Identification and cloning of differentially expressed genes involved in the interaction between potato and *Phytophthora infestans* using a subtractive hybridization and cDNA-AFLP combinational approach

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Abstract

Differentially expressed genes involved in the potato-*Phytophthora infestans* interaction were identified using a subtractive hybridization (SH)/cDNA-AFLP combinational approach. We detected *P. infestans* genes potentially controlling pathogenesis or avr genes, and potato genes potentially involved in resistance or susceptibility to this pathogen. Forty-one differentially expressed TDFs were cloned and sequenced. Two of these, suggested as potential pathogenicity factors, exhibited sequence similarity to N-succinyl diaminopimelate aminotransferase and a transcriptional regulator, TetR family gene, respectively. Two TDFs suggested as potential *P. infestans* avr genes had sequence similarity to an EST sequence from Avr4/Cf-4/Avr9/Cf-9, and a *P. infestans* avirulence-associated gene, respectively. Confirmation of expression and origin were done using Southern blot, Northern blot and qRT-PCR. I.e., potential resistance gene DL81 was induced at 12 hpi in the moderately resistant cultivar, whereas it was down-regulated as
early as 6 hpi in the susceptible cultivar. Another TDF DL21 induced at 6 hpi (3.38-fold) in
response to the highly aggressive isolate (US8) was strongly up-regulated thereafter (25.13-
fold at 120 hpi.). In contrast, this transcript was only slightly up-regulated in response to
the weakly aggressive isolate (US11) at 9, 12 and 96 hpi, with 3.75-, 3.86- and 3.82-fold
increase, respectively, suggesting its role as a potential susceptibility gene.

Running Title: Differential genes in potato-\textit{P. infestans} interaction

Key words: \textit{Phytophthora infestans}, potato, subtractive hybridization, defense suppression,
cDNA-AFLP.
Introduction

Potato late blight caused by the oomycete *Phytophthora infestans* (Mont.) de Bary (Fry and Goodwin 1997) results in economic losses of $3.25 billion per year in potato-growing areas worldwide (Pel et al. 2009). This disease can be initiated either directly with germinating sporangia or through zoospores, and affects leaves, stems, and tubers (Judelson et al. 2005).

The re-emergence of late blight as an important disease, for example in the US and Canada occurred in the 1980s and 1990s, respectively, due to changes in the population structure of *P. infestans*, with a shift from the pre-existing A1-mating type, metalaxyl-susceptible to new metalaxyl-insensitive, highly aggressive A1 and A2 strains (Daayf and Platt 2000). In Canada, the A1 genotype US-1 has been completely displaced by the A2 genotype US8 of *P. infestans* (Daayf et al., 2000, 2001), which is more virulent and exhibits highly increased aggressiveness (Kato et al. 1997).

An extensive literature is available about incompatible interactions between potato and *P. infestans* leading to plant resistance, whereas the molecular mechanisms of compatibility leading to disease have been investigated less (Restrepo et al. 2005). It has been suggested that potato susceptibility to *P. infestans* is a result of the suppression of potato resistance reactions by glucans released from *P. infestans* (Currier 1981; Doke et al. 1980; Garas et al. 1979; Ozeretskovskaya et al. 2001; Andreu et al. 1998). Also, *P. infestans* secretes both cytoplasmic and apoplastic effectors (Kamoun 2005). For example, serine protease inhibitors EPI1 and EPI10 are apoplastic effectors that are thought to function in the counter-defense inhibiting PR protein P69B, a subtilisin-like serine protease of tomato (Tian et al. 2005). In potato, Wang et al. (2004) reported that an aggressive *P. infestans*
US8 suppresses host defense mechanisms, through transcriptional inhibition of phenylpropanoid (PAL) and isoprenoid (HMGR) pathways (Wang et al. 2008).

In plant-pathogen interactions, up-regulation of defense-related genes such as those controlling chitinase, beta-glucanase, and other pathogenesis-related proteins in both compatible and incompatible interactions is well documented (Maleck et al. 2000). However, genes expressed in an incompatible interaction and represented in a cDNA library do not all correspond to critical information for future studies on resistance genes, especially when the same gene is also expressed in the compatible interaction counterpart.

Cases where up-regulation occurs only in the susceptible or the resistant line, and only in response to either the virulent or avirulent strain of the pathogen, have rarely been described (Valer et al. 2006). Also, during both incompatible and compatible interactions, many pathogens are invasive and establish pathogenic structures within the host tissue. As a result, it is difficult to separate the pathogen tissue from that of the host, and both are represented in the pool of expressed genes from the interaction.

The objective of the current study was the identification and cloning of differentially expressed genes involved in the interaction between potato and Phytophthora infestans using a combinational approach of subtractive hybridization and cDNA-AFLP in a quadratic potato-P. infestans system. This approach combines the advantages of subtractive libraries and cDNA-AFLP resulting in the removal of constitutively/commonly expressed sequences from simultaneously compared treatments, and the identification of uniquely
expressed genes. The quadratic system included one susceptible and one moderately resistant potato cultivars during their interactions with one weakly- and one highly-aggressive strains of *P. infestans*.

**Results**

**Inoculation results**

We used two isolates of *P. infestans*, D-03 (lineage US11, A1 mating type, weakly aggressive) and D1901 (lineage US8, A2 mating type, highly aggressive) to inoculate cultivars Russet Burbank (highly susceptible) and Defender (partially resistant). No symptoms were visible within the first 48 hpi on either cultivar and only small lesions became noticeable at 72 hpi. At 120 hpi, the US8 isolate caused spreading disease lesions and extensive tissue damage in Russet Burbank. By contrast, it only caused limited disease lesions in Defender. The US11 isolate caused limited lesions in Russet Burbank and failed to cause disease in Defender. The treatments composing this quadratic model were used for further analysis of differential gene expression in both the plant and the pathogen.

