceramide (Merrill et al. 1997). All three compounds are known in plants (Heinz 1996). Addition of either sphinganine or ceramide to animal cells causes apoptosis via regulation, respectively, of mitogen (MAPK)- and stress (SAPK)-activated protein kinases (Jarvis et al. 1997).

Apoptosis via the sphingolipid pathway may be activated by a variety of stresses, including oxidative stress (Peña et al. 1997). In addition, membrane receptors such as tumor necrosis factor receptor (TNFR), interleukin-1 receptor (IL-1 β), and p75 (neurotrophin receptor) activate cell death through ceramide signaling. Activation of SAPK by ceramide then leads to direct activation of the interleukin-1 β converting enzyme (ICE/CED3) family of cysteine proteases, which cause the characteristic cytoplasmic condensation of apoptosis (Merrill et al. 1997). Intriguingly, TIR domains of *R* genes *RPP5* from *A. thaliana*, *N* from tobacco, and *L6* from flax are shared with the cytoplasmic domain of the interleukin-1 receptor (van der

Table 1. Similarities at the DNA or protein level between cloned cDNA sequences from infected cv. Stirling and sequences in data bases

Clone no.	Clone length (bp)	Matching sequence from data base	Origin of matching sequence and accession nos.	DNA % match (FASTA) ^a	Protein % match (BLASTP) ^b
STLB1	275	Serine palmitoyltransferase (LCB2 or SPT2)	<i>Mus musculus</i> X95642		81 (5.9e-27)
STLB2	148	Cysteine protease	<i>Lycopersicon esculentum</i> M21444	85.0 (2.6e-25)	
STLB3	549	Cysteine protease	<i>L. esculentum</i> M21444	81.7 (5.9e-187)	96 (1.3e-118)
STLB4	207	EST	<i>Arabidopsis thaliana</i> AC005496		88 (1.4e-19)
STLB5	221	Phosphatidylinositol-4-phosphate-5-kinase	<i>A. thaliana</i> AF0193		66 (1.8e-06)
STLB6	171	Pathogenesis-related protein <i>prb-1b</i>	<i>Nicotiana tabacum</i> X66942	78.0 (2.9e-26)	75 (1.1e-14)
STLB7	56 (3 clones)	Pathogenesis-related protein (P14a)	<i>L. esculentum</i> X68738	88.7 (2.2e-07)	
STLB8	156	Proline-rich APG	<i>A. thaliana</i> Z97340		62 (2.0e-06)
STLB9	398	Proline-rich SAC51	<i>Brassica napus</i> X71618	63.1 (1.2e-31)	86 (1.1e-32)
STLB10	254	Stress-responsive cyclophilin	<i>Solanum commersonii</i> U92087	79.9 (1.8e-52)	
STLB11	134	Chloroplast carbonic anhydrase	<i>N. tabacum</i> M94135	95.5 (8.2e-24)	95 (7.8e-10)
STLB12	119	Chloroplast drought-induced stress protein (CDSP32)	<i>S. tuberosum</i> Y09987	90.2 (3.9e-09)	
STLB13	429	Metallothionein type II LEMTB	<i>L. esculentum</i> Z68138	91.9 (2.7e-125)	98 (3.7e-37)
STLB14	61	Metallothionein type II LEMTA	<i>L. esculentum</i> Z68310	97.1 (1.1e-16)	95 (2.7e-14)
STLB15	257	Ubiquitin carrier protein UBC1	<i>Medicago sativa</i> L06967	83.1 (1.0e-67)	100 (3.2e-58)
STLB16	244	Stress-responsive ubiquitin-ribosomal fusion protein (<i>ubi3</i>)	<i>S. tuberosum</i> L22576	97.2 (4.5e-90)	100 (2.8e-17)
STLB17	179	Tumor-related protein (TID617)	<i>N. tabacum</i> D26462	76.5 (6.3e-26)	
STLB18	281	Chitinase <i>ChitB1</i>	<i>S. tuberosum</i> U02605	81.8 (7.4e-30)	90 (1.2e-20)
STLB19	176	Contains actin-like region	<i>A. thaliana</i> U41998		70 (6.0e-08)
STLB20	54	Glycine-rich GRP2	<i>N. sylvestris</i> Z81320	72.3 (1.1e-04)	88 (8.4e-07)
STLB21	171 (2 clones)	RNA-binding glycine-rich protein	<i>N. tabacum</i> D16205	74.2 (1.9e-08)	
STLB22	142	Vitronection (also EF1 alpha-like)	<i>N. tabacum</i> U04632	72.8 (3.3e-16)	
STLB23	310	Elongation factor 2 (EF-2)	<i>Beta vulgaris</i> Y10991	83.2 (1.4e-95)	98 (7.5e-26)
STLB24	62	Glycine-rich protein	<i>N. tabacum</i> X74106	64.4 (1.1e-04)	65 (1.2e-03)
STLB25	229	Hyoscyamine 6-dioxygenase	<i>Hyoscyamus niger</i> P24397		55 (3.2e-05)
STLB26	62	Glyoxysomal malate dehydrogenase	<i>Citrullus vulgaris</i> M33148	72.6 (8.9e-09)	90 (2.0e-06)
STLB27	192 (2 clones)	Cytochrome C oxidase	<i>Plasmodium falciparum</i> M99416		72 (4.8e-05)
STLB28	64 (2 clones)	Electron transfer flavoprotein-ubiquinone	Yeast Z75264		88 (3.4e-03)
STLB29	43 (2 clones)	Ribosomal protein L8	<i>M. musculus</i> U67771		100 (1.0e-03)
STLB30	55	Ribosomal protein S3	<i>Petunia hybrida</i> U56910	93.8 (7.7e-09)	
STLB31	333	Glycyl-tRNA synthetase	<i>A. thaliana</i> AJ0020		71 (5.3e-11)
STLB32	133	CAB9 (CP29)	<i>L. esculentum</i> X61287	95.7 (1.1e-39)	100 (1.9e-18)
STLB33	170	Plastidic aldolase	<i>S. tuberosum</i> Y10380		93 (7.3e-04)
STLB34	70	Starch phosphorylase L-1	<i>S. tuberosum</i> X52385	85.0 (4.4e-14)	85 (7.1e-07)
STLB35	47	Ribulose 1,5 bisphosphate carboxylase	<i>S. tuberosum</i> Z69763	98.0 (9.0e-11)	
STLB36	292	Peptide transporter PTR2-B	<i>A. thaliana</i> L39082		55 (2.2e-09)

