Molecular characterization of potato defense suppression by *Phytophthora infestans*

BY

MARIA ANTONIA HENRIQUEZ

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University of Manitoba
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ABSTRACT

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Molecular mechanisms of potato defense suppression by *Phytophthora infestans*
Supervisor: Dr. Fouad Daayf.

Manitoba is the second largest potato producer in Canada after Prince Edward Island. This crop faces many disease problems including the most famous and devastating one, late blight, caused by the oomycete pathogen *Phytophthora infestans*. More than 150 years have elapsed since this disease caused the Irish potato famine, but strategies for managing it often remain unsustainable and costly. With the advent of molecular biology, genetic engineering, and now, genomics-based approaches, it may be possible to initiate studies which will address important questions regarding very complex host-pathogen interactions. Dr. Daayf's lab has shown that the most aggressive strains of *P. infestans* suppress potato defense mechanisms, through transcriptional inhibition of PAL and HMGR pathways. Therefore, the objective of the current study was to carry out a molecular characterization of potato defense suppression by *Phytophthora infestans*. Gene expression profiling was accomplished by developing a new subtractive hybridization (SH)/cDNA-AFLP combinational approach. This approach is a gel-based subtractive hybridization profiling technique that uses the advantages of cDNA-AFLP and subtractive hybridization in order to amplify cDNA products in a polyacrylamide gel and remove the constitutively/commonly expressed sequences. Using this approach differentially expressed genes involved in the potato-*Phytophthora infestans* interaction were identified. These included genes potentially controlling pathogenesis or avr genes in *P. infestans* as well as those potentially involved in potato resistance or susceptibility to

this pathogen. Using the subtractive hybridization (SH)/cDNA-AFLP combinational approach, DOXP-MEP pathway genes were identified for first time in potato and its regulation in response to the oomycete *P. infestans***.** The DOXP-MEP pathway, also called the non-mevalonate route is an alternative terpenoids' biosynthetic route that was discovered first in eubacteria and soon after in photosynthetic organisms such as higher plants, algae as well as in cyanobacteria. In addition, potato plants treated with glucans extracted from *P. infestans*, the elicitor eicosapentanoic acid (EPA) and *P. infestans* isolates were analyzed to study the accumulation of phenolic compounds and expression level of genes from the phenylpropanoid, mevalonate and DOXP-MEP pathway.

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ABBREVIATIONS

SH: subtractive hybridization

TDFs: transcript-derived fragments

ssor: potential pathogenicity factor

ssed: potentially suppressed potato gene

avr: potential *P. infestans* avirulence gene

rtant: potential resistance gene

ssept: potential susceptibility gene

RB: Russet Burbank.

DF: Defender

US8: *P. infestans* strains D1901 (lineage US8, A2 mating type, high aggressiveness)

US11: *P. infestans* strains D-03 (lineage US11, A1 mating type, low aggressiveness)

EPA: eicosapentanoic acid

GL: glucans

PAL: Phenylalanine ammonia-lyase

PAL-1: Phenylalanine ammonia-lyase

PAL-2*:* Phenylalanine ammonia-lyase

4CL*:* 4-coumarate:coenzyme A ligase

CHS*:* chalcone synthase

C4H: cinnamate 4-hyhrolase

HMGR: 3-hydroxy-3-methylglutaryl coenzyme A reductase

SQS: squalene synthase

SC*:* sesquiterpen cyclase

Efactor: Elongation factor

StDXS1: 1-deoxy-D-xylulose 5-phosphate synthase

DXR: 1-deoxy-D-xylulose 5-phosphate reductoisomerase

MCT: 2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase

CMK: 4-(cytidine 50-diphospho)-2-Cmethyl-D-erythritol kinase

MDS: 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase

HDS: (E)-4-hydroxy-3-methylbut-2-enyl diphosphate synthase

SDW: Sterile destilated water

Hpi: Hours post inoculation

IPP: Isopentenyl diphosphate

DMAPP: Dimethylallyl diphosphate

MVK: Mevalonate kinase

PMK: phosphomevalonate kinase

PMD: mevalonate diphosphate decarboxylase

FORWARD

This thesis has been written in manuscript style following the guidelines established by the Department of Plant Science at the University of Manitoba. A general introduction and review of the literatures precedes the four manuscripts that comprise the main part of the thesis. Each manuscript consists of an abstract, introduction, materials and methods, results, and discussion. Thereafter, a general summary and conclusions a list of references cited and appendices follow are presented.

CHAPTER 1

1.1. INTRODUCTION

Potato (*Solanum tuberosum* L.) is the most important food crop from the Solanaceae family (Friedman and McDonald 1997). According to FAO (2007), it is the fourth most important crop after maize, wheat and rice, with an annual production of more than 323 million tonnes. In Canada, Manitoba is the second potato producer after Prince Edward Island, and is followed by New Brunswick, Alberta, Quebec and Ontario (Statistics Canada 2006). Potatoes are vital to the food security of millions of people in the developing world (FAO 2006). Potato is an important source of carbohydrates, proteins, vitamins and antioxidants (Chauvin 2001). However, the negative difference between actual and potential potato yields is caused principally by insufficient inputs, low quality seeds, post-harvest losses, rural infrastructure, and biotic constraints (Zandstra 2002). In fact, among biotic constrains, late blight is the most damaging disease on potatoes, costing farmers $3 billion annually in lost harvest and fungicide expenses in developed countries alone (Zandstra 2002).

Potato late blight is caused by the oomycete *Phytophthora infestans* (Mont.) de Bary (Daayf et al. 2001; Kamoun 2003). The pathogen infects directly by sporangia or through zoospores, affecting leaves, stems, and potato tubers (Goodwin et al. 1998; Vleeshouwers et al. 2000; Judelson and Blanco 2005). Integrated management of late blight on potatoes includes the use of healthy seed, sanitation and cull clean-up, crop rotation, scouting, forecasting techniques, fungicide programs, storage monitoring and

the use of moderately resistant varieties (Pest Management Regulatory PMRA 1996). However, none of these practices is enough to control late blight. The use of fungicides is effective, but it is very expensive and contaminates the environment. New strains of *P. infestans* are more resistant to fungicides and more virulent, causing a worldwide reappearance of the potato late blight disease in the past 20 years (Fry and Goodwin 1997).

The most appropriate and practical way to manage late blight is using resistant varieties. However, developing potato varieties with stable resistance to late blight is complex, because specific known resistance genes can be rapidly overcome by mutants in local populations of *P. infestans* (Fry and Goodwin 1997). Therefore, it is necessary to have a better understanding of the mechanisms governing the interaction between potato and *P. infestans*, in order to develop durable resistance to late blight.

It is possible to initiate studies which may address important questions regarding the very complex host-pathogen interactions between *P. infestans* and its hosts, because of the advent of molecular biology, genetic engineering, and now, genomics-based approaches. Although much research has been conducted on defense mechanisms of potato against *P. infestans*, the molecular mechanisms regulating defense mechanisms remain unclear. However, it is still necessary to develop new approaches to generate solutions against *P. infestans.* In fact, it has shown that the most aggressive strains of *P. infestans* suppress potato defense mechanisms, through transcriptional inhibition of PAL and HMGR pathways (Wang et al. 2004b). Therefore, it becomes important to gain

insights into the molecular characterization of potato defense suppression by *Phytophthora infestans* isolates and effectors, which is the principal aim of the present research proposal.

1.2 LITERATURE REVIEW

1.2.1 Potato (*Solanum tuberosun* L.)

Potato (*Solanum tuberosum* L.) is the most important food crop the Solanaceae family (Friedman and McDonald 1997), which contains 3000 species, including; tomato *(Lycopersicon esculentum* Mill.), tobacco *(Nicotiana tabacum* L.), petunia *(Petunia hybrida* Hort. Vilm.-Andr.), eggplant *(Solanum melongena* L.) and garden pepper *(Capsicum annuum* L) (Bonierbale et al. 1988). According to the FAO (2007), it is the fourth most important crop after maize, wheat and rice, with an annual production of more than 323 million tonnes.

Potatoes were domesticated in the highlands of South America about 8000 years ago (Hawkes 1978). They had a monophyletic origin from a wild species of *Solanum brevicaule* in Peru (Spooner 2005). The first record of cultivated potato outside South America was in the Canary Islands in 1567 (Ríos et al. 2007) and the Spanish explorers brought the plant to Europe in 1573 (Romans 2005). Potato cultivation then expanded throughout Europe and worldwide.

Potato is an important source of carbohydrates, proteins, vitamins and antioxidants (Chauvin 2001). In addition, potato can be grown extensively under tropical,

subtropical and temperate environments, as well as at a range of altitudes due to its ability to adapt (Doehlonan and Sleper 1995). In all of theses environments, potato is affected by damaging diseases, such as *Phytophthora infestans, Alternaria solani, Sclerotinia sclerotiorum, Botrytis* sp., *Verticillium albo-atrum,* and *Verticillium dahliae, Streptomyces scabies*, *S. turgidiscabies*, *Fusarium sambucinum*, *Rhizoctonia solani, Pythium sp., , Helminthosporium solani, Erwinia carotovora,* Leaf Roll Virus, and Mosaic Virus (Stevenson et al. 2001) causing important losses in its production.

1.2.2 Potato Late Blight

Late blight caused by the oomycete *Phytophthora infestans* (Mont.) de Bary is the most devastating disease of potato and was responsible for the Irish potato famine in 1845 (Fry and Goodwin 1997). Annual potato crop losses due to late blight is estimated to be $6.7 billion per year in potato-growing areas worldwide (Haverkort et al. 2008). The first reported appearance of late blight in Canada was in the 1940's (Bourke 1969), but the emergence of late blight as an important disease in Manitoba and across Canada was in the 1990's resulting from a population shiff from the pre-existing A1, metalaxyl-susceptible strains to new metalaxyl-insensitive A1 and A2 strains (Goodwin et al. 1995; Chycoski and Punja 1996; Daayf et al. 2000). In particular, the A1 genotype US-1 was displaced by the A2 genotype US-8 of *P. infestans* (Peters et al. 1998; Peters et al. 1999). This later strain is more virulent and exhibits increased aggressiveness (Kato et al. 1997; Lambert and Currier 1997).