For convenience, we called an interaction “incompatible” when the host showed no or limited disease lesions (RB+US11, DF+US8, DF+US11), and “compatible” when spreading disease lesions were apparent (RB+US8). However, the moderately resistant cultivar “Defender” was also classified as “compatible” in (DF+US8) when late blight symptoms were prominent. Therefore, even though “Russet Burbank” is susceptible and
“Defender” moderately resistant, their interaction with the isolates was classified either as
“incompatible” or “compatible” based on their specific interaction with each strain.

Characterization of differentially expressed fragments

In order to identify both host and pathogen genes stimulated during the potato-
P. infestans
interaction, we used a combinational approach of subtractive hybridization and cDNA-
AFLP to simultaneously evaluate treatments involving potato cultivars differing in their
disease susceptibility after inoculation with each of two pathogen genotypes differing in
their pathogenicity levels. Due to space limitations, we only described the procedure for
one host cultivar and one pathogen genotype (Figure 1).

Four subtractive hybridizations were accomplished between inoculated (treatment) and
non-inoculated tissues (control, treated with sdH2O): (i) RB+US8 minus control, (ii)
RB+US11 minus control, (iii) DF+US8 minus control, and (iv) DF+US11 minus control.
This step allowed us to remove constitutive and commonly expressed transcripts
in/between different treatments. Subsequently, differential screening with cDNA-amplified
fragment length polymorphism (cDNA-AFLP) yielded a large number of polymorphic
bands.

A total of 23 AFLP primer combinations (EcoRI+3 selective bases/MseI+3 selective bases)
were used, generating differentially expressed transcript-derived fragments (TDFs) ranging
from approx. 100 bp to 500 bp. The average for each gel lane (treatment) typically
contained an average of 25 TDFs, exhibiting differences in the band intensity and
expression. Based on their expression patterns in the treatments, these fragments were grouped into five groups:

- Potential pathogenicity factors “ssor”: TDFs from the pathogen, exclusively expressed in the compatible interaction.

- Potentially suppressed potato genes “ssed”: TDFs from potato exclusively expressed in the incompatible interaction, neither present in the pathogen nor in the compatible interaction

- Potential P. infestans “avr” genes: TDFs from the pathogen constitutive expressed in the incompatible interaction

- Potential resistance genes “rtant”: TDFs from potato commonly expressed in partially resistant potato cultivar (Defender), neither present in the pathogen nor in the susceptible cultivar (Russet Burbank)

In addition, if the sequence analysis from a candidate “ssor” gene showed homology only with a host sequence and not with pathogens, we called this TDF potential plant disease susceptibility gene “ssept”. TDFs from constitutive genes and from those commonly expressed in both incompatible and compatible interaction, either from the pathogen or the host, were distinguished but not used for further characterization. The representative cDNA-AFLP display of each group is shown in Figure 2.
Fifty four TDFs (25 ssor, 18 ssed and 10 avr, 1 rtant), from 6 primer combinations, were excised from the polyacrylamide gels and successfully re-amplified by PCR, using the selective primers that were used to obtain them in SH-AFLP.

Gene sequence analysis

Fifty four TDFs re-amplified fragments were cloned using the pGEM-T Easy Vector system (Promega, Madison, WI, USA). To ensure that the correct bands were cloned, the plasmid was amplified with the appropriate AFLP primers and run adjacent to the original SH-AFLP reactions on a polyacrylamide gel. Three white colonies from each transformation event were selected and the respective inserts were sequenced (Macrogen, USA). Sequences were compared with existing databases. Thirteen sequences were discarded due to vector or adapter contamination. The remaining forty one sequences were singletons, representing unique sequences. Sequences have been submitted to the NCBI GenBank under the accession numbers described in the Table 1. Seven sequences were novel and showed no matches to sequences in public databases. Six sequences showed no significant matches at the expectation values (E) of < 10^-4 to sequences in public databases, but were included in Table 1. The remaining sequences had significant hits. The functional analysis of the forty one sequences showed no homology with genes involved in photosynthesis, respiration, energy conversion, metabolism, transport, or protein synthesis.

Table 1 shows the closest database similarities for the differentially expressed TDFs. Seven *P. infestans* TDFs potentially involved in pathogenicity, possibly in the suppression of potato defense genes showed an average GC content of 50.2 %. Two transcripts (DL119...
and DL95) shared homology with sequences from bacteria. In addition, DL119 and DL95 showed a similar expression pattern with N-succinyl diaminopimelate aminotransferase and a transcriptional regulator, TetR family respectively. Transcripts DL32 and DL12 shared homology with \textit{P. infestans} and \textit{P. sojae} respectively. Eight transcripts from a susceptible host (DL21, DL33, DL2, DL49, DL138, DL17, DL90 and DL54) showed homology only with other hosts’ sequences (i.e., \textit{Solanum tuberosum}, \textit{S. lycopersicum}, \textit{Triticum monococcum} subsp. \textit{Aegilopoides}, \textit{Capsicum annuum} and \textit{Aquilegia} sp.) and none with pathogens. We classified such fragments as potential plant disease susceptibility genes “ssept”. Fifteen transcripts from potentially suppressed potato genes (ssed) showed an average of GC content of 42.2%. Two sequences DL39 and DL10 had homology with EST sequences from the interaction potato-\textit{P. infestans}. DL10 also had homology with Beta-amylase. Transcripts DL16 and DL40 shared homology with Sweet potato (Ayamurasaki) and \textit{Lycopersicon esculentum}, respectively. DL91 and DL144 shared homology with EST sequences from \textit{Solanum chacoense} and \textit{Papaver somniferum}, respectively. In addition, the candidate resistance gene DL81 shared homology with an mRNA sequence from Potato callus. Finally, four cDNAs from potential \textit{P. infestans} elicitor genes (avr) showed an average GC content of 45.5%. The transcript DL24 had homology with an EST sequence from Avr4/Cf-4 and Avr9/Cf-9 cDNA-AFLP \textit{Solanum lycopersicum} cDNA. The sequence from DL41 had homology with a \textit{P. infestans} avirulence-associated gene, DL22 shared homology with \textit{Bradyrhizobium japonicum} inoculation \textit{Glycine max} cDNA and DL145 shared homology with \textit{P. infestans}. 
Identification of gene origin

