MOLECULAR ASSESSMENT OF BTH TREATMENT IN PETUNIA, POTATO AND TOMATO

A Thesis

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by

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ABSTRACT

Induced resistance by chemicals such as benzothiadiazole BTH (Syngenta Inc) mimics the biological activation of Systemic Acquired Resistance (SAR) by necrogenic pathogens, taking the place of salicylic acid (SA) in the SAR signal pathway, inducing the same molecular markers and range of resistance. Previous work in our laboratory found that BTH activates resistance against late blight caused by *P. infestans*, on petunias and tomatoes while it did not activate resistance against the same pathogen on potatoes, suggesting that the spectra of resistance activated by BTH are very crop and pathogen specific. My goal was to understand the molecular mechanism by which BTH mimics the SAR response and further understanding why BTH works in some plants and not others. To address this question I used microarray technology to identify the genes expressed in response to BTH in petunias, tomatoes and potatoes. I selected three candidate genes (cysteine protease, acidic chitinase and PR1-a) to characterize further by silencing using Virus Induced Gene Silencing (VIGS). My hypothesis was that silencing of these genes will reduce the resistance response in plants observed after BTH treatment against *P. infestans*. However, silencing of cysteine protease, PR1-a or acidic chitinase II individually did not reduce the effect of BTH on plants. The lack of phenotype after silencing PR1-a supports previous conclusions from our lab that partial resistance to *P. infestans* in tomato is not dependent of the SA pathway.

BIOGRAPHICAL SKETCH

The author was born in Bogotá, Colombia. She graduated from the Universidad de los Andes, in September 1999 with a Degree in Biology. She did her undergraduate thesis at CIAT (Centro Internacional de Agricultura Tropical) under the supervision of Dr. Valérie Verdier where she got interested in the field of Plant Pathology. After completing her studies, her career interests made her pursue graduate studies in this field. In January 2004 she enrolled in the Department of Plant Pathology at Cornell University to conduct her Master's studies under the guidance of Dr. William Fry.

A la Mamaina y mi Chandita

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CHAPTER 1

INTRODUCTION

Plants have evolved mechanisms to detect and respond effectively to an array of pathogens by innate or inducible defenses. Recognition of a pathogen through the detection of products of pathogen-encoded Avirulence (Avr) genes by plant Resistance (R) genes is often associated with a rapid localized programmed cell death called the hypersensitive response (HR). Following the HR, the plant accumulates salicylic acid (SA) and establishes a systemic acquired resistance (SAR) where uninfected parts of the plant develop resistance to further infection by some pathogens (Yang et al., 1997; Martin, G.B. 1999). During SAR, SA is required for pathogen resistance and induction of pathogenesis related (PR) genes (Zhang et al., 2003).

Induced resistance can be stimulated by chemicals mimicking the biological activation of SAR. This provides new opportunities to control plant diseases and to investigate disease resistance mechanisms in plants (Tally et al., 1999). Two different chemicals 2,6-dichloro isonicotinic acid (INA) and its derivatives (Métraux et al., 1991) and the benzol [1,2,3] thiadiazole-7-carbothiate (BTH) (acibenzolar-S-methyl -ASM-), are the best studied resistance activators (Oostendorp et al., 2001). INA derivatives were not commercialized due to their high toxicity in plants, while ASM derivatives have been commercialized as ACTIGARD™, BION® and BOOST® (Oostendorp et al., 2001).

It has been shown that in dicotyledonous plants such as tobacco and Arabidopsis, systemic translocation of these activators can take the place of

SA in the SAR signal pathway, inducing the same molecular markers and range of resistance (Ward et al. 1991; Uknes et al.1992, 1993; Vernooij et al. 1995; Lawton et al. 1996; Friedrich et al. 1996). However, in wheat, BTH treatment activated a set of genes different from the set of genes activated by the non-host pathogen *Erysiphe graminis* f. sp. *tritici* (Schaffrath et al 1997).

Application of BTH on the susceptible petunia cultivar White Madness and the susceptible tomato cultivar Sunrise activated resistance against late blight caused by *P. infestans*, while BTH application on potato did not activate resistance against the same pathogen (Tally et al., 1999; Si-Ammour et al 2003; Becktell et al 2005; this work). In contrast, potato plants treated with BTH acquired resistance to early blight (*Alternaria solani*) and powdery mildew (*Erysiphe cichoracearum*) (Bokshi et al 2003).

Therefore, it is clear that the spectra of resistance activated by chemicals mimicking the biological activation of SAR cannot be based on the relatedness of pathogens, but must be determined for each crop and pathogen (Oostendorp et al 2001; Bokshi et al 2003).

The *P. infestans* –potato interaction has been studied at the cytological level (Ferris 1955; Vleeshouwers et al 2000), indicating that potato cultivars carrying R genes lead to incompatible interactions when challenged with avirulent strains, inducing HR and preventing the pathogen growth. If the interaction is with a compatible strain, the HR is not induced, resulting in disease (Vleeshouwers et al 2000). Interestingly, Restrepo et al (2005), showed that in a compatible interaction between *P. infestans* and the potato cultivar Kennebec the plastidic carbonic anhydrase (CA) gene, which has an

antioxidant activity and is known to bind SA (Slaymaker et al 2002) was down regulated.

Using Virus Induced Gene Silencing (VIGS), consisting of a virus vector carrying a gene of interest which is inoculated into a plant to induce a post-transcriptional gene silencing (Ratcliff et al 2001; Liu et al 2002), Restrepo et al (2005) demonstrated that the suppression of CA in potato plants was correlated to an increased susceptibility to *P. infestans*.

In the tomato–*P. infestans* pathosystem, three types of host-pathogen interactions occur: 1) highly compatible which results from the interaction of a susceptible host and a tomato specialized isolate of *P. infestans* (Smart et al 2003), 2) partially compatible that occurs from the interaction between a tomato specialized isolate and a partially resistant host (Moreau et al 1998) or a non specialized isolate and a susceptible host (Vega-Sanchez et al., 2000) and 3) incompatible interactions that involve a resistant host and an isolate carrying the correspondent avirulence (Avr) gene (Gallegly and Marvel 1995 ; Turkensteen 1973). In the highly compatible interaction HR is delayed, whereas PR gene expression is not.

In contrast to the *P. infestans* –potato and tomato interactions, recent studies of the *P. infestans*- petunia pathosystem have shown that the HR was present in the susceptible, partially resistant and resistant interactions, although it was predominant in the resistant interaction (Becktell et al 2006).

Because a long term goal of our lab is to understand the resistance reaction to *P. infestans* in Solanaceous plants, I chose to investigate the resistance in petunias and tomatoes stimulated by BTH. Since the reaction of petunias to

BTH was so strong, it seemed that the *P. infestans*-petunia system combined with the tomato-*P. infestans* system might be used to develop a more comprehensive understanding of late blight resistance.

The approach I chose was to identify genes that might be involved in BTH-induced resistance (candidate genes) using microarray technology, and then to silence the selected candidates via VIGS. Two types of microarrays were used. Tomato microarrays (BTI) were used to identify tomato and petunia genes that were responsive to BTH because petunia microarrays were not commercially available. Potato cDNA was hybridized on potato arrays from TIGR. Genes that were most affected by BTH and which had previously been implicated in resistance were chosen for silencing. Silencing was accomplished in tomato using a tobacco rattle virus (TRV)-based vector pTRV2 as described by Liu et al (2002).

CHAPTER 2

MATERIALS AND METHODS

2.1 Plant Material and BTH Treatment

Four-week-old potato (*Solanum tuberosum*, cultivar Kennebec), petunia (*Petunia hybrida*, cultivar White Madness) and tomato (*Lycopersicon esculentum,* cultivar Sunrise) were used. Plants were grown in a greenhouse. Natural light was supplemented with 400W high pressure sodium lamps for 12 hours and temperatures maintained between 24 to 29 ºC. Plants were grown in a soil less mix (Cornell mix) consisting of a 1:1 (vol/vol) peat-vermiculite mix supplemented with nitrogen, phosphorus and potassium (0.4kg each) per cubic meter of mix.

On the fourth week after sowing, plants were separated into two sets, each set consisting of 9 plants per species. One set of plants was sprayed with water as control, while the other set was sprayed with BTH at a concentration of 37 mg/L until run-off with a hand held sprayer. One week after the first BTH treatment, a second BTH spray (at the same concentration), was done on the same 9 plants (per species) while control plants were sprayed with water. Immediately after the second BTH treatment plants were transferred to an inoculation chamber consisting of a PVC frame covered with semi-clear plastic sheeting, at 15ºC and 12hours light. To maintain a 100% relative humidity (RH), an automatic humidifier (Trion model 500 Hummert International, Earth City MO) ran periodically throughout the day and night. Leaf tissue of 3 plants per treatment was collected two days after the second BTH spray and frozen in liquid nitrogen. The remaining six plants were inoculated that same day with

P. infestans to determine the effect of BTH on the outcome of the plant-pathogen interaction (see below).

2.2 Inoculum preparation and *P. infestans* isolate

To determine the effect of BTH on potatoes, petunias and tomatoes, I inoculated BTH treated plants and water treated controls with the *P. infestans* isolate US940480 from the clonal lineage US-8 because it is compatible with potato (cultivar Kennebec), petunia (cultivar White Madness) (Becktell et al 2006) and tomato (cultivar Sunrise) (Restrepo et al 2005). Tomato leaflets with sporulating late blight lesions were detached and rinsed in 100ml of distilled water to collect the sporangia; the concentration of sporangia in water was determined by using a hemacytometer and then adjusted to 20,000 sporangia per ml. Subsequently, the sporangia were incubated at 4˚C for 1hour to release zoospores. This mixture of sporangia and zoospores was applied to plants with a hand held sprayer until run off. Plants were incubated at 15°C and 100% RH for the next 7 days when they were evaluated for disease.