^a Expect values of best match given in parentheses.

^b Expect value of best match given in parentheses.

Biezen and Jones 1998), raising the possibility of equivalent pathways in plants.

As yet, no ICE/CED3 analogues have been found in plants, but Greenberg (1996) has suggested that senescence-associated cysteine proteases may function in plant PCD. Sequences STL2/3, which match a senescence-associated cysteine protease in tomato (Drake et al 1996), have been isolated in this study. This protease strongly matches all known cathepsins in animals, particularly cathepsin K (P 9.1e-22). In addition, components of the proteasome, ubiquitin and ubiquitin carrier protein, showing protein matches with STL16 and STL15, respectively, are involved in proteolysis during apoptosis in animals (Soldatenkov and Ditschilo 1997) and are up-regulated by oxidative stress (Shang et al. 1997).

DNA fragmentation accompanies proteolysis during apoptosis and is observed during plant PCD (Greenberg 1997; Ryerson and Heath 1996). In animals, such nuclease activity is attributed to Ca^{2+} -induced cyclophilins A, B, and C (Montague et al. 1997). Clone STL10 matches (79.9%) a stress-responsive cyclophilin from *S. commersonii* at the DNA level. At the protein level, this cyclophilin shows 84% (P 1.9e-62) identity, across its entire length, with human cyclophilin A. In addition to cyclophilin, a match was made to ribosomal protein S3 (STLB30), which removes oxidatively damaged DNA bases (Deutsch et al. 1997) and may thus contribute to protection in cells surrounding tissues undergoing PCD.

Also implicated in apoptosis in animals are metallothioneins, although it is unclear whether they are active components or involved in protection against oxidative stress (e.g., Abdel-Mageed and Agrawal 1997). The latter role has recently been suggested for metallothioneins during senescence in plants (Buchanan-Wollaston and Ainsworth 1997). Two metallothionein matches were made in this work (STLBs 13 and 14).