To determine potato or pathogen origin of the TDF (DL81, Potential resistance gene) with significant homology to a gene in potato, specific primers were designed and the corresponding probe was prepared for this transcript. Southern blot and northern blot analyses (Figure 3A), confirmed that DL81 was of potato origin. Southern blot analysis was performed with genomic DNA from both potato cultivars, and *P. infestans* strains. Under low stringency conditions, DL81 identified the homologous sequence in the genomic DNA from both potato cultivars. Northern blot was performed at least twice in independent experiments and the differential expression was confirmed for the transcript. In this experiment, the RNA for each plant-pathogen treatment was the same time course mixture (3-144 hpi) as was used for the subtractive hybridization (SH)/cDNA-AFLP protocol. RNAs from RB+US8, RB+US11, DF+US8, DF+US11, RB control and DF control were included. To reconfirm the Southern blot results, the *P. infestans* RNA mixture (sporangia, zoospores, germinating sporangia, germinating zoospores, appressoria and mycelium) was also included. The signal for DL81 was detected again in DF+US8 and DF+US11, the same interactions from which the transcript was excised.

Validation of expression patterns using qRT-PCR

To investigate the reliability of SH/cDNA-AFLP for detecting differentially expressed genes and verify the expression patterns observed, qRT-PCR analyses were carried out for four TDFs. Similarly to results from Southern blot and northern blot analyses, DL81 was detected in DF+US8 and DF+US11 by qRT-PCR (Figure 3B). DL81 was induced in Defender 4- and 2-fold over the control plants (DF+H2O) in response to US8 and US11,
respectively. qRT-PCR showed a slight expression of DL81 in RB inoculated with US8 and US11 that was not shown in Northern blot analysis. This may be due to the sensitivity or stringency of the technique. Northern blot depends on high-quality template RNA that may be affected by extraction and storage, particularly when the transcript level is low. However, the DL81 presence in RB+US8, RB+US11 with a similar gene expression that the control (RB+H2O), also validated the SH/cDNA-AFLP methodology, because after subtractive hybridization (SH), constitutive and commonly expressed cDNAs were removed. Hybridized DL81 cDNA precursor from non-inoculated (RB+H2O) and inoculated plants (RB+US8 or RB+US11) were attached to magnetic beads. Nevertheless, un-hybridized cDNA from inoculated plants (transcripts of interest, supernatant) obtained from DF+US8 and DF+US11 were processed by cDNA-AFLP and detected in the polyacrylamide gel. These results indicate that the transcript DL81 is potentially involved in potato resistance against *P. infestans*, because it was specifically up-regulated in Defender and not in Russet Burbank.

In order to study the dynamics of gene expression during disease development, qRT-PCR analysis for the TDF DL81 was performed using two treatments and eight different times after inoculation similarly to those used for the SH/cDNA-AFLP analysis (Figure 3C). Treatments RB+US8 and DF+US8 were selected based on their contrasting expression patterns in the mixed RNA that was used in the SH/cDNA-AFLP and qRT-PCR (Figure 3B) experiments. DL81 transcript was induced at 12 hpi in DF+US8 (Figure 3C). The highest induction of this transcript occurred at 24 hpi. The transcript increased by 5.35 fold and 6.27 fold, respectively over the potato inoculated plant near 0
hpi. The expression decreased slightly thereafter and remained relatively constant from 48 to 120 hpi. In contrast, DL81 transcript was down-regulated as early as 6 hpi in RB+US8.

Parallel inoculation with sdH$_2$O was included as a control for each time point (RB+H$_2$O and DF+H$_2$O). We compared expression levels of DL81 in control samples across all time points and found that expression levels of DL81 did not change significantly without inoculation of _P. infestans_ (Figure 3C). The results from control treatments at all time points support the conclusion that this transcript changed its expression levels in response to inoculation with the pathogen.

Figure 4A, also validates the reliability of SH/cDNA-AFLP for detecting differentially expressed genes. DL21, as a potential plant disease susceptibility gene, was excised from the compatible interaction between Defender and US8 and it was not present in the incompatible interaction between Defender and US11. The qRT-PCR analysis using the mixed RNA that was used in the SH/cDNA-AFLP revealed an up-regulation of DL21 in DF+US8 6-fold over the control plant (DF+H$_2$O). Resembling DL81, only the up-regulated transcript DL21 in DF+US8 remains in the supernatant (SH product selected - transcript of interest) was used for further cDNA-AFLP analysis.

Similar to DL81, qRT-PCR analysis for the TDF DL21 was performed at two treatments and eight different times after inoculation similarly to those used for the SH/cDNA-AFLP analysis (Figure 4A). The treatments DF+US8 and DF+US11 were selected based on their contrasting expression patterns. DL21 transcript was induced at 6 hpi in DF+US8 (Figure
4A). The expression was strongly up-regulated thereafter, with a maximum induction at 96 and 120 hpi. The transcripts increased from 3.38 fold (6 hpi.) to 25.13 fold (120 hpi.) over those from potato inoculated plants near 0 hpi. In contrast, the transcript DL21 was slightly up-regulated in DF+US11 at 9, 12 and 96 hpi., with a 3.75, 3.86 and 3.82-fold increase, respectively, over the potato inoculated plant near 0 hpi. In addition, the expression levels of DL21 in DF control (DF+H2O) across all time points did not change their expression level significantly without inoculation with *P. infestans* (Figure 4A). Therefore, this transcript changed its expression levels in DF+US8 and DF+US11 in response to the pathogen infection.