The migration of metalaxyl resistant isolates of *P. infestans* from Mexico to North America and the new appearance of more aggressive genotypes of *P. infestans* changed

the late blight management strategies (Fry and Goodwin 1997). In orden to reduce the pathogen populations (survival, dispersal and reproduction), cultural control measures can be used to reduce pathogen. The cultural methods consist of: the use of clean seed, elimination of volunteer plants, management of plant nutrition, and the use of crop rotations (Garrett and Dendy 2001).

The most appropriate and practical way to manage late blight is using resistant varieties. Nevertheless, developing potato varieties with stable resistance to late blight is difficult, due to the ability of the funsgus to rapidly overcome specific resistance genes (Fry and Goodwin 1997).

1.2.3 The disease and life cycle of *Phytophthora infestans*

Phytophthora infestans is considered a specialized pathogen limited to potato (*Solanum tubersum*) and tomato (*Solanum lycopersicum*). However, natural infection of plants outside these genera has been reported (Erwin and Ribeiro 1996). According to van West and Vleeshouwers (2004), *P. infestans* infection generally starts in leaves and occasionally on stems. Leaf symptoms consist of water-soaked lesions that turn dark and expand rapidly, generating the total destruction of the plant. Infection typically begins when a sporangiophore is formed on the infected leaves and a zoosporangium or sporangium is released (Fig 1.1). Zoospores and sporangia penetrate the leaf surface either through stomata or directly through the epidermal cell wall. Sporangia germinate mainly at temperatures above 12°C. On the other hand, during wet conditions and temperatures below 12°C, zoospores are released, encyst and produce germ tubes. The germ tube is able to differentiate into an appressorium and a penetration peg is formed to

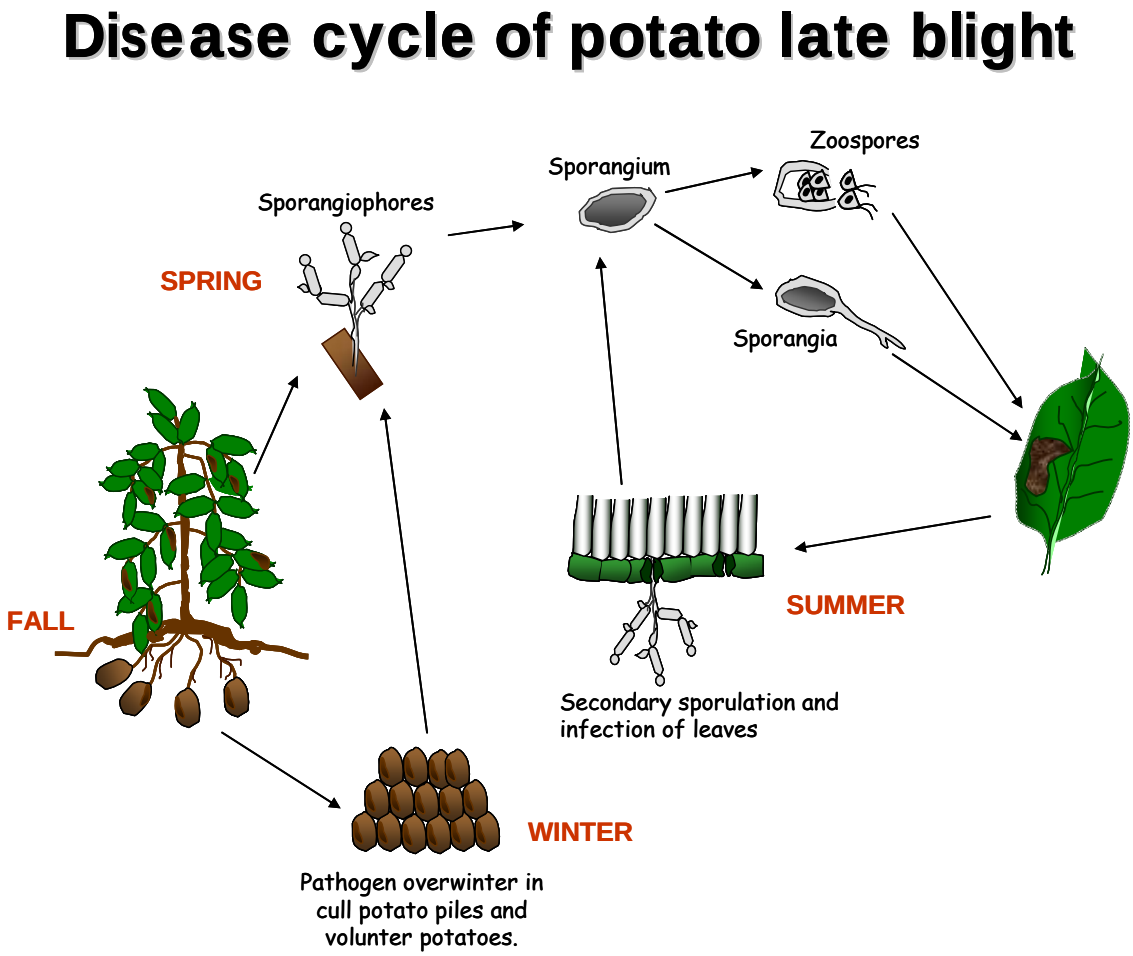

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Figure 1.1. Disease cycle of potato late blight.

aid the passage of the pathogen through the host cell wall. Occasionally, intracellular haustorial structures are formed. After three or four days, secondary sporulation and infection of leaves occurs, to initiate once more the disease cycle. *P. infestans* is heterothallic, with two known mating types, Al and A2. Therefore two strains from different mating types are required to complete the sexual cycle.

1.2.4. *Phytophthora infestans* effectors

Molecules named as avirulence, virulence, elicitor, and toxin are currently known as effectors. The term effector is neutral and does not involve a negative or positive impact in the plant-pathogen interaction. An effector with a virulence function in a specific host may have an avirulence activity in a different plant genotype (Espinosa and Alfano 2004). Therefore, it is feasible to state that plant pathogens have the ability to manipulate biochemical, physiological and morphological processes in their host plants through a diverse array of extracellular effector molecules that can either promote the infection or trigger defense responses (Torto et al. 2006a). Many of these effectors are soluble glucans, glycoproteins or proteinaceous compounds synthesized constitutively by the fungal pathogen. However, many can be surface components released from the cell wall of the microbe or the host (Cote et al. 1998; Radman et al. 2003; El Modafar et al. 2006; Wolski et al. 2006).

The *P. infestans* genome, with an estimated size of 240 megabases (Mb) has large complex families of effector genes encoding secreted proteins that are implicated in pathogenesis (Kamoun 2006; Haas et al. 2009) These effectors can accumulate in the

plant intercellular space (apoplastic effectors) or be translocated directly via exocytosis into the interfacial matrix, and some of them enter the plant cell (cytoplasmic effectors) (O'Connell and Panstruga 2006; Haas et al. 2009). Examples of *Phytophthora infestans* effectors include the apoplastic effectors EPI1, EPI10, EPIC1, EPIC2B and the cytoplasmic effectors Avr3a, CRN1, CRN2 and CRN8 (Torto et al. 2003; Tian et al. 2004; Tian et al. 2005; Bos et al. 2006; Win et al. 2006; Tian et al. 2007).

1.2.5 Potato defence against *Phytophthora infestans*

1.2.5.1 Innate immunity and effector-triggered immunity

In order to infect a plant, a pathogen should be able to make its way into and through the plant tissue, get nutrients, and counteract the defense reactions of the plant. The first barrier is the plant cell surface. Once pathogens are in contact with the host plant, the penetration could occur directly through the plant surface or by natural openings, e.g., stomata, hydathodes or wound sites (Agrios 2005). If pathogens get access into the host by penetrating the plant cuticle, the next barrier in the apoplast is plant-secreted degrading defense enzymes or antimicrobial compounds (Hückelhoven 2007). Additionally, pathogens need to suppress defense responses to access the cytosol and obtain nutrients from the plant (Göhre and Robatzek 2008). *Phytophthora infestans* forms appressoria that force penetration hyphae by turgor pressure through the cell wall to form feeding structures (haustoria) (van West and Vleeshouwers 2004).

