UNDERSTANDING THE INTERACTIONS BETWEEN PLANTS AND PATHOGENS USING INFECTION OF TOMATO (SOLANUM LYCOPERSICUM) BY PHYTOPHTHORA INFESTANS AS A MODEL PATHOSYSTEM

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Andrea Paola Zuluaga

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UNDERSTANDING THE INTERACTIONS BETWEEN PLANTS AND PATHOGENS USING INFECTION OF TOMATO (SOLANUM LYCOPERSICUM) BY PHYTOPHTHORA INFESTANS AS A MODEL PATHOSYSTEM

Andrea Paola Zuluaga Ph. D.

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The oomycete *Phytophthora infestans* causes devastating epidemics for both tomato (*Solanum lycopersicum*) and potato (*Solanum tuberosum*) worldwide (Fry 2008). There is a limited understanding of the mechanisms by which *P. infestans* overcomes plant defenses and how it regulates its pathogenicity factors; behaving as a biotroph during early stages of infection and switching to a necrotroph at later stages. To address these limitations, 454 sequencing was used to learn about the transcriptome of *P. infestans* (US-11 clonal lineage) in a compatible interaction with its host tomato (*Solanum lycopersicum* cv. M82), at three infection stages: biotrophic, the transition from biotrophy to necrotrophy and necrotrophic phase. This approach identified more than 550,000 high quality sequence reads, of which about 10% were derived from *P. infestans.* The tomato and *P. infestans* transcriptomes provided a comprehensive overview of the molecular interaction between plants and pathogens. On the host side, nearly 12,000 genes showed differential expression during the three infection stages analyzed. These genes corresponded to nearly 200 biochemical pathways, revealing a massive reorganization of the plant metabolism. Amongst these, probable components of resistance were up-regulated. For example, more than 100 putative resistance genes and more than 100 putative Pattern Recognition Receptor (PRRs) genes were induced. Transcript abundance of genes encoding

proteins in the SA pathway increased in the biotrophic phase, and subsequently declined. In contrast, transcript abundance of genes in the JA pathway gradually increased as infection progressed.

The expression of nearly 9,000 *P. infestans* genes was detected throughout the interaction. Of these, 800 had not been identified previously in the *P. infestans* genome. Many genes, including effectors, were stage-specific. It was determined that five candidate effector genes (three RXLRs, one CRN and one hypothetical protein), suppressed necrosis caused by the *P. infestans* necrosis inducing protein *PiNPP1.1* suggesting that these effectors might prolongue the biotrophic phase of *P. infestans*.

In addition, a model pathosystem (*Hyaloperonospora arabidopsidis* infection of *Arabidopsis thaliana*) was used to investigate the genetic basis and the mechanisms of action of quantitative resistance. Two putative quantitative resistant loci (QRL) were found, of which one is likely an R gene, but the other may be a quantitative resistance locus. The nature of this QRL awaits investigation.

BIOGRAPHICAL SKETCH

The author was born in Bogotá, Colombia. She graduated from the Universidad de los Andes, in September 1999 with a BS 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. For her undergraduate research, she studied the interaction between cassava (*Manihot esculenta Crantz*) and *Xanthomonas axonopdis pv manihotis*, looking for resistance gene analogs against cassava bacterial blight. After completing her studies, she taught Spanish to children in after school programs in Atlanta, Georgia.

Her passion for teaching and her interest in the interaction between plants and pathogens made her pursue graduate studies in plant pathology. In January 2004 she enrolled in the Department of Plant Pathology and Plant Microbe Biology at Cornell University to conduct her PhD studies under the guidance of Dr. William Fry, where she studied various aspects of *Phytophthora infestans*.

In her spare time, Paola likes to read, hike, ride bikes and swim, and has participated in the women swimmin' annual event in Ithaca, to get funds for terminal ill patients.

A mi mamaina, chandita y ratatouille, por ser mis fuegos

....hay fuegos grandes y fuegos chicos y fuegos de todos colores. Hay gente de fuego sereno que ni se entera del viento, y gente de fuego loco que llena el aire de chispas. Pero otros arden la vida con tantas ganas que no se puede mirarlos sin parpadear, y quien se acerca, se enciende.

De "El libro de los abrazos" Eduardo Galeano

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CHAPTER 1 – The Late blight pathogen *Phytophthora infestans*: literature review

1.1 Introduction

The Irish potato famine in the mid 1800's caused by the oomycete *Phytophthora infestans* permanently shaped Ireland's demographics, politics and culture (Bourke 1993). One million people died and one million emigrated to North America. Even today, after more than 150 years of studying this organism, devastating epidemics for both tomato (*Solanum lycopersicum*) and potato (*Solanum tuberosum*) can be seen worldwide (Fry 2008). During the summers of 2004 and 2009 the weather conditions were very conducive for late blight in the northeast of the USA and there were devastating epidemics of this disease. In this chapter, the pathogen, its life cycle, biology, and genomics of *P. infestans* as well as the plant mechanisms of defense response are introduced, and the stage is set for an investigation of host-pathogen interactions.

1.2 *Phytophthora infestans* biology

Phytophthora infestans (Mont.) de Bary is an oomycete. It was considered a fungus until recently because features such as reproduction by spores, feeding by absorption and filamentous growth are also characteristic of fungi (Money 1998). In the late 1990's, molecular and phylogenetic analyses showed that the oomycetes were unrelated to the true fungi and were more closely related to the brown algae, placing them in the kingdom Stramenopila (Harper 2005). Oomycetes are diploid for the major part of their life cycle; they have coenocytic hyphae (no septa) and their cell walls are mainly composed of cellulose and β–glucans with low levels of chitin (Kamoun 2003).

1.3 Life cycle of *P. infestans*

The life cycle of the late blight pathogen *P. infestans* is depicted in Figure 1.1. *P. infestans* can reproduce both asexually and sexually. Its asexual reproduction is via sporangia that can germinate either directly (germ tube) or indirectly (via zoospore formation). The difference in germination depends on environmental conditions such as temperature and humidity. Sporangia germinate directly via germ tubes at temperatures above 20°C, while at temperatures between 10-15°C the sporangia germinate indirectly forming zoospores. Zoospores are motile due to their two flagella (tinsel and whiplash flagella), which are typical of eukaryotes, with nine pairs of microtubules surrounding one pair of microtubules in the center (Hardham 1987). Sexual reproduction requires the presence of two mating types, labeled A1 and A2, and fertilization leads to the production of oospores, which act as resistant structures in unfavorable environmental conditions and as source of genotypic variation (Erwin and Ribeiro 1996). *P. infestans* is a hemibiotroph pathogen, requiring living cells to feed at the initial stages of the interaction with its host and at later stages of the interaction cause necrosis on the host tissue.

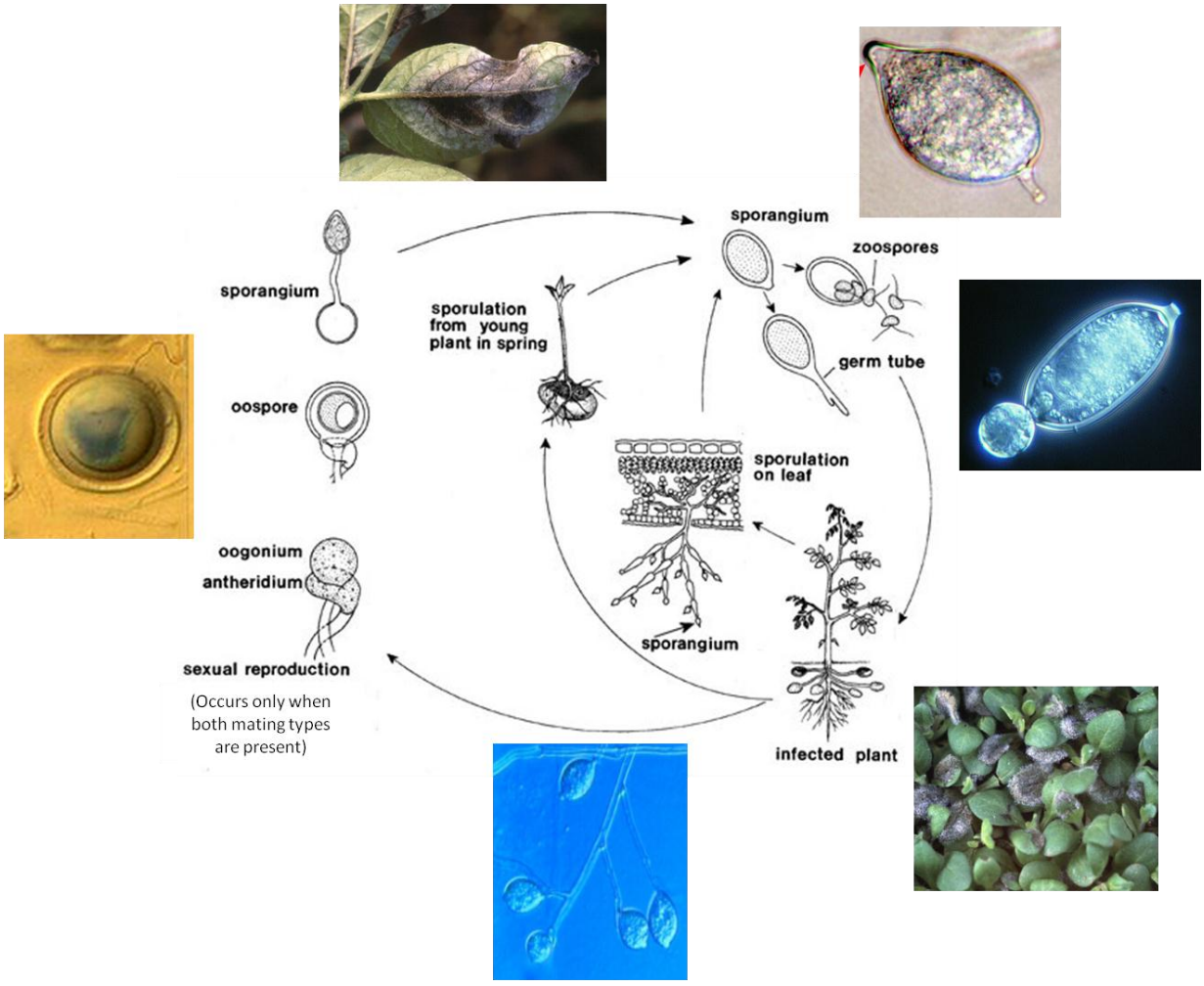

Figure 1.1 Life cycle of *Phytophthora infestans*. Life cycle adapted from Agrios (1997) and pictures were taken from http://www.plantpath.cornell.edu/Fry/lateblight_images.html.

Host-pathogen interactions

Host range and host adaptation

Although *P. infestans* is generally considered to have a narrow host range (potatoes and tomatoes), it is able to cause disease in many members of the *Solanaceae* family. Among the wide range of *Solanaceae* hosts are pear melon (*Solanum muricatum*) (Adler et al 2002), black nightshade (*S. nigrum*), woody nightshade (*S. dulcamara*), *S. sisymbriifolium* (Flier et al 2003), petunia (*Petunia x hybrida*), calibrachoa (*Calibrachoa x hybridus*), *Nicotiana benthamiana* (Becktell et al 2006), non-tuber bearing *S. caripense, S. montanum,* and wild tomato *S. hirsutum, S. peruvianum* (Garry et al 2005) among others.

Host adaptation or specialization has been studied widely, and results can differ, depending on the *P. infestans* population (Vega-Sanchez et al 2000; Lee et al 2002; Garry et al 2005). For example, it has been demonstrated that *N. benthamiana* is a host to common North American isolates (Becktell et al 2006), whereas, against European isolates of *P. infestans N. benthamiana* was regarded as a non-host (Kamoun et al 1998). In another example, Garry et al. (2005) studied isolates from *P. infestans* in Peru and found that there was a strong association of three lineages of *P. infestans* with their original hosts. One strain (EC-1) was isolated from all the hosts sampled and showed, in detached leaflets assays, more aggressiveness in the host where it was originally isolated when compared to the other hosts (Garry et al 2005).

Some clonal lineages are more commonly associated with potato than tomato or vice versa. For instance, the clonal lineage US-8 is associated with potatoes (Legard et al 1995) and is more aggressive on potato than tomato on detached leaflets, while the US-17 (Goodwin et al 1998;

Gavino et al 2000) is associated with tomato in the field. However, other clonal lineages such as US-6 and US-11can be found in both potato and tomato hosts in the field and did not show host specialization when evaluated on detached leaflets (Legard et al 1995). Host adaptation within a clonal lineage has also been reported (Vega-Sanchez et al 2000). Isolates of the US-1 clonal lineage in Uganda and Kenya showed differences of both aggressiveness and host specialization (Vega-Sanchez et al 2000).

In an effort to understand the basis of differences in aggressiveness to tomato in *P. infestans*, Lee et al (2002) analyzed hybrid progeny from five different crosses and concluded that there is a locus determining aggressiveness to tomato and that high aggressiveness is a recessive trait. Despite the importance of host specialization and aggressiveness differences in *P. infestans*, all the studies up to that date had only addressed these differences phenotypically making this the first study to address these factors at the genetic level.

Recent findings in *P. infestans* pathogenicity have broadened our understanding on how this organism influences its host. *P. infestans* secretes proteins and other molecules, termed effectors, which allow it to colonize and reproduce in the host tissue causing plant disease (Haas et al 2009). Pathogen effectors can be separated in two categories depending on whether they are secreted into the host apoplast or cytoplasm (Schneider and Collmer 2010). Apoplastic effectors of *P. infestans* include hydrolytic enzymes and necrotizing toxins that degrade plant tissue (Kanneganti et al 2006; Kamoun et al 1997), as well as enzyme inhibitors against host defenses (Tian et al 2004; Tian et al 2007; Damasceno et al 2008).

Cytoplasmic effectors are proteins with presumably diverse function. Characterization of

cytoplasmic effectors in *P. infestans* remained elusive until the discovery of the conserved RXLR domain in the predicted proteins of previously characterized oomycete effectors (Rehmany et al 2005). RXLR effectors in *Phytophthora* are modular proteins with a signal peptide and a conserved N-terminal domain required for translocation into the host cytoplasm (Whisson et al 2007) and a highly variable, fast-evolving C-terminal domain (Haas et al 2009). A second type of cytoplasmic effector is a group referred to as Crinklers (CRNs), which were first identified in a functional screening in *N. benthamiana* plants and named as CRinkler and Necrosis because of the phenotype they conferred in the plant after agro-infiltration (Torto et al 2003). Analogous to the RXLR effectors, CRNs have a predicted conserved N-terminal region, with a signal peptide and a conserved LXLFLAK domain, followed by a C-terminus region which is not conserved (Haas et al 2009). Similarly, the LXLFLAK domain in CRN effectors is required for translocation into the host cell (Schornack et al 2010).

However, whether translocation of effectors into the host cell is facilitated by the pathogen, the host or both is a current subject of study in oomycete biology. Support for a pathogen facilitated trafficking of effectors inside host cells comes from studies with the oomycete Avr3a protein which was unable to interact with the product of the potato gene R3, when it was secreted into the apoplast by *Pectobacterium atrosepticum* (Whisson et al 2007). In contrast, evidence for a translocation mechanism of oomycete effectors mediated by the host, came from the fact that in the absence of the pathogen, the RXLR and dEER motif sequences are sufficient for translocation into the plant cell (Dou et al 2008). In addition, the finding of the phospholipid phosphatidylinositol-3-phosphate (PI3P) in the outer surface of plants and some animal cell plasma membranes and the demonstration that binding of RXLR-dEER to the PI3P of the host

cell surface leads to the effector entry into the host cell by endocytosis, supports the idea of a host-mediated translocation of oomycete cytoplasmic effectors (Kale et al 2010). Likewise, subcellular localization of the CRN effectors inside the host nuclei is mediated by the host importin-α factor (Schornack et al 2010).

The *P. infestans* genome predicted more than 750 cytoplasmic candidate effectors (Haas et al 2009). A total of 563 were annotated as RXLRs and 196 as CRN effectors (Haas et al 2009); cytoplasmic effectors are often organized in clusters, in gene-sparse genomic regions rich in high copy repeat sequences and transposable elements (Haas et al 2009). The high number and the lack of orthology of these effectors when compared to *P. sojae* and *P. ramorum* genomes (only 16 are orthologous) suggest that this diversity might be driven by a co-evolutionary process with host plants (Haas et al 2009) in which plant defenses select for a pathogen genotype that can overcome such defenses, decreasing the plant fitness; as a counter adaptation, the plant evolves mechanisms to defend itself and reduce pathogen fitness, an evolutionary process called arms-race (Stahl and Bishop 2000).

The molecular targets and function of some of these cytoplasmic effectors have been studied. For instance, the *P. infestans* Avr3a effector, a member of the RXLR family, has two allelic variants (Bos et al 2009). $Avr3a^{KI}$ is recognized by the R3a resistance protein from *Solanum demissum*, triggering host defenses (Armstrong et al 2005). However, the allelic variant $Avr3a^{EM}$, suppresses the hypersensitive cell death (HR) induced by another *P. infestans* protein (Bos et al 2006; Bos et al 2009). Similarly, the ipiO RXLR effector causes an HR on some wild *Solanum* species (Vleeshouwers et al 2008). However, variants of the ipiO can be divided into three

classes (I, II and III) with distinct function (Champouret et al 2009; Halterman et al 2010). Classes I and II are present in the majority of *P. infestans* isolates and are recognized by the RB (or Rpi-blb1) resistance protein from *S. bulbocastanum* causing HR (Champouret et al 2009). In contrast, members of the class III are not recognized by the RB and inhibit HR elicited by members of class I (Halterman et al 2010). In addition, other effectors have been shown to either suppress (Kelley et al 2010; Oh et al 2009) or cause necrosis on the host (Schornack et al 2010; Haas et al 2009; Oh et al 2009). The recent characterization of CRNs showed that these effectors target the host nuclei, suggesting that CRNs might interfere in host nuclear processes (Schornack et al 2010).

The host response

There are three possible outcomes during the interaction between *P. infestans* and its hosts tomato and potato: compatible, partially compatible and incompatible interactions.

Compatible interactions: basal defense or PAMP Triggered Immunity

Basal defense is an active defense mechanism of plants triggered by virulent pathogens in susceptible hosts (Jones and Dangl 2006). It is also known as PAMP Triggered Immunity (PTI) (Jones and Dangl 2006) because it recognizes highly conserved microbial or pathogen associated molecular patterns (MAMPS or PAMPS respectively). Recognition of PAMPs is mediated by plant transmembrane domains called pattern recognition receptors (PRRs), which are leucine rich repeat (LRR)-receptor kinases (RK) (Jones and Dangl 2006). The two major PAMP receptors studied up to date are the kinase Flagellin sensing (FLS2) that recognizes bacteria flagellin epitope flg22 (Gomez-Gomez and Boller 2000) and the elongation factor receptor (EFR) which

binds the bacteria elongation factor Tu (Kunze et al 2004) from *Arabidopsis thaliana* (Segonzac 2011). Additionally, two protein kinases, the brassinostroid insensitive associated kinase (BAK1) and the *Botrytis* induced kinase (BIK1) are positive regulators of FLS2 and EFR (Segonzac et al 2011). After pathogen perception, PTI leads to the activation of ion fluxes including calcium (Ma and Berkowitz, 2007) which is perceived by calcium binding proteins including calcium-dependent protein kinases (Reddy and Reddy 2004), that have a role in the control of reactive oxygen species (ROS) and salicylic acid (SA) (Nicaise et al 2009). Two distinct signaling branches act downstream of calcium influx; one leads to the production of ROS and the reinforcement of plant cell walls and the other one to the activation of mitogen-activated protein kinases (MAPK) (Segonzac et al 2011) which induce WRKY-transcription factors, that are key regulators of plant defenses (Pandey and Somssich 2009).

Oomycetes PAMPs identified to date include Pep-13, a transglutaminase (Brunner et al 2002), involved in cell differentiation and tissue regeneration (Langston et al 2007), a cellulose binding domain CBEL (Gaulin et al 2006), INF1 a member of the elicitin family (Bonnet et al 1996) and the necrosis like protein –NLP family (Qutob et al 2006; Hein et al 2009). PRRs for some of these PAMPS have been identified as well. The lectin-like receptor kinase from *Nicotiana benthamiana* (NbLRK1) perceives INF1 (Kanzaki et al 2008) while in soybean, the β-glucan-binding protein (GBP), binds to a heptaglucoside from *P. sojae* eliciting PTI signaling defenses (Fliegmann et al 2004). Despite the characterization of many PRRs, current knowledge of PTI in the Solanacea family comes primarily from the model plant *N. benthamiana*, and there is scarce information on the tomato and potato response after PAMPs perception.

Incompatible interactions: effector triggered immunity

A second branch of plant defense response is intracellular, and is mediated by proteins with nucleotide binding sites (NBS) and LRR encoded by resistance (R) genes, which recognize pathogen effectors and was originally described as a gene for gene interaction (Flor 1971) and was subsequently termed effector triggered immunity (ETI) (Jones and Dangl 2006). Despite the ability of NBS-LRR proteins to recognize a wide array of pathogen effectors, the defense response is highly conserved regardless of the pathogen involved (Collier et al 2011). After recognition of pathogen effector(s) a localized programmed cell death, known as hypersensitive response (HR) is triggered by the plant. Following the HR, accumulation of salicylic acid (SA) stimulates a systemic acquired resistance (SAR) – a situation in which uninfected parts of the plant develop resistance to further infection by some pathogens (Martin 1999; Yang et al., 1997).

Because of the high economic impact of late blight on tomato and potato agriculture, resistance against *P. infestans* is a highly desired trait in breeding programs. The use of R genes has become a priority in breeding programs and three R genes found in the wild species *Solanum pimpinellifolium* (*Ph1*, *Ph2* and *Ph3*) have been incorporated into tomato (Chunwongse et al 2002). The potato breeding programs have identified at least 30 R genes from different wild species of *Solanum* (Hein et al 2009). However, most of these genes have been "overcome" by *P. infestans* even after several were deployed in a single cultivar (Tan et al 2010). Thus, the use of R genes has been difficult because of the high adaptability of *P. infestans* that can quickly overcome the effect of R genes. Sequencing of *P. infestans* has revealed that the likely mechanism by which *P. infestans* develop to avoid the effects of R genes is that the effectors seem to be in a rapid process of evolution (Haas et al 2009). Thus, improving our understanding

of both the host and the pathogen interaction will allow us to develop better strategies for disease control.

Partially compatible interactions (quantitative resistance)

Another type of resistance is quantitative (quantitative resistance loci --QRL) where several genes (with a small effect each) contribute to plant defense. QRL is thought to be more durable than qualitative resistance, because it will be less likely for the pathogen population to evolve resistance against several traits of small effect each (Poland et al 2009). For instance, QRLs conferring resistance to late blight have been identified for both potato (Gebhardt and Valkonen 2001; Simko 2002; Stewart et al 2003; Rauscher et al 2010; Mayton et al 2011 among others) and tomato (Frary et al 1998; Brouwer et al 2004; Smart et al 2007). These studies have broadened our understanding of the molecular components of QRL against late blight, demonstrating that some QRL co-localize with previously identified defense related genes in the phenylpropanoid pathway, WRKY regulatory genes and osmotin, among others (Trognitz et al 2002). In addition, "defeated" R genes, which are R genes that have been overcome by the pathogen, may contribute to resistance as well (Stewart et al 2003; Rauscher et al 2010). Because the gene components for some of these QRLs in potato and tomato are still unknown (Smart et al 2007), the use of model pathosystems such as *A. thaliana* and *Hyaloperonospora arabidopsidis* might be helpful in gaining an insight into the host response and to understand the underlying mechanisms of QRLs.

1.4 The scope of this dissertation

The second and third chapters of this dissertation focus on gene expression using 454 sequencing and reveal that thousands of genes are induced in both host and pathogen during this interaction. This data set provides insight on the PTI and ETI induced in tomato after *P. infestans* infection, and creates the basis for many hypotheses concerning factors controlling the interaction. Because I am particularly interested in factors that control the transition from biotrophy to necrotrophy (Figure 1.2), I tested some hypotheses on the pathogen side (chapter 2) and on the host side (chapter 3).