According to qRT-PCR data, the potentially suppressed plant gene DL39, excised from DF+US8 and DF+US11, was upregulated 5- and 3-fold over the control (DF+H2O), respectively (Figure 4B) and suppressed in RB+US8 and RB+US11. DL28, as a potential plant disease susceptibility gene, was excised from the compatible interaction between Russet Burbank and US8 (RB+US8) and was not expressed in RB+US11, DF+US8 or DF+US11. Consistently, the qRT-PCR analysis revealed an up-regulation of DL28 in RB+US8, 2-fold over the control and no expression in DF+US8 or DF+US11 (Figure 4C). Gene expression of DL28 in RB+US11 was similar to the control RB+H2O. As a consequence, it was hybridized with the control and attached to the magnetic particles. Only in RB+US8, the DL21 precursor remained in the supernatant and it was used for further cDNA-AFLP analysis and detected in the polyacrylamide gel.
**Discussion**

Using the subtractive hybridization (SH)/cDNA-AFLP technique, we successfully identified TDFs corresponding to (i) potential potato defense genes, (ii) potentially suppressed potato genes, (iii) potato genes potentially involved in susceptibility, (iv) *P. infestans* genes potentially involved in pathogenicity, and (v) potential *P. infestans* *avr* genes. With this gel-based subtractive hybridization profiling technique, it was possible to eliminate almost 100% of the host constitutive/commonly expressed sequences present in the interaction potato cultivar Russet Burbank inoculated with the *P. infestans* genotype US8. Using a traditional cDNA-AFLP methodology, the average of transcript-derived fragments (TDFs) previously reported for each gel lane was 55 to 203 (Avrova et al. 2003; Roy et al. 2008; Qin et al. 2000), due to the prevalence of constitutive (housekeeping genes) sequences.

Constitutive gene expression has been previously documented in *Arabidopsis* (Becker et al. 2003). From 1,620 genes analyzed, approximately 90% were expressed in pollen grains and in at least one of the vegetative tissues, whereas the remaining 10% were selectively expressed in the pollen. This means that it is necessary to sequence an extensive amount of cDNA clones from a host in order to fish out for a gene responsible for a specific function, because constitutive genes are the majority.

The advantage with potato, soybean and *Phytophthora*, is the bimodal distribution of ESTs based on GC content percentage, which can be used to determine the proportion of *Phytophthora* transcripts within the infected plant library. The average GC content of
soybean ESTs was 46%, whereas *P. sojae* zoospore and mycelium ESTs clustered around a mean of 58% GC (Qutob et al. 2000). The average GC content for potato is 42.7% GC as determined from 51,444 ESTs generated from non-pathogen-challenged tissues. In contrast, the average GC content of *P. infestans* is 56.6% GC as determined from a total of 4,314 publicly available late-blight pathogen ESTs (GenBank dbEST release 128, February 2002) (Ronning et al. 2003). However, this percentage of GC content should not be considered as an invariable number in the study of *P. infestans* or potato transcripts. In a random analysis of 40 full-length cDNA sequences from *P. infestans* (Win et al. 2006), we found that GC content ranged from 47% to 63%. For example, the elicitin INF4 (AF419841) has 49% GC. In planta-induced IPIO-1 (AY961430) showed 49% GC content and the elicitin INF1 (U50844) has 61% GC. Using the gel-based subtractive hybridization profiling technique described in this study, we identified *P. infestans* cDNAs potentially involved in pathogenesis with an average GC content of 50.2%, cDNAs from potentially suppressed potato genes with an average GC content of 42.2% and *P. infestans* potential avr genes with an average GC content of 45.5%.

During both incompatible and compatible interactions, many pathogens are invasive and establish pathogenic structures within the host tissue. As a result, it is difficult to separate the pathogen tissue from that of the host, and both are represented in the pool of expressed genes from the interaction. For example, in a comparative analysis of expressed sequences from infected soybean (*Glycine max* L. Merr) plants, there was a high representation (60%–70%) of the pathogen *P. sojae* cDNAs (Qutob et al. 2000). Therefore, it is necessary to integrate the associated pathogen into the analysis, in order to alleviate some of the
difficulties in differentiating between genes up-regulated in the host and those up-regulated in the pathogen.

Sequences obtained from our TDFs characterized as *P. infestans* cDNAs potentially involved in pathogenicity (DL119 and DL 95), shared homology with bacterial genes. It has been already reported that oomycetes contain sequences with homology to bacterial sequences (Morris and Phuntumart 2006). For example, the Nep1-like proteins (NLPs) are broadly distributed in bacteria, fungi, and oomycetes, sharing a high degree of sequence similarity with significant facility to induce cell death in dicotyledonous plants (Kamoun 2005).

The *P. infestans* transcript DL119 (ssor) exhibits sequence similarity to N-succinyl diaminopimelate aminotransferase. This is an important enzyme in the lysine biosynthesis via diamino-pimelate pathway (Tyler 2001). Oomycetes synthesize lysine via diamino-pimelate, whereas fungi synthesize it via alpha-amino-adipate pathway (Tyler 2001). Lysine is an important component in the *P. infestans* pathogenicity, because the inhibition of the lysine biosynthetic enzyme dihydrodipicolinate synthase (DHDPS) using dipicolinic acid reduces mycelial growth of *P. infestans* by 61% and completely inhibits blight infection of leaf discs when used at 1 mM (Walters et al. 1997). The *P. infestans* transcript DL95 showed a similar expression pattern as a transcriptional regulator, TetR family. The TetR Family of Transcriptional Repressors is mainly abundant in microbes exposed to environmental changes, such as *Streptomyces*, *Pseudomonas*, *Ralstonia* spp. and *Agrobacterium*, controlling genes involved in multidrug resistance, biosynthesis of
antibiotics and pathogenicity of gram-negative and gram-positive bacteria (Ramos et al. 2005). Finally, in the group of *P. infestans* cDNAs potentially involved in suppression of potato defense genes, sequences of DL32 and DL12 had homology with *Phytophthora infestans* and *Phytophthora sojae* respectively.