Once the host recognizes a pathogen via pathogen-associated molecular patterns (PAMPs), also called MAMPs, microbe-associated molecular patterns, it activates defense responses (Göhre and Robatzek 2008). PAMPs are essential for microbial survival or processes and cannot be lost without significant consequences (Gomez-Gomez and Boller 2002; Nurnberger and Brunner 2002). Numerous PAMPs have been isolated from bacteria, oomycetes, and fungi. In fungi, β-1,3-glucan, chitin, and ergosterol have been identified (Nurnberger et al. 2004; Altenbach and Robatzek 2007). The *Phytophthora* PAMP Pep-13 (Brunner et al. 2002), induces oxidative burst, the accumulation of salicylic acid (SA), jasmonic acid (JA), hydrogen peroxide, defense gene expression and hypersensitive response (HR) in potato (Halim et al. 2004). It has been shown that SA (Ellis and Amrhein 1971; Coquoz et al. 1998), JA, jasmonic methyl ester (Cohen et al. 1991), and nitric oxide (NO) radicals (Noritake et al. 1996) can induce local and systemic protection against *P. infestans.*

During infection, pattern recognition receptors (PRRs) in the plasma membrane recognize PAMPs and trigger defense responses. They are highly sensitive and specific recognition receptors for pathogen patterns. PRRs stimulate signaling cascades involving Ca^{2+} fluxes and mitogen activated protein kinases (MAPKs), leading to defense reactions. Therefore, PAMP-Triggered Immunity (PTI), basal resistance or innate immunity is activated, stimulating a transient immune response, which is not associated with visible symptoms (Göhre and Robatzek 2008; Métraux et al. 2009). Typical defense responses in PTI include, stomata closure (Melotto et al. 2006), thickening of the wall by formation of papilla, lignin or callose (Keshavarzi et al. 2004), oxidative burst, activation of

signaling cascades, alteration in gene expression and the release of antimicrobial products, for example phytoalexins, and pathogenesis-related (PR) proteins glucanases and chitinases (van Loon et al. 2006; Sels et al. 2008; Clay et al. 2009). In response to P. *infestans* attack, *N*-(hydroxycinnamoyl)-amines, such as *N*-feruloyltyramine and 4-*N*-coumaroyltyramine are incorporated into cell walls and are involved in cell wall fortification (Keller et al. 1996; Schmidt et al. 1998), as well as lignin also plays a role in potato resistance to *P. infestans* (Yao et al. 1995). Callose depositions in the form of collars and papillae, adjacent to invaded epidermal cells, have been identified in the potato-*P. infestans* interaction (Howles et al. 1996). In addition, superoxide anion generation in potato tissues have been associated with both host and non-host resistance to *P. infestans* (Doke 1983b, 1983a; Lamb et al. 1989; Pieterse et al. 1992; Wu et al. 1995; Vleeshouwers et al. 2000). Oxidative burst in potato is related to the activation of an NADPH oxidase (*gp91 phox*) (Doke and Miura 1995), and a change in Ca^{2+} flux in wich is important for the production of active oxygen species in potato (Yoshioka et al. 2001).

Arachidonic acid (AA) and eicosapentaenoic acid (EPA) released from *P. infestans,* are able to elicit the accumulation of phytoalexins (Bostock et al. 1981; Castoria et al. 1992; Coquoz et al. 1998), and several genes encoding key enzymes involved in the terpenoid pathways in potato have been cloned, such as 3-hydroxy-3-methyglutaryl CoA reductase (HMGR) (Choi et al. 1992). In addition, the pathogenesis-related (PR) proteins PR1, PR2, PR5 and PR9 were also studied in this interaction (Zhu et al. 1995; Collinge and Boller 2001; Hoegen et al. 2002; Tonon et al. 2002; Wang et al. 2006).

Successful pathogens suppress defense responses by the production of a range of effector molecules, allowing the pathogen to colonize the host (Grant et al. 2006). However, these effectors play a role as signals for the activation of R proteins encoded by the host resistance genes in order to reinforce the plant defense, resulting in effector-triggered immunity (ETI), classically called R gene-mediated, specific or vertical resistance (Göhre and Robatzek 2008). Subsequently, R protein triggers a local hypersensitive response (HR) leading to programmed cell death (PCD) and systemic acquired resistance (Chisholm et al. 2006; Bent and Mackey 2007; Da Cunha et al. 2007). This effector-triggered immunity can be defeated by some pathogens, that produce effectors that have evolved to inhibit the immunity reaction.

1.2.5.2. Resistance Genes

R genes from the Mexican wild species *Solanum demissum* were discovered and utilized in late blight resistant potato breeding programmes during the first half of the twentieth century. The genes, R3 (currently known as R3a and R3b), R5-R11, are located on chromosome 11 (El-Kharbotly et al. 1994; El-Kharbotly et al. 1996; Huang et al. 2004; Huang 2005; Bradshaw et al. 2006). R2 is located on chromosome 4 (Li et al. 1998) and R1 on chromosome 5 (Leonards-Schippers et al. 1994). R1, R2, R3 and R10 were introgressed into commercial potato varieties; however they were rapidly overcome after new isolates of *P. infestans* were more virulent to the previously resistant varieties (Umaerus and Umaerus 1994).

The recent research target in late blight resistance breeding programmes has been oriented to study Rpi genes (resistance to *P. infestans*) from other wild *Solanum* species conferring broad-spectrum resistance (van der Vossen et al. 2003). Rpi loci, include Rpi-ber1 from *S. berthaultii* on chromosome 10 (Ewing et al. 2000), RB/Rpi-blb1, Rpi-blb2 and Rpi-blb3 from *S. bulbocastanum* on chromosomes 8, 6 and 4, respectively (Naess et al. 2000; van der Vossen et al. 2003; Park et al. 2005; van der Vossen et al. 2005), Rpi-pnt1 from *Solanum pinnatisectum* on chromosome 7 (Kuhl et al. 2001), Rpi-mcq1 from *Solanum mochiquense* and Rpi-phu1 from *Solanum phureja* on chromosome 9 (Smilde et al. 2005; Sliwka et al. 2006). Quantitative trait loci (QTL) for resistance to *P. infestans* (Pi_QTL) have been identified on chromosome 3-9, 11 and 12 (Leonards-Schippers et al. 1994; Collins et al. 1999; Oberhagemann et al. 1999; Sandbrink et al. 2000; Ghislain et al. 2001; Kuhl et al. 2001; Trognitz et al. 2001; Gebhardt et al. 2004; Costanzo et al. 2005; Bradshaw et al. 2006).

1.2.5.3. Antimicrobial potato secondary metabolites

Antimicrobial secondary metabolites are different from the intermediary (primary) metabolism in that they are usually nonessential for the basic metabolic processes of the plant, but they are generally synthesized in complex pathways which involv the synthesis of pigments or other compounds that do not necessarily have a role in plant defense. The majority of antimicrobial secondary metabolites are derived from the phenylpropanoid, isoprenoid, alkaloid or fatty acid pathways (Dixon 2001). Phenolics compounds from the phenylpropanoid pathway that are linked to potato resistance from *Phytophthora infestans* are: chlorogenic acid (Kuc 1973; Mittelstra et al. 2006), p-

coumaroyloctopamine, p-coumaroylnoradrenaline (Mittelstra et al. 2006), caffeic acid, scopoletin, scopolin (Kuc 1973), *p*-coumaric acid, ferulic acid (Regnault-Roger et al. 2005), 4-hydroxybenzaldehyde, 4-hydroxybenzoate, N-4-coumaroyl-amine, n-feruloyltyramine (Schmidt et al. 1998), and salicylic acid (Coquoz et al. 1995). Compounds from the isoprenoid pathway or mevalonate pathway linked to potato resistance from *Phytophthora infestans* are; i) the sesquiterpenes rishitin (Kuc 1973; Walker and Wade 1978; Henfling et al. 1980; Bostock et al. 1981; Ghanekar et al. 1984; Engstrom et al. 1999), rishitinol, phytuberin (Kuc 1973), lubimin (Kuc 1973; Henfling et al. 1980; Bostock et al. 1981), cedrol, farnesol (Engstrom et al. 1999) and abscisic acid (Henfling et al. 1980; Engstrom et al. 1999) and ii) the steroidal glycoalkaloids – Saponins α-solanine, α-chaconine (Kuc 1973; Andrivon et al. 2003) and solanidine (Andrivon et al. 2003). The oxylipins from the octadecanoid pathway linked to potato resistance to *Phytophthora infestans* are jasmonic acid and jasmonic methyl ester (Cohen et al. 1993) and the fatty acid hydroperoxides derivatives colneleic acid (9-[10(E),30(Z)-nonadienyloxy]-8(E)-nonenoic acid and colnelenic acid (9-[10(E),30(Z),60(Z)-nonatrienyloxy]-8(E)-nonenoic acid (Bryant et al. 2006).

1.2.5.4 Antimicrobial Metabolic Pathways

1.2.5.4.1 Phenylpropanoid pathway

Plant phenolics play a variety of roles in the plant, ranging from scents (vanillin), pigments (anthocyanins), feeding deterrents (capsaicin, tannins), allelopathic compounds (syringin, caffeic acid), signaling molecules (salicylic acid), structural components

(lignin), UV protection (quercetin, kaempferol) and antimicrobial agents (medicarpin) (Bowsher et al. 2008). Phenolic compounds are substances that contain a hydroxyl, or phenolic group attached to a 6-carbon phenyl ring (Bowsher et al. 2008). The main phenolic groups are the simple phenylpropanoids, coumarins, benzoic acid derivatives and flavonoids.

The simple phenylpropanoids all share the same basic structure of a linear three-carbon side chain attached to a six-carbon phenyl ring e.g. caffeic, ferulic, cinnamic, and *p*-coumaric acid. The coumarins have the basic C_6-C_3 phenylpropanoid structure with a cyclized side chain e.g. coumarin, umbelliferone, scopoletin. The benzoic acid derivatives differ from the other groups by having C_6-C_1, rather than C_6-C_3 structure e.g. vanillin, salicylic acid. The more complex phenolics include the flavonoids, consisting of two six-carbon (ring A and ring B) phenyl ring linked by a three carbon bridge (ring C) (Bowsher et al. 2008).

The phenylpropanoid pathway occurs in the plastids. The first step in the core phenylpropanoid pathway is the formation of *trans*-cinnamic acid by the elimination of an ammonia molecule from phenylalanine (Fig 1.2). This reaction is catalyzed by the enzyme phenylalanine ammonia lyase (PAL). Then, cinnamate 4-hyhrolase (C4H) introduces a hydroxyl group into the phenyl group of *trans*-cinnamic, forming *p*-coumaric acid. The final step in the core phenylpropanoid pathway catalized by the enzyme 4-coumarate: CoA ligase (4CL) to form *p*-coumaroyl CoA from coumaric acid. This core phenylpropanoid pathway produces the ring B and C for the flavonoid

synthesis and the malonic acid/acetate pathway provides the ring A with the participation of enzyme chalcone synthase (CHS) (Dixon et al. 2002; Bowsher et al. 2008).