2.3 RNA extraction, probe preparation and hybridization on microarrays

RNA was extracted from each of three independent biological trials of each plant species. These biological trials were conducted at different times and the RNA from each trial was analyzed independently. Each sample consisted of leaflets of three plants of each treatment collected two days after the second BTH application. The leaflets of the three plants were pooled together at the moment of collection, and flash frozen in liquid nitrogen. Pooled plant tissue was ground in liquid nitrogen using a cold mortar and a pestle. Total

potato, petunia and tomato leaf RNAs were extracted using the hot-phenol protocol by Perry and Francki (1992) as modified by Gu et al (2000). mRNA was isolated using Dynabeads® mRNA Purification Kit (Dynal-Biotech) following the manufacturers' instructions.

cDNA was synthesized from 0.4-2.0 µg of mRNA by reverse transcriptase and subsequently labeled using SuperScript™ Indirect cDNA labeling Core kit (Invitrogen) following the manufacturers' instructions. Briefly, after cDNA synthesis and purification, 5µl of DMSO/dye solution (Cy3™ or Cy5™) was added to the cDNA, and incubated for one hour in the dark. Once the cDNA was labeled, it was purified to remove any un-reacted dye. cDNA probes were dried in a centrifugal evaporator (Jovan RC 10.10) at 1200 rpm and 55°C for 50 minutes (min) in the dark. Dried labeled cDNA Cy5™ (BTH) and Cy3™ (NO BTH) probes were resuspended in a final volume of 60µl of Corning Universal hybridization solution (Pronto!™ CORNING®) and combined into a single tube. To avoid potential dye-related differences in labeling efficiency the same procedure was followed for the correspondent dye-swap Cy5™ (NO BTH) and Cy3™ (BTH) probes.

In order to avoid nonspecific background during hybridization, microarray slides were pre-hybridized with 5X SSC buffer, 0.1% (w/v) SDS and 1%BSA for 45 minutes (min) at 42°C. Afterwards, the slides were washed for 5 min with room temperature milliQ water followed by a 2 min wash in isopropanol, and dried by centrifugation at 1500 rpm for 1min. cDNA probes were denatured at 95°C for 5 min and then centrifuged at 14000rpm for 1 min. Fifty-five µl of the probes were applied to the array; then it was carefully covered by a slip to avoid bubbles and transferred to a hybridization chamber (Corning

Microarray Technology) to be incubated overnight at 43 ℃ in a water bath. To finish the hybridization procedure, the slide was washed for 10 min at 42℃ with a low stringency buffer containing 2X SSC and 0.1% (w/v) SDS followed by a 10 min wash at room temperature in a high stringency buffer containing 0.05X SSC and 0.1% (w/v) SDS; finally slides were rinsed in a wash solution with 0.05X SSC at room temperature for 2 min repeating this last step 4 times with fresh 0.05X SSC solution each time. Slides were dried by centrifugation at 1500 rpm for 1 min.

Potato cDNA was hybridized on potato microarrays (TIGR: www.tigr.org), while petunia and tomato cDNAs were hybridized on tomato microarrays (BTI: www.sgn.cornell.edu).

2..4 cDNA Microarray analysis

Microarray slides were scanned using a GenePix Pro (4100) scanner (Axon Instruments Inc., Union City, CA, USA). The MIDAS computer program (Saeed et al 2003) was used to perform dye-swap filtering on GenePix results previously converted to TAV files with the CONVERTER program (www.tigr.org). Data were normalized using the local regression technique LOWESS (Locally Weighted Scatterplot Smoothing) with the MIDAS software (www.tigr.org). To identify genes with statistically significant changes in gene expression we analyzed the data using Significant Analysis of Microarrays (SAM) (Tusher et al 2001). SAM assigns a score to each gene on the basis of its change in gene expression relative to the standard deviation of repeated measurements for that gene (Tusher et al 2001). The threshold chosen was 1.5; genes with scores greater than that threshold, were considered to be

potentially significant. The false discovery rate (FDR) is the percentage of such genes identified by chance. The threshold can be adjusted to identify smaller or larger sets of genes by adjusting the FDR calculated for each set (Tusher et al 2001). I used a delta value 0.193 for tomato with a FDR between 0-4 percent, and I considered genes to be differentially expressed if they were selected by SAM in all three experiments. Because the FDR was higher than 20% at any delta value chosen for petunia and potato, it was not possible to identify any differentially expressed genes in potato or petunia (see Results).

2.5 cDNA Microarray validation using Northern blots

Total RNAs were separated electrophoretically on a 1.2% formaldehyde-agarose gel for 3 hours. Each RNA sample (34µl) contained 10 µg of total RNA in 44% Formamide, 6.5% Formaldehyde, 0.89X MOPS buffer and 0.89X sample dye. Subsequently, the gel was rinsed 3 times (10 min each) with DEPC treated water and subsequently soaked for 30 min in 10X SSC, pH 7.0. Finally, RNA was transferred to Hybond-N membrane in 10X SSC overnight (Amersham Biosciences, Piscataway, NJ, U.S.A.). The membrane was cleaned with 2X SSC for 3 min and crosslinked using UV crosslinker (Stratagene).

Because several genes were identified as differentially induced by BTH treatment (see Results), a subset of these (Cysteine protease, PR1-a and chitinase) were used as probes for northern analyses. DNA probes were obtained by Polymerase Chain Reaction (PCR) amplification from pBluescript SK+ plasmid. PCR conditions were 1X PCR reaction buffer (Invitrogen), 50mM $MgCl_2$, 10mM dNTPs, 2 µM T7 primer, 2 µM T3 primer, 5U/µl Taq in a final

volume of 30 µl with 25 ng of DNA template. PCR amplification conditions included an initial denaturation at 95°C for 5 min, followed by 35 cycles consisting of a denaturation at 95°C for 15 sec, annealing temperature at 55°C for 30 sec, an extension at 72°C for 1min and a final extension of 72°C for 5 min. PCR products were cleaned using the Wizard ® PCR Preps DNA purification System from Promega, following the manufacturer's instructions and sequenced at the at the Cornell University BioResource Center to confirm insert amplification. Homology of the sequences was determined at the NCBI website (www.ncbi.nih.gov).

Hybridizations were performed using Puregene Hyb-9 hybridization solution (Gentra Systems, Plymouth, MN, U.S.A.) preheated at 65°C. The northern blot membrane was placed in a hybridization tube with 25 ml of preheated Hyb-9 solution at 65°C for 30 min. Salmon sperm DNA (Invitrogen) was denatured at 95°C for 5 min and then placed on ice for 5 min. Subsequently, denatured salmon sperm DNA (250 µl) was added to the hybridization tube followed by a 3 hour incubation time. DNA probes (at least 25ng of DNA probe in a final volume of 23µl) were labeled with ATP^{P32} using the Random Primers DNA Labeling System according to the manufacturers' instructions (Invitrogen, Carlsbad, CA, U.S.A.). Probe synthesis was done at room temperature for 2 hours. Unused nucleotides were removed with Bio-Rad Micro Bio-spin P30 columns. The Eluted probe was denatured at 95°C for 5 min and then placed on ice for 5 min, and then it was added to the hybridization solution with the northern blot membrane and hybridized overnight in a Hybaid oven at 65°C. To finish the hybridization procedure, the membrane was washed with 2X SSC/0.1%SDS at 65°C for 5 min, followed by a second wash with 1X

SSC/0.1%SDS for 10min at 65℃. A final wash with 0.1X SSC/0.1% SDS for 10 min at 65℃ was performed. The blot was placed in a plastic sheet saver and then fit into film cassette with an X-O-Mat film and exposed overnight at -80℃.

2.6 Virus induced gene silencing (VIGS)

To study the function of Cysteine protease, PR1-a and chitinase in the defense response against *P. infestans* elicited by BTH in tomato, I silenced these 3 genes using a tobacco rattle virus (TRV)-based vector as described by Liu et al (2002). TRV is a bipartite virus where the RNA1 (TRV1) codes for a movement protein, replicase proteins and a cysteine-rich protein and the RNA2 (TRV2) encodes for a coat protein from the genomic DNA and two non-structural proteins from the subgenomic DNAs (MacFarlane 1999). To construct the vectors non-structural genes from TRV2 where replaced by a multiple cloning site (MCS) where the gene of interest is cloned (Liu et al 2002). I used the GATEWAY ® Technology system (Invitrogen, USA); in brief, a forward primer containing the attB1 sequence and a reverse primer containing the attB2 sequence were used to amplify the genes of interest from the plasmid by PCR. The conditions were initial denaturation at 94℃ for 2 min, followed by 35 cycles of amplification (denaturation at 94℃ for 15 sec, annealing at 55℃ for 30 sec, then extension at 68℃ for 1 min) followed by a final extension of 68℃ 5 min.