RT (reverse transcription)-PCR was used to confirm that sequences from eight clones—STLBs 1, 2, 4, 7, 10, 13, 16, and 18—were derived from 3' ends of mRNAs. Oligonucleotide primers designed to anneal to these (Table 2) were each used in combination with an oligo dT primer (Table 2) to PCR amplify cDNA prepared from either infected or uninfected cv. Stirling. In each case, a single amplification product was obtained from both cDNA populations (results not shown). While all sequences obtained from this library must be tested to confirm their plant origin, none of the sequences examined here generated amplification products from cDNA prepared from *P. infestans*. Each of the products amplified from plant cDNA was sequenced to the poly A tail, and confirmed the clones to be derived from regions close to the 3' ends of their respective mRNAs. Moreover, this result demonstrated PCR-

detectable levels of expression of all eight sequences in uninfected leaves. Given that RT-PCR is sensitive and non-quantitative, an alternative method was thus needed to test whether SSH had yielded differentially expressed Stirling genes.

Diatchenko et al. (1996) reported that, during normalization, rare cDNAs were enriched 1,000-fold in one round of SSH. They found that detection of some such sequences with Northern (RNA) analysis proved difficult, requiring high concentrations of RNA and over 2 weeks of film exposure. Recently, Bachem et al. (1996) have demonstrated that the kinetics of gene expression revealed by cDNA-AFLP are similar to those of Northern analysis. Given the speed of cDNA-AFLP and the small quantity of RNA required to make templates, we investigated whether such material would provide an independent test to see if Stirling sequences obtained by SSH are differentially expressed. In principle, PCR of cDNA-AFLP templates with M00/E00 (no selective nucleotides) primers should competitively amplify all cDNA sequences with two *EcoRI* sites or an *MseI* and an *EcoRI* site. Vos et al. (1995) have shown that the small *MseI*-*MseI* fragments present in AFLP templates are not amplified due to the formation of stem-loop structures during the PCR annealing step. Of the short sequences isolated in this study, many of which contain the AT-rich 3' UTR, 74% contain an *MseI* site, suggesting that, given one or more up-stream *EcoRI* sites, portions of most intact cDNAs would be expected to be amplified by E00/M00 primers. If so, then the amplification products of specific gene sequences should be present within the cDNA-AFLP pool of fragments and therefore detectable by hybridization.

cDNA-AFLP templates prepared from both cultivars, infected and uninfected, were therefore PCR amplified and 500 ng of each product electrophoresed and Southern blotted. Eleven such blots were made. DNA inserts from 10 clones (STLBs 1, 3, 4, 10, 13, 15, 16, 17, 18, and a clone, STL37, containing an unidentified 200 bp open reading frame) were ^{32}P -labeled and hybridized under high stringency to the filters. With the exception of STL3 (cysteine protease), and STL37 (unknown) all sequences matched 3' ends of genes. In addition, all sequences contained an *MseI* site, except for STL3, which contained an *EcoRI* site. In each case, strong hybridization was observed to one or more amplification product(s) derived from infected Stirling cDNA (Fig. 1A-J). STL18 (basic chitinase) hybridized to three products in the amplified cDNA from both infected Bintje and Stirling (Fig. 1I). However, only the largest was apparently differentially expressed. The experiment was repeated with cDNA-AFLP templates from fresh *P. infestans*-inoculated plant material and gave the same hybridization pattern (result not shown).

Four sequences—STLBs 1, 10, 13, and 18—hybridized to more than one amplification product. Given the large size of these products and that each probe matches the 3' end of a gene, hybridization is presumably to two or more distinct cDNAs in each case, either alleles or related genes. Hybridization to related genes may be expected of chitinase (STLB18), cyclophilin (STLB10), and metallothionein (STLB13), which are normally members of gene families. In the case of the last, weak hybridization was also observed to cDNAs amplified from both uninfected cultivars.