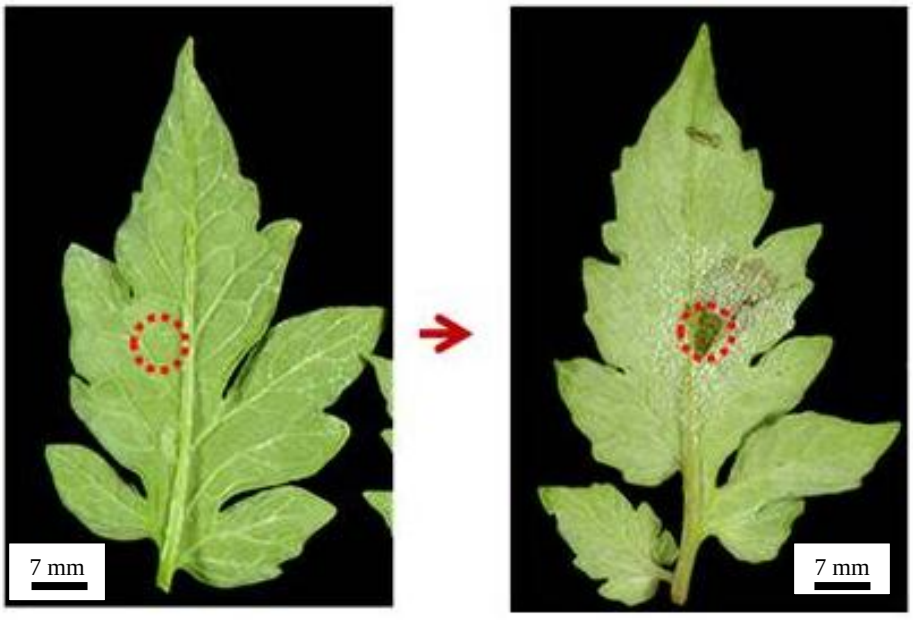

Figure 1.2 Left panel shows the biotrophic growth of a *P. infestans* tomato specialized isolate at early stages of infection and the right panel shows the necrotrophic growth at later stages of the interaction.

The fourth chapter describes some next steps in the continuing quest to understand the interaction between this pathogen and its tomato host.

The appendix addresses the interaction between two model organisms: the oomycete *H. arabidopsidis* and its natural host *A. thaliana*. The objective of this work was to identify quantitative resistance against this oomycete and try to elucidate the genetic mechanism of such resistance. We found two QRL that might explain the resistance in *Arabidopsis* against the tested strain of *H. arabidopsidis*. One of the QRL (LOD 3) is located on chromosome III and we provide some candidate genes that might be involved in resistance. The other QRL (LOD 17) identified in this study is located on chromosome V. This region has a cluster of R genes which might indicate that the resistance is due to an R gene rather than various genes contributing to resistance. This is in agreement with the high LOD value obtained for this QRL. Fine mapping will be the next step to reveal the components of resistance in both QRL.

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CHAPTER 2 – Analysis of transcriptional dynamics of *Phytophthora infestans*, during sequential stages of hemibiotrophic infection of tomato

A.P. Zuluaga*, J. C. Vega-Arreguín, Z. Fei, L. Ponnala, W.E. Fry and J.K.C. Rose

Abstract

There is little understanding of the mechanisms by which the oomycete *P. infestans* overcomes plant defenses and the factors that influence the biphasic nature of infection whereby it initially behaves as a biotroph and subsequently as a necrotroph. In order to understand better how this pathogen regulates its pathogenicity factors during host colonization and to learn how this pathogen switches from biotrophic to necrotrophic, we analyzed the transcriptome of *P. infestans* (US-11 clonal lineage) in a compatible interaction with its host tomato (*Solanum lycopersicum* cv. M82), at three infection stages: biotrophic, the transition from biotrophy to necrotrophy and necrotrophic phase using 454 sequencing. More than 550,000 sequence reads were identified, of which approximately 10% were from *P. infestans.* The expression patterns of putative pathogenicity effectors suggested a tight regulation over time. A total of 31 RXLR and 50 crinkler (CRN) putative effectors were expressed during the interaction. RXLRs reached the highest expression level at 96 hours after inoculation (hai), the time point at which the expression level for CRNs was the lowest. At later stages of infection, apoplastic effectors which presumably cause necrosis (i.e. including glycosyl hydrolases and *PiNPP*) were highly expressed. Six of the putative cytoplasmic effectors and seven secreted hypothetical proteins were evaluated to determine whether they were able to suppress necrosis caused by the *P. infestans* necrotic inducing protein (*PiNPP1.1*), or cause necrosis, using agro-infiltrations in *Nicotiana benthamiana*. Of these, five suppressed necrosis, suggesting that they might function to prolong the biotrophic phase. None of the RXLR, CRN and hypothetical candidate effectors that were studied caused necrosis. The current study suggests that effectors may modulate the

outcome of the interaction with tomato by prolonging the biotrophic phase by suppressing necrosis and at later stages of infection the apoplastic effectors induce necrosis.

* A.P. Zuluaga contribution: designed and performed experiments, analyzed the data and wrote the manuscript.

2.1 Introduction

Plant pathogens can be classified by their infection strategy into biotrophs, hemibiotrophs or necrotrophs. Biotrophic pathogens (e.g. *Hyaloperonospora arabidopsidis*) feed on living cells while necrotrophs (e.g. *Botrytis cinerea*) kill host tissue and feed on dead cells (Glazebrook 2005). Hemibiotrophic pathogens, such as the oomycete *Phytophthora infestans*, show an initial asymptomatic biotrophic phase of infection followed by a necrotrophic phase. During the biotrophic phase, *P. infestans* sequentially forms appressoria, primary and secondary hyphae and haustoria (Grenville-Briggs et al 2005). Haustoria are specialized feeding structures (Dou et al 2008), through which pathogens are thought to deliver proteins and small molecules called effectors, into the plant cell enabling pathogens to manipulate host metabolism and suppress host defenses (Hahn and Mendgen 2001; Abramovitch and Martin 2004; Axtell and Staskawicz 2003; Mackey et al 2003; Restrepo et al 2005; Tian et al 2004 among others). The subsequent necrotrophic phase is characterized by hyphal ramification and water soaking, followed by necrosis of the tissue (Grenville-Briggs et al 2005).

To date, little is known about how each pathogenicity stage is regulated, or the molecular mechanisms that trigger the transition from biotrophy to necrotrophy (Lee and Rose 2010). Characterization of some effectors supports the idea that *Phytophthora* actively induces or

suppresses cell death in plants by a temporal regulated mechanism, where effectors that are secreted at an early stage promote biotrophy (Kelley et al 2010; Lee and Rose 2010) while those that are secreted later induce necrosis (Lee and Rose 2010). Characterizaed examples of such secreted effectors include INF1, which causes a hypersensitive response (HR) cell death in *Nicotiana benthamiana*, and is highly expressed at later stages of the interaction of *P. infestans* with potato (Kamoun et al 1997); likewise, the necrosis inducing protein Nep-1 PiNPP1.1 (Kanneganti et al 2006) from *P. infestan*s induces necrosis in the later stages of the interaction between this pathogen and its hosts tomato and *N. benthamiana*. PiNPP1.1 acts synergistically with INF1 inducing cell death when the two proteins are expressed simultaneously (Kanneganti et al 2006). Similarly, the RXLR effector PexRD2 causes cell death in *N. benthamiana* (Oh et al 2009). In contrast, the AVR3aKI (Bos et al 2006), PexED8 and PexRD36 (Oh et al 2009) cytoplasmic effectors from *P. infestans* suppress the INF1 induced HR in *N. benthamiana*. In addition, the RXLX effector *SNE1* of *P. infestans* suppresses necrosis caused by either PiNPP1.1 or PsojNIP (Kelley et al 2010). SNE1 is secreted during the biotrophic phase of the tomato- *P. infestans* interaction and is hypothesized to promote biotrophy by suppressing necrosis (Kelley et al 2010; Lee and Rose 2010). Another example of the modulation of host responses by *P. infestans* comes from the interaction between the *P. infestans* effector *ipiO-1* and the *Solanum bulbocastanum* resistance gene *RB.* After recognition of *ipiO-1* by *RB,* HR is elicited in a typical gene for gene interaction (Halterman et al 2010). This effector-triggered immunity is suppressed by another pathogen effector, *ipiO-4*, abolishing the HR and leading to susceptibility (Halterman et al 2010). The crinkler (CRN) effectors were initially identified by their ability to cause necrosis *in planta* (Torto et al 2003). Recently, two CRN proteins were characterized by Liu et al (2011). One of these (PsCRN63) caused necrosis in *N. benthamiana*, while the other

(PsCRN115) suppressed necrosis caused by the necrosis inducing protein from *P. sojae* (*PsojNIP*) and PsCRN63 (Liu et al 2011). Despite recent progress in understanding the interaction of *P. infestans* with its hosts, the mechanisms that regulate hemibiotrophy remain largely unknown.

To study processes that mediate the hemibiotrophic infection, we used the infection of tomato (*Solanum lycopersicum*) by *P. infestans* as a model pathosystem, as the intereaction is characterized by a prolonged biotrophic phase and a distinct necrotrophic phase at later stages of the interaction. Specifically, the co-expressed transcriptomes of each species we evaluated using RNA-Seq profiling of *P. infestans* and its host tomato in a compatible interaction. The goal was to identify suites genes and pathways from both the pathogen and plant that could be associated with each stage of the interaction. A central hypothesis to be tested was that *P. infestans* secretes discrete suites of effectors during different stages of infection. We evaluated the ability of putative effectors that were induced during the interaction to suppress or induce necrosis in a heterologous system (*N. benthamiana*).

2.2 Materials and Methods

Plant Material

Four-week-old tomato (*Solanum lycopersicum* cv. M82) greenhouse grown plants were used. Natural light was supplemented with 400W high pressure sodium lamps for 12 hours and temperatures maintained between 24 and 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).

Inoculum preparation and *Phytophthora infestans* isolate

The *P. infestans* US-11 (US050007) isolate was grown on detached tomato leaflets. Sporangia were harvested in distilled water and the concentration was adjusted to 4,000 sporangia per ml using a hemacytometer. Subsequently, the sporangia were incubated at 4˚C for one hour to release zoospores. Inoculation was accomplished by applying a 20µl drop of this mixture of sporangia and zoospores to the abaxial side of the leaflet; the inoculated leaflet was placed in a Petri dish containing water agar as a humid chamber.

Assessment of the biotrophic, transition to necrotrophic and necrotrophic phase

Microscopic observation using Trypan blue staining

Trypan blue staining was based on an established technique, Knox-Davies, 1974, modified by Chung et al 2010. Briefly, leaflets were submerged in a clearing solution A (acetic acid:ethanol, 1:3 v/v) overnight. After 16 hours, the clearing solution A was discarded and replaced by clearing solution B (acetic acid:ethanol:glycerol, 1:5:1 v/v/v) for three hours. Clearing solution B was replaced by staining solution (0.01% trypan blue in lactophenol) overnight. The staining solution was removed and leaves were rinsed with sterile 60% glycerol. After rinsing, the glycerol was removed and new 60% glycerol was added to the leaflets for two hours prior to microscopic observation.

Reverse Transcription PCR (RT-PCR) for stage-specific genes of *P. infestans*

For the RT-PCR analyses, the oligonucleotide primers used were: *ipiO*-FW: 5’- GAA TTC CTG TTG ACC GTG CTT TTG AAC-3’, *ipiO*-RV: 5’-ggGGA TCC CAC CGG TGC AGT AAA GGA TG-3’; *SNE1*-FW: 5’-GCG CGC GAA TTC ATG ATC CCC ACC AAT GCC-3’, *SNE1*-

RV: 5'-GCG CGC GGT ACC CAC TCC CTG CTT CTG GTT CTG-3'; *PiNPP*-FW: 5'-GAA TTC ATG AAC ATC CTT CAA CTC TTC G-3', *PiNPP*-RV: 5'-TCT AGA CTA GGC GTA GTA GGC ATT GC-3' (Kelley et al 2010). Total RNA was extracted using the hot-phenol protocol (Perry and Francki 1992), as modified by Gu et al (2000). DNaseI-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 dNTPs, 2µM each of the primers, and 0.5 U *Taq* polymerase (Invitrogen). PCR conditions consisted of 1 cycle of 95ºC for 5 min, followed by 35 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 *P. infestans actin A* gene was performed with the following primers FW: 5′-CGGCTCCGGTATGTGCAAGGC -3′, RV: 5′-GCGGGCACGTTGAACGTCTC -3′ (Latijnhouwers and Govers 2003). The PCR reaction conditions for *actinA* were as described above.

Tissue collection and RNA extraction for 454 sequencing

Tissue was collected from *P. infestans*-inoculated leaves at 48, 96 and 144 hours after inoculation (hai). Tissue was also collected from mock inoculated plants with a 20µl drop of water at 48 hours after the droplet was added. Leaf discs from the drop inoculation sites were harvested using a paper puncher with a 7mm diameter, and immediately frozen in liquid nitrogen. Twenty-five tomato plants per time point were used in each experiment. The experiment was repeated three times and the leaf discs from the four experiments were pooled (100 plants per time point). The pooled plant tissue was ground in liquid nitrogen using a mortar and a pestle. Total RNA was extracted using the hot-phenol protocol.

RNA amplification

mRNA was isolated from 250ng of total RNA and amplified using TargetAmp™ One-Round aRNA Amplification Kit 103 (EpicentreBiotechnologies). First, poly-A RNA was transcribed into first strand cDNA starting from total RNA. The reaction was primed with a synthetic oligo (dT) primer containing a phage T7 RNA polymerase promoter sequence at its 5' end. The first strand cDNA synthesis was catalyzed by SuperScript III reverse transcriptase (Invitrogen) generating a cDNA:RNA hybrid. Next, the RNA component of the cDNA:RNA hybrid was digested into small pieces using the RNase H enzyme. The RNA fragments primed the second strand cDNA synthesis. The resulting product was a double stranded cDNA containing T7 transcription promoter in an orientation that generated anti-sense RNA. High yields of anti-sense RNA were produced in a rapid in vitro transcription reaction (amplified RNA) that utilized the double stranded cDNA previously produced (Epicentre Biotechnologies).

cDNA synthesis and FLX-454 sequencing

cDNA was synthesized from three reactions of 5 µg of amplified RNA for a total of 15µg of amplified RNA per sample, using 100 ng of random hexamers on each reaction.The first and second strands of cDNA were synthesized using the SuperScript ® choice system for cDNA synthesis (Invitrogen) following the manufacturers' instructions.

Once the second strand was synthesized, the cDNA was cleaned using PureLink™ PCR purification kit (Invitrogen) following manufacturers' instructions and quantified using a nanodrop, and a minimum of 9µg of cDNA was recovered. cDNA libraries construction and 454 sequencing took place at the Cornell University Life Sciences Core Laboratories Center (CLC: http://cores.lifesciences.cornell.edu).

cDNA sequence processing and assembly

The raw 454 sequence files in SFF format were base called using the Pyrobayes base caller (Quinlan et al 2008). The sequences were then processed to remove low quality regions and adaptor sequences using programs LUCY (Chou and Holmes 2001) and SeqClean (http://compbio.dfci.harvard.edu/tgi/software/). The resulting high quality sequences were then screened against the NCBI UniVec (ftp://ftp.ncbi.nih.gov/pub/UniVec/), *E. coli* genome sequences, and *Phytophthora* ribosomal RNA, to remove contaminants. Sequences shorter than 30bp were discarded. To distinguish *Phytophthora* transcript sequences from those of tomato, the cDNA sequences were aligned to genomes of *Phytophthora infestans*, *P. sojae* and *P. ramorum* (http://www.broadinstitute.org/annotation/genome/phytophthora_infestans/MultiHome.html) , respectively, using SPALN (Gotoh 2008) for those longer than 100 bp and BLAT (Kent 2002) for those shorter than 100 bp. Sequences that could be aligned to any of the three *Phytophthora* genomes with at least 90% sequence identity and 50% length coverage were regarded as derived from *P. infestans*, while the rest were treated as derived from tomato. *P. infestans* cDNA sequences, along with *P. infestans* transcripts predicted from the genome sequences (Haas et al 2009), were assembled into unigenes using the iAssembler program (http://bioinfo.bti.cornell.edu/tool/iAssembler).

Unigene annotation and pathway prediction

Phytophthora infestans unigenes were blasted against GenBank non-redundant protein (http://www.ncbi.nlm.nih.gov/genbank/) and UniProt (http://www.uniprot.org/) databases with a cutoff e value of 1e-5. The unigene sequences were also translated into proteins using ESTScan (Iseli et al 1999) and the translated protein sequences were then compared to InterPro

(http://www.ebi.ac.uk/interpro/) and pfam (http://pfam.sanger.ac.uk/) domain databases. The gene ontology (GO) terms were assigned to each unigene based on the GO terms annotated to its corresponding homologs in the UniProt database (Camon et al 2004), as well as those to InterPro and pfam domains using interpro2go and pfam2go mapping files provided by the GO website (http://www.geneontology.org), respectively.

Identification of differentially expressed genes

Following cDNA sequence assembly, digital expression information of each unigene was derived following normalization to the total number of sequenced transcripts per sample. The 454 reads were normalized with the calculation: number of ESTs of a unigene from the specific sample * 100,000 (that is, number of reads if 100,000 ESTs are collected) / total number of ESTs collected from that specific sample.

Significance of differential gene expression was determined using the R statistic described in Stekel et al (2000) and the resulting raw p values were adjusted for multiple testing using the False Discovery Rate (FDR, Benjamini and Hochberg 1995). Genes with fold change greater than two and FDR less than 0.05 were considered to be differentially expressed genes. GO terms enriched in the set of differentially expressed genes were identified using GO::TermFinder (Boyle et al 2004), requiring p values adjusted for multiple testing to be less than 0.05.

Cloning of *P. infestans* putative effector genes

P. infestans putative effector genes were cloned using total RNA from the three pathogenicity stages. The SuperScript^{TM}III One-step RT-PCR system with Platinum® *Taq* High Fidelity

(Invitrogen) was used to synthesize the full-length cDNA of each candidate gene with gene-specific primers (Table 2.1). PCR amplification conditions were as described above for the stage-specific primers. Amplified PCR fragments were purified using QIAquick® PCR purification kit (Qiagen) following the manufacturer's instructions. Candidates were cloned into pGEMT®-easy vector with the 2X rapid ligation buffer (Promega) and incubated at 4°C overnight. Plasmids were sequenced using T7 universal primers in the Cornell University Life Sciences Core Laboratories Center (CULSCLC). Once the sequence was confirmed, the candidate genes were cut out from the plasmid using their respective restriction enzymes (see table above) and purified in a 1.2% agarose gel to using the E.Z.N.A ™ Gel extraction kit (Omega bio-tek). Candidate genes cloning into pART-GFP plasmid (expression vector) was done at 15°C overnight. After cloning, plasmid was transformed into *E. coli* DH-5α quimio-competent cells. Minipreps were done using QIAprep® spin miniprep kit (Qiagen) following the manufacturer's instructions and plasmids were sent for sequencing to the CLC (http://www.brc.cornell.edu) using the 35S promoter primer. After sequence confirmation, the plasmid was subcloned into *Agrobacterium tumefaciens* strain GV3101.

Table 2.1 List of primers used to clone candidate effectors

Gene I.D.	Enzymes used for cloning	Forward Primer	Reverse Primer
PITG_09216 RXLR peak at 48 hai	*Eco*RI/ *Hind*III	5'-gGAATTCATGCGTTTCAGCGTTTTCGT-3'	5'-gggAAGCTTTTACGCTGAACCTGTCGTCG-3'
PITG_12612 CRN peak at 48 hai	*Xho*I/ *Hind*III	5'-ccgCTCGAGCTACACTGTAGAAATGGTGA-3'	5'-gggAAGCTTTTACTTCAATCGGGCAGCTA-3'
PITG_07892 Hypothetical protein peak at 48 hai	*Eco*RI/*Xba*I	5'-GAATTCATGACGTACCCAGCTCAGTG-3'	5'-TCTAGATTACAGCACGGTGAGCTCTC-3'
PITG_12766 Hypothetical protein peak at 48 hai	*EcoRI/XbaI*	5'-GAATTCATGAGCAGTCATGACCGTGT-3'	5'-TCTAGATTAGCCGTCCATCGTTGTCG-3'
PITG_13452 RXLR peak at 96 hai	*Xho*I/ *Hind*III	5'-ccgCTCGAGATGCGCCTTAGTTACATCCT-3'	5'-gggAAGCTTTTATCTCCAGCTTCTGCCTT-3'
PITG_18215 RXLR peak at 96 hai	*Xho*I/ *Hind*III	5'-ccgCTCGAGATGCGAGCCTACTTTGTCCT-3'	5'-gggAAGCTTTTAGAAATTGTTCTTTGCGG-3'
PITG_15638 Hypothetical protein peak at 96 hai	*EcoRI/XbaI*	5'-GAATTCATGTCGACGATCACCAAGGA-3'	5'-TCTAGATTAGTAGTCCTCGTCGCCAC-3'
PITG_04742 CRN peak at 144 hai	*Eco*RI/*Xba*I	5'-GGAATTCATGGCGGCGCGAAAATGGTT-3'	5'-gcTCTAGACTACCGGAGCAAATCCCAGT-3'
PITG_17176 CRN peak at 144 hai	*Eco*RI/*Xba*I	5'-gGAATTCATGGTCAAGCTTGTTTGTGC-3'	5'-gcTCTAGACAATTCATTACGCACCTCGGG-3'
PITG_03583 putative secreted protein peak at 144 hai	*Xho*I/ *Xba*I	5'-ccgCTCGAGATGATGCGTCTCCTCTCCTGT-3'	5'-gcTCTAGACTAAGCAAAGTTCATCACGA-3'
PITG_07285 Hypothetical protein peak at 144 hai	*Eco*RI/*Xba*I	5'-GAATTCATGGACGCCAAGCAGACCGC-3'	5'-TCTAGATTACATCTCCATCAGAGCAT-3'
PITG_10543 Conserved hypothetical protein peak at 144 hai	*Eco*RI/*Xba*I	5'-gGAATTCATGGTACTGTCTGTGAAGAA-3'	5'-gcTCTAGATTACACCGACCCTTCCACAA-3'
PITG_13919 Hypothetical protein peak at 144 hai	*Eco*RI/*Xba*I	5'-GAATTCATGGACAAGCTGTTGGAGTA-3'	5'-TCTAGATTACTGAGACTTCTCCTCGA-3'

Transient expression assays in *Nicotiana benthamiana*

Transient expression of recombinant proteins in *N. benthamiana* was performed as described previously (Bos et al 2006). *A. tumefaciens* strains were grown at 29°C for approximately 24 hours in induction medium (Sessa et al 2000). After the 24 hours growth, cells were centrifuged for 10min at 3000rpm and the pellet was re-suspended in 5ml of infiltration medium (10mM $MgCl_2$, 10mM MES pH 5.5 and 200µM acetosyringone) and centrifuged again for 10 min at

3000rpm. Cells were re-suspended in new infiltration medium and the OD_{600} adjusted to 0.3. For cell death suppression assays, *A. tumefaciens* GV3101 carrying the gene of interest was infiltrated into *N. benthamiana* leaves with a1ml needle-less syringe. One day later, the necrosis inducer *PiNPP1-1* (kindly provided by Dr. S. Kamoun, Sainsbury Laboratory, John Innes Centre UK) was infiltrated at the same OD. The plants were scored for necrosis symptoms every 24 hours for 4 days.

Immunoblotting to detect expression of PiNPP1.1-HA protein

To show the expression of the PiNPP1.1 protein on the overlapping region where necrosis was suppressed by the candidate effectors, a one cm in diameter leaf disc was excised from three different agro-infiltrated sites per candidate gene. Agro-infiltrated leaf discs were ground with liquid N_2 and 50µl of SDS gel loading buffer were added to each sample. Samples were vortexed and centrifuged at 14,000 rpm for 1 minute. Next, samples were denatured in boiling water for 5 min. After denaturation, 10 µl of each sample was loaded on a 12% SDS-PAGE and run for 3 hours. Membrane transfer (Immobilion-P Millipore) was done following manufacturer's instructions (http://www.millipore.com/) using electroblotting apparatus (Bio-Rad, http://www.bio-rad.com/). After transfer, membranes were incubated overnight with a rat anti-HA- horseradish peroxidase-high affinity 3F10 monoclonal antibody (Roche, https://www.roche-applied-science.com) at a 1:5000 dilution in TBS containing 5% dry-milk. The following day membranes were washed three times with TBS for 10 min. Chemiluminescent detection was done using Amersham ECL Plus Western blotting detection kit (Amersham-Pharmacia, http://www5.amershambiosciences.com/) following manufacturer's instructions.

2.3 Results

Dynamics of *Phytophthora infestans* infection of tomato plants

We first defined the time frame over which the various stages of hemibiotrophic growth were exhibited by *P. infestans* (US-11 clonal lineage) during infection of tomato (*Solanum lycopersicum*, cultivar M82). Samples of material analyzed from each time point were derived from the pooled tissue of four biological replications (25 plants per time point repeated four times, giving a total of 100 plants per time point).

Three methods were used to select the time points corresponding to the biotrophic phase, the transition to necrotrophic growth and the necrotrophic phase: macroscopic observation, microscopic evaluation, and molecular assessment of the interaction based on the expression of *P. infestans* biotrophic and necrotrophic stage-specific genes.

In the macroscopic assessment of the pathogen development in tomato leaflets we observed no symptoms at the inoculation site up to 60 hours after inoculation (hai) (Figure 2.1A). Water soaking was visible at the inoculation site starting at 96 hai (Figure 2.1B), indicating the transition from biotrophy to necrotrophy. Necrosis was seen at the center of the lesion and sporulation at the edges at 144 hai (Figure 2.1C), which is symptomatic of a well-established necrotrophic phase.