The resulting PCR products were cleaned using Wizard ® PCR Preps DNA purification System from Promega, following the manufacturer's instructions and then sequenced at the BioResource Center at Cornell University to confirm the insert. PCR products with the attB1 and attB2 terminal sequences

were incubated with the p-DONOR vector that contains the attP1 and attP2 recombination site and the BP CLONASE enzyme. Then the pTRV2 destination vector containing the attR1-attR2 recombination sites and the LR CLONASE enzyme were added. This mixture was transformed into TOP10 *E. coli* cells (Invitrogen, USA) by heat shock following the manufacturer's instructions (Invitrogen USA) and selected in 50µg/ml Kanamycin low salt LB plates. Two clones per construct were sequenced to verify the insertion of the gene of interest. pTRV1 and pTRV2 were transformed into *Agrobacterium* strain GV3101 by freezing in liquid nitrogen for 5 minutes, then leaving at room temperature until thawed, followed by a 4 hour incubation in 1ml low salt Luria broth (LB) with no antibiotic in a shaker at 28˚C. Transformed *Agrobacterium* cells (100 µl) were plated into 100 µg/ml Rifampicin, 30µg/ml Gentamicin and 50µg/ml Kanamycin low salt LB plates and incubated overnight at 28˚C. One colony was selected and grown in 5 ml low salt LB medium with the same antibiotic selection overnight at 28˚C and 250rpm. The next day, *Agrobacterium* cells were centrifuged for 5 minutes at 5000rpm, rinsed with 10mM $MgCl_2$ and resuspended in 5 ml of infiltration media (10 mM $MgCl_2$, 10mM MES pH 5.5 and 150 µM acetosyringone), adjusted to an O.D. of 1.5 and maintained at room temperature for 3 hours. *Agrobacterium* carrying pTRV1 was mixed with the *Agrobacterium* carrying pTRV2 in a 1:1 ratio into a final volume of 10ml. *Agrobacterium* infiltration was done using a 1ml needle-less syringe in cotyledons and the first true leaves of two-week-old tomato plants. After inoculation, plants were kept at 18˚C with 12 hours light. Generally, silencing was accomplished 20 days after the inoculation with *Agrobacterium*.

2.7 Vectors used for VIGS and Experiment design

Vectors: The experiment involved three vectors; each vector is identified by the name of the gene that it carried for silencing. These are: pTRV2: Cysteine protease; pTRV2:PR1-a and pTRV2: Chitinase. In addition, I had two negative controls for the experiment: plants that were inoculated with pTRV2: empty vector (vector which does not carry any gene) and plants that were not inoculated with *Agrobacterium*.

Plants carrying a vector with the phytoene desaturase (PDS) gene (pTRV2:PDS), were used as a visual guide to determine when gene silencing was achieved.

2.8 Experiment design

I had three independent trials of the experiment for each vector. I used a complete randomized block design with five replications. Each block contained 10 plants that were separated in two sets of 5 plants each. One set was treated with BTH and the other set was treated with water as control. Each set consisted of one plant carrying one of the vectors of interest (pTRV2:Cysteine protease; pTRV2:PR1-a and pTRV2:Chitinase) and the two controls (pTRV2:empty vector and no vector). The pTRV2:PDS plants were distributed around the greenhouse. When the plants were two weeks old, I inoculated them with the appropriate vector mentioned above. Two weeks later, the plants were separated in 2 sets; one set of plants was treated with BTH and the other set was treated with water as a control. One week after the first BTH treatment, a second BTH spray was done on the same plants.

Once silencing was accomplished as determined by the PDS phenotype (in our case five-week old plants) we collected the two youngest leaflets from each of the five replicates per treatment and flash froze them in liquid nitrogen. Tissue from each replicate was analyzed separately. Immediately after collecting the tissue, plants were inoculated with the *P. infestans* isolate US970001 from the clonal lineage US17 at a concentration of 5000 sporangia/ml to determine the effect of gene silencing in tomato plants.

A general linear model was used to determine the effect of BTH and gene silencing in plants. Statistical analyses were carried out using MINITAB version 14.

2.9 Reverse Transcriptase (RT) PCR to confirm gene silencing

To determine the degree of gene silencing I used RT-PCR. The primers used were: Cysteine protease FW: 5'AGG TTG CGA TGG TGG TCT TAT GGA-3', RV: 5'- GCT TCA AGT GCA ATG CTC ACA GGT-3'; PR1-a: FW: 5'-CTG GAT CGG ACA ACG TCC TTA CTA-3', RV: 5'-GGA AAC AAG AAG ATG CAG TAC TTA-3' and Chitinase: FW: 5'- ATT GGA CAA TGG ACG CCAT CCC -3', RV:5'- TAG CCC TGG GCG AAG TTC TTT -3'. DNase-treated RNA (1 µg) was used for cDNA synthesis, using the ImProm-IITM Reverse Transcription System (Promega), following manufacturer's instructions. PCR was carried out with 2 µl of the cDNA synthesis reaction in a 30-µl volume containing 0.2 mM each of the four dNTPs, 2µM each of the primers, and 0.5 U *Taq* polymerase. PCR conditions consisted of 1 cycle of 95ºC for 5 min, followed by 30, 35 or 45 cycles of a three-step procedure: 1 min at 94°C, 1 min at 55°C, and 1 min at 72°C, and a final step of 5 min at 72°C. As a control, RT-PCR of the tomato

actin gene was performed with the following primers FW: 5′- CCA AAA GCC AAT CGA GAG AA -3′, RV: 5′- GGT ACC ACC ACT GAG GAC GA -3′. The same PCR reaction conditions were used for the actin.

CHAPTER 3

RESULTS

3.1 Differentially expressed genes

In order to determine gene expression changes in potato, petunia and tomato after BTH treatment, we analyzed microarray data probed with RNA from plants after two applications of BTH. The data were analyzed using a bootstrap analysis with SAM. SAM estimates the fold change and significance of the differences in gene expression. I set the false discovery rate (FDR) at four percent. Because I did not have differentially expressed genes for potato and petunia at that FDR, I lowered the stringency and analyzed potato and petunia genes at a 20% FDR. Tomato FDR was left at 4%. The fold change cutoff was 1.5 with 1000 permutations.

I was not able to detect any difference in gene expression due to BTH treatment for potato or petunia, even at a FDR of 20%.

BTH induced up-regulation of 13 genes in tomato (Table 2). Twelve of these showed similarity to previously known proteins, and just one had no significant similarity to sequences in the databases. Differentially expressed genes were classified according to their functional categories derived from Swiss-Prot (http://ca.expasy.org/sprot/).

Some of these genes were similar to genes coding proteins previously suggested to be involved in plant response to biotic or abiotic stress. These included several PR proteins. The first was chitinase precursor, which is involved in chitin hydrolysis and has been tested for both in vitro and in vivo pathogen inhibition (Brunner et al 1998; Loon and Strien 1999; Anand et al

2003). Another was a lipid transfer protein (LTP) belonging to the PR-14 family that has been implicated in systemic resistance signaling in *Arabidopsis* (Maldonado et al 2002), but which may also have a role in wax or cutin deposition in the cell walls of expanding epidermal cells (by similarity: www.ncbi.nlm.nih.gov). A third was PR1-a which has been shown to increase plant tolerance against oomycetes (Heitz et al 1993; Alexander et al 1993; Niderman et al 1995). Also, enzymes that are involved in programmed cell death, like Cysteine proteases which degrade proteins into individual amino acids by hydrolysis and antioxidants involved in the protection of cells against oxidative damage such as Glutathione S- transferase were up regulated (Apel and Hirt, 2004). A gene with homology to the high mobility group protein in potato, which is involved in the regulation of transcription, was also up regulated. Calmodulin, a major transducer of calcium signals was induced. Also, cyclophilin which is an enzyme involved in protein folding was induced. Two proteins involved in transport activities were up regulated; these were Chloroplast phosphate transporter involved in inorganic phosphate transporter activity and a putative alpha-coat protein which has been proposed to play a role in different steps of intracellular transport, forming a coat around vesicles budding from the Golgi.

Table1. List of differentially expressed genes in tomato plants treated with BTH

Gene	Fold Change	TUS#	Clone I.D.
High mobility group protein potato	4.11	TUS44N7	1-1-2.2.16.4
Chloroplast phosphate transporter	4.1	TUS45O7	1-1-2.3.14.10
Pathogenesis-Related 1-a	4	TUS29C12 TUS44P7 TUS44J4	1-1-5.3.4.17 1-1-2.4.16.4 1-1-5.2.16.3
Chlorophyll A-B binding protein	3	N.A.	1-1-1.4.7.7
Acidic Chitinase II	2.58	TUS47J16	1-1-1.2.9.4
unspecific lipid transfer protein PR-14	1.8	TUS19L19	1-1-6.4.15.14
Glutathione S-transferase-like protein	1.75	TUS32C3	1-1-6.3.1.10
Unknown protein	1.7	TUS16L19	1-1-6.4.18.17
Cysteine protease	1.6	TUS29D7 TUS46D13 TUS21N14	1-1-2.4.4.13 1-1-4.4.12.17 1-1-3.2.13.17
anthranilate phosphoritiosyltransferase (transferase glycosyl groups)	1.56	TUS17L19	1-1-6.4.17.8
Calmodulin, signal transduction Ca signaling	1.54	TUS42D17	1-1-8.4.1.6
Peptidyl-prolyl cis-trans isomerase Cyclophilin	1.5	TUS45M14 TUS45A22 TUS45M7 TUS45K18	1-1-3.1.14.14 1-1-3.1.14.15 1-1-2.1.14.10 1-1-7.3.14.17
Putative alpha-coat protein	1.5	TUS27A7	1-1-2.1.6.7

3.2 Confirmation of microarray results

Three up-regulated genes from tomato that have been previously proposed to be involved in plant response against pathogens were chosen for further investigation. These genes are Cysteine proteinase (TUS 21N14), Pathogenesis-Related 1-a (PR1-a) (TUS 29C12) (Heitz et al 1993; Alexander et al 1993; Niderman et al 1995) and Acidic Chitinase II (TUS 47J16) (Zhu et al 1994; Heitz et al 1994; Brunner et al 1998; Anand 2003). To confirm the reliability of our microarray results I selected the three genes of interest that were differentially expressed and performed a Northern blot analysis with total RNA from one of the biological replicates. Each gene was used as a probe to determine its expression after BTH treatment and water (NO BTH). As can be seen in figure 1, the differential expression was confirmed for the 3 genes; BTH treated plants showed a higher expression of the 3 genes, while No BTH treated controls showed a lower expression. To make sure that the difference in gene expression between the BTH treated plant and the water treated control was due to the BTH treatment, we show underneath the radiograph an ethidium bromide stained gel showing equal loading of the RNA samples (Figure1).