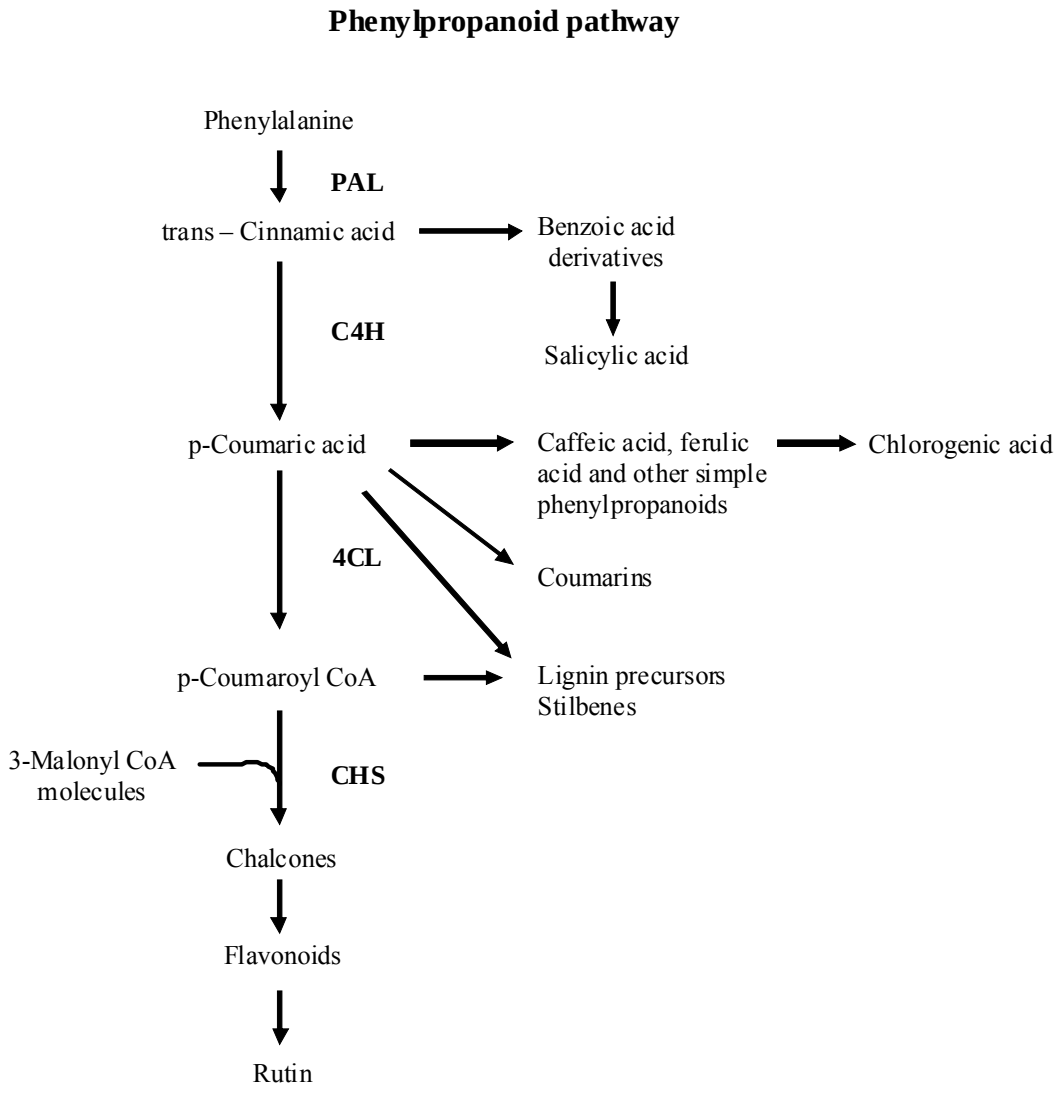

Figure 1.2. Schematic representation of the Phenylpropanoid pathway. PAL: Phenylalanine ammonia-lyase, 4CL*:* 4-coumarate:coenzyme A ligase CHS*:* chalcone synthase, C4H: cinnamate 4-hyhrolase.

1.2.5.4.2 Terpenoids pathways

Terpenoids, also recognized as isoprenoids, participate in essential plant processes such as respiration (ubiquinone), photosynthesis (carotenoids, chlorophylls, plastoquinone), regulation of growth and development (gibberellic acid, abscisic acid, cytokinins) and protection against pathogens (rishitin). Without the terpenoids plants would not be able to function (Wanke et al. 2001; Rodriguez-Concepcion and Boronat 2002).

Terpenoids are synthesized from two common precursors, isopentenyl diphosphate (IPP) and its isomer, dimethylallyl diphosphate (DMAPP) by two separate routes; the cytoplasmic mevalonate (Ac-MVA) and the plastidial 2*C*-methyl-D-erythritol-4-phosphate (DOXP-MEP) pathways (McGarvey and Croteau 1995). Until recently, it was thought that the mevalonate (Ac-MVA) pathway was the only route for the synthesis of isoprenoid precursors in all organisms (Chappell 1995). However, the DOXP-MEP pathway was discovered as an alternative terpenoids biosynthetic route, first in eubacteria (Flesch and Rohmer 1988), and soon after in photosynthetic organisms such as higher plants, algae as well as in cyanobacteria (Cvejic and Rohmer 2000). This pathway also occurs in the malaria parasite *Plasmodium falciparum* (Jomaa et al. 1999) but not in humans, animals or archaebacteria.

In the mevalonate (Ac-MVA) pathway that proceeds in the cytosol, 3-hydroxy-3-methylglutaryl-CoA (HMG CoA) is formed from three acetyl-CoA In the mevalonate (Ac-MVA) pathway that occurs in the cytosol. Then, mevalonic acid (MVA) is formed

from HMG CoA by a reaction catalyzed by the enzyme 3-hydroxy-3-methylglutaryl-CoA reductase (HMGR). This enzyme is encoded by a small family of genes with different pattern of expression, ranging from constitutive to tissue-specific induction. After two remaining consecutive steps IPP is released and isopentenyl diphosphate (DMAPP) is formed by the isomerization of IPP (Rodriguez-Concepcion and Boronat 2002; Eisenreich et al. 2004; Phillips et al. 2008) (Fig. 1.3).

The DOXP-MEP pathway, also called the non-mevalonate route, consists of eight reactions catalyzed by nine enzymes, seven of which are characterized structurally (Hunter et al. 2003). Briefly, 1-deoxy-D-xylulose 5-phosphate (DXP) obtained by condensation of pyruvate and D-glyceraldehyde 3-phosphate (GAP) in a reaction catalyzed by the enzyme 1-deoxy-D-xylulose 5-phosphate synthase (DXS) undergoes a reorganization associated with a reduction step. The next step of the DOXP-MEP pathway is the conversion and reduction of DXP to 2C-methyl-D-erythritol 4-phosphate (MEP) by the enzyme 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR). MEP is subsequently transformed to IPP and DMAPP by five-independent steps by the action of the enzymes 2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase (MCT), 4-(cytidine 50-diphospho)-2-Cmethyl-D-erythritol kinase (CMK) and 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase (MDS). In two final steps catalyzed by (E)-4-hydroxy-3-methylbut-2-enyl diphosphate synthase (HDS) and reductase (HDR), IPP and DMAPP are formed (Rodriguez-Concepcion and Boronat 2002; Eisenreich et al. 2004; Phillips et al. 2008) (Fig. 1.4).

Isopentenyl diphosphate (IPP) is the activated five-carbon building block of terpenes. Terpenoids are classified according to the number of isoprene units in their structure:

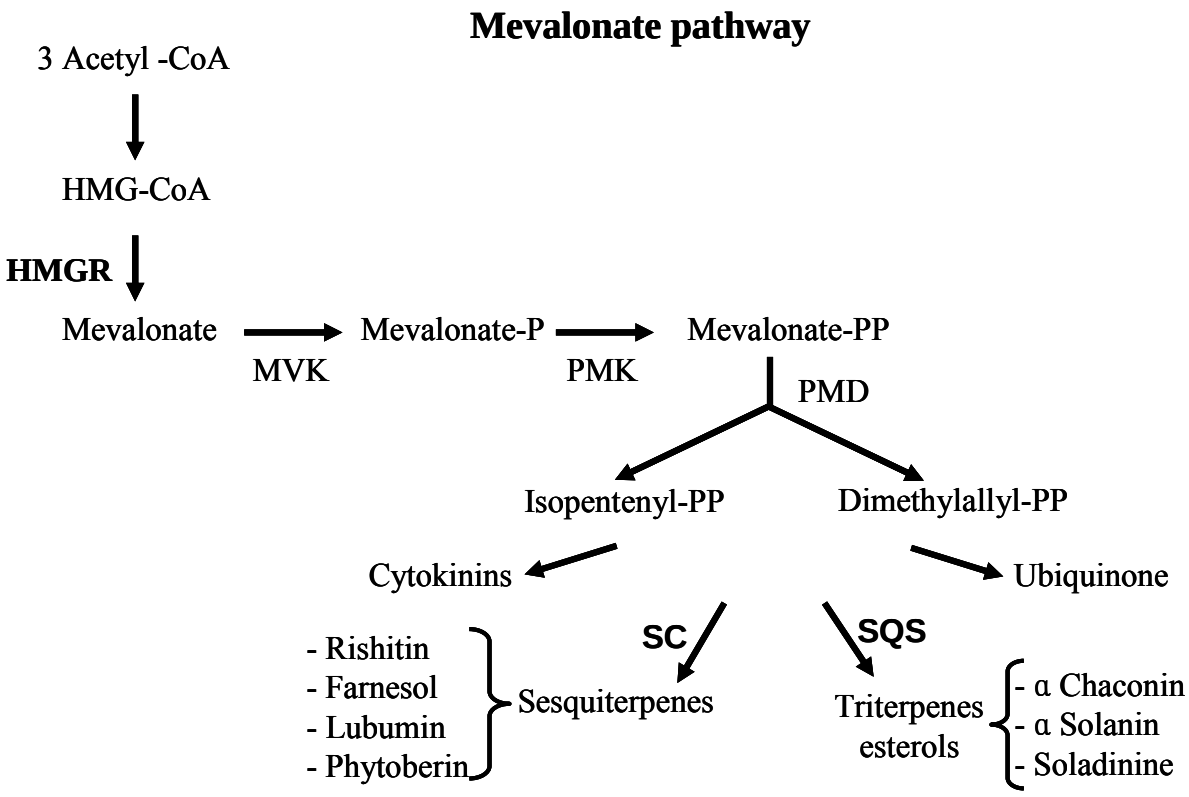

Figure 1.3. Schematic representation of the Mevalonate pathway. HMGR: 3-hydroxy-3-methylglutaryl coenzyme A reductase, SQS: squalene synthase, SC: sesquiterpen cyclase, MVK: Mevalonate kinase, PMK: phosphomevalonate kinase, PMD: mevalonate diphosphate decarboxylase.