To further assess the developmental stage of the pathogen we observed the inoculation site microscopically using the vital stain trypan blue (Knox-Davies 1974; Chung et al 2010). In the biotrophic phase (48 hai) germinating sporangia and hyphae were seen penetrating plant tissue (Figure 2.1D). In the transition from biotrophic to necrotrophic hyphal growth (96 hai), some

mycelial branching was apparent (Figure 2.1E). Finally, in the necrotrophic phase (144 hai) there was abundant mycelial growth and ramification and the emergence of sporangiophores bearing new sporangia (Figure 2.1F).

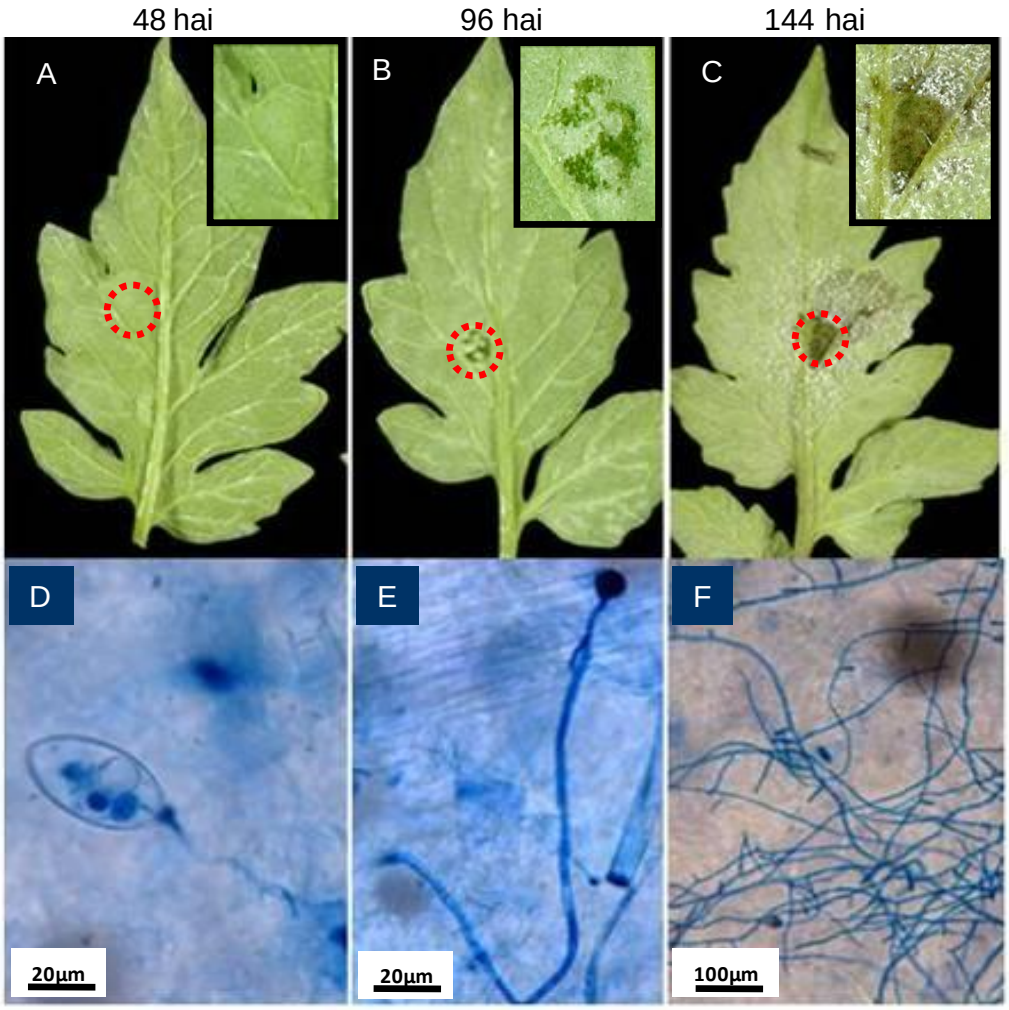

Figure 2.1 Macroscopic and microscopic assessment of symptoms of pathogen development on tomato leaflets. The upper panel shows the macroscopic symptoms of the pathogen development on the tomato leaflets, with a magnified inset for each stage of infection in the upper right corner of each panel. The pathogen developmental stages (trypan blue staining) are shown in the lower panel. The M82 tomato line was used for inoculation with *P. infestans* (US-11). Detached leaves were inoculated with 20 µl droplets containing 4000 sporangia/ml. Inoculated leaves were kept at 15˚C in a humid chamber and symptoms were monitored at 48 hai (A and D), 96 hai (B and E) and 144 hai (C and F). The red circle denotes an example of the 7mm diameter sample areas from which RNA was extracted. Approximately 100 plants per time point were used.

The expression of three *P. infestans* marker genes at the three stages of the infection was assessed using reverse transcription (RT)-PCR. Two *P. infestans* RXLR/RXLX effector genes, *ipiO* (van West et al 1998) and *SNE-1* (Kelley et al 2010) respectively, both of which have previously been shown to be expressed during the biotrophic phase of the interaction, reaching a peak of expression followed by a decrease during the transition to the necrotrophic phase were used as biotrophic markers. The *PiNPP-1* gene was used as the necrotrophic marker since its expression is restricted to the necrotrophic phase (Kanneganti et al 2006). The expression of both biotrophic markers was detected at 48 hai during the early stages of the interaction and transcript accumulation peaked during the biotrophic and transition phase, before decreasing at the necrotrophic phase (Figure 2.2). In contrast, the *PiNPP-1* was first detected at 96 hai and was predominantly expressed in the late necrotrophic phase. Expression of the *P. infestans actinA* gene was used to show that the reduction of expression of *SNE-1* and *ipiO* was not due to lack of pathogen biomass. Collectively, these results indicate that the time points chosen in this study correspond to the three different phases of the interaction.

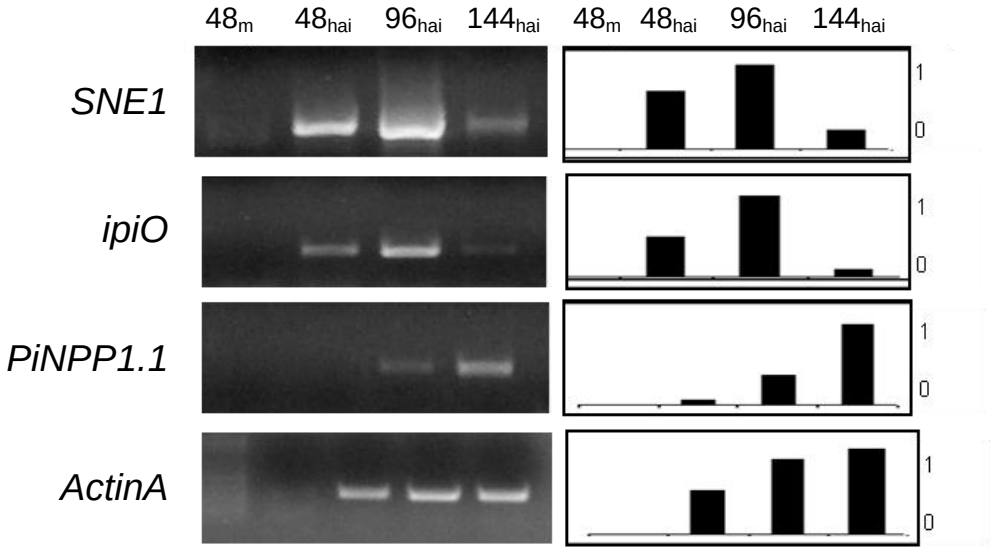

Figure 2.2 Expression profile of the molecular markers used to characterize *P. infestans* developmental stage. M82 plants were inoculated with a *P. infestans* isolate of the clonal lineage US11. The expressions of biotrophic (*ipiO* and *SNE-1*) and necrotrophic (*PiNPP-1*) stage specific markers in the water control (48_{mock}) and at 48, 96 and 144 hours after inoculation (hai) are shown in this figure. The left panels show a gel of the reverse transcription (RT-PCR) products and the right panels show corresponding quantification using Image J. *P. infestans actin A* was used as loading control.

Analysis of 454 reads and comparison to *P. infestans* unigenes

Having defined the time points at which *P. infestans* shows clear biotrophic and necrotrophic phases of the infection, as well as the transitional stage, we generated cDNA libraries from tomato tissue inoculated with *P. infestans* at 48, 96 and 144 hai, which were then sequenced using FLX-454 technology to generate transcriptome profiles over the infection time course.

Low quality sequences (<30bp) and those with polyadenylated tails were eliminated from the analyses. A summary of the number of 454 reads before and after trimming, as well as the average length in nucleotides (nt) for the three samples, is shown in Table 2.2. Based on these criteria 98-99% of the sequences from each sample were high quality. The cDNA sequences were aligned to genomes of *P. infestans*, *P. sojae* and *P. ramorum* (as described in materials and methods). Sequences that could be aligned to any of the three *Phytophthora* genome sequences with at least 90% sequence identity and 50% length coverage were regarded as derived from *P. infestans*, and further considered in the analysis.

At early stages of the interaction only 0.3% of the sequenced reads at 48 hai were from *P. infestans*. This proportion increased at 96 hai to approximately 6% and to 28% at 144 hai. At 144 hai when the tissue was showing advanced necrosis, still over half of the 454 sequences were derived from tomato.

Table 2.2 Summary of number of reads from 454 sequencing and BLAST hits to the *P. infestans* genome data (http://www.broadinstitute.org/annotation/genome/phytophthora_infestans/MultiHome.html). [a]nt: nucleotides; [b]hai: hours after inoculation.

Sample	Total number of reads	High quality reads	Length average (nt[a])	BLAST *P. infestans* Gene Models Hits (evalue ≤ 9e-7)
48 hai[b]	248,172	245,631	225	737
96 hai	154,842	151,863	209	8,472
144 hai	187,459	184,620	201	51,732

Inferring transcription levels using EST abundance

In order to confirm the accuracy of the RNA-Seq data we analyzed the expression of the four *P. infestans* genes that were used as markers to characterize the stage of pathogenesis (Figure 2.2). A similar pattern of expression for these genes was seen using either RT-PCR or 454 reads (Figure 2.3), although it appeared that RT-PCR was more sensitive, since *SNE-1* was detected at 48 hai using RT-PCR but was not identified until 96 hai in the 454 sequence profiles. A peak of expression of *SNE-1* at 96 hai, was observed using either technique, and expression then declined at 144 hai. Similarly, *ipiO* was first detected at 48 hai using RT-PCR and at 96 hai using the RNA-Seq data. With both techniques the peak of expression for this marker was at the transition

phase, while the necrotrophic marker *PiNPP1-1* showed a peak of expression at 144 hai. Finally, when we compared the *P. infestans actinA* expression profile we observed an increase of expression of this gene over time with both methods, reflecting the increasing amount of pathogen biomass. Thus, RNA-Seq based EST abundance analysis was an accurate measure of expression of these genes, but was apparently somewhat less sensitive than RT-PCR.

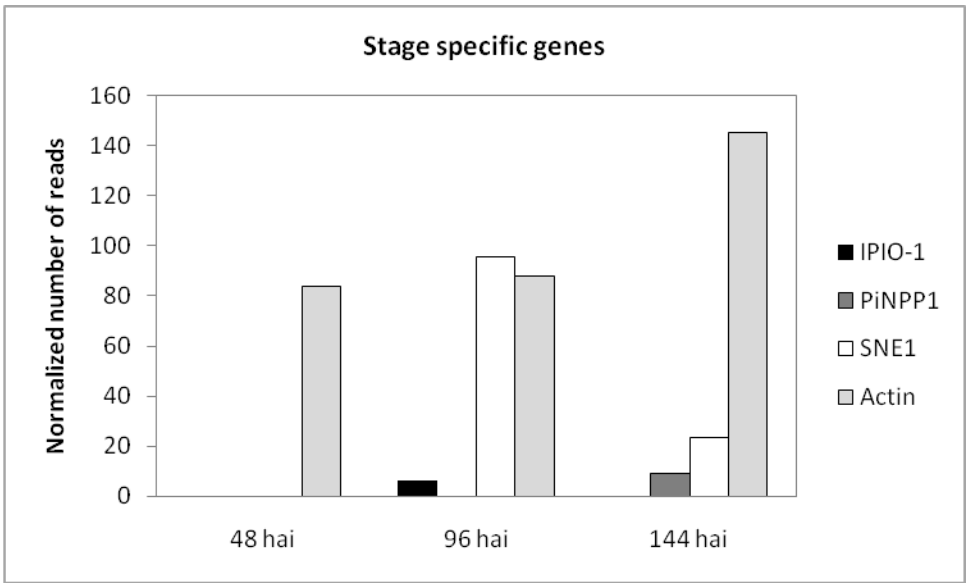

Figure 2.3 Normalized 454-reads for the three stage specific genes *SNE-1*, *ipiO*, *PiNPP-1* and *P. infestans actinA*. Units are normalized to the number of reads.

Transcriptome of *P. infestans*

In order to determine the relative gene expression in *P. infestans* and account for the increase in pathogen biomass throughout the time course of the interaction, we investigated the transcript abundance *Phytophthora* genes that have previously been described as constitutively expressed (Yan and Liou 2006). In our analysis, some of these were first detected at 96 hai (β-tubulin, glucose-6 phosphate dehydrogenase, secretory protein OPEL and phospholipase A2), while others were first detected only at 144 hai (TATA box binding protein and peptidyl prolyl isomerase). For the constitutively expressed genes that were detected at 48 hai we found between four to eight homologs for each that had differences in their expression profile (actin, α-tubulin, ubiquitin-conjugating enzyme, translation elongation factor 1-α and glyceraldehydes-3-phosphate dehydrogenase) (Figure 2.4). Therefore, we did not normalize our data using reference genes, but rather using the total number of genes per sample, as described in materials and methods. This allowed us to account for the increase in pathogen biomass, enabling a linear comparison of gene expression.

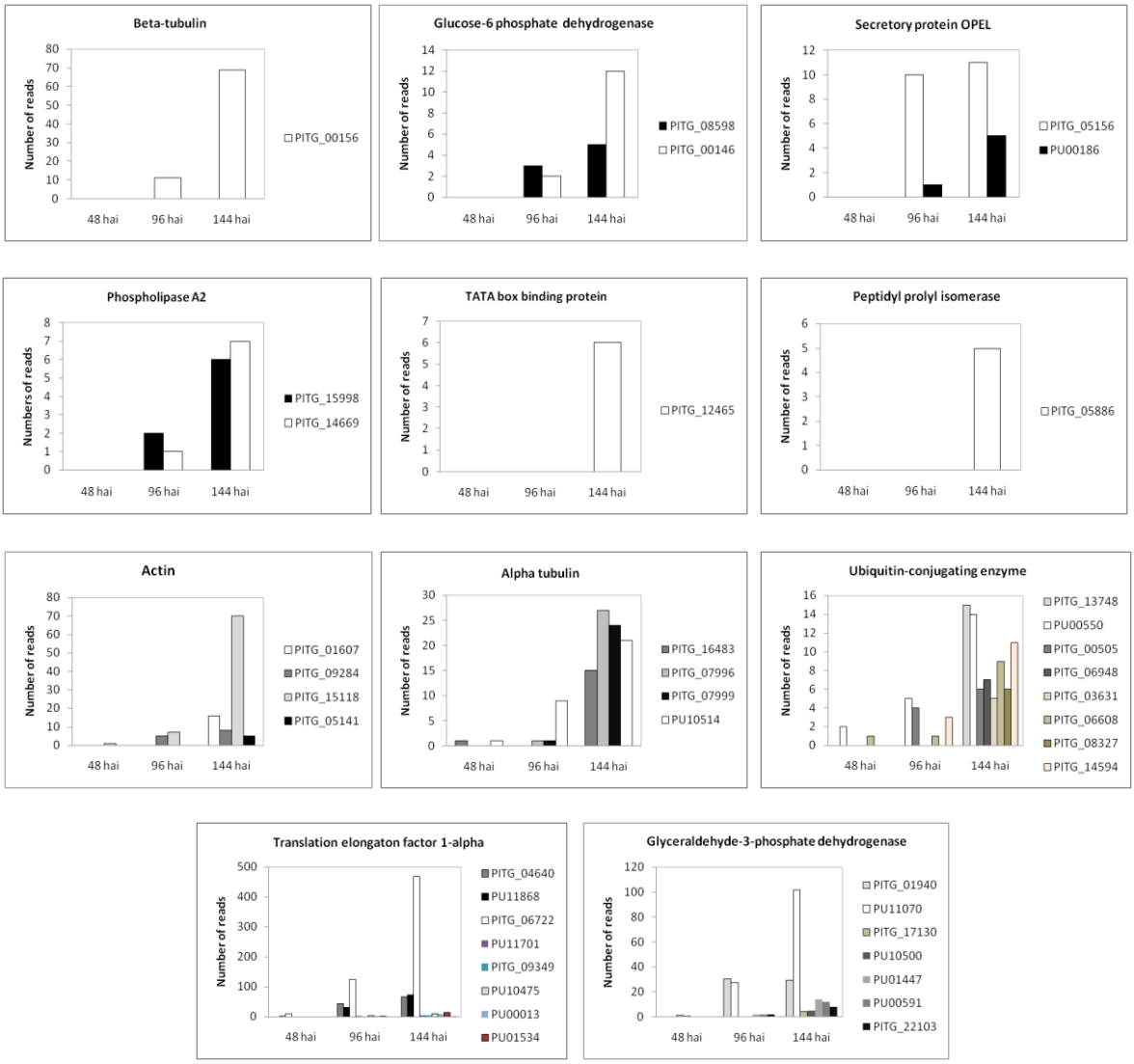

Figure 2.4 Expression of putative housekeeping genes of *P. infestans* during the interaction with tomato. Units are numbers of reads of each homolog at each time point.

Of the 18,178 predicted genes for *P. infestans* (Haas et al 2009), we found 9,109 to be expressed during the time course of the interaction. In subsequent analyses, only unigenes that had at least five reads were considered, reducing the number of genes studied to 3,495 (filtered file: ftp://ftp.solgenomics.net/secretom/Secretome_Phytophthora_tomato_interactions/supplementalfile2.1.xls). All the analyses in this work were done using the filtered file. Approximately 38% of these, corresponded to hypothetical proteins, of which 10% are predicted to be secreted based on the presence of signal-peptide, as determined using the SignalP software (Bendtsen et al 2004).

A total of 818 genes (23%) were identified from those listed in the filtered file, which were not previously identified in the *P. infestans* genome sequence. Of these, 29% had no homology to known genes, while 43% corresponded to hypothetical or unknown proteins using BLAST. A total of 11% were predicted ribosomal proteins, one gene corresponded to a carbonic anhydrase and this subset included putative effectors as well (six RXLR, six CRN and 12 elicitins; ftp://ftp.solgenomics.net/secretom/Secretome_Phytophthora_tomato_interactions/supplementalfile2.2.xls).

Ten percent of the *P. infestans* genes were differentially expressed based on a false discovery rate (FDR) of 0.05 and a two-fold change in transcript abundance (for a complete list of genes see ftp://ftp.solgenomics.net/secretom/Secretome_Phytophthora_tomato_interactions/supplementalfile2.3.xls). Of these, 34% were classified as hypothetical proteins, 6% were putative effectors (14 RXLR, five CRNs, three elicitins and one NPP-1-like protein), 3% were categorized as detoxification genes, including ATP-binding cassette and cytochrome P-450, and 10%

corresponded to ribosomal proteins. Of the differentially expressed genes 8% showed no homology to known genes in public databases. The remaining genes are associated with primary metabolism. At 48 hai the genes showing the highest fold change in expression were a putative nuclear LIM interactor-interacting protein, which mediates protein-protein interactions and appears to function as a transcription factor (Jurata et al 1996), a carbonic anhydrase, three RXLRs, one CRN and an ATP-binding cassette. At 96 hai genes showing the highest fold change in expression are predicted to encode proteins involved in translation as well as ten RXLRs (including *SNE1* and a member of the *avrblb2* family) and one NPP1-like protein. Finally, at 144 hai the genes with a major fold change in expression are putatively involved in ubiquitination and proteasomal degradation processes, in addition to cytochrome P-450, three CRNs and one elicitin (INF6-like).

Understanding hemibiotrophy in *P. infestans*

P. infestans acts as a biotroph pathogen at early stages of the interaction and as a necrotroph at later stages of the interaction. We hypothesize that *P. infestans* effectors modulate the outcome of the interaction by either blocking host defense mechanisms, or avoiding recognition by the plant, or a combination of both. To address this, we examined a temporal profile of the expression of some of the genes that are known or that have been previously hypothesized to be involved in the *P. infestans*-tomato or *P. infestans*-potato interactions, including putative effector genes, using the scheme adopted by Torto-Alalibo et al (2007). We examined the expression of putative pathogenicity genes from eight functional categories (Figure 2.5) in each of the three stages of the interaction. Putative effectors, elicitins and elicitin-like (*INF*s), CRNs, Necrosis inducing (*NPP*) and RXLRs were analyzed separately and shown in figure 2.6.

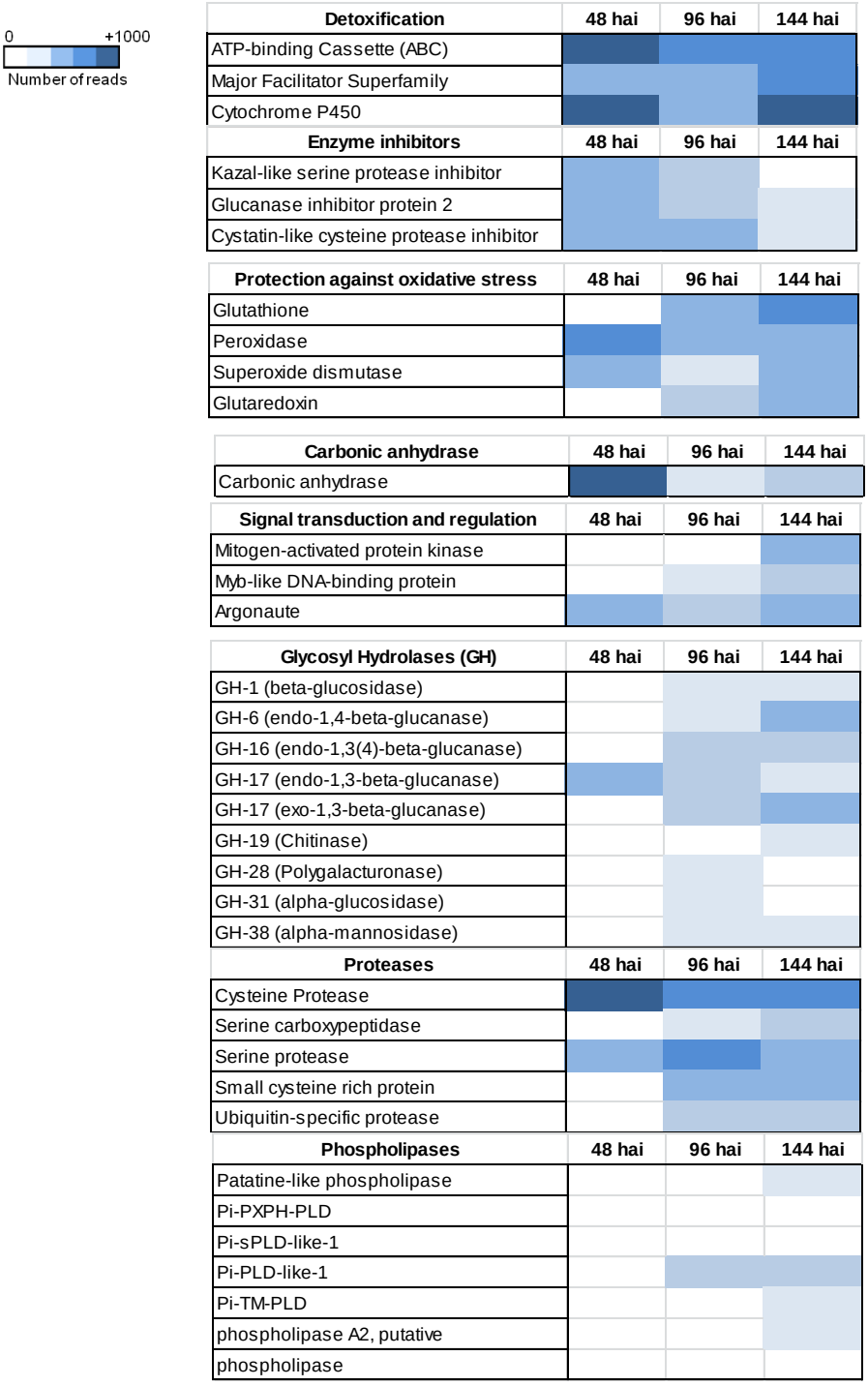

Figure 2.5 Putative pathogenicity genes of *P. infestans* throughout the interaction with tomato. The units are the number of normalized reads for each category, representing all the homologs for each gene. The CAZy database was used to define the members of each glycosyl hydrolase (GH) family nomenclature (http://www.cazy.org/).