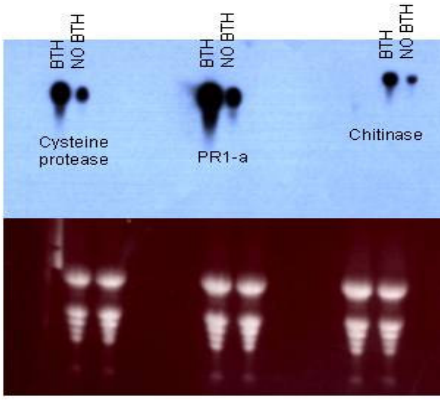


Figure 1. Northern analysis of Cysteine protease, PR1-a and Chitinase gene expression in BTH treated and control tomato plants (No BTH). The image of an ethidium bromide-stained gel, shown below the blot, demonstrates equal loading of the RNA samples.

Once I confirmed that these genes were differentially expressed due to the BTH treatment, I asked investigated whether the silencing of these genes would reduce the effect of BTH on treated plants making tomato more susceptible or as susceptible as the water treated controls to *P. infestans*.

3.3 Silencing of genes of interest using VIGS and silencing determination by RT-PCR

I used Virus Induced Gene Silencing (VIGS) as an approach to silence the tomato genes. In order to predict the timing of silencing, I used the Phytoene desaturase (PDS) gene which causes the plants to exhibit a photo-bleached phenotype by inhibiting carotenoid biosynthesis (Kumagi et al., 1995) as a visual aid to determine when silencing was achieved. Silencing of PDS typically occurred 20 days after inoculation with *Agrobacterium* (Fig. 2).

Figure 2. Tomato plant inoculated with VIGS using the Phytoene Desaturase gene (PDS) as a visual marker and positive control for gene silencing.

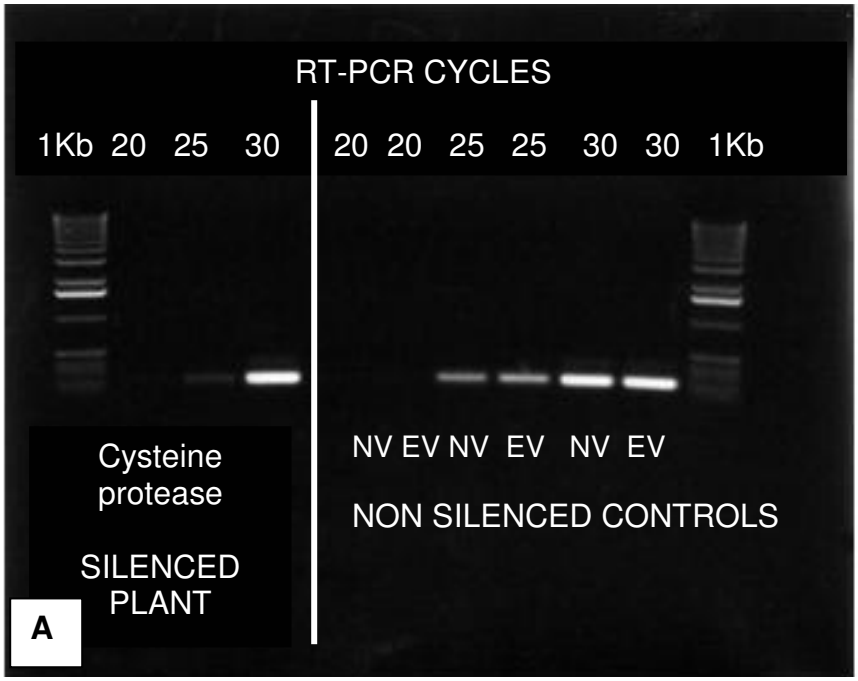
Inoculations with *P. infestans* were done two days after the second BTH spray (21 days after VIGS treatment). This time coincided when the PDS phenotype was widely spread on the leaflets (see materials and methods) ensuring that the genes were silenced at the time of pathogen inoculation.

Before the inoculation with *P. infestans* I collected one leaflet per plant to extract the RNA and determine whether silencing had occurred. Silencing for each gene of interest was investigated by Reverse Transcriptase (RT)-PCR for at least two independent trials and at least two replicates per trial (Figure 3). Based on the RT-PCR results, I was able to determine that silencing of the target gene was accomplished in approximately 40% of the plants. I did RT-PCR for each gene of interest with their respective controls (empty vector and

no vector) at three different cycles: 20, 25 and 30. Amplification of silenced plants at 20 and 25 cycles showed a low amount of PCR product which indicates a reduced expression due to silencing as opposed to the control plants. To make sure that the difference in RT-PCR amplification was due to the gene silencing, and not to differences in the amount of RNA used, each gene was compared with the actin transcript (30 cycles), whose expression was constant under BTH treatment (Figure 3).

I did a time course evaluation of the disease incidence during 5 days rated as the percentage of disease in silenced and control plants (empty vector and no vector) after inoculation with *P. infestans*. Phenotypic assessment of these plants is shown in Figure 4. These pictures were taken six days after inoculation. On the left side are the water treated plants and on the right side are BTH treated plants. I analyzed the data with a general linear model (GLM) and determined that there was a significant effect (P=0.000) of BTH conferring resistance to *P. infestans* as has been shown previously by Becktell et al (2005). Strikingly, the effect of BTH on tomatoes was not altered by the silencing of any of these three genes, e. g. silencing caused no reduction in the susceptibility of BTH-treated plants (P=0.421) as can be seen in Figure 4.

Figure 3. The product of RT-PCR at 20, 25 and 30 cycles showing gene silencing for the three genes of interest Cysteine protease, PR1-a and acidic chitinase and their positive controls, plants containing no vector (NV) or inoculated with an empty vector (EV). Actin amplification (30 cycles) was used as control to show equal amounts of RNA samples. (A) Amplification for Cysteine protease followed by its actin control; (B) PR1-a with actin control; (C) acidic chitinase with its respective actin control.


RT-PCR CYCLES
1Kb 20 25 30
20 20 25 25 30 30 1Kb
Cysteine protease
SILENCED PLANT
NV EV NV EV NV EV
NON SILENCED CONTROLS
A

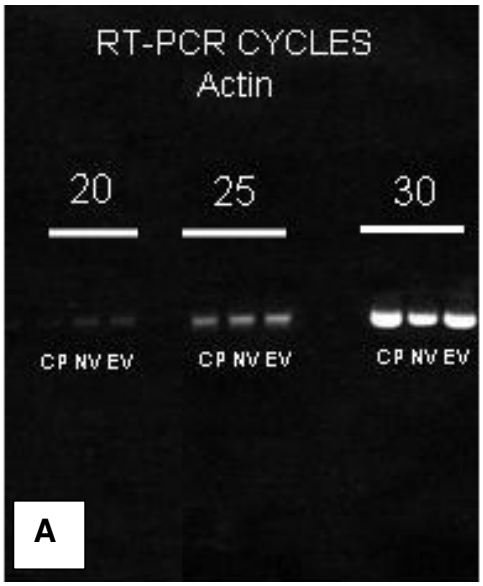

RT-PCR CYCLES
Actin
20
25
30
CP NV EV
CP NV EV
CP NV EV
A

Figure 3 (continued)

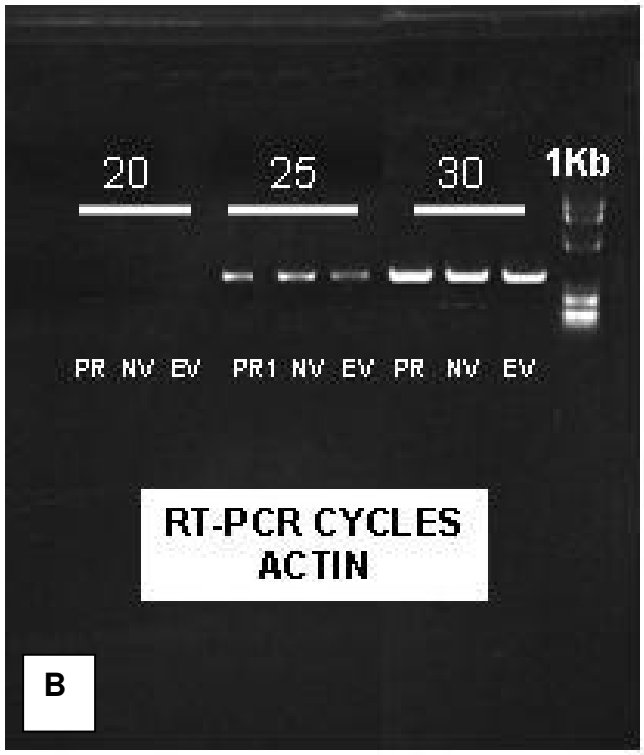
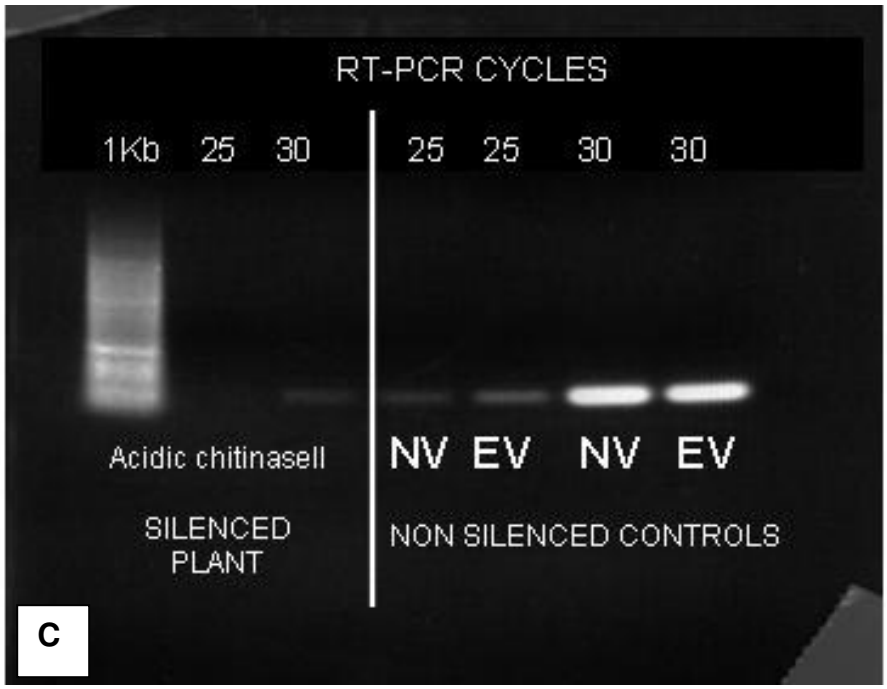