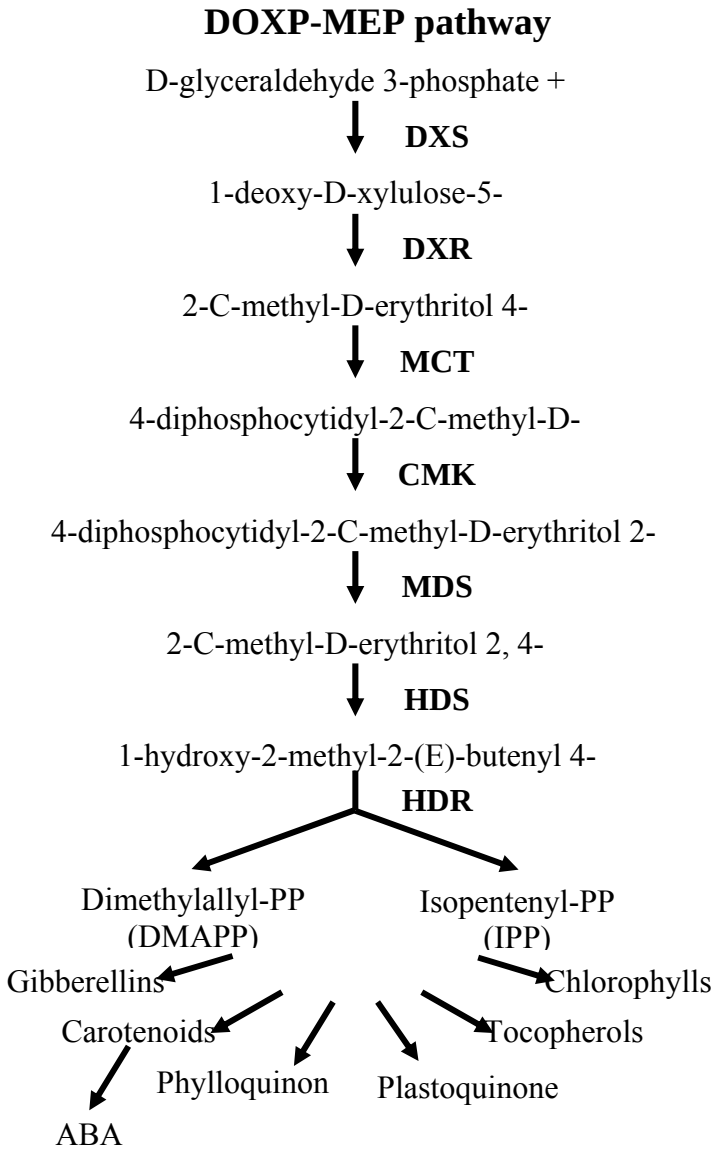

Figure 1.4. Schematic representation of the DOXP-MEP pathway. DXS: 1-deoxy-D-xylulose 5-phosphate synthase, DXR: 1-deoxy-D-xylulose 5-phosphate reductoisomerase, MCT: 2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase, CMK: 4-(cytidine 50-diphospho)-2-Cmethyl-D-erythritol kinase, MDS: 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase , HDS: (E)-4-hydroxy-3-methylbut-2-enyl diphosphate synthase

hemiterpenes C_5 (1 isoprene unit), monoterpenes C_{10} (2 isoprene units), sesquiterpenes C_{15} (3 isoprene units), diterpenes C_{20} (4 isoprene units), triterpenes C_{30} (6 isoprene units), tetraterpenes C_{40} (8 isoprene units) (Dubey et al. 2003). In the mevalonate (Ac-MVA) pathway cytokinins, sesquiterpenes, triterpenes (sterols) and the polyterpene ubiquinone are synthesized. In the DOXP-MEP pathway the isoprene, monoterpenes, diterpenes (phytol) included the side chain of chlorophyll, tocopherol and gibberellin are synthesized, as well as the tetraterpenes (carotenoids) include the side chain of ABA and the polyterpene plastoquinone (Lichtenthaler 2000; Rodriguez-Concepcion and Boronat 2002; Eisenreich et al. 2004; Phillips et al. 2008).

1.2.6. Defense suppression by pathogens

Plants activate defense mechanisms that help prevent invasion and microorganisms spread in response to pathogen attack. Plants produce signals, structural barriers, hypersensitive cell death and inhibitors of pathogen growth. On the other hand, only after overcoming the host defenses, can pathogenic microorganisms benefit from the host nutrients by degradation of host high molecular weight constituents and absorvance of low molecular weight metabolites. For these purposes, pathogens produce enzymes, toxins and suppressors (Agrios 2005).

Suppressors are inhibitory molecules that suppress the expression of defense responses in the host plant. They allow some pathogens to interrupt or evade the host defense responses. As a result, the host cells become susceptible. However, experimental validation of this assumption has been scarce (Shiraishi et al. 1994).

Defense suppressors may:

1) Inhibit the interactions between the elicitor from the pathogen and the corresponding receptor from the plant by blocking the receptor site;

2) Block the signal transduction pathway during the elicitor-mediated activation of the defense response;

3) Affect the formation of binding complexes in the promoter region and suppress the expression of specific genes (Shiraishi et al. 1994).

For example, saponin-degrading enzymes have been isolated from a number of plant pathogenic fungi. *Gaeumannomyces graminis* var. *avena* produces the degrading enzyme avenacinase that detoxifies the phytoanticipin avenacin (Bowyer et al. 1995) and *Septoria lycopersici* produces the degrading enzyme tomatinase that detoxifies the phytoanticipin tomatine (Bouarab et al. 2002). A recent report by Rose *et al*. (2002) provided the first molecular evidence that fungi may suppress antifungal proteins. A class of glucanase inhibitor proteins (collectively called GIPs) has been characterized in the soybean oomycete pathogen *Phytophthora sojae* (Rose et al. 2002). Also, pathogens can inactivate reactive oxygen species (ROS) with peroxidases, catalases and superoxide dismutase (Mayer et al. 2001).

Inhibition of tomato proteases by EPI1 and EPI10 could form a novel type of defense-counterdefense mechanism between plants and microbial pathogens. EPI1 and EPI10 are extracellular protease inhibitors, which inhibited and interacted with the pathogenesis-related P69B subtilisin-like serine protease of tomato in intercellular fluids

(Tian et al. 2005; Tian et al. 2007). On the other hand, *P. infestans* secretes several inhibitors of the Cystain and Kazal family of protease inhibitors: EPIC2B inhibits the cysteine protease PIP1, and its homolog EPIC1 is predicted to act in a similar manner (Göhre and Robatzek 2008).

Inoculation of bean with *P. syringae* pv. *phaseolicola* inhibited key enzymes in the phenylpropanoid pathway, phenylalanine ammonia-lyase (PAL), chalcone synthase (CHS) and chalcone isomerase (CHI) (Jakobek et al. 1993). AvrPtoB Type III effector protein from *Pseudomonas syringae* pv. tomato suppresses hypersensitive response (HR) base on programmed cell death (PCD) (Abramovitch et al. 2003). AvrPto Type III effector protein from *Pseudomonas syringae* pv. *tomato* suppresses callose production in Arabidosis (Hauck et al. 2003) and *Xanthomonas campestris* pv. *vesicatoria* suppresses papillae production in pepper plants (Brown et al. 1995).

It has been suggested that susceptibility in potato to *P. infestans* is produced from the suppression and/or interruption of the production or activation of resistance reactions by glucans released from *P. infestans* (Garas et al. 1979; Doke et al. 1980; Currier 1981; Andreu et al. 1998; Ozeretskovskaya et al. 2001). All of these investigations have been realized *in vitro* using potato tubers and biochemical analysis. On the other hand, at the molecular level, Wang et al. (2004a) found that the most aggressive strains of *P. infestans* suppresses potato defense mechanisms through transcriptional inhibition of phenylpropanoid (PAL) and isoprenoid (HMGR) pathways.

1.3 OBJECTIVES

My hypothesis is that *Phytophthora infestans* suppresses defense responses in potato (*Solanum tuberosum*) by alterating the expression of defense-related genes, accumulation of secondary metabolites and secondary metabolism pathways genes. This hypothesis will be tested by i) developing a new molecular biology approach that will help to identify novel genes in the potato-*P.infestans* interaction, such as genes potentially controlling pathogenesis or avr genes in *P. infestans,* as well as those potentially involved in potato resistance or susceptibility to this pathogen, ii) studying the gene expression of the genes identified in this research, in order to identify a possible defense suppression, iii) evaluating the suppression of known potato genes by *Phytophthora infestans* effectors and iv) assessing the accumulation of potato secondary metabolites.