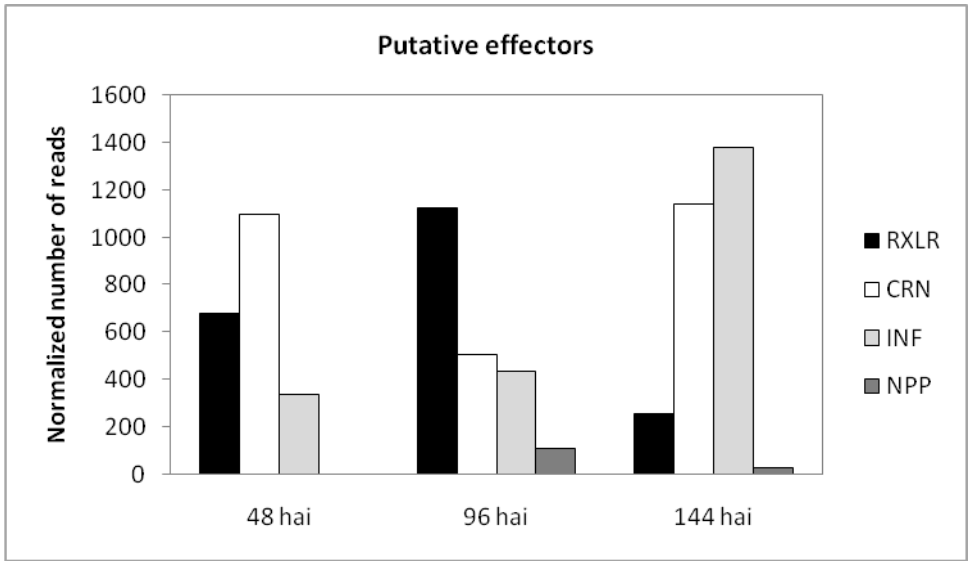

Figure 2.6 Temporal profile of the expression of *P. infestans* putative effectors: Elicitins and elicitin-like (*INF*), Crinkler (*CRN*), Necrosis inducing-like (*NPP1.1*) and RXLR. Units are normalized to the number of reads.

Transcriptome of *P. infestans* at the biotrophic stage: 48 hai

After penetration of the host tissue, the pathogen must overcome the preformed or induced defenses, such as degradative enzymes, enzyme inhibitors and toxic secondary metabolites. Accordingly, the *P. infestnas* RNA-Seq data revealed high levels of expression during the biotrophic phase of genes associated with detoxification (cytochrome P450 and ATP-binding cassette transporters ABC-transporters, Coleman et al 2011; Matthews and van Etten1983), protection against oxidative stress (peroxidase and superoxide dismutase) (Figure 2.5) and enzyme inhibitors. This latter class included a Kazal-like serine protease inhibitor, which inhibits a subtilisin-like serine protease from tomato (Tian et al 2004), glucanase inhibitor proteins that inhibit host endo-β-1,3 glucanases (Damasceno et al 2008) and a cystatin-like cysteine preotease inhibitor that targets a tomato papain-like apoplastic protease (Tian et al 2007; Figure 2.5).

In contrast to necrotrophic pathogens, which secrete abundant hydrolases into host cells to obtain nutrients (Govrin and Levine 2002), hemibiotrophic pathogens, such as *P. infestans*, avoid causing cell death to its host at early stages of the interaction. Accordingly, few hydrolases were expressed at the biotrophic stage. One endo-1,3-β-glucanase (glycosyl hydrolase (GH) family 17; Figure 2.5) was expressed at this time point, and is presumably involved in remodeling the *P. infestans* cell wall for hyphal tip growth and branching in the initial colonization steps (McLeod et al 2003). Serine protease homologs were also abundantly expressed at this stage, reaching a peak at 96 hai, while members of the family of cysteine proteases (cathepsin-B and papain-like) showed a peak of expression at the biotrophic stage (Figure 2.5). Genes that are involved in pathogen cell wall formation are also highly expressed at this stage. In addition, elicitin-like (*INF*) genes, which are sterol-carriers (Mikes et al 1998) and are also known to cause HR-like

cell death in *N. benthamiana* (Kamoun et al 1997), were abundantly expressed at this stage (Figure 2.6). In addition, five RXLR and nine CRN cytoplasmic putative effectors were highly expressed at this stage.

One of the *P. infestans* genes with the highest transcript abundance at 48 hai was a putative carbonic anhydrase (CA) (Figure 2.5), an enzyme that has been reported to be involved in sensing CO_2 concentrations and that catalyzes formation of bicarbonate, CO_2 and water (Schlicker et al., 2009).

Transcriptome of *P. infestans* at the transition stage: 96 hai

During the transition to necrotrophy (96 hai) we observed a shift in the categories of *P. infestans* genes that were expressed (Figure 2.5). For example, at this stage, expression of genes that have been previously associated with necrosis (i.e. small cysteine rich proteins and NPP-like family) were detected for the first time (Figures 2.5 and 2.6). There was also an increase in the number and diversity of families of GHs that likely facilitate plant cell wall modification and breakdown, including members of GH-1 (β-glucosidase), GH-6 (endo-1,4- β-glucanase), GH-16 (endo 1,3(4)-β-glucanase), GH-17 (exo and endo-1,3-β-glucosidases), GH-19 (chitinase), GH-28 (polygalacturonase), GH-31 (alpha-glucosidase) and GH-38 (alpha-mannosidase). Other putative plant cell wall modifying enzymes such as lipases, serine proteases and pectin esterases were also detected for the first time in this time course (Figure 2.5).

In addition, members of four phospholipase D sub-families, including the putatively secreted Pi-sPLD-like-1, were detected at 96 hai and their transcript abundance increased at144 hai (Figure

2.5). Recently the characterization of some members of the phospholipase D family of *P. infestans* suggested that some members are secreted and might be involved in pathogenicity (Meijer et al 2011). Likewise, the Major Facilitator Superfamily (MFS) transporters which are involved in pathogen protection were detected for the first time at this stage.

The RXLR cytoplasmic effectors, which are hypothesized to modulate the host defenses, were expressed at their highest levels at 96 hai (Figure 2.4). Among the RXLRs that showed a peak of expression in this transitional stage were the previously characterized *ipiO* (also known as avrblb1) (Vleeshouwers et al 2008), *SNE-1* (Kelley et al 2010), *avr1*, *avr2* and two members of the *avrblb2* superfamily (Oh et al 2009). In contrast, the CRN effectors showed the lowest relative transcript abundance at this stage (Figure 2.4). Other genes that had a reduction in transcript abundance were genes related to protection against oxidative stress (Figure 2.5).

Transcriptome of *P. infestans* at the necrotrophic stage: 144 hai

In the necrotrophic phase, there was a peak in the number and expression levels of necrosis inducing genes, including NAPDH oxidases and GHs, some of which were initially induced at the transition stage but reached a peak of expression at 144 hai. Similarly, transcript abundance of genes involved in signal transduction, protection against oxidative stress, detoxification, and the cytoplasmic effectors CRNs and the INFs was highest at this stage, while that of the RXLRs was the lowest (Figures 2.5 and 2.6). Among the RXLRs found is the PexRD2 effector that has been shown to cause cell death (Oh et al 2009).

One of the *P.infestans* genes (PITG_02623) that showed the highest level of transcript expression at 144 hai corresponds to a homolog of a recently identified aspartyl protease Plasmepsin V (PMV), which was characterized from the human malaria parasite *Plasmodium falciparum*. This protease recognizes and cleaves the RXL motif of this parasite effectors (Boddey et al 2010; Russo et al 2010), and is responsible for the specificity of export of the *Plasmodium* effectors into the host erythrocyte (Boddey et al 2010; Russo et al 2010).

Cluster analysis

A hierarchical cluster analysis was done in order to determine the genes and pathways that show a similar expression profile. Regardless of the level of expression, profiles were characterized as the number of reads for each gene at each time point compared to the mean expression of that gene across all the time points (Figure 2.7). Red represents values above the mean, black represents the mean, and green represents values below the mean of a row (gene) across all columns (time points). Homologs of a particular gene might have differences in their transcription profile, thus not all homologs will be in the same cluster. Using all the genes of the filtered file, five clusters were identified based on the expression profile (P1-P5) (Figure 2.7). Clusters P2 (76 genes) and P6 (362 genes) include genes for which expression was predominant at the biotrophic stage (Figure 2.7; for a complete list of the genes for each cluster see ftp://ftp.solgenomics.net/secretom/Secretome_Phytophthora_tomato_interactions/supplementalfile2.4.xls). Among the genes that were primarily expressed were those corresponding to unknown or hypothetical proteins (24%). Other are predicted to be involved in transcription, such as helicases, translation (ribosomal proteins and elongation factors), and the initiation of protein synthesis, including eukaryotic initiation factor 4A-III, DEAD/DEAH box RNA helicase and

eukaryotic translation initiation factor 3 subunit C. Clusters P1 (947 genes) and P4 (695 genes) include genes whose expression peaked at the transition stage. Finally, cluster P3 (1,415 genes) include genes that are mostly expressed at the necrotrophic stage (Figure 2.7). Approximately 38% of the genes expressed in the necrotrophic stage correspond to unknown or hypothetical proteins.

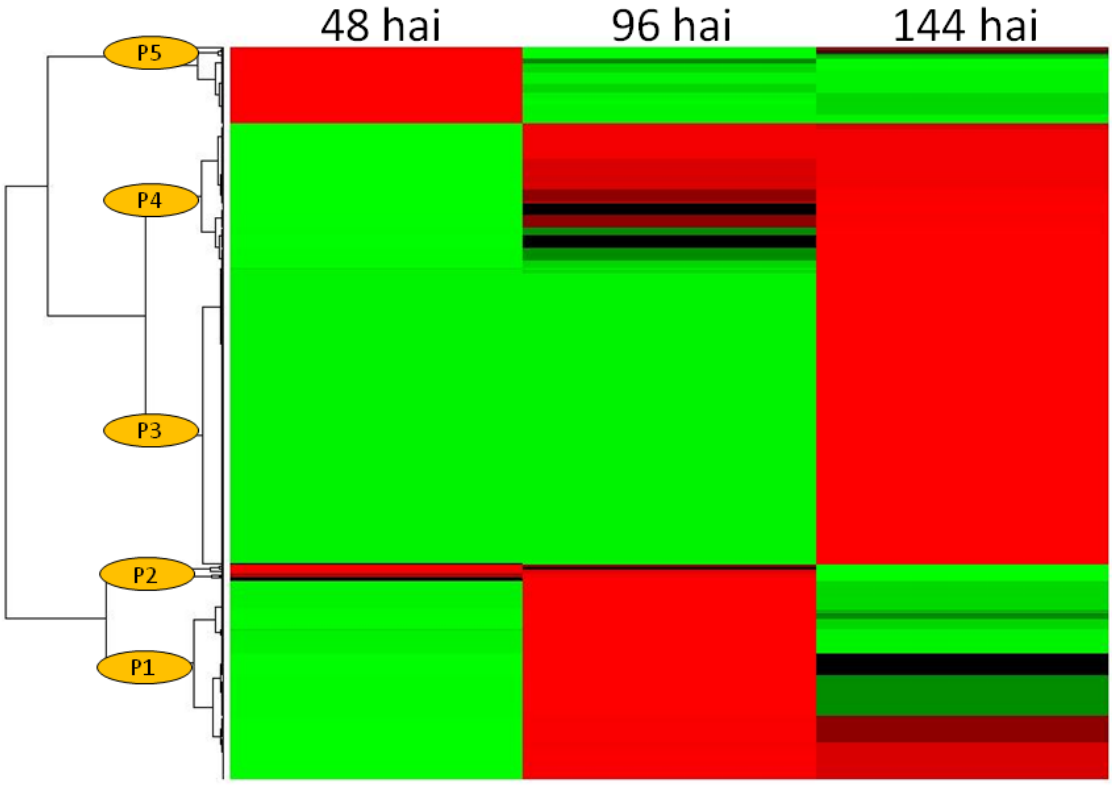

Figure 2.7 Hierarchical cluster analysis of *Phytophthora infestans* gene expression using the total number of genes from the filtered file. Based on the transcript profile, five clusters were generated identified in yellow circles (P1-5). Red represents values above the mean, black represents the mean, and green represents values below the mean of a row (gene) across all columns (time points).

A hierarchical cluster analysis was also performed done based on transcript abundance. The expression data were divided into quartiles: low transcript abundance genes (fewer than 20.5 reads, corresponding to 50% of the genes, from 0 to 50% quartiles: 1,735 genes Figure 2.8A); medium transcript abundance (between 21.9 and 144.2 reads, representing 40% of the genes, between the 50% and 90% quartiles: 1,412 genes, Figure 2.8B); and high transcript abundance genes: (the top 10% of the genes, corresponding to those with more than 144.2 reads, or 348 genes, Figure 2.8C).

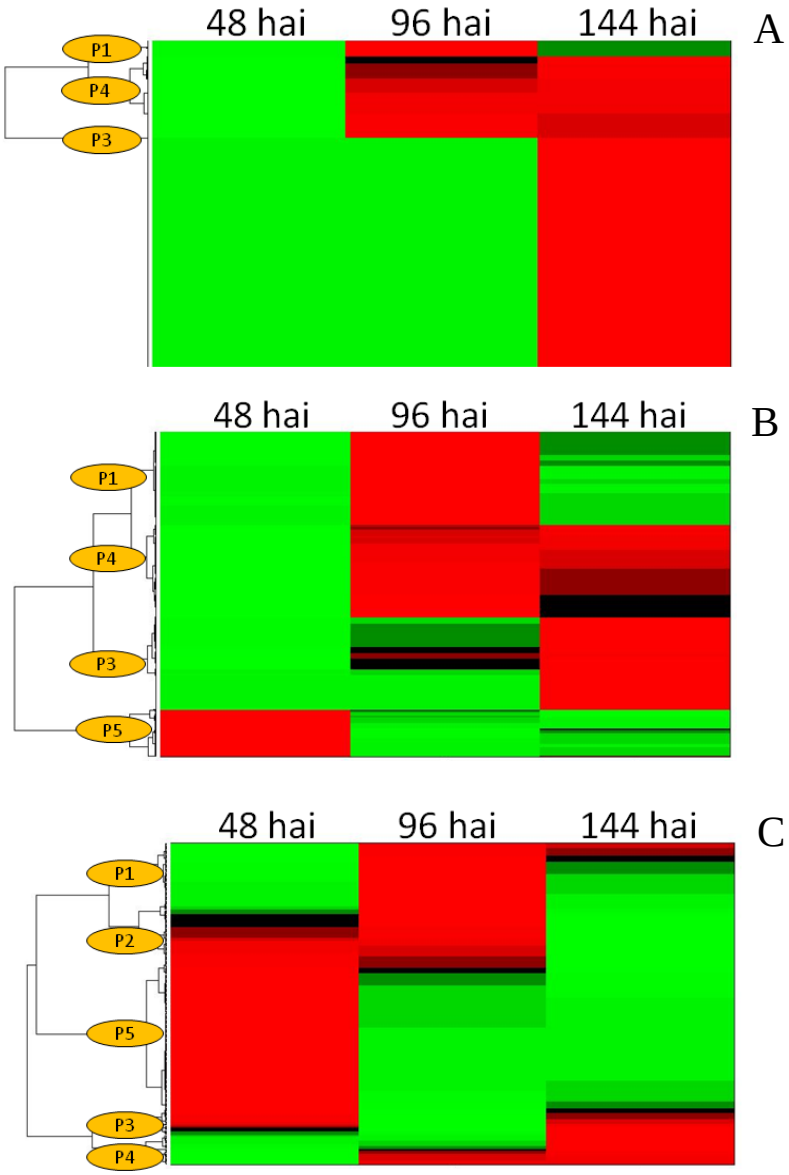

Figure 2.8 Hierarchical cluster analysis of *P. infestans* gene expression based on three data sets divided according to transcript abundance: low transcript abundance (A), medium transcript abundance (B) and high transcript abundance genes (C). Based on the transcript profile, five clusters were generated depicted as yellow dots (P1-5). Red represents values above the mean, black represents the mean, and green represents values below the mean of a row (gene) across all columns (time points).

Functional Characterization of putative effectors: RXLR, CRN and hypothetical proteins

To test the hypothesis that *P. infestans* effectors modulate the outcome of the interaction with tomato, the function of a subset of putative effectors identified in this study was evaluated by transiently expressing them using agro-infiltrations in *N. benthamiana* leaves. Each of the target genes was expressed together with the gene encoding the necrosis inducing PiNPP1-1 effector, which was expressed in an overlapping region (Figure 2.9A). Three outcomes were predicted: i) necrosis would occur in the entire agro-infiltrated region if the putative effector causes necrosis; ii) cell death would only occur in the region infiltrated with the *PiNPP1-1* if the putative effector does not have suppressing activity or cause necrosis; iii) the overlapping region would not develop necrosis (Figure 2.9A) if the putative effector suppresses necrosis caused by *PiNPP*1-1.

Three putative RXLRs effectors, one expressed at 48 hai (PITG_09216), and the other two at 96 hai (PITG_13452 and PITG_18215), as well as one CRN expressed at 48 hai (PITG_12612) were selected for functional analysis, based on transcript abundance at each stage. We hypothesized that effectors expressed during the biotrophic and transition phase might suppress cell death in the plant and delay the onset of necrosis. In agreement with our model, each of these four candidates suppressed necrosis caused by the *PiNPP*1-1 effector (Figure 2.9B).
Since CRN families have been shown to cause necrosis *in planta* (Haas et al 2009), two CRN candidates that were expressed at 144 hai (PITG_04742 and PITG_17176) were similarly tested. We hypothesized that these effectors might cause necrosis; however, expression of these two candidate genes neither suppressed nor induced necrosis in the *N. benthamiana* infiltrations assays (Figure 2.9B).

Because cytoplasmic effectors of *P. infestans* have a signal peptide for secretion into the host cell cytoplasm (Haas et al 2009), some of the secreted hypothetical proteins found in the current study, were selected to investigate whether they had a role as effectors, by either suppressing or inducing necrosis in *N. benthamiana*. Two secreted hypothetical proteins with the highest transcript abundance at 48 hai (PITG_07892, and PITG_12766), one at the transitional stage (PITG_15638) and four at the necrotrophic phase (PITG_03583, PITG_07285, PITG_10543 and PITG_13919) were chosen. Candidates were agro-infiltrated in *N. benthamiana* and assessed whether they suppressed necrosis caused by *PiNPP*1.1 or caused necrosis. None of the proteins that we studied caused necrosis and only one putative effector, the hypothetical secreted protein, PITG_03583 which was expressed at the necrotrophic stage, suppressed necrosis in the majority of trials (13/15) (Figure 2.9B).

As a negative control, transformation with the empty vector pART27 did not suppress or induce necrosis (Figure 2.9A and B). In addition, leaf discs from the overlapping region where suppression of cell death was observed were collected and analyzed using Western blot to determine whether the PiNPP1.1 protein was expressed (Figure 2.10). Presence of PiNPP1.1 protein in the agro-infiltrated region indicates that the candidate effectors are indeed suppressing necrosis but not interfering with PiNPP1.1 expression.

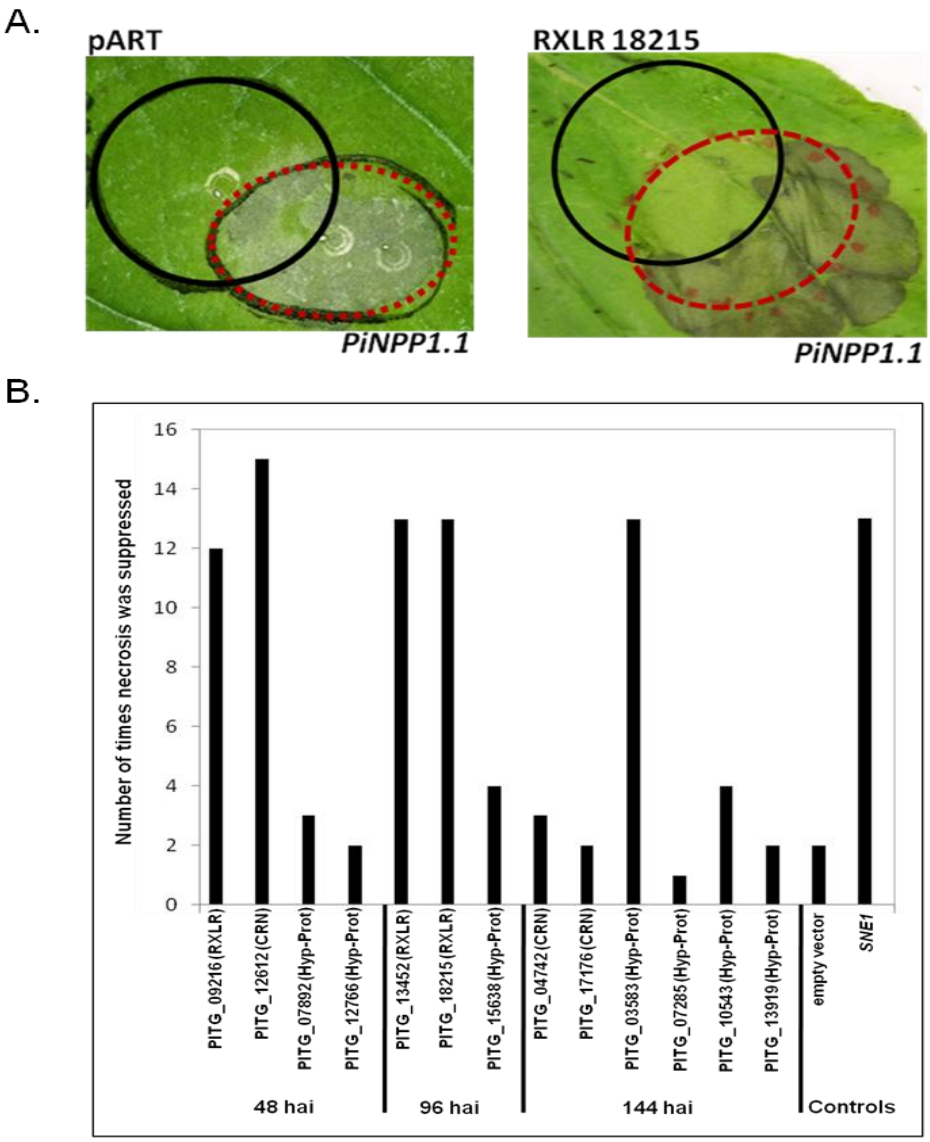

Figure 2.9 Agroinfiltrations of *N. benthamiana* leaves to determine whether the candidate genes contributed to suppress or cause necrosis. Candidate genes were infiltrated onto *N. benthamiana* leaves (black circle) and 24 hours later, the inducer of necrosis *PiNPP1.1* was infiltrated (red circle). No cell death is visible on the overlapping region infiltrated with the candidate genes that suppress necrosis. In contrast, cell death in the overlapping region can be seen with the pART27 empty vector control which does not suppress necrosis (A). Number of times a candidate gene (RXLRs, CRNs and hypothetical proteins) suppressed cell death caused by *PiNPP1.1* (results of at least 15 infiltrations per gene) (B).

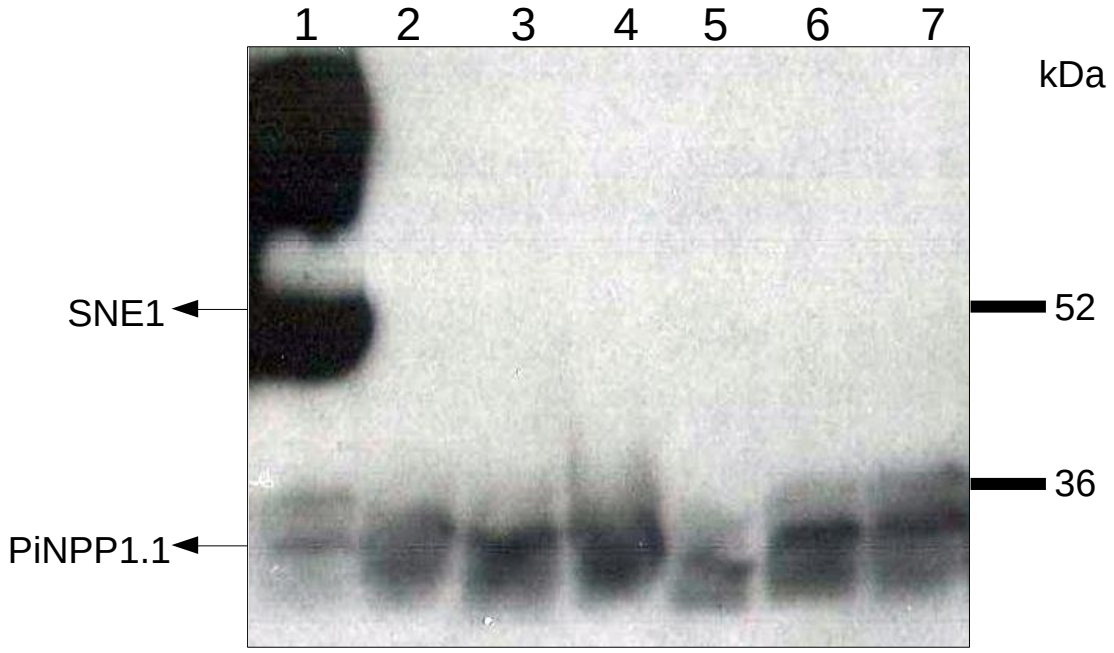

Figure 2.10 Western blot analysis of total protein extracts of leaves of *N. benthamiana*, showing *in planta* expression of PiNPP1.1-HA protein in the overlapping region where necrosis was suppressed. Controls: (1) SNE1-HA; (2) empty vector; (3) PITG_09216; (4) PITG_12612; (5) PITG_13452; (6) PITG_18215; and (7) PITG_03583.