Figure 3 (continued)



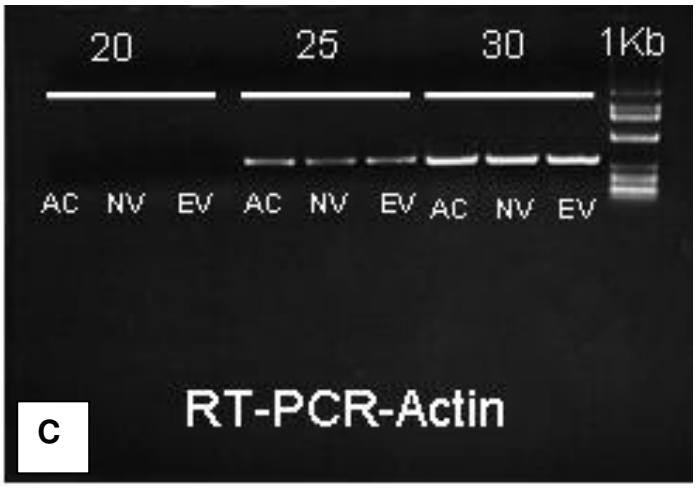
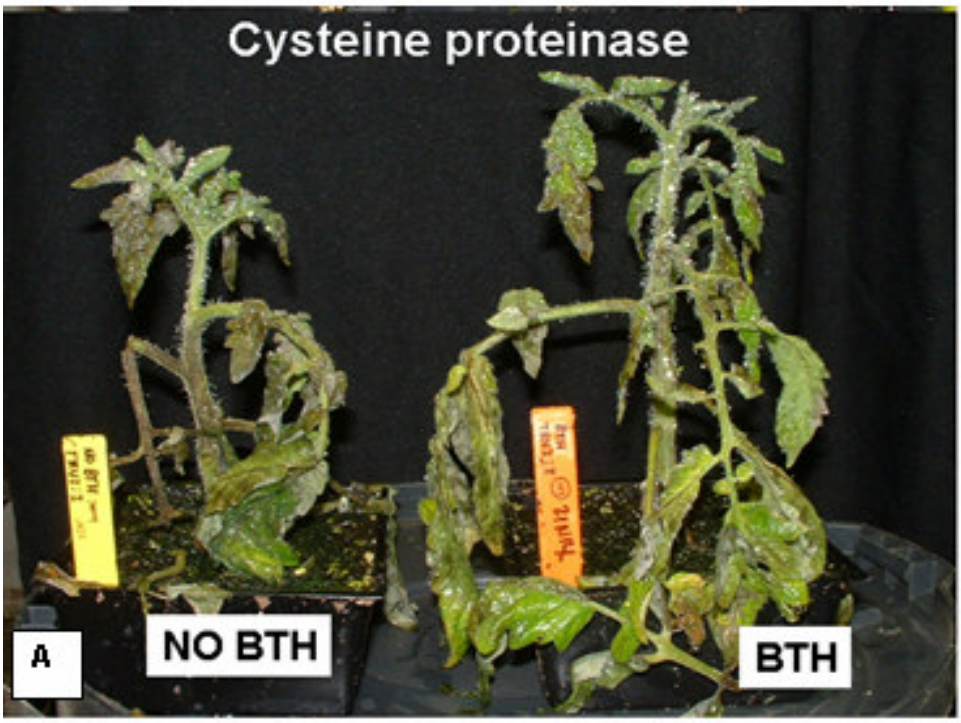


Figure 4. Phenotypic evaluation of VIGS silenced plants (water control and BTH) for the three genes of interest: Cysteine protease (A), PR1-a (B) and Chitinase (C), with their respective controls, plants carrying an empty vector (D-E) and plants with no vector (F-G).


Cysteine proteinase
A
NO BTH
BTH

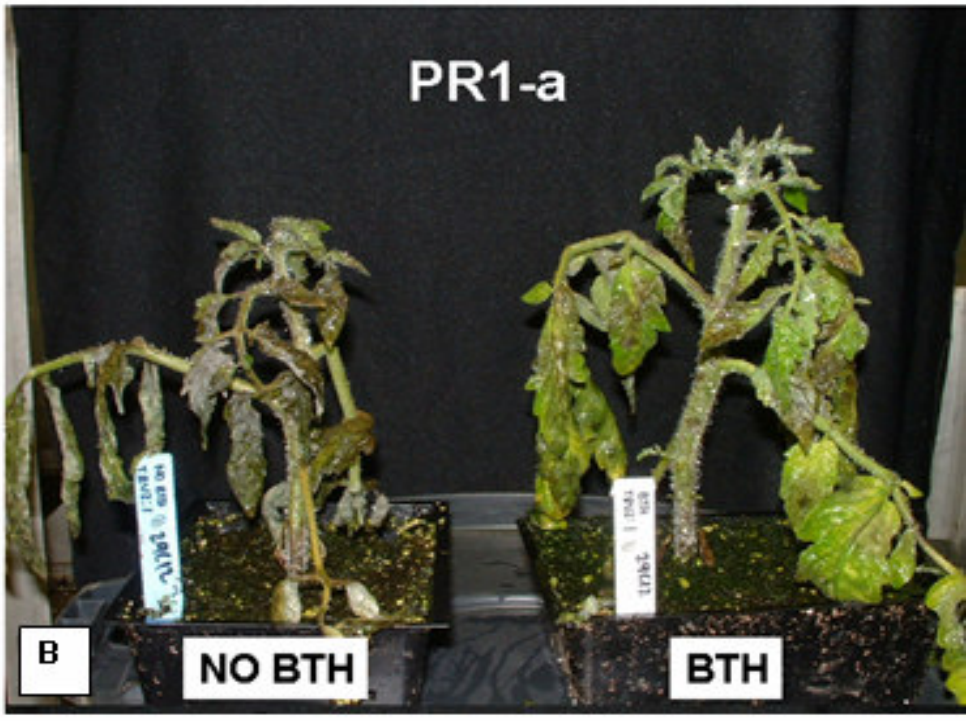

PR1-a
B
NO BTH
BTH

Figure 4 (continued)

Figure 4 (continued)