The sequences obtained from our TDFs, characterized as potentially suppressed potato genes, as well as the potential resistance gene DL81, did not match with any sequence from microorganisms. These results confirm the effectiveness of the subtractive hybridization (SH)/cDNA-AFLP protocol to separate genes from the pathogen and from the host. DL39 and DL10 matched with EST sequences from the interaction of potato with *P. infestans*. DL10 also showed homology with Beta-amylase, which is an important protein of tuberous storage root of sweet potato and accumulation of starch in leaves with a possible role in defense (Ohto et al. 1992).

Sequences obtained from our TDFs suggested as potential *P. infestans* *avr* genes matched with EST sequences from Avr4/Cf-4 and Avr9/Cf-9., determining the interaction between a tomato *Cf* resistance gene and a matching *C. fulvum* *Avr* gene (Wang et al. 2005). Also, the *P. infestans* potential *avr* gene DL41 had homology with a *P. infestans* avirulence-associated gene.

Little is known about the plant genes required for susceptibility. In 2002, Nancy A. Eckardt reported that in a search of the ISI Web of Science identified 524 documents related to “plant disease resistance” and just 1 match for the phrase “plant disease susceptibility”.

Seven years later, our search of the ISI Web of Science identified 1,130 documents related to “plant disease resistance” and just 11 matches for the phrase “plant disease susceptibility”. Also, we found 2,254 documents to “resistance gene” and 5 documents to “susceptibility gene” in Plant Sciences. In our research, we found TDFs classified as potential plant disease susceptibility genes (DL21, DL33, DL2, DL49, DL138, DL17, DL90 and DL54) that showed homology with *Solanum tuberosum*, *Solanum lycopersicum*, *Triticum monococcum* subsp. Aegilopoides, *Capsicum annuum* and *Aquilegia* sp., and none with pathogens. Further studies of these potential susceptibility genes would represent a novel form of disease resistance derived from the failure of a gene that is necessary during a compatible interaction, rather than the activation of known host defense pathways (Vogel et al. 2006).

Further validation of the subtractive hybridization (SH)/cDNA-AFLP technique for detecting differentially expressed genes was performed for four randomly selected transcripts (DL81, DL21, DL39 and DL28) by qRT-PCR, southern blot and northern blot analysis. The results were in agreement with our characterization criteria and confirmed that the data obtained by SH/cDNA-AFLP reliably reflected the differential expression of those genes involved in potato - *P. infestans* interaction. In addition, the study of DL81 and DL21 using two treatments and eight different times after infection, similarly to those used for the SH/cDNA-AFLP analysis, illustrated the dynamics of these transcripts’ expression during disease development. Future work is necessary to determine the biological functions of transcripts. Nevertheless, based on these results, we can suggest that DL81 is a good candidate for futures studies of gene resistance by gene silencing and Marker Assisted
Selection (MAS) studies, as well as DL21 as an interesting potential plant disease susceptibility gene.

The gel-based subtractive hybridization profiling technique uses the advantages of cDNA-AFLP and subtractive hybridization in order to amplify cDNA products in a polyacrylamide gel and remove the constitutively/commonly expressed sequences. Using subtractive hybridization with paramagnetic particles, we did not have problems with re-annealing processes, because the tester (inoculated plant) is represented by single stranded cDNAs. In addition, the driver (control plant) is also single strand cDNAs that attach to paramagnetic particles in the process. Therefore, there were no problems related to kinetics of hybridization and as a consequence, no need for a double hybridization, making the process simple and reliable. The present study has shown that using the subtractive hybridization (SH)/cDNA-AFLP approach, it was not necessary to sequence hundreds of samples to detect P. infestans genes potentially controlling pathogenesis or avr genes, and potato genes potentially involved in resistance or susceptibility to plant disease.

Materials and methods

Phytophthora infestans strains and inoculation

Phytophthora infestans strains D-03 (lineage US11, weakly aggressive) and D1901 (lineage US8, highly aggressive) were grown on rye agar supplemented with 2% sucrose at 18°C (Caten and Jinks 1968). High quality tubers of two potato cultivars, Russet Burbank (RB, susceptible) and Defender (DF, moderately resistant) were inoculated with a sporangia suspension (4 X 10^4 sporangia/ml), and with water for non-inoculated control plants.
following the method described by Wang et al. (2004). This resulted into four inoculation treatments; (A) RB+US8, (B) RB+US11, (C) DF+US8, (D) DF+US11, plus two controls; non-inoculated Russet Burbank (RB+H2O) and non-inoculated Defender (DF+H2O) which are parallel inoculations with sterile water. The treatments composing this quadratic model were used for further analysis of differential gene expression in both the plant and the pathogen.

Combinational approach of subtractive hybridization and cDNA-AFLP

RNA was extracted from both inoculated and control tissues as well as the *P. infestans* isolates used in the interaction. Consequently, mRNA was extracted from the host alone, the pathogen alone and their interacting tissues. First and double-stranded cDNA was synthesized and the first-strand cDNA from the non-inoculated plant (control) was combined with the second-strand cDNA (inoculated plant) in order to perform a subtractive hybridization (SH). Transcripts of interest, obtained from the subtractive hybridization are single-stranded DNAs with 3’ polyA; therefore, a synthesis of a complementary DNA strand was performed for each interaction (SH-Second strand). Then, the SH-Second Strand was used for AFLP analysis, followed by SH-Digestion, SH-Ligation, SH-Preamplification (SH+1) and SH-Selective (SH+3) amplifications. The products were separated on 5% PAGE and bands detected by AgNO3 staining. Differentially expressed products were then characterized (Figure 1).