2.4 Discussion

Using next generation sequencing we identified 818 unigenes (filtered file) that were not present in the *P. infestans* sequenced genome. There are several possible explanations for this: 1) these "orphan" genes are located in areas of the *P. infestans* genome that were not sequenced, or incorrectly assembled and annotated; 2) they correspond to non-coding RNAs; 3) they represent untranslated (UTR) sequences that were not included in the predicted genes and 4) they are the product of alternative splicing. Indeed, approximately one third of the *P. infestans* genes have introns (Win et al 2006). These results are in agreement with what has been reported for other well annotated genomes where 454 sequencing derived ESTs cannot be mapped to the predicted genes, including humans (36%; Mane et al 2009), *Arabidopsis thaliana* (13%; Weber et al 2007) and cucumber (28%; Guo et al 2010).

Ten percent of the genes (371) in this study were differentially expressed in at least one of the developmental stages, with at least a twofold change in expression and a FDR of 0.05. The majority of these genes were classified as hypothetical proteins, and 23 belonged to the putative effectors category (RXLR, CRN, elicitins and NPP-like). The majority of genes differentially expressed at the biotrophic stage are predicted to be involved in detoxification and protection against preformed plant defenses. The high abundance of elongation factors suggests a high rate of protein biosynthesis at the transition from biotrophy to necrotrophy. The RXLR effectors are also highly expressed at this stage and ten were differentially expressed. Finally, during the necrotrophic stage, ubiquitination and proteasomal degradation processes were highly induced.

To date, many of the analyses of *P. infestans* effectors was been based on selecting candidate effectors *in silico* and screening for a function by expressing then in a heterologous system (Oh

et al 2009; Haas et al 2009; Whisson et al 2007). This has been a successful strategy for both discovering expressed effectors and assigning function (Oh et al 2009; Haas et al 2009; Whisson et al 2007). However, this current study showed that of the 31 RXLRs expressed during tomato-*P. infestans* interaction (this study), only 12 were identified in the potato-*P. infestans* interaction (Haas et al 2009) and only three were found in common with Oh et al 2009. In contrast with the *P. infestans*-potato analysis, where only ten CRN were induced during infection (Haas et al 2009), predicted CRN proteins were the most abundant family of effectors expressed in the tomato-*P. infestans* interaction (51). None of the 51 CRN effectors found in this current study was present in the expression profile of *P. infestans* genes expressed during infection of potato (Haas et al 2009). This highlights the importance of using different approaches when studying plant-pathogen interactions to get a more comprehensive coverage of putative *in planta* expressed effectors.

The expression patterns of predicted pathogenicity effectors observed in this current study suggests that there is a coordinated regulation of these effectors over time. During the biotrophic phase the pathogen has to overcome potential plant defenses. Accordingly, we observed a large induction of genes involved in detoxification, enzyme inhibitors and protection against oxidative stress during the biotrophic stage. In addition, the pathogen modulates the plant response by secreting predicted cytoplasmic effectors.

Two families of putative cytoplasmic effectors (RXLR and CRN proteins) have been the subject of many studies because of their presumed role in pathogenicity. We found that many of these genes were expressed during tomato infection, with a higher proportion of the annotated CRN

genes (25%) than of the RXLR genes (5%). Additionally, two of these expressed CRNs had their sequence disrupted by gypsy transposons (Haas et al 2009).

The timing of expression of previously characterized effectors coincided with their predicted function. The RXLR genes that are either associated with the biotrophic phase or that have been shown to suppress necrosis (such as *ipiO*: van West et al 1998; and *SNE-1*: Kelley et al 2010) were detected early in the interaction (48 hai and 96 hai) in this study, while transcript abundance for genes thought to be involved in necrosis (*PexRD2*: Oh et al 2009; *PiNPP*-like proteins: Kanneganti et al 2006; *INF*-like elicitins: Kamoun et al 1997) and GHs was higher at later stages (144 hai). Through functional studies we showed the three RXLR and the CRN expressed at 48 and 96 hai suppressed necrosis caused by the *PiNPP1.1* effector, supporting the hypothesis that effectors secreted at early stages of the interaction may allow the pathogen to extend its biotrophic phase. However, contrary to our expectations that proteins expressed at 144 hai would contribute to necrosis, the hypothetical protein (PITG_ 03583) expressed at 144 hai suppressed necrosis rather than causing necrosis. None of the effectors we evaluated caused necrosis in *N. benthamiana*.

The use of agro-infiltration proved to be an effective tool to assess the putative function of several candidate effector proteins, although there are limitations to its use for the study of effectors. It is possible that the putative effectors interact with other effectors or with plant specific genes, in which case the use of heterologous systems would not be appropriate. A next step in the characterization of these candidate effectors is to find their targets in the affected plant cell and elucidate how they function in the context of other virulence genes.

Our expression data give support to other investigations of pathogenesis by *P. infestans*. For example, Meijer and co-workers (Meijer et al 2011) suggested that members of the *P. infestans* phospholipase D (PLD)-like family may have a role in pathogenicity and our data show that the expression of phospholipases is induced at 96 hai after the interaction with the host. As another example, one of the *P. infestans* transcripts that was most abundant during the biotrophic phase corresponds to a putative carbonic anhydrase (CA). This class of enzyme has been widely studied in mammalian pathogenic microbes such as the yeasts *Cryptococcus neoformans*, *Candida albicans* and the malaria pathogen *Plasmodium* (Schlicker et al 2009). Silencing of CA in *N. benthamiana* resulted in a faster *P. infestans* growth, suggesting a role for this gene in resistance (Restrepo et al 2005). Thus, CA may be important in both host and pathogen. We hypothesize that CO_2 sensing in *P. infestans* might trigger a change in the developmental stage or the formation of some virulence factors.

To conclude, these results suggest that effectors may modulate the outcome of the interaction with tomato by prolonging the biotrophic phase by suppressing necrosis and at later stages of infection the apoplastic effectors (i.e. including glycosyl hydrolases and *PiNPP* among others) induce necrosis.

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CHAPTER 3 – Analysis of the tomato transcriptome during a compatible interaction with the hemibiotrophic pathogen *P. infestans*

A.P. Zuluaga*, J. C. Vega-Arreguín, Z. Fei, G.M. López-Casado, A.J. Matas-Arroyo, W.E. Fry and J.K.C. Rose

Abstract

The transcriptome of tomato leaves (*Solanum lycopersicum* cv. M82) was analyzed during a compatible interaction with the oomycete *P. infestans* during three infection stages: biotrophic (48 hours after inoculation, hai), the transition from biotrophy to necrotrophy (96 hai) and necrotrophic phase (144 hai). The tomato transcriptome provided an overview of the physiological changes during infection. Transcript abundance of nearly 12,000 genes showed differential expression during the three infection stages analyzed. Similarly, genes encoding proteins in nearly 200 biochemical pathways were differentially expressed in tomato upon pathogen infection, revealing a massive reorganization of the plant metabolism. There was a strong decrease in transcript abundance of genes related to the photosynthesis and oxidative stress pathways. In contrast, there was an increase of transcript abundance of genes related to fermentation and of putative components of resistance. For example, more than 100 putative R genes and putative Pattern Recognition Receptors (PRRs) genes were induced. Signaling hormones such as salicylic acid (SA) and jasmonic acid (JA) that regulate resistance pathways causing significant changes in gene expression (Glazebrook 2005) were also studied. Transcript abundance of genes in the SA pathway were induced in the biotrophic phase, and subsequently declined. Whereas transcripts of genes in the JA pathway gradually increased as infection progressed. In agreement with the transcriptome data, SA levels increased during the biotrophic phase, but decreased in the necrotrophic phase. In contrast, JA levels increased as infection progressed. Consistent with this observation we demonstrated that JA tomato mutants *def*-1 and

*spr*2 were more susceptible to *P. infestans* than was the wild type. The tomato transcriptome proved to be a rich resource for data mining and testing hypotheses.

* A.P. Zuluaga contribution: designed and performed experiments, analyzed the data and wrote the manuscript.

3.1 Introduction

Plants have evolved an array of mechanisms to detect and respond to the wide range of potential pathogens that constantly challenge them (Cohn et al 2001). One of these mechanisms is known as PTI (PAMP Triggered Immunity) because it recognizes highly conserved microbial or pathogen-associated molecular patterns (MAMPS or PAMPS, respectively) of oomycetes, bacteria and fungi using trans-membrane pattern recognition receptors (PRR) (Jones and Dangl 2006). PRRs are Leucine rich repeat (LRR)-receptor kinases (RK) such as β-glucan-binding protein (GBP) (oomycete perception), Flagellin-sensing 2 (FLS2), Xa21 and the elongation factor (EF-Tu) receptor (EFR) (bacteria perception) and ethylene-inducing xylanase (EIX1/2) and LysM receptor kinase (fungal perception) (Boller and Felix 2009). Some PRRs are present in several plant families (e.g. FLS2), whereas EFR has been found in the Brassicaceae family only. However, LRR-RKs homologs to EFR are also encoded in poplar and rice genomes (Boller and Felix 2009). Although EFR has been exclusively found in the Brassicaceae family the recent finding that heterologous expression of EFR from *Arabidopsis* in tomato and *N. benthamiana* conferred broad spectrum resistance to bacteria (Lacombe et al 2010) suggests that downstream elements of PRR resistance are conserved in these members of the Solanaceae family.

As a counter to PTI defense, pathogens have evolved so-called effectors which are secreted proteins and other molecules that enable them to suppress basal defense and cause disease on plants (Schneider and Collmer 2010). However, as a counter-measure plants have evolved intracellular defense mechanisms that either directly or indirectly recognize pathogen effectors (McHale et al 2006). Molecular recognition of the pathogen effector proteins is typically mediated by host proteins with nucleotide binding sites (NBSs) and LRRs that are encoded by resistance (R) genes (Jones and Dangl 2006). The resulting resistance is referred to as effector triggered immunity (ETI), which results in a strong and fast induction of defense responses that often triggers a localized programmed cell death, known as the hypersensitive response (HR) (Jones and Dangl 2006). Following the HR, SA accumulation stimulates a systemic acquired resistance (SAR), wherein uninfected parts of the plant develop resistance to further infection by some pathogens (Martin 1999; Yang et al 1997).

Induced resistance pathways are regulated by signaling hormones such as SA, JA and ethylene (ET) causing significant changes in gene expression (Glazebrook 2005). A current model of host resistance indicates that the defense mechanism deployed by plants depends on the type of pathogenicity (Glazebrook 2005). Plants respond to biotrophic pathogens, which require living host cells for survival by activating the SA signaling pathway. In contrast, responses to necrotrophic pathogens, which kill host cells by activating the JA and ET mediated signaling pathways (Glazebrook, 2005). Accordingly, when plants are faced with hemibiotrophic pathogens, which have a biotrophic phase at the beginning of the interaction but a necrotrophic phase later in the interaction, the SA pathway is induced at early stages and the JA/ET pathways are activated at later stages (Glazebrook, 2005).

Transcriptomic studies of plant-pathogen interactions have, until recently, been limited in the number of genes that could be studied simultaneously. Earlier studies of the *P. infestans*-potato/tomato interaction at the level of gene expression involved the characterization of a few genes (Smart et al 2003; Choi et al 1992 among others). The use of suppression subtractive hybridization (SSH) proved to be a valuable tool to increase the number of genes (those transcribed after pathogen inoculation) that could be studied (Beyer et al 2001; Avrova et al 1999; Birch et al 1999; Tian et al 2003 among others). The introduction of microarrays allowed the simultaneously analysis of thousands of genes and provided insights into the complex crosstalk between defense and other signaling pathways in the *P. infestans*-potato/tomato interaction (Lindqvist-Kreuze et al 2010; Retrepo et al 2005). However, the use of such closed architecture systems exclude the study of genes that are not represented in the closed system, and is limited to genes that are already known, and species that are sequenced at a genome wide level or have very well characterized transcriptomes (Wang et al 2010).

The use of an open architecture system (RNA-Seq) to study plant-pathogen interactions circumvents these limitations allowing a simultaneous wide assessment of both plant and pathogen transcriptomes. To this end, in this study we evaluated the transcriptome of tomato leaves during a compatible interaction with *Phytophthora infestans*, the pathogen that causes late blight of tomato and potato. Plant transcriptomic analyses will improve our understanding of plant-pathogen interactions, from which we hope to develop strategies for disease control.

3.2 Materials and Methods

Plant Material

Four week-old tomato (*Solanum lycopersicum*, cv. M82) greenhouse grown plants were used for inoculation with *Phytophthora infestans*. Natural light was supplemented with 400W high pressure sodium lamps for 12 hours and temperatures maintained between 24 and 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).

Inoculum preparation and *Phytophthora infestans* isolate

The *P. infestans* US-11(US050007) isolate was grown on detached tomato leaflets. Sporangia were harvested in distilled water and the concentration was adjusted to 4,000 sporangia per ml using a hemacytometer. Subsequently, the sporangia were incubated at 4˚C for 1hour to release zoospores. Inoculation was accomplished by applying a 20µl drop of this mixture of sporangia and zoospores to the abaxial side of the leaflet; the inoculated leaflet was placed in a Petri dish containing water agar as a moist chamber.

Assessment of the biotrophic, transition to necrotrophic and necrotrophic phase

The methods used to define the three stages were: 1) macroscopic observation of the tomato symptoms in a time course after *P. infestans* inoculation, 2) microscopic observation of the *P. infestans* development using trypan blue and 3) the use of molecular markers from the biotrophic and necrotrophic stage of *P. infestans*.

Macroscopic observation

The biotrophic period was defined to extend from the time of inoculation until just before the tissue was first observed to be water soaked. The period from just before the appearance of water soaking to just before the appearance of necrosis was the transition phase. The necrotic phase was defined to occur when the entire inoculation site (7mm diameter) was necrotic.

Microscopic observation using Trypan blue staining

Trypan blue staining was based on an established technique of Knox-Davies, 1974, modified by Chung et al (2010). Briefly, leaflets were submerged in a clearing solution A (acetic acid:ethanol, 1:3 v/v) overnight. After 16 hours, the clearing solution A was discarded and replaced by clearing solution B (acetic acid:ethanol:glycerol, 1:5:1 v/v/v) for three hours. Clearing solution B was replaced by staining solution (0.01% trypan blue in lactophenol) overnight. The staining solution was removed and leaves were rinsed with sterile 60% glycerol. After rinsing, the glycerol was removed and new 60% glycerol was added to the leaflets for two hours prior to microscopic observation.

Reverse Transcription PCR (RT-PCR) for stage-specific genes of *P. infestans*

For the RT-PCR analyzes the oligonucleotide primers used were: *ipiO*-FW: 5'- GAA TTC CTG TTG ACC GTG CTT TTG AAC-3', *ipiO*-RV: 5'-ggGGA TCC CAC CGG TGC AGT AAA GGA TG-3'; *SNE1*-FW: 5'-GCG CGC GAA TTC ATG ATC CCC ACC AAT GCC-3', *SNE1*-RV: 5'-GCG CGC GGT ACC CAC TCC CTG CTT CTG GTT CTG-3'; *PiNPP1.1*-FW: 5'-GAA TTC ATG AAC ATC CTT CAA CTC TTC G-3', *PiNPP1.1*-RV: 5'-TCT AGA CTA GGC GTA GTA GGC ATT GC-3'. As a control, RT-PCR of the *P. infestans actin-A* gene was

performed with the following primers FW: 5′-CGGCTCCGGTATGTGCAAGGC -3′, RV: 5′-GCGGGCACGTTGAACGTCTC -3′ (Latijnhouwers and Govers, 2003). Total RNA was extracted using the hot-phenol protocol by Perry and Francki, (1992), as modified by Gu et al., (2000). DNaseI-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 dNTPs, 2µM each of the primers, and 0.5 U *Taq* polymerase (Invitrogen). PCR conditions consisted of 1 cycle of 95ºC for 5 min, followed by 35 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.

Tissue collection and RNA extraction for 454 sequencing

Tissue was collected from *P. infestans*-inoculated leaves at 48, 96 and 144 hours after inoculation (hai). These time points corresponded to the biotrophic, transition and necrotrophic stages, respectively (see Results). Mock-inoculated tissue with a 20µl drop of water was collected at 48 hours after the droplet was added. Leaf discs from the drop inoculation sites were harvested using a paper puncher with a 7 mm diameter, and immediately frozen in liquid nitrogen. Twenty-five tomato plants per time point were used in each experiment. The experiment was repeated three times and the leaf discs from the four experiments were pooled (100 plants per time point). The pooled plant tissue was ground in liquid nitrogen using a mortar and a pestle. Total RNA was extracted using the hot-phenol protocol.

mRNA isolation and RNA amplification

mRNA was isolated from 250ng of total RNA and amplified using TargetAmp™ One-Round aRNA Amplification Kit 103 (EpicentreBiotechnologies). First, poly-A RNA was transcribed into first strand cDNA starting from total RNA. The reaction was primed with a synthetic oligo (dT) primer containing a phage T7 RNA polymerase promoter sequence at its 5' end. The first strand cDNA synthesis was catalyzed by SuperScript III reverse transcriptase (Invitrogen) generating a cDNA:RNA hybrid. Next, the RNA component of the cDNA:RNA hybrid was digested into small pieces using the RNase H enzyme. The RNA fragments primed the second strand cDNA synthesis. The resulting product was a double stranded cDNA containing T7 transcription promoter in an orientation that generated anti-sense RNA. High yields of anti-sense RNA were produced in a rapid in vitro transcription reaction that utilized the double stranded cDNA previously produced (Epicentre Biotechnologies).

cDNA synthesis and FLX-454 sequencing

The first and second strands of cDNA were synthesized using the SuperScript ® choice system for cDNA synthesis (Invitrogen) following the manufacturers' instructions. For each sample, first strand cDNA was synthesized from three reactions of 5 µg of the amplified anti-sense RNA (aRNA) for a total of 15 µg of aRNA per sample, using 100 ng of random hexamers on each reaction.

Once the second strand was synthesized, the cDNA was cleaned using PureLink™ PCR purification kit (Invitrogen) following manufacturers' instructions and quantified using a nanodrop (http://www.nanodrop.com/), and a minimum of 9µg of cDNA was recovered. cDNA

libraries construction and 454 sequencing took place at the Cornell University Life Sciences Core Laboratories Center (CLC: http://cores.lifesciences.cornell.edu).

cDNA sequence processing and assembly

The raw 454 sequence files in SFF format were base called using the Pyrobayes base caller (Quinlan et al 2008). The sequences were then processed to remove low quality regions and adaptor sequences using programs LUCY (Chou and Holmes, 2001) and SeqClean (http://compbio.dfci.harvard.edu/tgi/software/). The resulting high quality sequences were then screened against the NCBI UniVec database (ftp://ftp.ncbi.nih.gov/pub/UniVec/), *E. coli* genome sequences, and *Phytophthora* ribosomal RNA, to remove contaminants. Sequences shorter than 30bp were discarded. To distinguish *Phytophthora* transcript sequences from those of tomato, the cDNA sequences were aligned to genomes of *Phytophthora infestans*, *P. sojae* and *P. ramorum* (http://www.broadinstitute.org/annotation/genome/phytophthora_infestans/MultiHome.html), respectively, using SPALN (Gotoh, 2008) for those longer than 100 bp and BLAT (Kent, 2002) for those shorter than 100 bp. Sequences that can be aligned to any of the three *Phytophthora* genomes with at least 90% sequence identity and 50% length coverage were regarded as derived from *P. infestans*, while the rest were treated as derived from tomato.

Unigene annotation and pathway prediction

Tomato unigenes were blasted against GenBank non-redundant protein (http://www.ncbi.nlm.nih.gov/genbank/) and UniProt (http://www.uniprot.org/) databases with a cutoff e value of 1e-5. The unigene sequences were also translated into proteins using ESTScan (Iseli et al 1999) and the translated protein sequences were then compared to InterPro

(http://www.ebi.ac.uk/interpro/) and pfam (http://pfam.sanger.ac.uk/) domain databases. The gene ontology (GO) terms were assigned to each unigene based on the GO terms annotated to its corresponding homologs in the UniProt database (Camon et al 2004), as well as those to InterPro and pfam domains using interpro2go and pfam2go mapping files provided by the GO website (http://www.geneontology.org), respectively.

Identification of differentially expressed genes

Following cDNA sequence assembly, digital expression information of each unigene was derived following normalization to the total number of sequenced transcripts per sample. The 454 reads were normalized with the calculation: number of ESTs of a unigene from the specific sample * 100,000 (that is, number of reads if 100,000 ESTs are collected) / total number of ESTs collected from that specific sample.

Significance of differential gene expression was determined using the R statistic described in Stekel et al (2000) and the resulting raw p values were adjusted for multiple testing using the False Discovery Rate (FDR, Benjamini and Hochberg, 1995). Genes with fold change greater than two and FDR less than 0.05 were considered to be differentially expressed genes. GO terms enriched in the set of differentially expressed genes were identified using GO::TermFinder (Boyle et al 2004), requiring p values adjusted for multiple testing to be less than 0.05.

Tomato mutant plants growth and inoculation

Tomato mutants *spr2* (Li et al 2003), and *def-1* (Howe et al 1996) and the wild type control cv Castlemart were kindly provided by Dr. Peter Moffett of the Boyce Thomson Institute. Growth conditions were the same as described for the tomato cv. M82.

Four- week old tomato plants were transferred to an inoculation chamber consisting of a PVC frame covered with semi-clear plastic sheeting, at 15ºC and 12hours light at 100% relative humidity (RH), maintained by an automatic humidifier (Trion model 500 Hummert International, Earth City MO) that ran periodically throughout the day and night. A total of three biological replications, with three plants per genotype each, were inoculated with the *P. infestans* isolate US-11 at a concentration of 4,000 sporangia/ml until run-off with a hand held sprayer.

Macroscopic quantification of late blight infection on tomato mutants

Inoculated tomato mutant plants (*spr2* and *def-1*) and the wild type control Castlemart were monitored daily for seven days and qualitatively rated for the percentage of leaves with disease symptoms at seven days after inoculation. A student's t-test (jmp9: http://www.jmp.com/software/jmp9/) was used to determine if there were differences in susceptibility between the tomato mutants and the wild type control.

3.3 Results

Defining the time frame of the tomato-*P. infestans* interaction

In order to define the time frame for the various stages of hemibiotrophic growth of *P. infestans* (US-11 clonal lineage) during infection of tomato (*Solanum lycopersicum*, cv. M82) three different approaches were used as described in chapter 2: 1) macroscopic assessment (evaluation of symptom development on leaflets); 2) microscopic evaluation of pathogen developmental stage; and 3) analysis of the expression of molecular markers expressed in either the biotrophic or necrotrophic phase of the interaction (Chapter 2). At 48 hours after inoculation (hai) under our conditions, the pathogen was interacting with the plant biotrophically. At 144 hai, the interaction was necrotrophic and at 96 hai, the interaction was in transition. Mock-inoculated tomato tissue served as control (tissue collected 48 h after mock-inoculated with water -48_{mock}-).

At each of these stages we generated cDNA libraries which were then sequenced using FLX-454 technology to generate high resolution transcriptome profiles of both pathogen and host over the infection time course. A summary of the number of 454 reads before and after trimming, as well as the average length in nucleotides (nt) for the four samples, are shown in Table 3.1. Approximately 98-99% of the sequences from each sample were high quality. In both the water control (48_{mock}) and 48 hai samples, approximately 90% of the sequences showed a match to tomato unigenes. There was a slight increase (~2%) in the number of tomato genes that were detected at 48 hai when compared to the water control. Some of the 2% corresponded to plant biotic-stress responding genes. At 144 hai when the tissue was necrotic, still more than half of the 454 sequences were derived from tomato.