The research accomplished in this thesis is presented in several chapters:

Chapter 2: Identification and cloning of differentially expressed genes involved in the interaction between potato and *Phytophthora infestans* using a subtractive hybridization and cDNA-AFLP combinational approach (Henriquez, M.A. and Daayf F. 2010. *Journal of Integrative Plant Biology* 52: 453–467)

Chapter 3: Cloning and characterization of a novel gene from the plastid-localized 2-c-methyl-d-erythritol 4-phosphate (doxp-mep) pathway in potato infected by *phytophthora infestans*. (Henriquez, M.A. and Daayf F. 2010. Ms. Ref. No.: *Journal of Integrative Plant Biology* /2010/021261; 24p. Submitted Sept 29, 2010).

Chapter 4: *Phytophthora infestans* effectors suppress secondary metabolism pathways in potato.

Chapter 5: Alterated metabolic profile of secondary metabolites in potato leaves after inoculation with *Phytophthora infestans*

CHAPTER 2: IDENTIFICATION AND CLONING OF DIFFERENTIALLY EXPRESSED GENES INVOLVED IN THE INTERACTION BETWEEN POTATO AND *PHYTOPHTHORA INFESTANS* USING A SUBTRACTIVE HYBRIDIZATION AND CDNA-AFLP COMBINATIONAL APPROACH

(Henriquez, M.A. and Daayf F. 2010. *Journal of Integrative Plant Biology* 52: 453–467)

2.1. ABSTRACT

Using a subtractive hybridization (SH)/cDNA-AFLP combinational approach, differentially expressed genes involved in the potato-*Phytophthora infestans* interaction were identified. These included genes potentially controlling pathogenesis or avr genes in *P. infestans* as well as those potentially involved in potato resistance or susceptibility to this pathogen. Forty-one differentially expressed transcript-derived fragments (TDFs), resulting from the interaction, were cloned and sequenced. Two TDFs, suggested as potential pathogenicity factors, have sequence similarity to N-succinyl diaminopimelate aminotransferase and a transcriptional regulator, TetR family gene. Two other TDFs, suggested as potential avr genes, have sequence similarity to an EST sequence from Avr4/Cf-4/Avr9/Cf-9 and a *P. infestans* avirulence-associated gene. The expression and origin of the genes were confirmed using Southern blots, Northern blots and qRT-PCR. Potential resistance gene DL81 was induced at 12 hpi in the moderately resistant cultivar, whereas it was down-regulated as early as 6 hpi in the susceptible cultivar. On the other hand, DL21 was induced at 6 hpi (3.38-fold) in response to the highly aggressive isolate (US8) and strongly up-regulated thereafter (25.13-fold at 120 hpi.), whereas it was only

slightly up-regulated in response to the weakly aggressive isolate US11 (3.82-fold at 96 hpi), suggesting its potential involvement as a susceptibility gene.

2.2. INTRODUCTION

Potato late blight caused by the oomycete *Phytophthora infestans* (Mont.) de Bary (Fry and Goodwin 1997) results in economic losses of $3.25 billion per year in potato-growing areas worldwide (Pel et al. 2009). This disease can be initiated either directly with germinating sporangia or through zoospores, and affects leaves, stems, and tubers (Judelson and Blanco 2005). The re-emergence of late blight as an important disease in the US and Canada occurred in the 1980s and 1990s, respectively, due to changes in the population structure of *P. infestans*, with a shift from the pre-existing A1-mating type, metalaxyl-susceptible to new metalaxyl-insensitive genotypes, highly aggressive A1 and A2 genotypes (Daayf and Platt 2000). In Canada, the A1 genotype US-1 has been completely displaced by the A2 genotype US8 of *P. infestans* (Daayf et al. 2000; Daayf et al. 2001), which is more virulent and exhibits highly increased aggressiveness (Kato et al. 1997).

Extensive literature is available about incompatible interactions between potato and *P. infestans* leading to plant resistance, whereas the molecular mechanisms of compatibility leading to disease have been investigated less (Restrepo et al. 2005). It has been suggested that potato susceptibility to *P. infestans* is a result of the suppression of potato resistance reactions by glucans released from *P. infestans* (Garas et al. 1979; Doke et al. 1980; Currier 1981; Andreu et al. 1998; Ozeretskovskaya et al. 2001). Also, *P.*

infestans secretes both cytoplasmic and apoplastic effectors (Kamoun 2005). For example, serine protease inhibitors EPI1 and EPI10 are apoplastic effectors that are thought to function in the counter-defense inhibiting PR protein P69B, a subtilisin-like serine protease of tomato (Tian et al. 2005). In potato, Wang et al. (2004b) reported that an aggressive *P. infestans* US8 strain suppresses host defense mechanisms through transcriptional inhibition of phenylpropanoid (PAL) and isoprenoid (HMGR) pathways (Wang et al. 2008).

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

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

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

2.3. MATERIALS AND METHODS

2.3.1. *Phytophthora infestans* strains and inoculation

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

treatments composing this quadratic model were used for further analysis of differential gene expression in both the plant and the pathogen.

2.3.2. Combinational approach of subtractive hybridization and cDNA-AFLP

RNA was extracted from both inoculated and control potato tissues as well as the *P. infestans* isolates used in the interaction. Consequently, mRNA was extracted from the host alone, the pathogen alone and their interacting tissues. First and double-stranded cDNA was synthesized and the first-strand cDNA from the non-inoculated plant (control) was combined with the second-strand cDNA (inoculated plant) in order to perform a subtractive hybridization (SH). Transcripts of interest, obtained from the subtractive hybridization are single-stranded DNAs with 3' polyA; therefore, a synthesis of a complementary DNA strand was performed for each interaction (SH-Second strand). Then, the SH-Second Strand was used for AFLP analysis, followed by SH-Digestion, SH-Ligation, SH-Preamplification (SH+1) and SH-Selective (SH+3) amplifications. The products were separated on 5% PAGE and bands detected by AgNO3 staining. Differentially expressed products were then characterized (Fig. 2.1).

2.3.3. Total RNA and mRNA extraction

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

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

2.3.4. Subtractive Hybridization (SH)

Four subtractive hybridizations were completed between inoculated (treatment) and non-inoculated tissues (control): (i) RB+US8 *minus* control, (ii) RB+US11 *minus* control, (iii) DF+US8 *minus* control and (iv) DF+US11 *minus* control, modifying the method described by Krista and Pauls (2001) and superscript II reverse transcriptase (Invitrogen) protocol. In brief, in a 1.5 ml tube, the mRNA extracted using paramagnetic particles was mixed with 4 µl of reverse transcriptase (RT) 5X buffer (250 mM Tris-HCl, pH 8.3 at room temperature; 375 mM KCl; 15 mM MgCl2), 2 µl of 0.1 M DTT, 1 µl of 10 mM dNTP mix, and 1 µl of 200 units of M-MLV (RT) enzyme. The reaction was incubated 1h at 37 ºC and the first-strand cDNA was collected using a magnetic separation stand, because it has magnetic beads. This first cDNA strand was then used as a template for the synthesis of a second cDNA strand. The first-strand cDNA was mixed in a 200 µl PCR tube with 2X DNA polymerase buffer (10 mM Tris-HCl, 50 mM NaCl, 10 mM $MgCl_2$,

1 mM DTT, pH 7.9), 0.2 mM of dNTP mix, 5 U/µl of *E. coli* DNA Ligase, 20 U/µl of *E. coli* DNA polymerase and 5 U/µl of *E. coli* RNAse H. The reaction was completed with water to 150 µl and incubated for 2h at 16ºC in a thermocycler (Techne Flexigene, Inc., Canada). The reaction was transferred into a 1.5 ml tube, the reaction mixture was removed by pipetting, and the double-stranded cDNA was collected using a magnetic separation stand. Then, the double-stranded cDNA was re-suspended in 50 µl of sterile water and boiled for 10 min to denature the cDNA. Without delay, using a magnetic separation stand, the second-strand cDNA (supernatant) was separated from the first-strand (collected particles). The first-strand cDNA from the non-inoculated plant (control) was combined with 18 µl of the second-strand cDNA (inoculated plant) in 30 µl of hybridization buffer (30 mM HEPES, 1 mM EDTA, 1 M NaCl). The mixture was incubated for 24 h at 37°C. At the end of the incubation, 3 cDNA species were present in the mixture: (i) un-hybridized cDNA from non-inoculated plants (attached to magnetic beads), (ii) hybridized cDNA from non-inoculated and inoculated plants (attached to magnetic beads) and (iii) un-hybridized cDNA from inoculated plants was in the supernatant (no magnetic beads). The latter is the SH product selected (transcripts of interest) that was precipitated with 3 volumes of isopropanol, washed with 70% ethanol and dissolved in a final volume of 20 µl of sterile water. In addition, for each genotype of *P. infestans*, the same procedures described above for first and second strands were used without hybridization. Finally, a 2-µl aliquot from products of interest was visualized in a 1.2 % Petri dish agarose gel with Et-Br in order to corroborate the presence of cDNA.