Expression of sequences from eight clones was detected in cDNA from infected and uninfected Stirling by RT-PCR (see above). However, here five of these clones hybridized to

Table 2. Oligonucleotide primers used for reverse transcription-polymerase chain reaction analyses

Primer	Sequence name	Annealing temp.
5'-ATTCGCGGCCGAGGAT ₁₅ -3'	PolyA tail	
5'-GTAGTGATAGTTGGTTTCCAGC-3'	STLB1	62°C
5'-CAGCAGAAAGGTGTAGAGACAG-3'	STLB2	64°C
5'-GAGGAGATTGAGGAGGAATTCG-3'	STLB4	65°C
5'-TATTTCCCTTCGATGTTGCTAG-3'	STLB7	61°C
5'-GTCTCAGTCCCAAATAAGTTGG-3'	STLB10	62°C
5'-TCTTTTAGTGTCTGTCTGTTGG-3'	STLB13	61°C
5'-TGCTGCTATACTTACATCCATTC-3'	STLB16	61°C
5'-AACAGCAGTTCAAAGTGGACC-3'	STLB18	62°C

Fig. 1. cDNA-amplified fragment length polymorphism (AFLP) template from (lane 1) uninfected cv. Stirling, (lane 2) cv. Stirling 24 h after inoculation with *Phytophthora infestans*, (lane 3) uninfected cv. Bintje, and (lane 4) cv. Bintje 24 h after inoculation with *P. infestans*, were amplified with M00/E00 primers and hybridized to (A) ³²P-labeled STLBI, (B) an unidentified open reading frame in STLBI37, (C) STLBI3, (D) STLBI10, (E) STLBI16, (F) STLBI15, (G) STLBI13, (H) STLBI4, (I) STLBI18, (J) STLBI17, and (K), as a control, a potato β -tubulin gene. Sizes of hybridizing fragments are indicated to the left of the panels (bp). Southern blotting was on to Hybond N membrane (Amersham, Little Chalfont, UK); hybridizations were performed under high stringency conditions as described by Sambrook et al. (1989). ³²P-radiolabeled probes for hybridizations were prepared with the Random Primed Labeling kit (Pharmacia, St. Albans, UK). cDNA-AFLP templates were prepared by a modification of the method described by Bachem et al. (1996). Five hundred nanograms of double-stranded cDNA was digested with both *Eco*RI and *Mse*I and appropriate adapters (sequences given in Vos et al 1995) ligated. Templates were nonselectively amplified for 35 cycles with E00 (5'-GACTGCGTACCAATTC-3')/M00 (5'-GATGAGT CCTGAGTAA-3') primers.

AFLP-amplified total cDNA from infected, but not uninfected, Stirling. This could be explained if each cDNA-AFLP PCR is competitive, with less abundant sequences being out-competed by those of greater abundance during the reaction. It is unlikely that failure to detect amplification products in cDNA from Bintje was due to RFLPs (restriction fragment length polymorphisms), as only 3.7% differences were observed between the uninfected cultivars (P. R. J. Birch, A. O. Avrova, G. D. Lyon, and R. L. Toth, *unpublished results*). It is thus likely that, for the 10 clones examined, hybridizing sequences were out-competed during amplification of cDNA from uninfected Stirling and from either infected or uninfected Bintje, whereas more abundant sequences were amplified in cDNA from infected Stirling, allowing their detection. In contrast, a constitutive, abundantly expressed potato β -tubulin (control) gene (*tubst1*; Taylor et al. 1994) is not out-competed in these amplifications (Fig. 1K).

To accurately quantify temporal regulation of transcription, Northern analysis will be required. Further work is needed to test whether all the isolated sequences are up-regulated in the HR. However, sequences matching ribulose 1,5 bisphosphate carboxylase (STLB35) and carbonic anhydrase (STLB11) were found, and these genes are known to be highly constitutively expressed and may thus have escaped subtraction. One would predict that glycyl tRNA synthase (STLB31) was also not up-regulated during infection. However, a number of sequences identified in this work, including that which encode nucleotide-binding proteins (matched by STLBI20 and STLBI21) are glycine rich. In the case of the former, GRP2 contains two zinc fingers and a cold-shock domain (Kingsley and Palis 1994) and may thus be stress responsive.

In conclusion, SSH has allowed us to generate a cDNA library highly enriched for defense-related cDNAs from the potato cv. Stirling. Further sequencing of clones in this library, and more information up-stream of unidentified clones, obtained by rapid amplification of 5' cDNA ends (5' RACE), will reveal pathways involved in resistance to late blight and in the HR in general. Already, evidence has emerged to relate mechanisms in the plant HR with apoptosis in animals. In addition, the sphingolipid pathway is implicated in signaling during plant defense. Finally, SSH and cDNA-AFLP in parallel may be used to target ESTs specific to a particular tissue,

metabolic, or developmental process and thus complement existing EST programs.

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