Table 3.1 Summary of number of reads from 454 sequencing and BLAST hits to Solanacea Genomics Network (SGN: http://solgenomics.net/). [a]nt: nucleotides; [b]hai: hours after inoculation.

Sample	Total number of reads	High quality reads	Length average (nt[a])	BLAST to tomato unigenes Hits (evalue ≤ 9e-7)
(48_{mock})	171,569	168,217	229	151,137
48 hai[b]	248,172	245,631	225	224,966
96 hai	154,842	151,863	209	128,706
144 hai	187,459	184,620	201	102,230

Tomato transcriptome analyses

Sequencing of cDNA made from infected tomato leaves at the three different stages chosen and the mock inoculated control generated a total of 93,978 tomato unigenes, among which 24,093 were singletons. This number is remarkably higher than the 35,000 genes that are currently predicted in the tomato genome (http://solgenomics.net/genomes/Solanum_lycopersicum/genome_data.pl#annotation).

For statistical purposes, we only analyzed unigenes that had at least five reads in at least one time point (filtered file, ftp://ftp.solgenomics.net/secretom/Secretome_Phytophthora_tomato_interactions/supplementalfile3.1). All the analyses in this work used the filtered file, which contained 20,220 tomato genes.

Genes with a fold change of transcript abundance greater than two and a false discovery rate (FDR) less than 0.05 (Benjamini and Hochberg 1995) were defined as differentially expressed. Using these criteria we identified 11,855 genes (58% of the filtered file) that were differentially expressed. Overall, 75% of the sequences showed homology to previously described genes, and approximately 16% were predicted to encode hypothetical proteins.

Gene expression in tomato from biotrophy to necrotrophy during the interaction with *P. infestans*

Biotrophic phase

There were several changes in the tomato transcriptome at 48 hai despite the absence of symptoms on the leaflets (for a complete list of genes induced/repressed, see ftp://ftp.solgenomics.net/secretom/Secretome_Phytophthora_tomato_interactions/supplemntalfile 3.1). First, transcripts associated with photosynthesis were down regulated when compared to the mock inoculated plants. Expression of photosynthesis-related genes (such as Ribulose bisphosphate carboxylase -RuBisCO, chlorophyll A/B-binding protein and photosystem b genes) showed an overall 50% reduction in transcript abundance (Figure 3.1A). This suppression was retained for the duration of the interaction. In addition, there was suppression of genes involved in glycolysis (Figure 3.1B). In contrast, there was an induction of transcripts related to fermentation (Figure 3.1C) and mitochondrial related genes (such as NADH dehydrogenase, proline oxidase, glycine cleavage system H protein, mitochondrial electron transport complex III), but this induction weakened over time (Figure 3.1D).

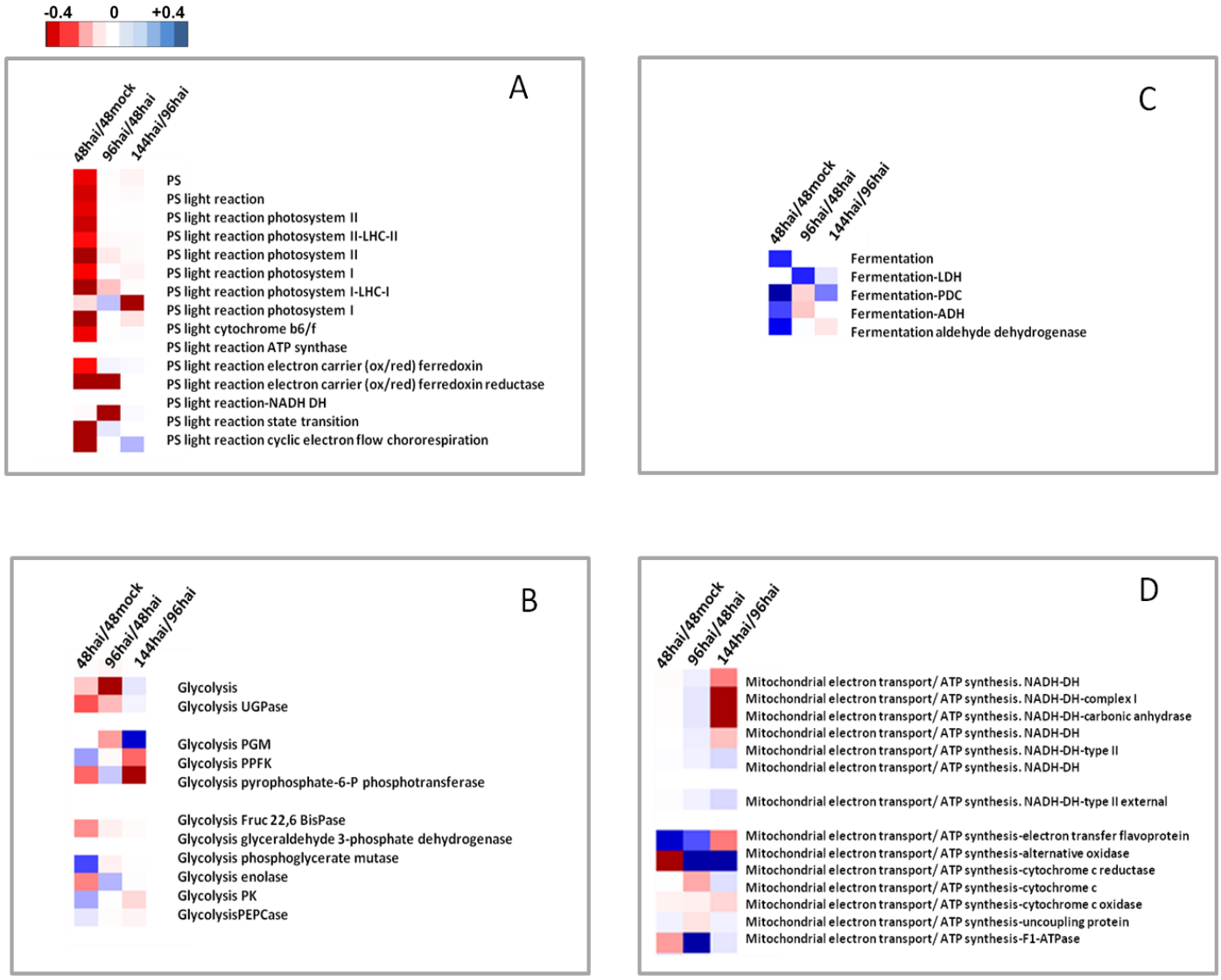


Figure 3.1 Change in transcript abundance of primary metabolism associated genes after infection: photosynthesis- related genes genes (A), glycolysis (B), fermentation (C) and mitochondria genes (D) (mapman: http://mapman.gabipd.org). The units are the Log_2 values of the changes from 48 $_{mock}$ to the first 48 hours after inoculation (hai), 48 hai to 96 hai and 96 hai to 144 hai. Red denotes down-regulated gene expression and blue up-regulated gene expression.

Genes encoding proteins in at least 181 tomato biochemical pathways responded to pathogen infection by 48 hai and 44 pathways were uniquely detected at this stage (Figure 3.2). Some of the pathways that were either induced or repressed after pathogen inoculation include those involved in osmotic regulation (proline, glutathione, trehalose biosynthesis), cell structure (suberin biosynthesis) and phytohormone production (for a complete list of genes see ftp://ftp.solgenomics.net/secretom/Secretome_Phytophthora_tomato_interactions/supplementalfile3.1.xls). A total of 6,808 genes were differentially expressed at 48 hai when compared to the water control (48_{mock}). Of these 3,361 genes were up-regulated while 3,447 were down-regulated. Roughly 38% of the genes induced more than two fold at 48 hai were classified as unknown, predicted, hypothetical or unnamed, suggesting that many specialized genes are expressed during the biotrophic phase of the interaction.

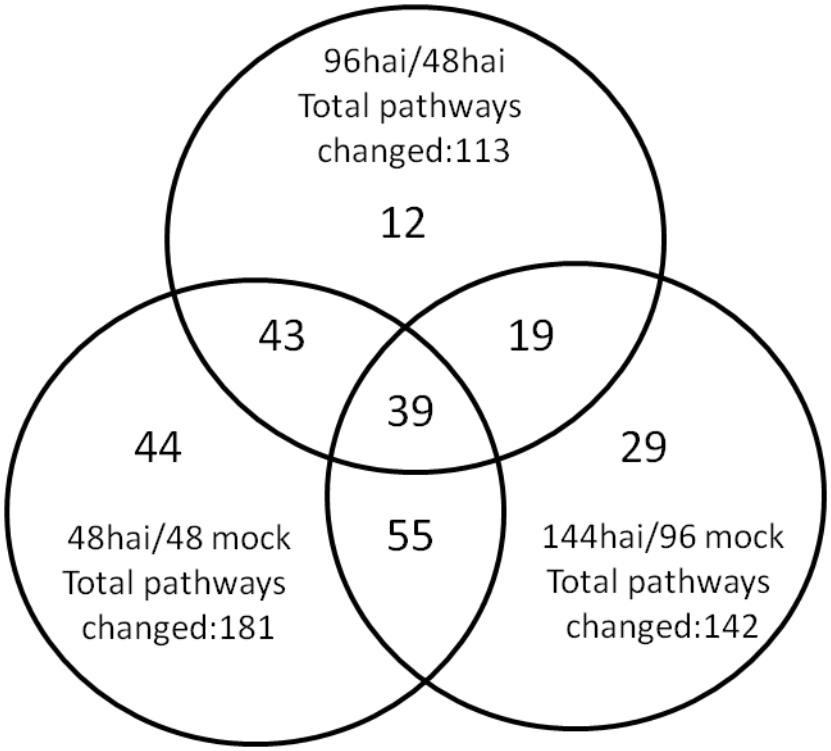


Figure 3.2 The Venn diagram shows the number of significantly changed transcript abundance of genes in the tomato biochemical pathways with at least a two fold change in expression and a p-value ≤0.05 at the different stages during the *P. infestans*-tomato interaction.

Some of the genes that have the highest change in transcript abundance at 48 hai have been previously suggested to be involved in the plant response to biotic stress (i.e. PR1, endo-β-1,3-glucanase (glycoside hydrolase (GH) family 17), GH-19 (chitinase), hevein-like protein and acidic thaumatin-like protein; Bowles 1990) (Figure 3.3). The increase in transcript abundance for some of these genes at 48 hai was over 200-fold when compared to the mock inoculated plants (Figure 3.3). In contrast the transcript abundance for some genes was reduced after pathogen infection. During the biotrophic phase, the expression of genes such as defensin (PDF1.2), chalcone synthase and some involved in ROI production was reduced (Figures 3.3 and 3.4).

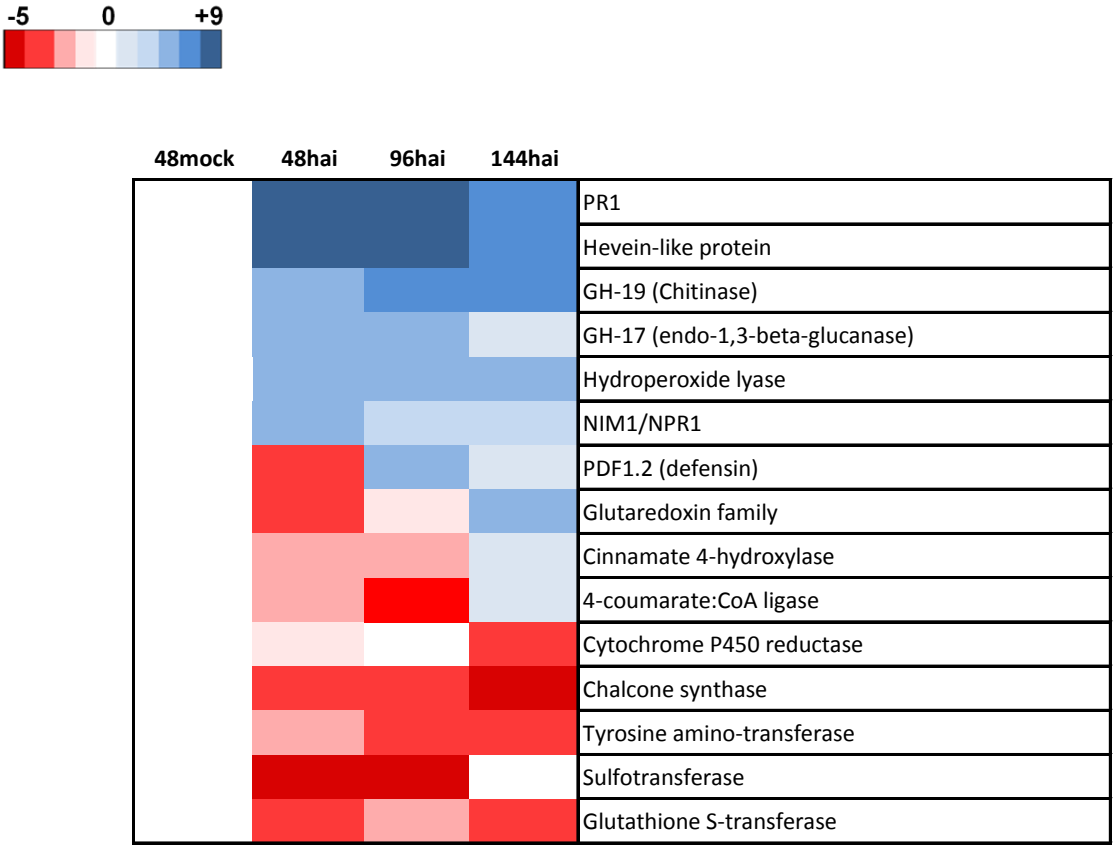


Figure 3.3 Heat map of genes that showed the highest and lowest transcript abundance in tomato after inoculation with a compatible isolate of *P. infestans*. The units are the Log_2 value of each sample compared to 48 $_{mock}$. Red denotes down-regulated gene expression and blue up-regulated gene expression.

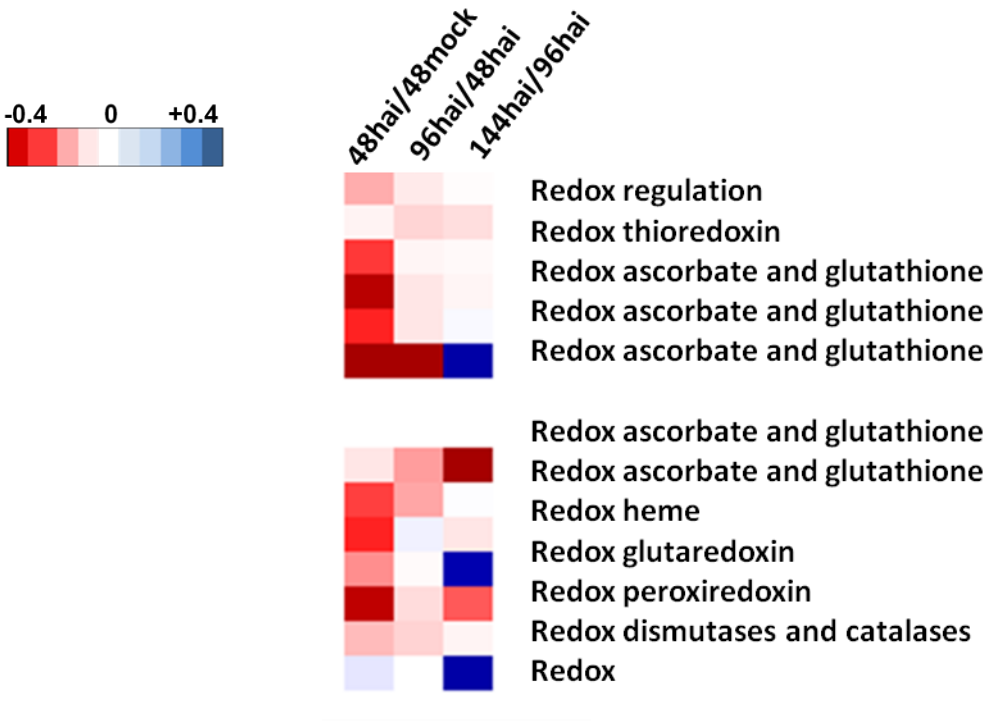


Figure 3.4 Tomato redox metabolism overview using Mapman (http://mapman.gabipd.org); for the significantly changed genes during the time course of the interaction. The units are the Log_2 values of the changes from 48 $_{mock}$ to the first 48 hours after inoculation, 48 hai to 96 hai and 96 hai to 144 hai. Red denotes down-regulated and blue up-regulated.

Tomato gene expression during the transition stage

A total of 2,909 genes were differentially expressed when comparing the transition stage (96 hai) with the biotrophy stage (48 hai) of which 1,485 were up-regulated and 1,424 were down-regulated. There were differences in genes encoding proteins in 113 pathways in this comparison (Figure 3.2; for a complete list of genes that were differentially expressed, see ftp://ftp.solgenomics.net/secretom/Secretome_Phytophthora_tomato_interactions/supplementalfile3.2.xls). There were 12 pathways detectable only at the transition stage, these included: production of secondary metabolites; 2,3-dihydroxybenzoate biosynthesis; fatty acid biosynthesis initiation; glutathione biosynthesis; purine biosynthesis; triacylglycerol degradation; urea degradation; and xylose degradation. Transcript abundance for three families of transcription factors bZIP, MYB and WRKY increased at 96 hai and continued to raise until 144 hai (Jakoby et al., 2002; Yanhui et al., 2006; Zhang and Wang, 2005). The genes that showed highest transcript abundance at this transitional stage included: β-1,3-glucanase (GH-17); a lipoxygenase, chitinase (GH-19); and PR1 (Figure 3.4 and ftp://ftp.solgenomics.net/secretom/Secretome_Phytophthora_tomato_interactions/supplementalfile3.2.xls). After an initial down-regulation of the defensin (*PDF1.2*) gene during the biotrophic stage, an induction of expression of this gene was observed at this transition stage (Figure 3.3). In addition to the primary metabolism genes for which transcript abundance was highly reduced at this stage, others that showed a similar pattern included ROI, calcium sensing receptors and hypothetical proteins (Figures 3.3 and 3.4; for a complete list of genes see ftp://ftp.solgenomics.net/secretom/Secretome_Phytophthora_tomato_interactions/supplementalfile3.2.xls).

Necrotrophic stage

Finally, during the necrotrophic phase genes encoding proteins in 142 pathways were differentially expressed and 29 were exclusively detected at this stage (Figure 3.2; ftp://ftp.solgenomics.net/secretom/Secretome_Phytophthora_tomato_interactions/supplementalfile3.2.xls). A total of 4,045 genes were differentially expressed when compared to the transitional stage. A slight majority of the genes were down-regulated (2,254) and 1,791 were up-regulated. We classified the genes that were down-regulated at this stage using the gene ontology (GO) terms. Most of the known genes showing the highest transcript abundance at 144 hai have been previously isolated from plant-pathogen interactions and are involved in redox homeostasis and antioxidant signaling, including the glutathione S-transferase, ascorbate, dismutases, catalases, and peroxidases (Foyer 2005; Figure 3.4); additionally, genes involved in production of secondary metabolites (Figure 3.3) and those related to biotic cell death had a high transcript abundance. Transcript abundance of genes involved in protein degradation via ubiquitination was highest at this time point. Finally, the transcription factors bZIP, MYB and WRKY reached the highest level of transcript abundance at the necrotrophic phase.

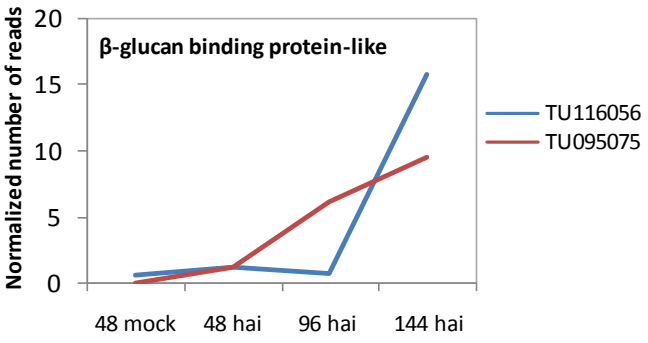
Identification of components of resistance in response to *P. infestans*

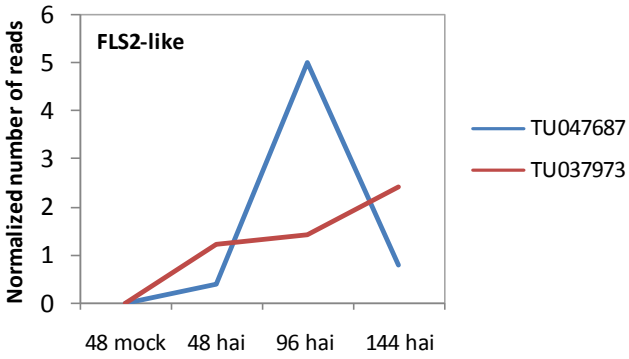
To characterize the defense mechanisms induced after pathogen infection we first investigated the expression of putative pathogen recognition receptors (PRRs). Analysis using a Hidden Markov Model (HMMER: http://hmmer.janelia.org/ version 3.0) for each PRR protein model revealed a total of 106 putative LRR-RK to be expressed at some point during the interaction, which could be divided into seven categories; however, we selected only the best matches for each of the putative homologous genes (the total number selected is in parenthesis for each

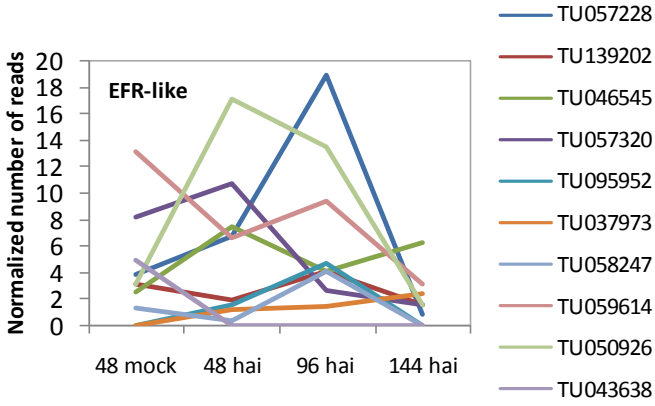
category): GBP (2), FLS2 (2), EFR (10), EIX (1), LysM (8), Xa21 (6) and NbLRK1 (10). Most of these putative PRRs (67%) are currently annotated as hypothetical proteins or unnamed proteins in the tomato database and their expression profiles are shown in Figure 3.5. The transcript abundance of most of these putative receptors increased after *P. infestans* infection. However, under each category, the putative homologs showed differences in transcript abundance throughout the interaction (Figure 3.5).

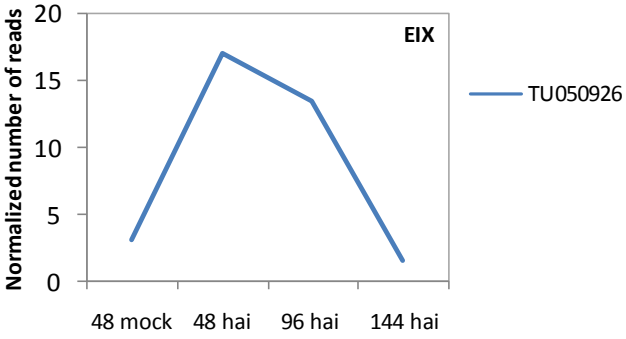
The list of transcripts showing differential accumulation included a total of 108 putative disease resistance genes with TIR/CC-NBS-LRR domains. Among the putative R genes, we found genes that can be associated with resistance to bacteria: *Pto* and *RPM1*(*Pseudomonas syringae*; Chang et al., 2002; Grant et al., 1995); oomycetes: *RPP8* and *RPP13* (*Hyaloperonospora arabidopsidis*; Bittner-Eddy and Beynon, 2001; Cooley et al., 2000) and a homolog to *RGA4* (*P. infestans*; Song et al., 2003); and virus: *N*-like gene (Whitham et al., 1996). Most of them were gradually up-regulated and their expression increased at least until 144 hai (Figure 3.5). In addition, other components of the R-gene mediated defense response such as *Rar1* (Muskett et al., 2002), *EDS1*(Falk et al., 1999) and *NPR1*-like (Rairdan and Delaney, 2002) genes were found to be expressed during this interaction. Our results suggest that the tomato transcriptome can be used as a data mining tool to identify putative PRR as well as putative disease resistance genes.

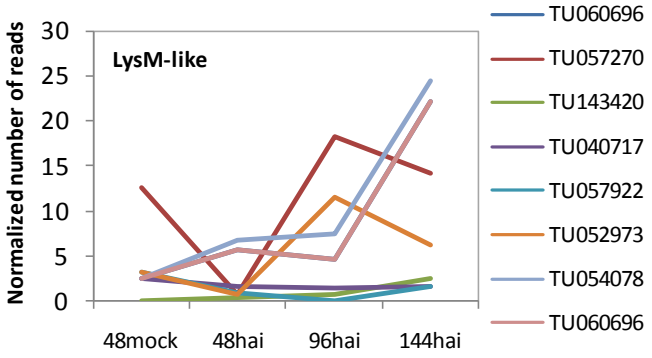
Figure 3.5 Expression profiles of putative Pathogen Recognition Receptors (PRR) genes and putative Resistance genes (TIR/CC)-NBS-LRR after pathogen infection. Units are normalized to the number of reads.