2.3.5. SH- Second Strand synthesis

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

2.3.6. SH-AFLP

The SH-Second Strand was used for AFLP analysis, using a modified protocol of Vos et al. (1995) and Bachem et al. (1996). In brief, the SH-Digestion was prepared with 10 µl of SH-Second Strand, 10 units of EcoRI Enzyme (Invitrogen), 5 units of MseI enzyme (Invitrogen) and 1X MseI Buffer in a 14.5 ul reaction volume. The reaction was incubated 2h at 37ºC and inactivated for 10 min at 70ºC. The SH-Ligation was done by adding 1.25 µM of MseI adapter (50µM), 0.125 µM of EcoRI adapter (5µM), 1.25X of T4 ligase buffer (5X), and 400 units of T4 DNA ligase, to the total SH-Digestion. The reaction was incubated 2h at 37ºC. For the SH-Pre-amplification (SH+1), 5 µl of SH-Digestion/Ligation product was mixed with 50 ng of ECORI + A (100 ng/µl), 50 ng of

MseI + C (100 ng/µl), 1X Buffer (200 mM Tris-HCl (pH 8.3), 15 mM $MgCl_2$, 500 mM KCl), 0.25 µl of dNTP mix, 1 unit of Taq DNA polimerase (Invitrogen) and SDW to achieve a 25 µl reaction volume. Amplification was performed in a programmed thermocycler for 25 cycles of 30 s at 94°C, 60 s at 56°C, and 60 s at 72°C, followed by a final extension for 60 sec at 72°C. The PCR products were run on a 1.2% agarose gel following staining with EthBr. The selective amplification (SH- Selective (SH+3)) was generated in a 25 µl reaction volume containing 5 µl of SH+1 product diluted 1:50, 50 ng EcoRI (3 selective bases) (100 ng/µl), 50 ng of MseI (3 selective bases) (100 ng/µl), 1X Buffer (200 mM Tris-HCl (pH 8.3), 15 mM $MgCl_2$, 500 mM KCl), 0.25 mM of dNTP mix, 2.5 mM of $MgCl_2$ and 1 unit of Taq DNA polymerase (Invitrogen). The PCR reactions were performed for 36 cycles of 30 s at 94°C, 30 s at 56°C and 60 s of extension at 72°C. The annealing temperature in the first cycle was 65°C and was subsequently reduced each cycle by 0.7°C for the following 12 cycles. The annealing temperature was then maintained at 56°C for the remaining 23 cycles. Amplification products were separated on 5% polyacrylamide gels, using the Sequigel system (Biorad) and bands were detected by $AgNO_3$ staining. All oligonucleotides were synthetized by Invitrogen Canada Inc. The AFLP sequence for the adapters and primers for the SH+1 and SH+3 were similar to Vos et al. (1995). However, the three selective bases for the SH+3 were; AAC, AAG, ACA, ACT, ACC, ACG, AGC, AGG for the ECoRI Core and CAA, CAC, CAG, CAT, CTA, CTC, CTG, CTT for the MsE I Core.

The SH-AFLP also can be performed using the AFLP® Analysis System I or the AFLP® Analysis System for Microorganisms (Invitrogen), starting with 10 µl of SH-

Second Strand for digestion. However, we reduced the reagents for digestion, ligation and pre-amplification to half the manufacturer's recommendations. The selective amplification is performed using 5 µl of SH+1 product diluted 1:50, 0.5 µl of EcoRI primer, 6 µl of MseI primer, 2 µl of 10X buffer and 1 unit of Taq DNA polymerase (Invitrogen) (data not shown).

2.3.7. Sequence analysis

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

Center for Biotechnology Information (http: // www.ncbi.nlm.nih.gov), *Phytophthora infestans Database* (http://www.broad.mit.edu/annotation/genome/phytophthora_infestans/Home.), VBI Microbial Database (http://annuminas.vbi.vt.edu/), The Solanaceae Genomics Resource at MSU (http://solanaceae.plantbiology.msu.edu/), the GABI Primary Database, GabiPD (http://www.gabipd.org/) and the Oomycete Genomics Database (http://www.oomycete.org/ogdg/blasttool.html).

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

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

CAGCTACTTGGGAGGCTGAG-3' and DL81-R 5'-TAGGGCGAGTTTGCATCTT-3'. In addition, for northern blot, the 18S RNA probe was prepared from potato DNA using the primers 18S-F 5'-TAGATAAAAGGTCGACGCGG-3' and 18S-R 5'-TCATTACTCCGATCCCGAAG -3' (GenBank accession number X67238).

2.3.9. qRT-PCR and data analyses

Similar to those treatments used for the subtractive hybridization (SH)/cDNA-AFLP analysis, five micrograms of mixed RNA from each plant-pathogen treatment (3-144 hpi) and *P. infestans* RNA mixture (spores, zoospores, germination, appressoria and mycelium) were treated with Deoxyribonuclease I (Invitrogen) in order to preserve the integrity of RNA by degrading any possible residual genomic DNA. The DNase-treated RNA was reverse transcribed following the M-MLV (RT) enzyme (Invitrogen) manufacturer's recommendations. Reverse transcription was also performed from five micrograms of nine different times after infection (0, 6, 9, 12, 24, 48, 72, 96, 120 hpi), similar to those used for the subtractive hybridization (SH)/cDNA-AFLP analysis in order to study the dynamics of gene expression during disease development. Gene expression was quantified using a Stratagene Mx3005p cycler. Each 20 µL qPCR reaction contained 2 µL of cDNA (1:3), 6.5 µL of IQ SYB Green Supermix (Biorad), and 0.375 µM of each primer. The following qPCR cycling program was used for all sets of primers: The thermocycle program included 95 °C (2 min), followed by 40 cycles of 95 °C (15 s), 50 °C (45 s) and 72 °C (45 s). Melt-curve analysis was performed to observe primer-dimer formation and to check amplification of gene-specific products. All PCR reactions were performed from triplicate biological samples. The $2^{-\Delta\Delta C(T)}$ method

(Livak and Schmittgen 2001) was used to calculate the fold expression relative to the controls. Primers were designed, based on the sequence of the transcripts, DL81-F 5'-CAGCTACTTGGGAGGCTGAG-3', DL81-R 5'-TAGGGCGAGTTTGCATCTT-3' (184bp), DL21-F 5'-AAAGGTGCACGCCTGTTTAC-3', DL21-R 5'-TTGCTTTTGCAACATTAGGG-3'(101 bp), DL39-F 5'-GCTCACCAAATCACCAAACA -3', DL39-R 5'- GGGAAGAGTTGGGGATCTTC -3'(102 bp) and DL28-F 5'- GAAGAAACGCTAGGAAAAGTCG -3', DL28-R 5'-TCTATTATTGCTTACACAGCACTCAG -3 (103 bp). In addition, primers specific for elongation factor gene; Efactor-F 5'- GATGGTCAGACCCGTGAACAT -3' and Efactor-R 5'- GGGGATTTTGTCAGGGTTGT-3' (180 bp) (Genbank accession number; AB061263) were used to normalize small differences in template amounts.

2.3.10 Statistical analysis

The statistical analyses were performed with the Statistical Analysis Software (SAS) (SAS Institute, Cary, NC; release 9.1 for Windows). Prior to analysis, expression levels of DOXP-MEP pathway gene data sets were checked for normality (PROC Univariate). Thus, data were normalized by log+0.5 transformations for analysis. ANOVA analysis was performed using PROC GLM. Treatment means were separated using the Tukey's Studentized Range test. A $p < 0.05$ was considered to indicate significant differences.

2.4. RESULTS

2.4.1. Inoculation results

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

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

2.4.2 Characterization of differentially expressed fragments

In order to identify both host and pathogen genes stimulated during the potato-*P. infestans* interaction, we used a combinational approach of subtractive hybridization and cDNA-AFLP to simultaneously evaluate treatments involving potato cultivars differing in their disease susceptibility after inoculation with each of two pathogen genotypes differing in their pathogenicity levels. Due to space limitations, we only describe the procedure for one host cultivar and one pathogen genotype (Fig. 2.1).

Four subtractive hybridizations were accomplished between inoculated (treatment) and non-inoculated tissues (control, treated with sdH_2O): (i) RB+US8 *minus* control, (ii) RB+US11 *minus* control, (iii) DF+US8 *minus* control, and (iv) DF+US11 *minus* control. This step allowed us to remove constitutive and commonly expressed transcripts in/between different treatments. Subsequently, differential screening with cDNA-amplified fragment length polymorphism (cDNA-AFLP) yielded a large number of polymorphic bands.