Total RNA and mRNA extraction
RNA was extracted from all treatments including inoculated; (A) RB+US8, (B) RB+US11, (C) DF+US8, (D) DF+US11, and control tissues; non-inoculated Russet Burbank (RB+H2O) and non-inoculated Defender (DF+H2O) with TRIZOL (Invitrogen), from 400 mg of plant material at 3, 6, 9, 12, 24, 48, 72, 96, 120 and 144 hour post-inoculation (hpi).

In addition, RNA was extracted from *P. infestans* collected after five days growth in Pea Broth Medium at 18°C and from a *P. infestans* mixture of spores, zoospores, spores in germination, zoospores in germination, appressoria and mycelia, following the protocol of Ebstrup et al. (2005). These control treatments are necessary for further subtraction of genes that are constitutively expressed in *P. infestans* before inoculation. Consequently, mRNA was extracted from 100 μg total RNA of the time course mixture (3-144 hpi) for each treatment and from the *P. infestans* mixture using the straight A’s mRNA Isolation System (Novagen). This method uses paramagnetic particles, which are beads containing covalently attached oligo d[T]25 to bind the poly-A tail of mRNA.

**Subtractive Hybridization (SH)**

Four subtractive hybridizations were completed between inoculated (treatment) and non-inoculated tissues (control): (i) RB+US8 *minus* control, (ii) RB+US11 *minus* control, (iii) DF+US8 *minus* control and (iv) DF+US11 *minus* control, modifying the method described by Krista and Pauls (2001) and superscript II reverse transcriptase (Invitrogen) protocol. In brief, in a 1.5 ml tube, the mRNA extracted using paramagnetic particles was mixed with 4 μl of reverse transcriptase (RT) buffer (5X), 2 μl of 0.1 M DTT, 1 μl of 10 mM dNTP mix, and 1 μl of 200 units of M-MLV (RT) enzyme. The reaction was incubated 1h at 37 °C and the first-strand cDNA was collected using a magnetic separation stand, because it has
magnetic beads. This first cDNA strand was then used as a template for the synthesis of a second cDNA strand. The first-strand cDNA was mixed in a 200 μl PCR tube with 2X DNA polymerase buffer, 0.2 mM of dNTP mix, 5 U/μl of *E. coli* DNA Ligase, 20 U/μl of *E. coli* DNA polymerase and 5 U/μl of *E. coli* RNAse H. The reaction was completed with water to 150 μl and incubated for 2h at 16°C in a thermocycler (Techne Flexigene, Inc., Canada). The reaction was transferred into a 1.5 ml tube, the reaction mixture was removed by pipetting, and the double-stranded cDNA was collected using a magnetic separation stand. Then, the double-stranded cDNA was re-suspended in 50 μl of sterile water and boiled for 10 min to denature the cDNA. Without delay, using a magnetic separation stand, the second-strand cDNA (supernatant) was separated from the first-strand (collected particles). The first-strand cDNA from the non-inoculated plant (control) was combined with 18 μl of the second-strand cDNA (inoculated plant) in 30 μl of hybridization buffer (30 mM HEPES, 1 mM EDTA, 1 M NaCl). The mixture was incubated for 24 h at 37°C. At the end of the incubation, 3 cDNA species were present in the mixture: (i) un-hybridized cDNA from non-inoculated plants (attached to magnetic beads), (ii) hybridized cDNA from non-inoculated and inoculated plants (attached to magnetic beads) and (iii) un-hybridized cDNA from inoculated plants was in the supernatant (no magnetic beads). The latter is the SH product selected (transcripts of interest) that was precipitated with 3 volumes of isopropanol, washed with 70% ethanol and dissolved in a final volume of 20 μl of sterile water. In addition, for each genotype of *P. infestans*, the same procedures described above for first and second strands were used without hybridization. Finally, a 2-μl aliquot from products of interest was visualized in a 1.2 % Petri dish agarose gel with Et-Br in order to corroborate the presence of cDNA.
**SH- Second Strand synthesis**

The SH products selected in the step above, that represent transcripts of interest from (A) RB+US8, (B) RB+US11, (C) DF+US8, (D) DF+US11, are single-stranded DNA with 3’ polyA. Therefore, synthesis of a complementary DNA strand was performed. Ten microliters from the transcripts of interest (single strand) were mixed in a 200 μl PCR tube with 100 ng/μl of primer d[T]25 V, 0.1X of Buffer RT, 0.5 mM of dNTP mix and 200 U/μl of M-MLV (RT) enzyme. The reaction was completed with water to 50 μl. The reaction was incubated 1h at 37°C. The pathogen genotype second strand (supernatant) was also used in the synthesis of the complementary DNA strand. The resulting double-stranded (SH-Second strand) was chloroform:isoamyl alcohol (24:1)-extracted, precipitated with 4 volumes of isopropanol, washed with ethanol and dissolved in a final volume of 20 μl of SDW.