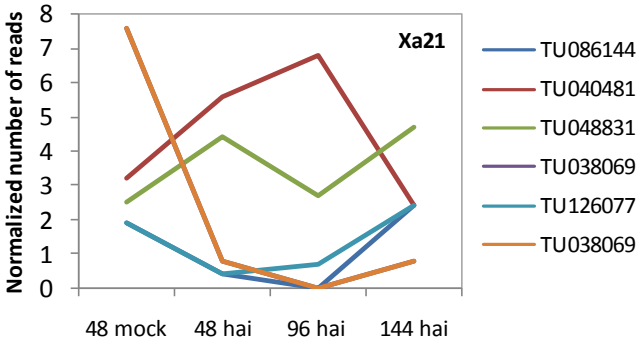

β-glucan binding protein-like
Normalized number of reads
20
15
10
5
0
48 mock
48 hai
96 hai
144 hai
TU116056
TU095075

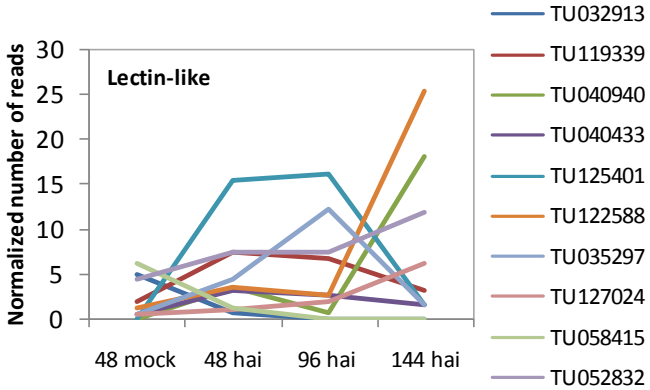

FLS2-like
Normalized number of reads
6
5
4
3
2
1
0
48 mock
48 hai
96 hai
144 hai
TU047687
TU037973

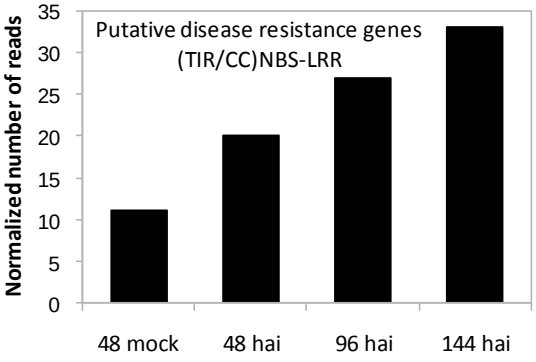

EFR-like
Normalized number of reads
20
18
16
14
12
10
8
6
4
2
0
48 mock
48 hai
96 hai
144 hai
TU057228
TU139202
TU046545
TU057320
TU095952
TU037973
TU058247
TU059614
TU050926
TU043638


EIX
Normalized number of reads
20
15
10
5
0
48 mock
48 hai
96 hai
144 hai
TU050926


LysM-like
Normalized number of reads
30
25
20
15
10
5
0
48mock
48hai
96hai
144hai
TU060696
TU057270
TU143420
TU040717
TU057922
TU052973
TU054078
TU060696


Xa21
Normalized number of reads
8
7
6
5
4
3
2
1
0
48 mock
48 hai
96 hai
144 hai
TU086144
TU040481
TU048831
TU038069
TU126077
TU038069


Lectin-like
Normalized number of reads
30
25
20
15
10
5
0
48 mock
48 hai
96 hai
144 hai
TU032913
TU119339
TU040940
TU040433
TU125401
TU122588
TU035297
TU127024
TU058415
TU052832


Putative disease resistance genes
(TIR/CC)NBS-LRR
Normalized number of reads
35
30
25
20
15
10
5
0
48 mock
48 hai
96 hai
144 hai

Hormone signaling during *P. infestans*-tomato compatible interaction

The pattern of transcript levels associated with the signaling hormones JA and SA which regulate resistance pathways causing significant changes in gene expression (Glazebrook 2005) was determined. There was a modest induction of the transcripts associated to the JA pathway (Figure 3.6A). In contrast there was a large induction of transcripts associated to the SA pathway with a peak observed at 96 hai (Figure 3.6B). To determine whether gene expression correlated with the phytohormone levels we measured the amounts of JA and SA during the time course of the interaction using the method described by Thaler et al 2010. The levels of both JA and SA were significantly different at a p-value <0.05 when compared to the non-inoculated control (Figure 3.6 C and D). The accumulation of JA increased at least three-fold at 48 hai and continued to increase throughout the interaction (Figure 3.6C). There was a five-fold induction of SA hormone level at 48 hai but this was followed by a gradual decline from 96 hai through 144 hai (Figure 3.6D).

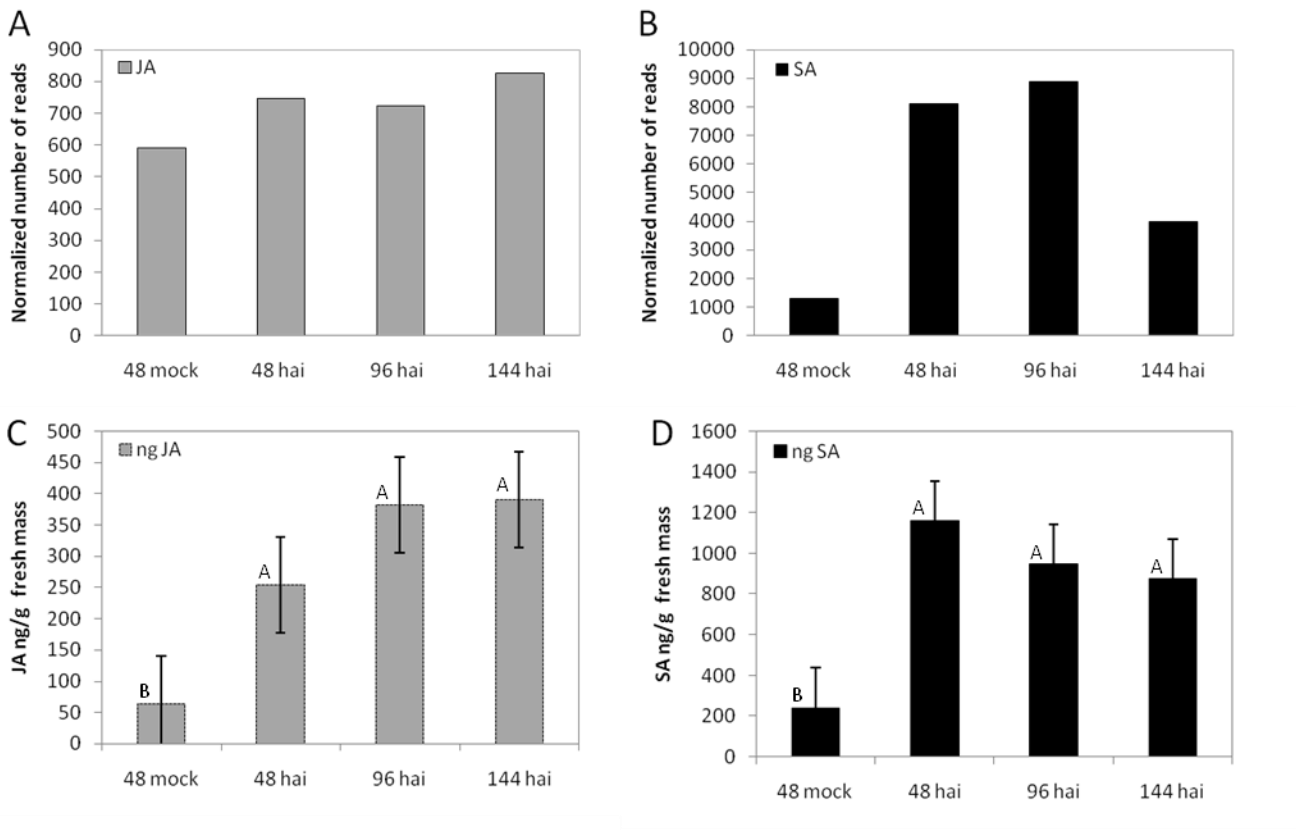


Figure 3.6 Quantification of jasmonic acid (JA) and salicylic acid (SA) levels by two different methods. A and B: normalized number of reads for genes previously described to be involved in the synthesis of JA and SA. Lower graphs (C and D) show the quantification by LC/MSMS analysis (n=3). The data were transformed with log10 and a student's-t test was used to analyze the difference in phytohormone production. Different letters represent significant differences at a $p<0.05$.

Evaluation of tomato mutants in jasmonic acid pathway

Because of the high induction of JA in response to infection by *P. infestans,* we hypothesized that JA production might be a component of tomato defense against this pathogen. To test this hypothesis we used two mutants. The first was the fatty acid desaturase mutant (*spr2*) required for the biosynthesis of JA (Li et al 2003), and the second was the *def-1* mutant which is defective in octadecanoid metabolism and is unable to accumulate JA (Howe et al 1996). We used a student's t-test to determine whether tomato plants hampered in biosynthesis and accumulation of JA were more susceptible to *P. infestans* than the wild type counterpart. Measuring percentage of diseased area, seven days after infection, it was determined that JA mutant plants were significantly more susceptible at a p-value <0.05 when compared to the wild type Castlemart inoculated control (Figure 3.7) suggesting a role for JA in defense to this pathogen.

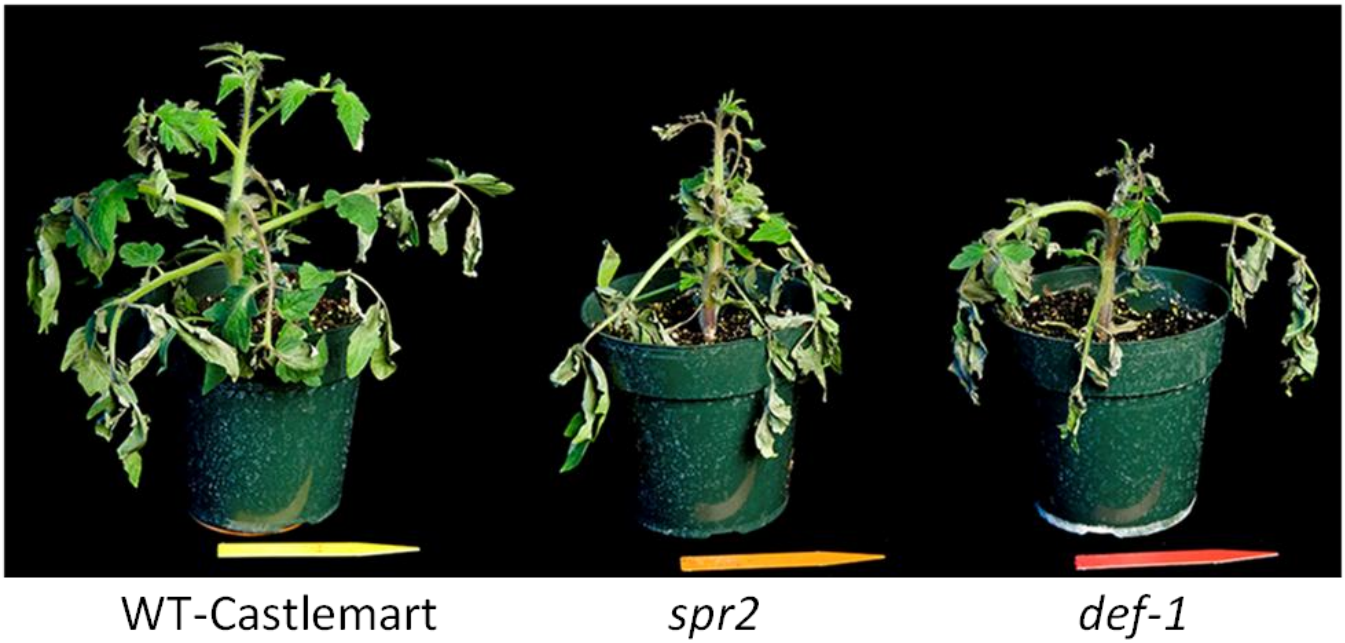


Figure 3.7 Tomato (cultivar Castlemart) wild type (wt) and mutants impaired in the biosynthesis (*spr2*) and accumulation (*def-1*) of jasmonic acid infected with *P. infestans*. Both mutants were significantly more severely infected than was wild type (n=6 per genotype in two independent trials $p<0.05$). Photo was taken 7 days after inoculation. All plants were the same size when inoculated.

3.4 Discussion

Analysis of the tomato transcriptome during the interaction with *P. infestans* using the 454 pyrosequencing platform generated nearly 94,000 unigenes, most of which were singletons. This number is much higher than the 35,000 genes currently predicted for the tomato genome (http://solgenomics.net/genomes/Solanum_lycopersicum/genome_data.pl#annotation). It is likely that many of these sequences are the product of alternative splicing or alternatively the unmatched sequences may reflect: incomplete coverage of the tomato genome sequence, non-coding RNAs, fusion transcripts, UTR sequences that were not included in the predicted genes or relatively short and low quality singletons. It is important to note that these numbers of unmatched sequences is consistent with what has been reported for other annotated genomes (humans: Mane et al 2009; Arabidopsis: Weber et al 2007; and cucumber: Guo et al 2010; *P. infestans*: Chapter 2).

This study confirmed that infection of a plant by a pathogen involves a complex regulation of gene expression for both sides of the interaction. However, our study expanded the understanding of this complexity, demonstrating that thousands of genes (close to 12,000) and genes encoding proteins for almost 200 pathways were responsive. Transcript reduction of genes involved in primary metabolism (photosynthesis and glycolysis) and the induction of transcripts of genes related to fermentation, starch degradation and mitochondrial related genes suggested a major shift in host physiology in response to infection by *P. infestans*. The reduction of photosynthesis-related gene expression at 48 hai is unlikely to be directly related to plant cell death, since there were no apparent symptoms at this point. It has been well documented that defense induction in plants leads to rearrangements in the plant physiology and metabolism after

pathogen infection (Berger et al 2007); with these data we can begin to quantify that response in terms of gene expression.

Several layers of defense response are activated in the plant after pathogen infection. Putative tomato PRRs involved in basal defense, were identified, most of which were annotated as hypothetical proteins and collectively showed variation in the timing of expression. The abundance of some of the putative PRR transcripts increased throughout the interaction (e.g. GBP-like involved in oomycete recognition in soybean against *P. sojae* Fliegmanna et al 2005). This late activation (up to 144 hai) supports the idea of a potential sustained activation of PRRs (Lu et al 2009). FLS2 and EFR have been implicated in bacteria perception by recognizing bacteria flagellin and bacterial elongation factor-Tu respectively (Gomez-Gomez and Boller 2000; Zipfel et al 2006). Thus, future work will be directed towards addressing possible *P. infestans* PAMPS targeting FLS2-like and EFR-like homologs in tomato. Although EFR has been found only in the Brassicaceae family (Segonzac and Zipfel 2011), the finding of a putative EFR-like in tomato is consistent with Boller and Felix, who found LRR-RKs homologs to EFR encoded in the poplar and rice genomes (Boller and Felix 2009).

One of the earliest responses to a pathogen is the induction of oxidative stress. ROI have been associated with calcium signaling, papillae formation and reinforcement of the cell wall (basal defense), the induction of HR (Heath, 1998; Richberg et al., 1998) and with SAR (Bolwell and Daudi 2009; Torres et al 2006). After pathogen infection there was a decrease in transcript abundance of genes associated with the production of ROI. The suppression was maintained through the transition stage, but was somewhat relieved in the necrotrophic stage. The mechanism for this repression is not yet known.

Pathogens that are able to suppress or evade basal defense, encounter another layer of defense mediated by R genes. In this study, transcript abundance of 108 putative disease resistance genes (homologs to TIR- and CC-NBS-LRR genes) increased after pathogen infection. However, in this case they were not able to prevent pathogen infection. This might be because none of these *R* gene products recognized any of the effectors secreted by this genotype of *P. infestans* or that if they were recognized, the defense responses triggered in the gene for gene interaction were blocked by another pathogen effector(s) as described by (Halterman et al 2010) for *ipiO1*-RB. Our results seem to suggest that the plant activates an array of defense mechanisms as early as 48 hai. However, in this compatible interaction, *P. infestans* is able to overcome or avoid such defenses.

Another important component of the plant defense responses is their regulation by SA and JA. It has been reported that plants challenged with hemibiotrophic pathogens induce the SA pathway at early stages of the interaction and the ET/JA pathways are activated at later stages (Glazebrook, 2005). While there was a significant increase in SA levels at 48 hai, JA levels were also highly induced at the biotrophic stage and continued to increase throughout the interaction until the necrotrophic stage. Because of the high levels of SA and JA after *P. infestans* infection, we inferred that the resistance pathways regulated by these phytohormones were induced, but the plant was unable to arrest pathogen growth. Similar results have been found in the *Arabidopsis* response against a hemibiotrophic pathogen *Colletotrichum higginsianum*; after inoculation with a virulent strain there was induction of both *PR1* and *PDF1.2* genes suggesting that the SA and the JA pathways were activated (Liu et al 2007), differing from the model proposed for an incompatible interaction (Glazebrook 2005). Therefore, future work determining the regulation

of both SA and JA in an incompatible or partially compatible interaction between *P. infestans* and tomato will give insight into how tomato regulates these phytohormones against this hemibiotrophic pathogen.

While it has been reported that SA deficient plants are more susceptible to growth of *P. infestans* (Halim et al 2007), it has been suggested that this is not the case for JA deficient potato plants (Halim et al 2009). In addition, JA was not induced in either the susceptible potato cultivars or in a partially resistant potato cultivar after inoculation with *P. infestans* (Weber et al 1999; Gobel et al 2002), while JA was induced after the incompatible interaction between *Pseudomonas syringae* and potato leading the authors to hypothesize that JA mediated defense responses occur only in non-host pathogen interactions. These results differ from studies by Cohen et al (1993), who demonstrated that induction of JA enhanced the resistance of tomato and potato against *P. infestans*.

In this study, we provide evidence that JA levels are induced during a compatible interaction between *P. infestans* and tomato. Therefore, to evaluate whether JA mediated defense responses are important in tomato against *P. infestans*, two tomato mutants that are deficient in JA accumulation *spr2* (Li et al 2003), or biosynthesis *def-1* (Howe et al 1996) were used. We determined that JA mutants were significantly more susceptible to *P. infestans* than the wild type. Together, these data suggest that the JA pathway may play a role in an effective defense against *P. infestans*. Four-week-old tomato *NahG* mutants (Brading et al 2000) showed such a severe necrotic phenotype, that it was not possible to determine whether mutants deficient in SA accumulation were compromised in their response to *P. infestans*. This study provides evidence

for a difference in JA regulation and requirement for host resistance between tomato and potato against *P. infestans*.

The key outcome of this study was the generation a comprehensive tomato transcriptome in a time course of the interaction with *P. infestans.* It allowed us to gain insight into the activation of different components of defense in a susceptible tomato, against *P. infestans*. Transcripts of genes for both PTI and ETI were induced as early at 48 hai. In addition, SA and JA levels increased after inoculation, suggesting that defenses mediated by these phytohormones were induced as well and a difference in response between tomato and potato during a compatible interaction with *P. infestans* was determined. Future investigations on the regulation of this complex response should provide insights for creating durably resistant plants.

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CHAPTER 4 – Future work

4.1 Pathogen investigations

The transcriptomes of the interaction between tomato and *P. infestans* provided insights into both the host response against this pathogen as well as the pathogen's mechanisms for overcoming the host's defenses. These data provide many additional options for continuing investigations. An obvious next step in the characterization of *P. infestans* would be to study the expressed cytoplasmic effectors obtained in the time course of the interaction. Using *N. benthamiana* as a heterologous system one could test function by agro-infiltrations of all the RXLRs and CRNs identified in this study. Some of the hypothetical secreted proteins with a high number of transcripts at each stage should be assessed as well.

Subsequently, one could investigate the targets of the candidate effectors in the plant cells. One approach would be to use a yeast two hybrid system to determine which plant or pathogen proteins interact with effectors. To this end, one would generate cDNA libraries from each of the three different stages of pathogenicity. Results from these studies will contribute in the understanding of how *P. infestans* overcomes the plant defenses.

Despite the importance of host specialization and aggressiveness differences in *P. infestans*, there are currently no reported studies that address host specificity across species at the molecular level. An approach to address this type of specificity might be to study the pathogen's effectors and then determine whether there is a correlation with the type, timing and/or abundance of the effectors secreted into the host. One could apply this approach to all of the diverse hosts, but because tomato and potato pathogenicity are so important, it would be

interesting to investigate difference in effector patterns in these two hosts. In addition, because there are also differences among isolates of *P. infestans* it would be interesting to determine if the differences in effector profiles during infection of potatoes and tomatoes by isolates that are specialized, or not specialized, to tomato, or potato. One would learn if there is a difference in the effectors secreted in tomato versus potato isolates, or if there is a core of effectors among clonal lineages that might explain host specificity or aggressiveness. Probes of some putative effectors obtained in this study can be designed to perform real time PCR (RT-PCR) and used to investigate differences of both expression differences among isolates and timing of expression in different hosts.

There is a difference of pathogenicity in *P. infestans* when it was growing in plant tissue compared to growing in culture (Mizubuti et al 2000). When *P. infestans* grows in plants, it is very aggressive and sporulates profusely, while *P. infestans* growing on culture loses its virulence. I am currently addressing this observation by testing several candidate genes, obtained from this work and determining whether there is a difference in expression of these candidates between culture-grown isolates versus *in planta* grown isolates.

Carbonic anhydrase (CA) seems to have a complex role in the interaction between *P. infestans* and its hosts tomato and potato (Chapters 2 and 3; Restrepo et al 2005). One could test whether this enzyme might be a target to be regulated by *P. infestans*. CA inhibitors could be used to investigate the role of CA in the growth of *P. infestans* in vitro as an initial approach. Acetazolamide and sulfanilamide, two such carbonic anhydrase inhibitors that have been used to

successfully inhibit *P. falciparum* growth (Krungkrai et al 2001), can be tested in *P. infestans* to determine if they have an inhibitory effect on growth of this pathogen as well.

4.2 Host investigations

The tomato transcriptome provided a broad overview of the physiological changes occurring in the plant; having this information, we can start to address the differences between compatible and partially compatible interactions. We started the project with two tomato isogenic lines: IL 6-2 and M82, which are partially resistant and susceptible to *P. infestans*, respectively (Smart et al 2007). In addition to collecting *P. infestans*-M82 leaves during the biotrophic, transition and necrotrophic stages of the interaction, *P. infestans*-infected leaves of IL 6-2 were also collected. However, 454 sequencing was done only on the susceptible host (M82). Since the IL 6-2 RNA is available, it possible to do comparative analyses for both the host and pathogen genes expressed overtime in this host. By using real time PCR with some candidate genes, it can be determined whether some plant genes are either expressed earlier, or at higher levels, in the partially resistant cultivar than in the susceptible cultivar, or if they have some mutations that allow evasion of pathogen effectors that suppress the defense response. Likewise, a comparison among pathogen genes that are expressed during the interaction with these two different hosts can be made as well as determination of whether are differences in abundance and timing of expression in the partially resistant host.

Salicylic acid (SA) and jasmonic acid (JA) are important components of disease resistance in plants. We have the SA and JA profile in a compatible interaction, but to understand how SA and JA are regulated in a partially compatible interaction, and to determine if there is a temporal

regulation of these phytohormones after inoculation with *P. infestans*, we can compare how SA and JA are regulated in a partially resistant tomato host after inoculation with *P. infestans*.

4.3 Quantitative resistance investigations in *A. thaliana*

To determine the genetic basis and the mechanisms of action of quantitative resistance loci (QRL) in *A. thaliana*, the next step in the characterization of the QRL obtained on chromosome III, which spans a region of 1Mb and the QRL on chromosome V that spans a region of 9Mb, would be fine mapping (combined with association mapping) to narrow the region to a smaller number of candidate loci. Once the candidate list is reduced to few genes, *Arabidopsis* mutants can be evaluated for disease resistance or susceptibility against *Hpa*. However, mutants in the defensin-like gene and the two *ZAR1*genes from the QRL on chromosome III can be tested to determine their contribution on the resistance phenotype against *Hpa*.