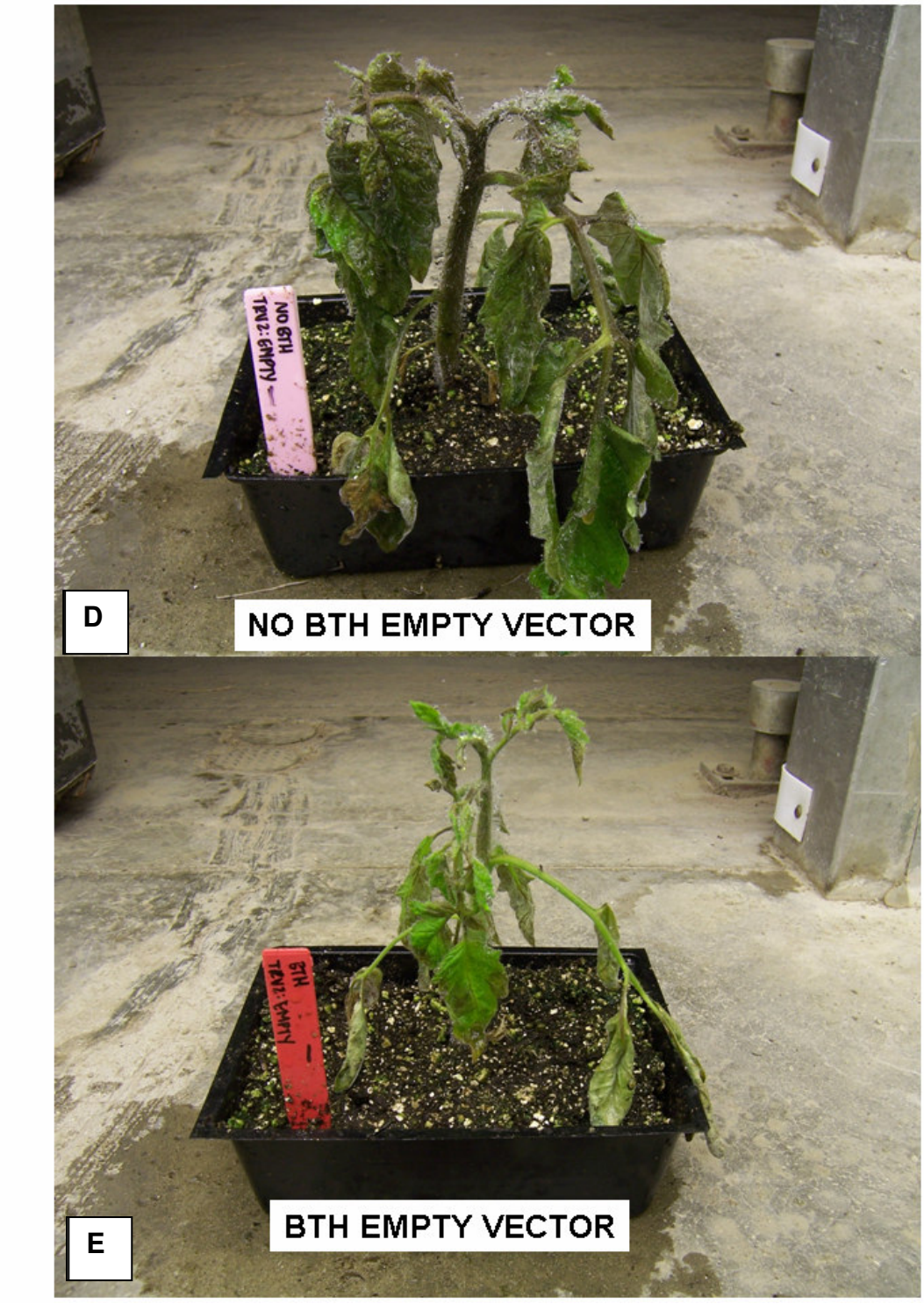
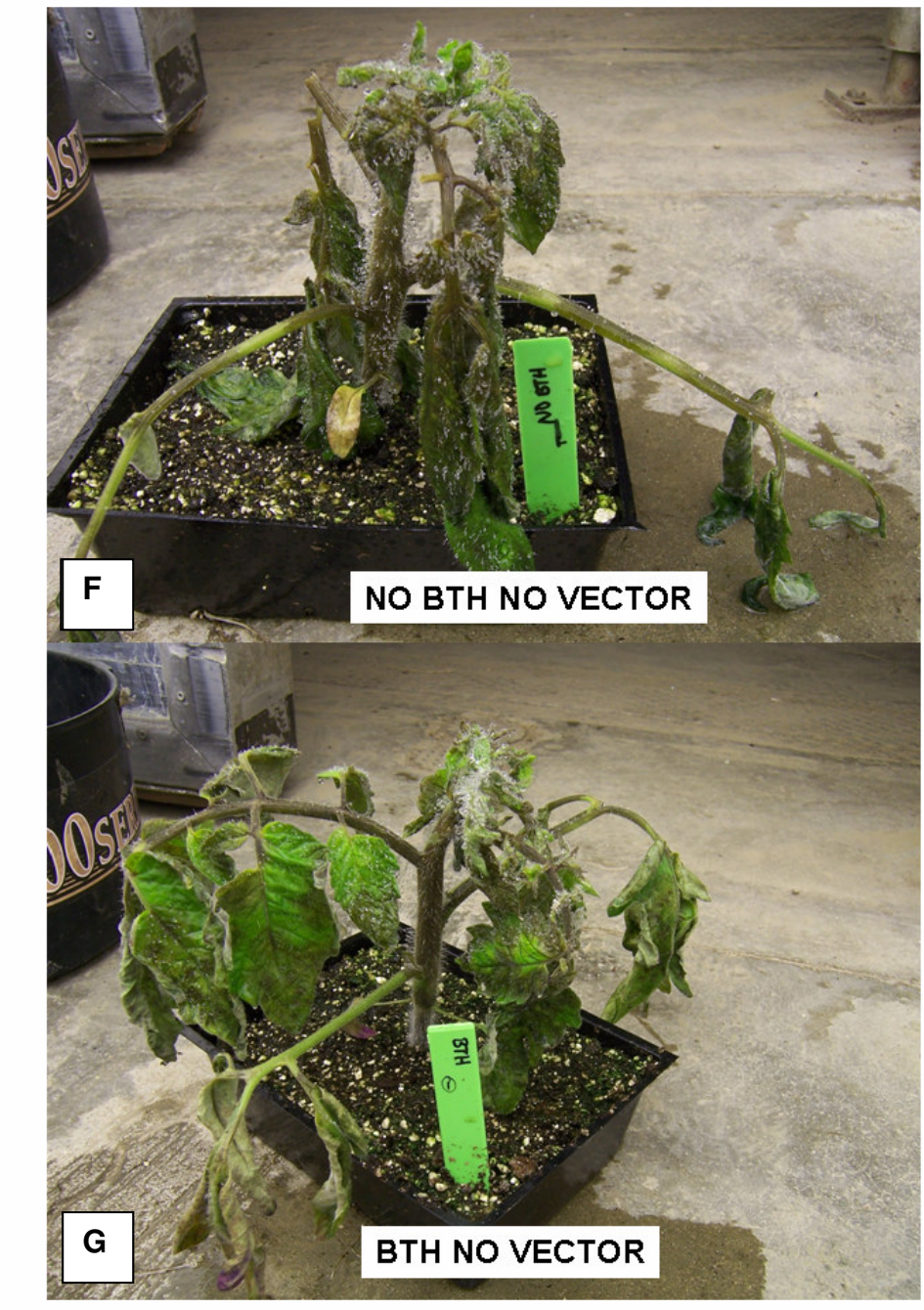


Figure 4 (continued)



CHAPTER 4
DISCUSSION

In this study I used microarrays to assess the response of three plant species of the Solanaceae family, potato, petunia and tomato to BTH, a chemical inducer of the SA defense response pathway.

I was not able to detect any differentially expressed genes after BTH treatment for potato or petunia, but BTH induced up-regulation of 13 genes in tomato. The absence of differentially expressed genes in potato is not surprising because our greenhouse and field experiments showed no phenotypic difference in disease susceptibility between BTH treatment and the controls. Also, it has been reported that BTH does not induce resistance in potatoes against *P. infestans* (Si-Ammour et al 2003).

In contrast, I had expected to detect differentially-expressed genes in petunia, because this plant responds noticeably to BTH. Several factors could explain my inability to detect petunia genes responding to BTH. It seems likely that similarity of petunia genes with tomato genes might be too low to allow hybridization on the tomato microarray. Additionally, microarrays are closed architecture systems, limited by the availability of existing EST collections present on the slide. Specific genes from the petunia genome involved in the resistant phenotype against *P. infestans* observed after the BTH application might be absent from the tomato array.

An alternative approach to detecting petunia genes could be the use of an open architecture system such as AFLPs or differential display, which would allow us to find those rare and specific genes from one species which might be the ones involved in the resistance response.

I found thirteen genes to be up-regulated in response to BTH treatment in tomato.

Some of these genes have been previously identified in the plant response against biotic and abiotic stress. Glutathione S- transferase was up regulated after BTH treatment. This gene has been implicated in the protection of cells against oxidative damage (Appel and Hirt, 2004) as well as in the induction of plant defense genes ((Wingate et al 1988). A gene with homology to the high mobility group protein in potato, which is involved in the regulation of transcription, was also up regulated. Calmodulin, a major transducer of calcium signals was induced. Also, cyclophilin which is an enzyme involved in protein folding was induced. Two proteins involved in transport activities were up regulated; these were Chloroplast phosphate transporter involved in inorganic phosphate transporter activity and a putative alpha-coat protein which has been proposed to play a role in different steps of intracellular transport, forming a coat around vesicles budding from the Golgi.

Three genes corresponded to PR genes, chitinase III, a lipid transfer protein (LTP) and PR1-a. Chitinase expression is a plant defense strategy against fungi, nematodes and herbivorous insects (Brunner et al 1998). LTPs are peptides with antimicrobial activity and have been proposed to be involved in systemic resistance signaling in *Arabidopsis* (Broekaert et al 1997; Maldonado

et al 2002). The PR1-a gene is a marker of the pathogen induced systemic acquired resistance (SAR) in plants.

I decided to investigate further the role of three genes (cysteine protease, PR1-a and chitinase) in the defense response of BTH treated tomato. I selected these genes based on the fold change of gene expression (more than 1.5) and because they have been previously identified in the plant response against pathogens.

Plants defend themselves from hemibiotrophic pathogens by activating SA and ethylene pathways (Glazebrook 2005). Early in the interaction SA impedes the biotrophic phase inducing the hypersensitive response (HR). The activation of the ethylene pathway later in the interaction reduces the development of the necrotrophic phase of the pathogen. BTH treatment induced the systemic acquired resistance (SAR) in tomato plants. Induction of the SA pathway during the biotrophic phase of *P. infestans* could slow its development, resulting in reduced disease.

Cysteine protease and PR1-a have been previously shown to be involved in the induction of HR in plants (D'Silva et al 1998; Solomon et al 1999). It has been proposed that the hypersensitive response (HR) is involved in the potato defense response to *P. infestans* (Kamoun et al 1999). Cysteine proteases have been shown to be up regulated in Programmed Cell Death (PCD) processes in plants (D'Silva et al 1998; Solomon et al 1999; Heath 2000). During an incompatible interaction between potato and *P. infestans* a cysteine protease was found to be up regulated (Avrova et al 1999). If SAR enhances

expression of such genes and suppresses pathogen growth, silencing these genes should lessen the effect of SAR. Therefore, I hypothesized that tomato plants silenced for these genes will have a reduced SAR (reduced resistance).

However, BTH-treated plants in which cysteine protease was silenced did not show a susceptible phenotype when challenged with *P. infestans*. These plants were as resistant to *P. infestans* as were the BTH-treated plants that were not silenced. There are several potential explanations. The first is that cysteine protease might be a member of a gene family. For example, the cysteine protease cathepsin L family in Arabidopsis has 21 members with an identity that ranges from 64% to 43% (Tatusov et al 1997; Tatusov et al 2003). Therefore, it might be possible that the tomato cysteine protease that I silenced belongs to a gene family in tomato with members having a redundant function. Even though VIGS has been successfully used sometimes to silence several members of a gene family, (Burch-Smith et al 2004), that is not always the case, and the vector I constructed might have been specific to one or a few members of a gene family.