**SH-AFLP**

The SH-Second Strand was used for AFLP analysis, with a modified protocol of Vos et al. (1995), and Bachem et al. (1996). In brief, the SH-Digestion was prepared with 10 μl of SH-Second Strand, 10 units of EcoRI Enzyme (Invitrogen), 5 units of MseI enzyme (Invitrogen) and 1X MseI Buffer. The reaction was incubated 2h at 37°C and inactivated for 10 min at 70°C. The SH-Ligation was done by adding 0.1 μM of MseI adapter (5μM), 1 μM of EcoRI adapter (50μM), 2.5X of T4 ligase buffer (5X), and 400 units of T4 DNA ligase, to the total SH-Digestion. The reaction was incubated 2h at 37°C. For the SH-Pre-amplification (SH+1), 5 μl of SH-Digestion/Ligation product was mixed with 50 ng of
ECORI + A (100 ng/μl), 50 ng of MseI + C (100 ng/μl), 1X Buffer (200 mM Tris-HCl (pH 8.3), 15 mM MgCl₂, 500 mM KCl), 0.25 μl of dNTP mix, 1 unit of Taq DNA polymerase (Invitrogen) and SDW to complete 25 μl reaction volume. Amplification was performed in a programmed thermocycler for 25 cycles of 30 s at 94°C, 60 s at 56°C, and 60 s at 72°C, followed by a final extension for 60 sec at 72°C. The PCR products were run on a 1.2% agarose gel following staining with EthBr. The selective amplification (SH- Selective (SH+3)) was generated in a 25 μl reaction volume containing 5 μl of SH+1 product diluted 1:50, 50 ng EcoRI (3 selective bases) (100 ng/μl), 50 ng of MseI (3 selective bases) (100 ng/μl), 1X Buffer, 0.25 mM of dNTP mix, 2.5 mM of MgCl₂ and 1 unit of Taq DNA polymerase (Invitrogen). The PCR reactions were performed for 36 cycles of 30 s at 94°C, 30 s at 56°C and 60 s of extension at 72°C. The annealing temperature in the first cycle was 65°C and was subsequently reduced each cycle by 0.7°C for the following 12 cycles. The annealing temperature was then maintained at 56°C for the remaining 23 cycles. Amplification products were separated on 5% polyacrylamide gels, using the Sequigel system (Biorad) and bands were detected by AgNO₃ staining. All oligonucleotides were synthetized by Invitrogen Canada Inc. The AFLP sequence for the adapters and primers for the SH+1 and SH+3 were similar to Vos et al. (1995). However, the three selective bases for the SH+3 were; AAC, AAG, ACA, ACT, ACC, ACG, AGC, AGG for the ECoRI Core and CAA, CAC, CAG, CAT, CTA, CTC, CTG, CTT for the MsE I Core.

The SH-AFLP also can be performed using the AFLP® Analysis System I or the AFLP® Analysis System for Microorganisms (Invitrogen), starting with 10 μl of SH-Second Strand for digestion. However, we reduced the reagents for digestion, ligation and pre-
amplification to half the manufacturer’s recommendations. The selective amplification is performed using 5 μl of SH+1 product diluted 1:50, 0.5 μl of EcoRI primer, 6 μl of MseI primer, 2 μl of 10X buffer and 1 unit of Taq DNA polymerase (Invitrogen) (data not shown).

Sequence analysis

Fragments corresponding to differentially expressed transcripts were excised from the dried polyacrylamide gel with a sterile scalpel, eluted in 20 μl of 1X buffer, separated from the polyacrylamide gel for incubation at 95°C for 10 min and re-amplified under the conditions used for selective amplification, but adding 2 μl of BSA (bovine serum albumin 1mg/ml) to the reaction. The PCR products were isolated with the Qiaex II gel-extraction kit (Qiagen Inc., Alameda, CA, USA) following the manufacturer's instructions. The isolated fragments were cloned into the bacterial plasmid pGEM-T Easy Vector (Promega, Madison, WI, USA) following the manufacturer's instructions. The plasmids were then transformed into *E. coli* DH5α, sequenced and analyzed with Seqman within the DNAStar program (DNAStar, Madison, WI, USA). To ensure the correct bands had been cloned, the isolated plasmid was amplified with the appropriate AFLP primers and run adjacent to the original SH-AFLP reactions on a polyacrylamide gel. Three white colonies from each transformation event were selected and the respective inserts were sequenced (Macrogen, USA). cDNA sequences were analyzed with Seqman within the DNAStar program (DNAStar, Madison, WI, USA). If the sequences of the three clones were identical, the cDNA sequence was analyzed with BLAST programs at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov), *Phytophthora infestans*

Origin identification of a TDF homologous to a gene in potato by Southern and Northern Blot analyses

Southern blot was performed by EcoRI digestion of 15 μg of genomic DNA from potato RB, DF, and from P. infestans US8 and US11, and electrophoresis through 1% agarose gel. The DNA was transferred to a Hybond-N+ membrane (Hoffmann-La Roche Ltd., Mississauga, ON, Canada) according to Sambrook and Russell (2001). For northern blot, 25 μg of total RNA was denatured and separated in a formaldehyde gel (Sambrook and Russell 2001), then blotted onto a Hybond N nylon membrane (Roche). The probe labeling and detection was accomplished using a DIG DNA Labeling and Detection Kit (Roche) following the manufacturer’s recommendations. DIG-labeled hybrids were detected with an anti-DIG-alkaline phosphatase conjugate and the substrates NBT (nitroblue tetrazolium salt) and BCIP (5-bromo-4-chloro-3-indolyl phosphate, toluidinium salt), which result into a light-blue precipitate. For the presented example, primers were designed based on the sequence of the transcript DL81 and their corresponding probes were prepared with the primers; DL81-F 5’-CAGCTACTTGGGAGGCTGAG-3’ and DL81-R 5’-TAGGGCGAGTTTGCATCTT-3’. In addition, for northern blot, the 18S RNA probe was prepared from potato DNA using the primers 18S-F 5’-TAGATAAAAGGTCGACGCGG-
3’ and 18S-R 5’- TCATTACTCGATCCCGAAG -3’ (GenBank accession number X67238).