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APPENDIX A – Characterizing the *Arabidopsis thaliana-Hyaloperonospora arabidopsidis* interaction

Abstract

The objectives of this project were to identify quantitative resistance loci (QRL) to *Hyaloperonospora arabidopsidis* in *Arabidopsis thaliana* and determine the genetic basis and the mechanisms of action of these QRLs. In order to identify QRLs, we screened 30 *Arabidopsis* ecotypes with three strains of *H. arabidopsidis* to find differences in resistance to these strains. From a total of 90 *At-Hpa* interactions, more than 50% were resistant. Two ecotypes that showed differences in their response to the *H. arabidopsidis* strain *Emwa*-1 were chosen as the parents for the cross. *Bay-0* was the partially resistant parent and *Tsu-1* was the susceptible parent. From this cross, the F_2 (207 individuals) was evaluated phenotypically for the segregation of resistance to *Emwa*-1. Using composite interval mapping we found two QRL-- one on chromosome III and the other on chromosome V.

A.1 Introduction

Plants have evolved mechanisms to detect and respond effectively to an array of pathogens. In an agricultural setting the durability of resistance is subjectively defined as a resistance that is widely used, for a long period of time and under pathogen pressure (Johnson 1981). Two main categories for disease resistance have been identified in plants: qualitative resistance (also known as complete resistance, vertical resistance and gene for gene, among others; Flor 1971; Maor and Shirasu 2005) and quantitative resistance (known as incomplete resistance, horizontal resistance and quantitative resistance loci-QRL among others; Young 1996). In qualitative resistance the direct or indirect recognition of pathogen-encoded effectors by plant resistance (R) genes is often

associated with a rapid localized programmed cell death called the hypersensitive response (HR) that inhibits pathogen growth. Quantitative resistance on the other hand is associated with many genes of small effect that reduce pathogen infection and/or colonization (Young 1996). Research in QRL suggests that in some cases these two types of disease resistance might be controlled by the same genetic mechanisms instead of two completely different ones (for a review see Poland et al 2009). The concept of defeated R-genes is where an R gene that has been overcome by the pathogen (blocking the defense response of the plant or avoiding recognition) has a residual effect (Tan et al 2008; Rauscher et al 2010; Mayton et al 2011).

For instance, short durability of resistance has been seen when studying the *Phytophthora infestans*-potato/tomato pathosystem (Hein et al 2009). Despite the effort of potato breeding programs to screen wild *Solanum* accessions and the cloning of more than 30 R genes against *P. infestans*, resistance against this pathogen remains elusive (Hein et al 2009). The high rate of pathogen evolution and adaptability has overcome the deployed R genes (Hein et al 2009). Because QRLs have a smaller effect on resistance and might be conferred by several genes with partial effects it is presumed that the pathogen will be under a lower selection pressure and pathogens that overcome the QRLs will gain only a small advantage (Niks and Rubiales 2002; Poland et al 2009).

Thus, QRLs might be more durable in the field which is a desirable trait in breeding programs for agriculturally important crops like potato and tomato against *P. infestans* (Brower et al 2004; Bradshaw et al 2008; Smart et al 2007; Rauscher et al 2010). However, genetic bases of the mechanisms of QRLs resistance are not well understood (Richardson et al 2006; Poland et al

2009) and there is evidence that several components are responsible for the QRLs. Poland et al (2009) reviewed six different hypotheses that explain QRLs: defeated R-genes; morphological and developmental phenotypes; the production of toxic compounds or inhibitors that detoxify the plant against these toxic compounds; mutations of genes involved in basal defense; defense signal transduction; and previously unidentified genes without a sequence similarity to any reported defense gene.

The availability of the genome sequences from model plants could allow us to determine the genetic basis of quantitative resistance (Richardson et al 2006). Studying the model pathosystem *Arabidopsis thaliana* (*At*) with its natural pathogen *Hyaloperonospora arabidopsidis* (*Hpa*) can help us understand some of the mechanisms of QRL resistance. *Hpa* is an obligate biotrophic oomycete, which causes downy mildew of *At* in the wild. There is a high level of phenotypic variation in this pathosystem that could reflect the natural co-evolution that has taken place between the host and pathogen (Coates and Beynon 2010). Single dominant R genes, partial dominance, additive and genetic epistasis have been postulated as the main factors driving this high level of phenotypic and genotypic variation in this pathosystem (Holub et al 1994; Nemri et al 2010). This pathosystem has been used as a model to study qualitative resistance. One of the major contributions of studying this host-microbe interaction has been the mapping of 27 R-genes on the five chromosomes, and six have been cloned (Coates and Beynon 2010). Nemri and coworkers (2010) used linkage and genome wide association mapping to study natural variation to *Hpa* in *At*. They surveyed 96 ecotypes with five different *Hpa* strains and found that resistance is mainly attributed to R genes. However, some QRLs were also found and the advantage is that since the *At* genome is sequenced, many markers can be utilized to narrow the QRL region to a

few candidate genes, thus enabling us to study the genetic basis of quantitative resistance. Here we report the occurrence of two QRL in *At* against *Hpa* -- one in chromosome III and the other in chromosome V. The genes in each QRL provide candidates for additional investigation.

A.2 Materials and Methods

Plant material

Thirty Arabidopsis thaliana ecotypes were kindly provided by Dr. M. Nasrallah and Dr. R. Loria at Cornell University. Seeds were vernalized at 4°C for 3 days in the dark on petri dished with a humid filter paper. After the vernalization period, seeds were sown on the surface of 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 and then transferred to a growth chamber at 18-20 °C with short days light (10 hours light/14 hours dark).

Inoculum preparation and *Hyaloperonospora arabidopsidis* isolates

H. arabidopsidis (*Hpa*) isolates Emwa-1, Emco-5 and Noco-1 (kindly provided by Dr. D. Klessig) were maintained continuously on seedlings to provide consistent inoculum for the inoculations. Arabidopsis leaflets with sporulating downy mildew lesions were detached and submerged into 25ml of chilled distilled water and then vortexed vigorously for two minutes to detach conidiophores. The concentration of conidiophores was determined by using a hemocytometer and then adjusted to 1×10^6 conidiophores per ml. Two weeks-old seedlings were sprayed-inoculated until run-off. Plants were incubated for two more weeks at 15 °C and covered with a clear plastic lid to maintain high humidity.

Pathogen observation using Trypan blue staining

Hpa development in the leaflet was observed using trypan blue staining, Knox-Davies, (1974) modified by Chung C. (2010). Briefly, leaflets were submerged in a clearing solution A (acetic acid:ethanol, 1:3 v/v) overnight. After 16 hours, the clearing solution A was discarded and replaced by clearing solution B (acetic acid:ethanol:glycerol, 1:5:1 v/v/v) for three hours. Clearing solution B was replaced by staining solution (0.01% trypan blue in lactophenol) overnight. The staining solution was removed and leaves were rinsed with 60% sterile glycerol. After rinsing, the glycerol was removed and new 60% glycerol was added to the leaflets for two hours before microscopic observation.

Evaluation of disease resistance

Thirty *Arabidopsis* ecotypes were inoculated with three *Hpa* isolates and evaluated for disease resistance (see table1). Two weeks after inoculation, plants were assessed qualitatively and quantitatively. For the qualitative assessment, plants were rated as resistant (no symptoms seen), partially resistant when either necrosis (brown patches) or chlorosis (yellow discoloration) was present and susceptible if some conidiophores were produced. The quantitative assessment was done by determining the number of conidiospores produced on each plant two weeks after inoculation. To this end, the whole plant was harvested and then vortexed vigorously in a 2 ml eppendorf tube with one ml of distilled water for one minute to detach conidiospores. Subsequently the conidiospore concentration was determined using a hemocytometer.

DNA extraction

Genotyping was performed on DNA from one leaflet of each of the 207 individual F_2 progeny. Prior to inoculation, a leaflet was harvested from a two-week-old seedling and flash frozen in liquid nitrogen. The frozen leaflet was then ground using a mortar and a pestle in liquid nitrogen. DNA was extracted using CTAB (cetyltrimethylammonium bromide) extraction buffer (2% CTAB, 1.4 M NaCl, 100mM Tris at pH 8, and 20mM EDTA at pH8 with 0.2% β-mercaptoethanol). Five hundred µl of CTAB were added to each sample and incubated for 15min at 60°C. DNA was extracted with 400µl of phenol chloroform isoamyl-alcohol 25:24:1 and centrifuged for 5min at 12000xg. The supernatant was transferred to clean tubes. Isopropanol (300 µl) was added to the mixture and held for one hour at -20°C to precipitate the DNA. Samples were then centrifuged for 5min at 600xg. DNA was cleaned with 70% ice cold ethanol and centrifuged for 5min at 600xg followed by an additional clean up step using 300 µl of 90% ethanol and centrifuged for 5min at 600xg. DNA was resuspended in100µl of H_20. For the PCR, a total of 20ng was used per sample.

Genotyping

Molecular markers were selected from TAIR (www.arabidopsis.org). We selected CAPS, SSLP, and SNPs on each of the five chromosomes of Arabidopsis. The polymorphic markers on *Bay-0* and *Tsu-1*(parents) were assessed on the 207 individuals of the F_2 progeny (Table A.2).

Mapping and QRL analysis

QRL mapping was done for one component of resistance, the spore count. Linkage analysis was performed using MapDisto (Lorieux 2007). Haldane map was done with a minimum LOD score

of 3.0 and a recombination frequency of 0.3 (see figure 3). A composite interval mapping was done to identify QRL using QTL Cartographer (Xu and Hu 2008) (Figure A.4).

A.3 Results

Resistance in *A. thaliana (At)* against *H. arabidopsidis (Hpa)* isolates

We assessed disease resistance in 30 *At* ecotypes with three *Hpa* isolates for a total of 90 *At-Hpa* interactions (Table 4.1). Of these, only 16 showed susceptibility (S) as measured by conidiophore formation. Twenty seven interactions were partially resistant (intermediate: I), defined as the capacity of the pathogen to cause necrosis and/or chlorosis but with no conidiophores. However, in more than 50% of the interactions, the outcome was resistance.

Table A.1 Resistance of 30 *Arabidopsis thaliana* ecotypes in response to three isolates of *Hyaloperonospora arabidopsidis*. The reactions were Susceptible (S), partially (intermediate) resistant (I) and resistant (R), as defined in the text.

Ecotype / Strain	Emco-5	Emwa-1	Noco-1
BAY-0	S	I	I
C-24	R	R	R
Cta	R	R	R
Ct-1	R	R	R
COL	I	R	S
CONO-1	I	R	S
CS3879	I	R	S
CTA-0	R	R	R
CVI	R	I	S
DI-3	R	R	R
DI-17	R	R	R
EST-0	R	R	R
EST-1	I	I	I
GY-0	R	S	R
HODJA	R	R	I
KAS	R	I	I
KASHMIR	R	R	I
KIN-O	R	R	S
LER	R	R	R
LLO	I	I	I
MT-0	R	R	R
MZ-0	I	I	I
Nd-1	I	S	S
NO	S	I	R
SHAH	R	R	R
SORBO	I	I	I
TSU1	S	S	S
VAR-0	I	R	R
WS	I	S	I
WT-5	R	R	R

Parents and mapping population

Two ecotypes that showed a distinct response to the *Hpa* strain *Emwa*-1 were selected as the parents to generate a mapping population. These were *Bay-0* and *Tsu-1*. *Bay-0* was partially resistant showing some chlorosis and necrosis after inoculation but no conidiophores were seen after inoculation with the *Emwa*-1 isolate (Figure A.1). *Tsu-1* was the susceptible parent; abundant conidiophore formation was seen consistently after inoculation with *Emwa*-1 (Figure A.1). The F_2 progeny consisted of 207 individuals. For DNA extraction, we collected one leaflet from each F_2 plant before inoculation (two weeks old) and flash froze it in liquid nitrogen immediately after collection. This tissue was saved until the genotyping was performed. Two weeks-old F_2 plants were inoculated with *Emwa*-1 and evaluated for disease two weeks later. Quantitative and qualitative ratings were done as we described for the parents. For the quantitative evaluation, the whole plant was collected and the total number of conidiophores was counted. We obtained a normal distribution of the number of conidiophores present in the F_2 progeny suggesting the quantitative inheritance of this trait (Figure A.2).

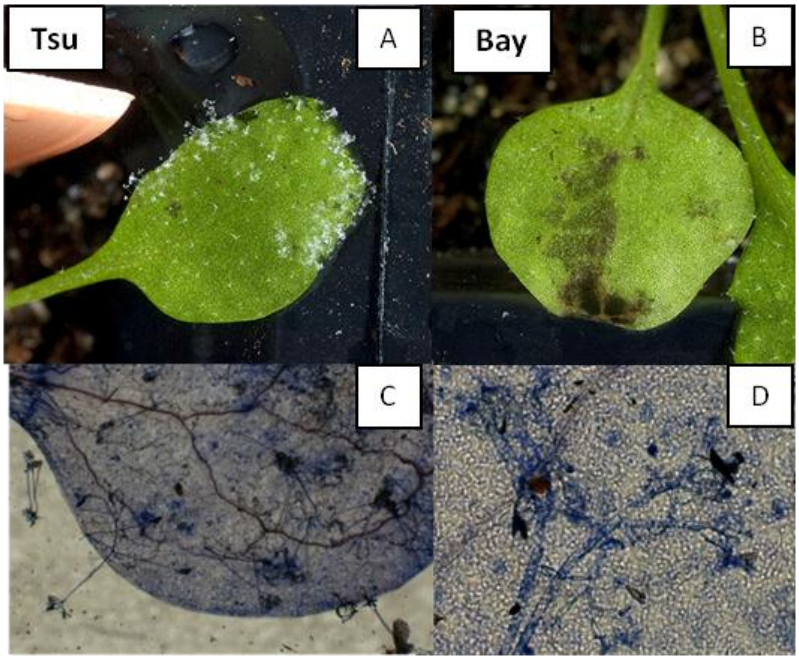

Figure A.1 Difference in susceptibility between *Tsu-1* (susceptible parent) and *Bay-0* (partially resistant parent) after inoculation with *H. arabidopsidis* strain *Emwa*-1. The upper panels A and B show the macroscopic difference in symptoms at two weeks after inoculation. The lower panels C and D show the difference in pathogen colonization and growth between both Ecotypes (trypan blue staining).

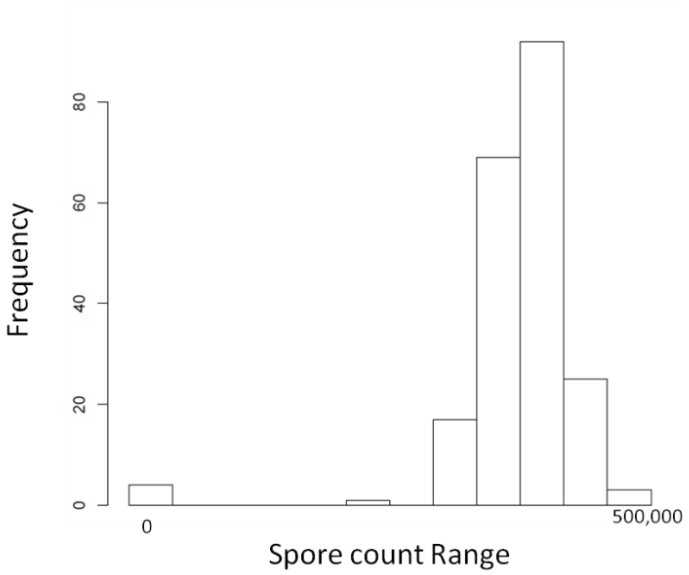

Figure A.2 Phenotypic evaluation of the 207 F_2 individuals from the Tsu-1 x Bay-0 progeny. The total number of conidiospores per plant was counted and the range in numbers of conidiospores for the F_2 individuals is presented.

Mapping and QRL analysis

In order to make the genetic map, we selected molecular markers from the TAIR public available database (http://www.arabidopsis.org/). We selected CAPS, SSLP, and SNPs associated with each of the five chromosomes of *Arabidopsis*. Polymorphic markers between *Bay-0* and *Tsu-1* were evaluated in the 207 F_2 progeny (Table A.2).

Table A.2 Polymorphic markers for *Bay-0* and *Tsu-1* by chromosome (Chr).

Chr-I	Chr-II	Chr-III	Chr-IV	Chr-V
NGA59	G009	NGA32	NGA8	NGA158
NGA5	F7B19	LUGSSLP714	CIW6	NIT4
JV28/29	T10J7	LUGSSLP815	NGA1139	NGA139
ZPFG		ALS	ACM1	CIW9
CIW12		F24M12	CIW7	CIW10
NF5I14		CIW28		JV57/58
		CIW22		
		NGA6		

Linkage analysis was performed using MapDisto (Lorieux 2007). Maps were made using a minimum LOD score of 3.0 and a recombination frequency of 0.3 (Figure A.3). QRL analysis was done using composite interval mapping with QTL Cartographer (Xu and Hu 2008) (Figure A.4).

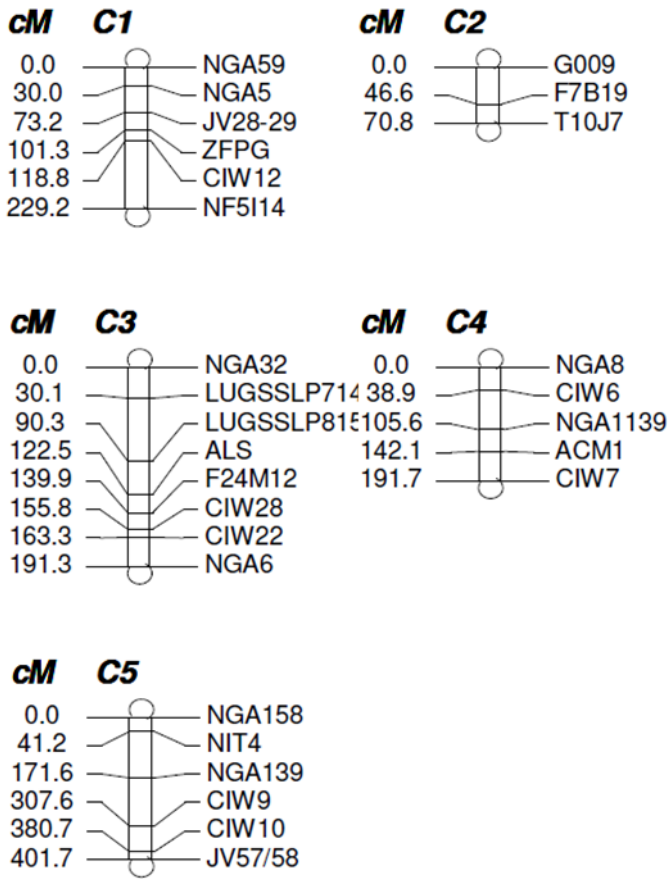

Figure A.3 Genetic map of *A. thaliana* using polymorphic markers for the 207 F_2.

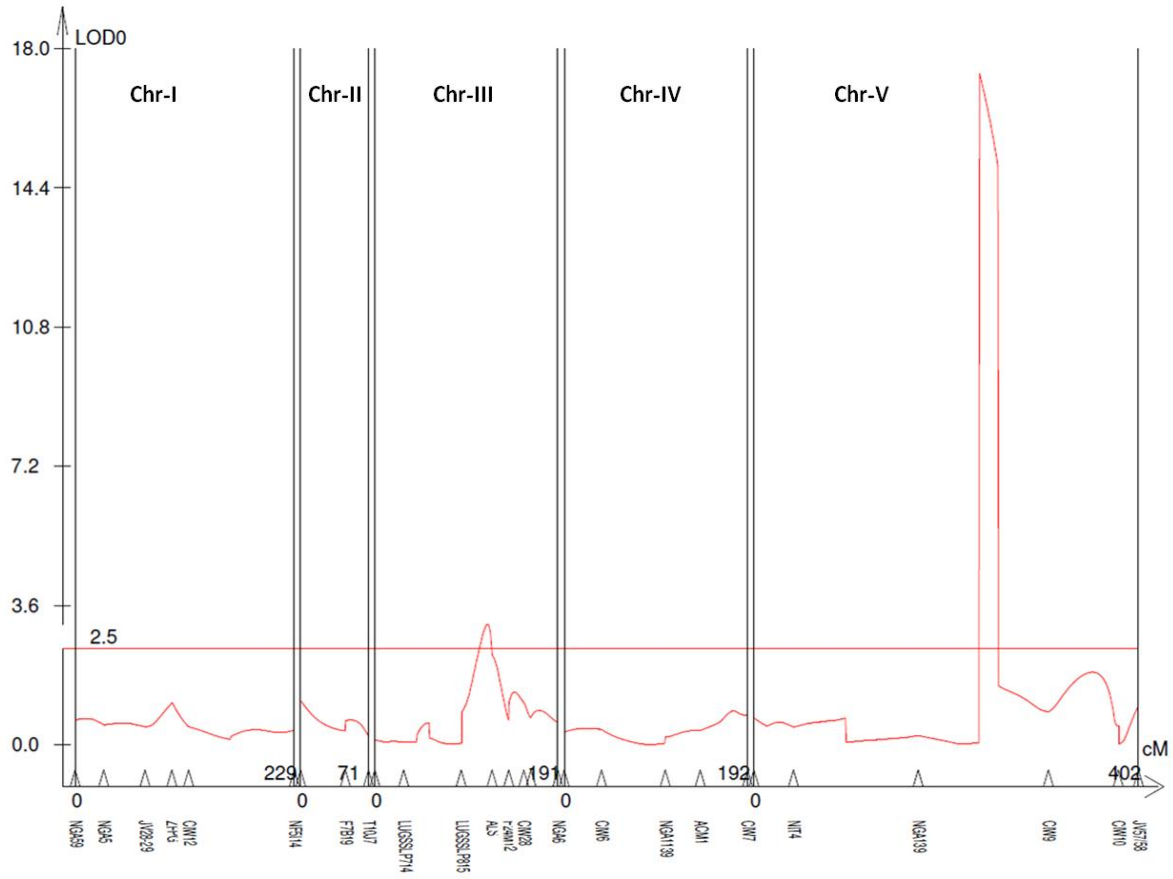

Figure A.4 Composite interval mapping results showing LOD values for two QRL identified in *Arabidopsis thaliana*. Chromosomes (Chr) III and V have a QTL with a LOD of 3 and 17 respectively.

Two QRL were identified using spore number as a criterion. One QRL with a minor effect (LOD of 3) was in chromosome III between markers F24M12 and ALS spanning a region of one Mb composed of 86 loci (supplemental table 1). These 86 loci can be divided into 46 functional categories, 22% were described as unknown proteins/unknown function, one is a defensin-like protein previously involved in biotic stress (De Coninck et al 2010), two ZAR1 which are CC-NBS-LRR type R-proteins which have been involved in the recognition of the HopZ1a Type III effector of *Pseudomonas syringae* (Lewis et al 2010). The other QRL had an LOD of 17 and was found in chromosome V between markers NGA158 and CIW9. This region is very big; it has 2308 loci covering 9Mb of chromosome V (supplemental table 2). Among the genes present in the interval there is a cluster of 16 TIR-NBS-LRR spanning the region between 14.5Mb and 17Mb and one CC-TIR-LRR gene at 12Mb. Close to that region, although outside of the markers interval is the LZ-NBS-LRR type RPP8 R-gene that confers resistance against *H. arabidopsidis Emco5* in Ler (McDowell et al 1998).

A.4 Discussion

Our study is consistent with several others in that we found that resistance was the dominant interaction. In the 90 interactions we observed, 54% were resistant and 30% were intermediate. This is comparable to what has been reported previously for this pathosystem where Nemri and coworkers found that resistance was observed 60% of the time (Nemri et al 2010). However, the percentage of intermediate resistance was higher in our study when compared to the one reported by Nemri and coworkers (4.5% Nemri et al 2010). Differences in rating criteria might account for this difference.

The fact that we obtained a QRL (LOD 3) on chromosome III in a region with defense related genes (defensin and ZAR1) raises the possibility that these genes might contribute to the

resistance phenotype. Very close to this region is *RPP13,* an R-gene which conceivably could contribute to this QRL. This R-gene confers resistance against several strains of *H. arabidopsidis* (Bittner-Eddy et al 2000), and has been identified in *Col-5, Rld-2* and *Nd-1 Arabidopsis* ecotypes. This region of chromosome III has also been identified by Nemri et al (2010) as providing additive, epistatic and direct effects to resistance to different isolates of *H. arabidopsidis*. All these together suggest that a homolog of *RPP13* in the *Bay-0* accession could confer some resistance to the *Emwa-1* strain.

The other region associated with resistance is in chromosome V. This region had a very high LOD score (17) and this high LOD might be an indication that the actual resistance is due to an R-gene rather than several genes contributing to the resistance phenotype. This is entirely possible because there is an R-gene cluster with 16 members of the TIR-NBS-LRR class of resistance genes within the interval. In addition, the *RPP8* which confers resistance to *H. arabidopsidis* (McDowell et al 1998) is located very close to this region. However, the facts that this segment spans 9Mb and includes more than 2000 loci mean that there are many potential contributors. Fine mapping (combined with association mapping) is required to narrow the region to a smaller number of candidate loci.

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