A second hypothesis is that the BTH-induced cell death associated genes might belong to a defense pathway that is not effective against *P. infestans.* This is a reasonable hypothesis because Avrova et al (2004) found evidence for two independent defense pathways that allow differentiation between field resistance and R gene mediated resistance in a potato –*P. infestans* interaction.

I was also interested in PR1-a because it has been implicated in the tolerance of tomato and tobacco plants against oomycetes including *P. infestans* (Alexander et al 1993; Niderman et al 1995; van Loon et al 2006). I found that plants silenced for PR1-a and then treated with BTH, were as resistant as wild-type tomato plants treated with BTH. (Both were more resistant to P. infestans than were tomatoes not treated with BTH.) This observation supports previous conclusions that partial resistance to this pathogen in tomato is independent of SA, JA and ethylene pathways (Smart et al 2003). Interestingly, Edgar et al (2006) demonstrated that *Arabidopsis* plants treated with SA showed enhanced resistance to *Fusarium oxysporum*, but there was no induction of the PR1 gene after inoculation with *F. oxysporum.* The authors hypothesized that the pathogen could be involved in the suppression of PR1 expression. An alternative explanation is that although PR1-a might be involved in the defense response, the effect of other genes activated during SAR are also important in conferring a resistant phenotype and these other genes might overcome the effect of the silenced gene. In addition, PR1a is a member of a gene family and other members that were not silenced by our VIGS vector might have been over expressed by BTH conferring resistance against this pathogen.

Finally, I was interested in investigating further the role of chitinase in the *P. infestans* –tomato interaction. Even though oomycete cell walls lack chitin, there is a commercial product, Electra 4 with Chitosan as its active ingredient which claims that it induces defense responses in tomato against *P. infestans*. Also, chitinase was found to be induced in potato leaves that have horizontal resistance when challenged with *P. infestans* (Tian et al 2006). Because of these previous findings, I was interested in testing chitin's role as possible

marker for the defense response of tomato against *P. infestans*. However, I found that plants silenced for chitinase III and then treated with BTH, were as resistant as wild-type tomato plants treated with BTH. (Both were more resistant to *P. infestans* than were tomatoes not treated with BTH.)

Because partial resistance to *P. infestans* in tomato is quantitative rather than qualitative (Smart et al in press) it might be possible that the enhanced resistance phenotype observed after BTH treatment was due to the additive effect of multiple genes with relatively small effects on disease resistance acting together. It may be that each gene contributes a small effect that was not detectable in our assays. More accurate tests of this hypothesis await experiments that can detect differences of very small effect.

CHAPTER 5

REFERENCES

Alba R., Fei Z., Payton P., Liu Y., Moore S.L., Debbie P., Cohn J., D'Ascenzo M., Gordon J.S., Rose J.K., Martin G. Tanskley S.D., Bouzayen M., Jahn M.M and Giovannoni J. (2004). ESTs, cDNA microarrays, and gene expression profiling: tools for dissecting plant physiology and development. The Plant Journal: 39, 697-714.

Alexander D., Goodman RM., Gutrella M., Glascock C., Weymann K, Friedrich L., Maddox D., Ahlgoy P., Luntz T., Ward E., Ryals J. (1993). Increased Tolerance to 2 Oomycete Pathogens in Transgenic Tobacco Expressing Pathogenesis-Related Protein-1A. PNAS 90 (15): 7327-7331.

Anand A., Zhou T., Trick HN., Gill BS., Bockus WW., Muthukrishnan S. (2003). Greenhouse and field testing of transgenic wheat plants stably expressing genes for thaumatin-like protein, chitinase and glucanase against Fusarium graminearum. Journal of Experimental Botany 54 (384): 1101-1111.

Apel K. and Hirt H. (2004). Reactive oxygen species: Metabolism, oxidative stress, and signal transduction. Annual Review of Plant Biology 55: 373-399

Avrova AO, Taleb N, Rokka VM, Heilbronn J, Campbell E, Hein I, Gilroy EM, Cardle L, Bradshaw JE, Stewart HE, Fakim YJ, Loake G, Birch PRJ. (2004). Potato oxysterol binding protein and cathepsin B are rapidly up-regulated in independent defence pathways that distinguish R gene-mediated and field resistances to *Phytophthora infestans*. Molecular Plant Pathology 5 (1): 45-56.

Avrova AO., Stewart HE., De Jong W., Heilbronn J., Lyon G.D. and Birch PRJ. (1999). A Cysteine Protease Gene is Expressed Early in Resistant Potato Interactions with Phytophthora infestans. MPMI Vol 12, No 12: 1114-1119.

Becktell, M.C., Daughtrey, M.L., and Fry, W.E. (2002). Petunia late blight, an emerging disease? Focus on Floriculture, Cornell University. Vol.1, No 2.

Bokshi A. I., Morris S. C. and Deverall B. J. (2003). Effects of benzothiadiazole and acetylsalicylic acid on-1,3-glucanase activity and disease resistance in potato. Plant Pathology 52: 22–27.

Broekaert WF., Cammue BPA., DeBolle MFC., Thevissen K., DeSamblanx GW., Osborn RW. (1997). Antimicrobial peptides from plants. Critical Reviews in Plant Sciences 16 (3): 297-323.

Brunner F., Stintzi A., Fritig B., Legrand M. (1998). Substrate specificities of tobacco chitinases. Plant Journal 14 (2): 225-234.

Burch-Smith TM, Anderson JC, Martin GB, Dinesh-Kumar SP. (2004). Applications and advantages of virus-induced gene silencing for gene function studies in plants. Plant Journal 39 (5): 734-746.

D'Silva I., Poirier GG., Heath MC. (1998). Activation of cysteine proteases in cowpea plants during the hypersensitive response - A form of programmed cell death. Experimental Cell Research 245 (2): 389-399.

Delaney, T.P., Uknes, S., Vernooij, B., Friedrich, L., Weymann, K., Negretto, D., Gaffney, T., Gut-Rella, M., Kessmann, H., and Ward, E. (1994). A central role of salicylic acid in plant disease resistance. Science 266: 1247–1249.

Durner, J. and Klessig, D.F. (1995). Inhibition of ascorbate peroxidase by salicylic acid and 2,6-dichloroisonicotinic acid, two inducers of plant defense responses. Proc Natl Acad Sci USA 92: 11312–11316.

Edgar, C. I., McGrath, K. C., Dombrecht, B., Manners, J. M., Maclean, D. C., Schenk, P. M., Kazan, K. (2006). Salicylic acid mediates resistance to the vascular wilt pathogen Fusarium oxysporum in the model host Arabidopsis thaliana. Australasian Plant Pathology 35 (6): 581-591.

Farmer, E.E., Weber, H., and Vollenweider, S. (1998). Fatty acid signaling in Arabidopsis. Planta 206: 167–174.

Friedrich L., Lawton K., Ruess W., Masner P., Specker N., Rella MG, Meier B., Dincher S., Staub T., Uknes S., Métraux JP., Helmut Kessmann, John Ryals. (1996). A benzothiadiazole derivative induces systemic acquired resistance in tobacco. The Plant Journal 10 (1), 61–70.

Fry, W.E. and Goodwin, S.B. (1997). Re-emergence of potato and tomato blight in the United States. Plant Dis. 81, 1349-1357.

Gallegly, M. E., and Marvel, M. E. (1955). Inheritance of resistance to tomato race 0 of *Phytophthora infestans*. Phytopathology 45:103-109.

Glazebrook. (2005). Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. Annual Review of Phytopathology 43: 205-227

Gu YQ., Yang C., Thara VK., Zhou J., Martin GB. (2002). The Pti4 gene is regulated by the ethylene and salicylic acid and its product is phosphorylated by the Pto kinase. Plant Cell 12: 771-785.

Heath MC. (2000). Hypersensitive response-related death. Plant Molecular Biology 44 (3): 321-334.

Heitz T., Segond S., Kauffmann S., Geoffroy P., Prasad V., Brunner F., Fritig B., Legrand M. (1994). Molecular Characterization of a Novel Tobacco Pathogenesis-Related (PR) Protein- A New Plant Chitinase/Lysozyme. Molecular and General Genetics 245 (2): 246-254

Heitz T., Geoffroy P., Stintzi A., Fritig B., Legrand M. (1993). cDNA Cloning and Gene-Expression Analysis of the Microbial Protienase-Inhibitor of Tobacco. Journal of Biological Chemistry 268 (23): 16987-16992

Judelson, H.S. (1997). The genetics of Phytophthora infestans: modern approaches to a historical challenge. Fungal Genet. Biol. 22, 65-76.

Kamoun S., Huitema E., Vleeshouwers VGAA. (1999). Resistance to oomycetes: a general role for the hypersensitive response? Trends in Plant Science 4 (5): 196-200.

Kerr K.M. and Churchill G.A. (2001). Experimental design for gene expression microarrays. Biostatistics, **2**, 2 183-201.

Kuc, J. (1982) Induced immunity to plant diseases. BioScience 32: 854–860

Kumagi, M.H., Donson, J., Della-Cioppa, G., Harvey, D., Hanley, K. and Grill, L.K. (1995) Cytoplamic inhibition of carotenoid biosynthesis with viru-derived RNA. Proc. Natl Acad. Sci. 92, 1679-1683.

Kumar, D., and Klessig, D.F. (2003). High-affinity salicylic acid-binding protein 2 is required for plant innate immunity and has salicylic acid-stimulated lipase activity. Proc Natl Acad Sci USA vol. 100, no.26, 16101-16106.