qRT-PCR and data analyses

Similar to those treatments used for the subtractive hybridization (SH)/cDNA-AFLP analysis, five micrograms of mixed RNA from each plant-pathogen treatment (3-144 hpi) and *P. infestans* RNA mixture (spores, zoospores, germination, appressoria and mycelium) were treated with Deoxyribonuclease I (Invitrogen) in order to preserve the integrity of RNA by degrading any possible residual genomic DNA. The DNase-treated RNA was reverse transcribed following the M-MLV (RT) enzyme (Invitrogen) manufacturer’s recommendations. Reverse transcription was also performed from five micrograms of eight different times after infection (0, 6, 9, 12, 24, 48, 72, 96, 120 hpi), similar to those used for the subtractive hybridization (SH)/cDNA-AFLP analysis in order to study the dynamics of gene expression during disease development. Gene expression was quantified using a Stratagene Mx3005p cycler. Each 20 μL qPCR reaction contained 2 μL of cDNA (1:3), 6.5 μL of IQ SYB Green Supermix (Biorad), and 0.375 μM of each primer. The following qPCR cycling program was used for all sets of primers: The thermocycle program included 95 °C (2 min), followed by 40 cycles of 95 °C (15 s), 50 °C (45 s) and 72 °C (45 s). Melt-curve analysis was performed to observe primer-dimer formation and to check amplification of gene-specific products. All PCR reactions were performed from triplicate biological samples. The $2^{-\Delta\Delta C(T)}$ method (Livak and Schmittgen 2001) was used to calculate the fold expression relative to the controls. Primers were designed, based on the sequence of the transcripts, DL81-F 5’-CAGCTACTTGGGAGGCTGAG-3’, DL81-R 5’-
TAGGGCGAGTTTGCATCTT-3’ (184bp), DL21-F 5’-AAAGGTGCACGCCTGTTC-3’, DL21-R 5’- TTGCTTTTGCAACATTAGGG-3’(101 bp), DL39-F 5’- GCTCACCAAATCACCAAAC -3’, DL39-R 5’- GGGAAGAGTTGGGGATCTTC -3’(102 bp) and DL28-F 5’- GAAGAAACGCTAGGAAAAGTCG -3’, DL28-R 5’- TCTATTATTGCTTTACACAGCACTCAG -3 (103 bp). In addition, primers specific for elongation factor gene; Efactor-F 5’- GATGGTCAGACCCGTGAACAT -3’ and Efactor-R 5’- GGGGATTTTGTCAGGGTTGT-3’ (180 bp) (Genbank accession number; AB061263) were used to normalize small differences in template amounts.

Acknowledgements

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References


putative pathogenicity factors from the potato cyst nematode *Globodera rostochiensis*. 


**Figure Legends**

**Figure 1.** Schematic representation of the subtractive hybridization (SH)/cDNA-AFLP approach.

**Figure 2.** Example of TDFs in a polyacrylamide gel with one primer combination (EcoRI+ACT/MseI+CAG). a) cDNA from potential *P. infestans* avr genes (avr), b) potential potato resistance gene (rtant), c) cDNA from potentially suppressed potato genes (ssed), d) *P. infestans* cDNA potentially involved in pathogenicity (ssor). Subtractive hybridization products from RB inoculated with US8 (RB+US8 minus control), RB inoculated with US11 (RB+US11 minus control), DF inoculated with US8 (DF+US8 minus control) and DF inoculated with US11 (DF+US11 minus control). US8, *P. infestans* genotype US8 and US11, *P. infestans* genotype US11.

**Figure 3.** Validation of the potential resistance gene transcript DL81. Southern blot and Northern blot (A), qRT-PCR for mixed RNA from each plant-pathogen treatment (3-144 hpi) similar to those used for the (SH)/cDNA-AFLP analysis (B) and different times after inoculation similar to those used for the (SH)/cDNA-AFLP analysis (C). In Southern blot analysis: DF, genomic DNA of cultivars Defender (DF) and Russet Burbank (RB) and of *P. infestans* genotypes US8 and US11 were digested with EcoRI. Northern blot analysis and
qRT-PCR show Russet Burbank inoculated with US8 (RB+US8), Russet Burbank inoculated with US11 (RB+US11), Defender inoculated with US8 (DF+US8), Defender inoculated with US11 (DF+US11), Russet Burbank control (RB+H2O), Defender control (DF+H2O), and *P. infestans* genotypes US8 and US11. In Real rime qRT-PCR, all PCR reactions were performed from triplicate biological samples. The $2^{-\Delta \Delta C(T)}$ method (Livak and Schmittgen 2001) was used to calculate the fold expression relative to the controls. The elongation factor gene was used to normalize small differences in template amounts.

Figure 4. Validation of the TDFs DL21, DL39 and DL28 using qRT-PCR. DL21 qRT-PCR for mixed RNA from each plant-pathogen treatment (3-144 hpi) similar to those used for the (SH)/cDNA-AFLP analysis (A) and different times after inoculation similar to those used for the (SH)/cDNA-AFLP analysis (A). DL39 and DL28 qRT-PCR for mixed RNA from each plant-pathogen treatment (3-144 hpi) similar to those used for the (SH)/cDNA-AFLP analysis (B and C, respectively). RB+US8, Russet Burbank inoculated with US8; RB+US11, Russet Burbank inoculated with US11; DF+US8, Defender inoculated with US8; DF+US11, Defender inoculated with US11; RB+H2O, Russet Burbank control; DF+H2O, Defender control; US8, *P. infestans* genotype US8; US11, *P. infestans* genotype US11. All PCR reactions were performed from triplicate biological samples. The $2^{-\Delta \Delta C(T)}$ method (Livak and Schmittgen 2001) was used to calculate the fold expression relative to the controls. The elongation factor gene was used to normalize small differences in template amounts.
Table 1. Similarities between the identified sequences and genes available in different databases

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1 NCBI Database, 2 *Phytophthora infestans* Database (Broad Institute), 3 Virginia Bioinformatics Institute - VBI Microbial Database, 4 The Solanaceae Genomics Resource at MSU, 5 GabiPD - The Max Planck Institute of Molecular Plant Physiology, 6 Oomycete genomic database, 7 Oomycete genomic database.

* Sequences have been submitted to the NCBI GenBank database.
** Potential pathogenicity factor (ssor), potentially suppressed potato gene (ssed), potential *P. infestans* avirulence gene (avr), potential resistance gene (rtant), potential plant disease susceptibility gene (ssept).
Figure 1.
Figure 2.
Figure 3.
Figure 4.