Lawton KA, Friedrich L., Hunt M., Weymann K., Delaney T., Kessmann H., Staub T., Ryals J. (1996). Benzothiadiazole induces disease resistance in *Arabidopsis* by activation of the systemic acquired resistance signal transduction pathway. The Plant Journal 10 (1), 71–82.

Liu YL., Schiff M., Dinesh-Kumar SP. (2002). Virus-induced gene silencing in tomato. Plant Journal 31 (6): 777-786.

MacFarlane. (1999). Molecular biology of the tobraviruses. Journal of General Virology 80: 2799-2807.

Maldonado AM., Doerner P., Dixon RA., Lamb CJ., Cameron R. (2002). A putative lipid transfer protein involved in systemic resistance signalling in Arabidopsis. Nature 419 (6905): 399-403.

Martin, G.B. (1999). Functional analysis of plant disease resistance genes and their downstream effectors. Current Opinion Plant Biology 2:273-279.

Métraux, J.P., Ahl-Goy, P., Staub, T., Speich, J., Steinemann, A., Ryals, J. and Ward, E. (1991). Induced systemic resistance in cucumber in response to 2,6-dichloro-isonicotinic acid and pathogens. In: Hennecke H and Verma DPS (eds) Advances in Molecular Genetics of Plant–Microbe Interactions Vol 1 (pp 432–439).

Métraux, J.P., Nawrath, C. and Genoud, T. (2002). Systemic acquired resistance. Euphytica 124: 237–243.

Mölders, W., Buchala, A., and Métraux, J.P. (1996). Transport of salicylic acid in tobacco necrosis virus-infected cucumber plants. Plant Physiol 112: 787–792.

Moreau P., Thoquet P., Olivier J., Laterrot H., Grimsley N. (1998). Genetic mapping of Ph-2, a single locus controlling partial resistance to Phytophthora infestans in tomato. MPMI 11 (4): 259-269.

Niderman T., Genetet I., Bruyere T., Gees R., Stintzi A., Legrand M., Fritig B., Mosinger E. (1995). Pathogenesis-Related PR-1 Proteins are Antifungal – Isolation and Characterization 3 14-Kilodalton Proteins of Tomato and of a Basic PR-1 of Tpbacco with Inhibitory Activity against *Phytophthora infestans*. Plant Physiology 108 (1): 17-27

Oostendorp, M., Kunz, W., Dietrich, B., and Staub, T. (2001). Induced disease resistance in plants by chemicals. European Journal of Plant Pathology 107: 19–28.

Rasmussen, J.B., Hammerschmidt, R., and Zook, M.N. (1991). Systemic induction of salicylic acid accumulation in cucumber after inoculation with *Pseudomonas syringae* pv *syringae*. Plant Physiol 97:1342–1347.

Rathbone, M.C., Smart, C.D. and Fry, W.E. (2001). Isolates of *Phytophthora infestans* that infect *Petunia* x *hybrida* and *Nicotiana benthamiana* also produce INF1. Phytopathology 92(6):S145.

Saeed Al., Sharov V., White J., Li J., Liang W., Bhagabati N., Braisted J., Klapa M., Currier T., Thiagarajan M., Sturn A., Snuffin M., Rezantsev A., Popov D., Ryltsov A., Kostukovich E., Borisovsky I., Liu Z., Vinsavich A., Trush V., Quackenbush J. (2003). TM4: a free, open-source system for microarray data management and analysis. Biotechniques 34 (2): 374-374.

Schaffrath U., Freydl E., Dudler R. (1997). Evidence for different signaling pathways activated by inducers of acquired resistance in wheat. MPMI 10 (6): 779-783.

Seskar, M., Shulaev, V., and Raskin, I. (1998). Endogenous methyl salicylate in pathogen-inoculated tobacco plants. Plant Physiol 116: 387–392.

Shulaev, V., Leon, J., and Raskin, I. (1995). Is salicylic acid a translocated signal of systemic acquired resistance in tobacco? Plant Cell 7: 1691–1701.

Shulaev, V., Silverman, P., and Raskin, I. (1997). Airborne signaling by methyl salicylate in plant pathogen resistance. Nature 385: 718–721.

Si-Ammour, A., Mauch-Mani B. and Mauch F. (2003). Quantification of induced resistance against Phytophthora species expressing GFP as a vital marker: β-aminobutyric acid but not BTH protects potato and Arabidopsis from infection. Molecular Plant Pathology (4) , 237–248.

Smart, C.D., Myers, K.L., Restrepo, S., Martin, G.B., and Fry, W.E. (2003). Partial Resistance of Tomato to *Phytophthora infestans* Is Not Dependent upon Ethylene, Jasmonic Acid, or Salicylic Acid Signaling Pathways. MPMI Vol. 16, No. 2, 2003, pp. 141–148.

Solomon M., Belenghi B., Delledonne M., Menachem E., Levine A. (1999). The involvement of cysteine proteases and protease inhibitor genes in the regulation of programmed cell death in plants. Plant Cell 11 (3): 431-443.

Tally, A., Oostendorp, M., Lawton, K., Staub, T., and Bassi, B. (1999). Commercial development of elicitors of induced resistance to pathogens. In: Agrawal AA, Tuzun S and Bent E (eds) Induced Plant Defenses against Pathogens and Herbivores (pp 357–369). APS Press, St. Paul.

Tatusov RL, Fedorova ND, Jackson JD, Jacobs AR, Kiryutin B, Koonin EV, Krylov DM, Mazumder R, Mekhedov SL, Nikolskaya AN, Rao BS, Smirnov S, Sverdlov AV, Vasudevan S, Wolf YI, Yin JJ, Natale DA. (2003). The COG database: an updated version includes eukaryotes. BMC Bioinformatics 4: Art. No. 41.

Tatusov RL., Koonin EV., Lipman DJ. (1997). A genomic perspective on protein families. Science 278 (5338): 631-637.

Tian, Z. D., Liu, J., Wang, B. L., Xie, C. H . (2006). Screening and expression analysis of Phytophthora infestans induced genes in potato leaves with horizontal resistance. Plant Cell Reports 25 (10): 1094-1103.

Turkensteen, L. J. 1973. Partial resistance of tomatoes against *Phtophthora infestans*, the late blight fungus. Pages 1-88 in: Mededeling no. 633. Instituut Voor Plantenziektenkundig Onderzoek, Wageningen, The Netherlands.

Uknes S., Winter AM., Delaney T., Vernooij B., Morse A., Friedrich L., Nye G., Potter S., Ward E., Ryals J. (1993). Biological Induction of Systemic Acquired-Resistance in Arabidopsis. MPMI 6 (6): 692-698.

Uknes S., Mauchmani B., Moyer M., Potter S., Williams S., Dincher S., Chandler D., Slusarenko A., Ward E., Ryals J. (1992). Acquired-Resitance in Arabidopsis. Plant Cell 4 (6): 645-656.

van Loon, L. C., Rep, M., Pieterse, C. M. J. (2006). Significance of inducible defense-related proteins in infected plants. Ann. Rev. of Phytopathology 44: 135-162.

van Loon L.C. and van Strien E.A. (1999). The families of pathogenesis-related proteins, their activities, and comparative analysis of PR-1 type proteins. Physiological and Molecular Plant Pathology 55, 85±97

Vega-Sanchez, M. E., Erselius, L. J., Rodriguez, A. M., Bastidas, O., Hohl, H. R., Ojiambo, P. S., Mukalazi, J., Vermeulen, T., Fry, W. E., and Forbes, G. A. (2000). Host adaptation to potato and tomato within the US-1 clonal lineage of *Phytophthora infestans* in Uganda and Kenya. Plant Pathol. 49:531-539.

Vernooij B., Friedrich L., Goy PA., Staub T., Kessmann H., Ryals J. (1995). 2,6-Dichloroisonicotinic Acid-Induced Resistance to Pathogens Without the Accumulation of Salicylic-Acid. MPMI 8 (2): 228-234.

Vernooij, B., Friedrich, L., Morse, A., Reist, R., Kolditz Jawhar, R., Ward, E., Uknes, S., Kessmann, H., and Ryals, J. (1994). Salicylic acid is not the

translocated signal responsible for inducing systemic acquired resistance but is required in signal transduction. Plant Cell 6: 959–965.

Vleeshouwers A.A.V., van Dooijeweert W. Govers F. Kamoun S. and Colon L.T. (2000). The hypersensitive response is associated with host and nonhost resistance to *Phytophthora infestans*. Planta 210: 853-864.

Ward ER., Uknes SJ., Williams SC., Dincher SS., Wiederhold DL., Alexander DC., Ahlgoy P., Metraux JP., Ryals JA. (1991). Coordinate Gene Activity in Response to Agents that Induce Systemic Acquired-Resistance. Plant Cell 3 (10): 1085-1094.

Wingate VPM, Lawton MA., Lamb CJ. (1988). Glutathione Causes a Massive and Selective Induction of Plant Defense Genes. Plant Physiology 87 (1): 206-210.

Yang, Y., Shah, J., and Klessig, D.F. (1997). Signal perception and transduction in plant defense responses. Genes Dev. 11, 1621-1639.

Zhang, Y., Tessaro, M.J., Lassner, M., and Li, X. (2003). Knockout Analysis of Arabidopsis Transcription Factors *TGA2*,*TGA5*, and *TGA6* Reveals Their Redundant and Essential Roles in Systemic Acquired Resistance. The Plant Cell, Vol. 15, 2647–2653.

Zhu Q., Maher EA., Masoud S., Dixon RA., and Lamb CJ. (1994). Enhanced Protection Against Fungal Attack by Consitutive Coexpression of Chitinase and Glucanase Genes in Transgenic Tobacco. BIO-TECHNOLOGY 12 (8): 807-812.