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

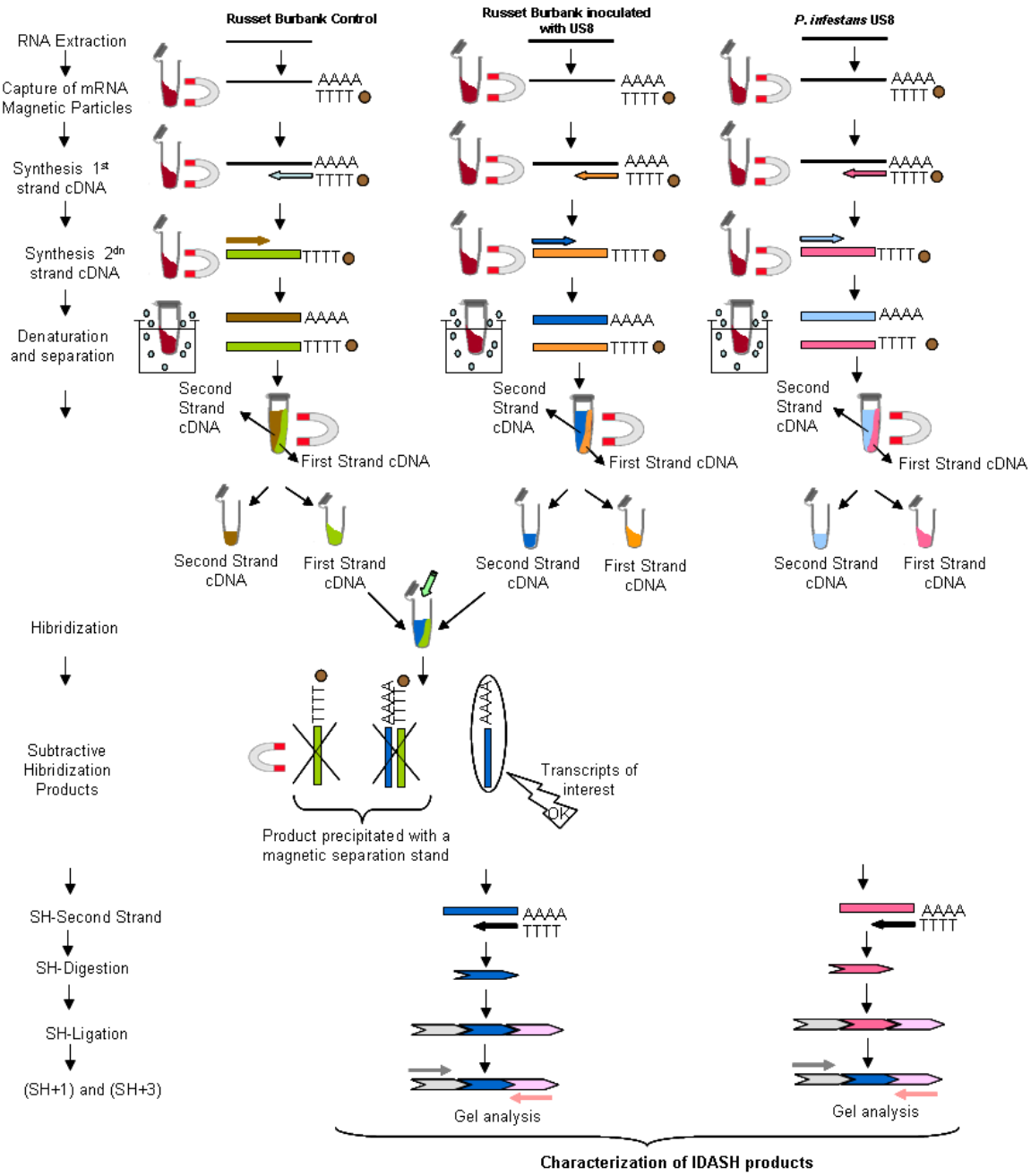

Figure 2.1. Schematic representation of the subtractive hybridization (SH)/cDNA-AFLP approach.

- Potential pathogenicity factors “ssor”: TDFs from the pathogen, exclusively expressed in the compatible interaction,
- Potentially suppressed potato genes “ssed”: TDFs from potato exclusively expressed in the incompatible interaction, neither present in the pathogen nor in the compatible interaction,
- Potential *P. infestans* “avr” genes: TDFs from the pathogen constitutive expressed in the incompatible interaction,
- Potential resistance genes “rtant”: TDFs from potato *commonly* expressed in partially resistant potato cultivar (Defender), neither present in the pathogen nor in the susceptible cultivar (Russet Burbank).

In addition, if the sequence analysis from a candidate “ssor” gene showed homology only with a host sequence and not with pathogens, we called this TDF potential plant disease susceptibility gene “ssept”. TDFs from constitutive genes and from those commonly expressed in both incompatible and compatible interaction, either from the pathogen or the host, were distinguished but not used for further characterization. The representative cDNA-AFLP display of each group is shown in Figure 2.2.

Fifty four TDFs (25 ssor, 18 ssed and 10 avr, 1 rtant), from 6 primer combinations, were excised from the polyacrylamide gels and successfully re-amplified by PCR, using the selective primers that were used to obtain them in SH-AFLP.

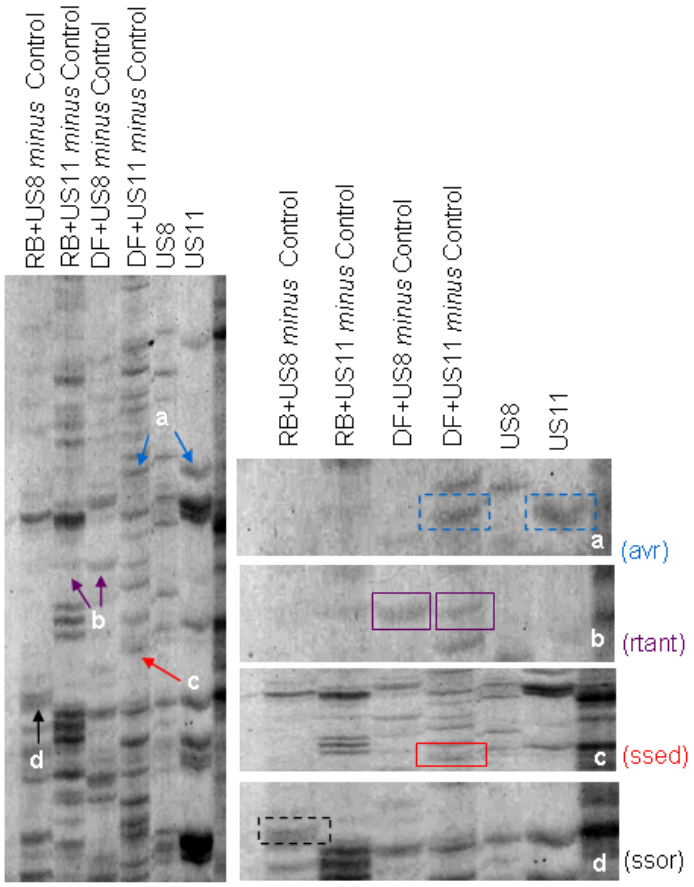

Figure 2.2. Example of TDFs in a polyacrylamide gel with one primer combination (EcoRI+ACT/MseI+CAG). a) cDNA from potential *P. infestans* avr genes (avr), b) potential potato resistance gene (rtant), c) cDNA from potentially suppressed potato genes (ssed), d) *P. infestans* cDNA potentially involved in pathogenicity (ssor). Subtractive hybridization products from RB inoculated with US8 (RB+US8 *minus* control), RB inoculated with US11 (RB+US11 *minus* control), DF inoculated with US8 (DF+US8 *minus* control) and DF inoculated with US11 (DF+US11 *minus* control). US8: *P. infestans* genotype US8 and US11: *P. infestans* genotype US11.

2.4.3. Gene sequence analysis

Fifty four TDFs re-amplified fragments were cloned using the pGEM-T Easy Vector system (Promega, Madison, WI, USA). To ensure that the correct bands were cloned, the plasmid was amplified with the appropriate AFLP primers and run adjacent to the original SH-AFLP reactions on a polyacrylamide gel. Three white colonies from each transformation event were selected and the respective inserts were sequenced (Macrogen, USA). Sequences were compared with existing databases. Thirteen sequences were discarded due to vector or adapter contamination. The remaining forty one sequences were singletons, representing unique sequences. Sequences have been submitted to the NCBI GenBank under the accession numbers described in Table 2.1. Seven sequences were novel and showed no matches to sequences in public databases. Six sequences showed no significant matches at the expectation values (E) of $< 10^{-4}$ to sequences in public databases, but were included in Table 2.1. The remaining sequences had significant hits. The BLAST analysis of the forty one sequences showed no homology with genes involved in photosynthesis, respiration, energy conversion, metabolism, transport, or protein synthesis.

Table 2.1 shows the closest database similarities for the differentially expressed TDFs. Seven *P. infestans* TDFs potentially involved in pathogenicity, possibly in the suppression of potato defense genes showed an average GC content of 50.2 %. Two transcripts (DL119 and DL95) shared homology with sequences from bacteria. In addition, DL119 and DL95 showed a similar expression pattern with N-succinyl diaminopimelate aminotransferase and a transcriptional regulator, TetR family

respectively. Transcripts DL32 and DL12 shared homology with *P. infestans* and *P. sojae* respectively. Eight transcripts from a susceptible host (DL21, DL33, DL2, DL49, DL138, DL17, DL90 and DL54) showed homology only with other hosts' sequences (i.e., *Solanum tuberosum*, *S. lycopersicum*, *Triticum monococcum* subsp. *Aegilopoides*, *Capsicum annuum* and *Aquilegia* sp.) and none with pathogens. We classified such fragments as potential plant disease susceptibility genes "ssept". Fifteen transcripts from potentially suppressed potato genes (ssed) showed an average of GC content of 42.2 %. Two sequences DL39 and DL10 had homology with EST sequences from the interaction potato-*P. infestans*. DL10 also had homology with Beta-amylase. Transcripts DL16 and DL40 shared homology with Sweet potato (Ayamurasaki) and *Lycopersicon esculentum*, respectively. DL91 and DL144 shared homology with EST sequences from *Solanum chacoense* and *Papaver somniferum*, respectively. In addition, the candidate resistance gene DL81 shared homology with an mRNA sequence from Potato callus. Finally, four cDNAs from potential *P. infestans* elicitor genes (avr) showed an average GC content of 45.5%. The transcript DL24 had homology with an EST sequence from Avr4/Cf-4 and Avr9/Cf-9 cDNA-AFLP *Solanum lycopersicum* cDNA. The sequence from DL41 had homology with a *P. infestans* avirulence-associated gene, DL22 shared homology with *Bradyrhizobium japonicum* inoculation *Glycine max* cDNA and DL145 shared homology with *P. infestans.*

Table 2.1. Similarities between sequences identified in this study and those available from different databases.

*Accession No.**	*TDF ***	*Closest similarity*	*BLAST*	*Score*	*Length (pb)*	*% GC*
GH456578	DL32-ssor	*P. infestans* supercont1.3326 of *Phytophthora infestans* [DNA] 2432-2485	[2]blastn	2.4e-20	118	48
GH456575	DL119-ssor	N-succinyl diaminopimelate aminotransferase, *Rhodococcus sp*. RHA1, YP_705882.1	[1]blast x	9e-18	217	54
GH456580	DL95-ssor	Transcriptional regulator, TetR family, *Psychrobacter sp*., YP_001280803.1	[1]blastx	3e-07	229	44
GH456571	DL12-ssor	*Phytophthora sojae* , CL356Contig1, Hypothetical Protein	[3] blastn	2e-15	132	52.7
GH456577	DL21-ssept	Late Blight-Challenged Tubers *S. tuberosum* var. Shepody cDNA clone , DR037839.1	[1]est	6e-30	117	44
GH456579	DL33-ssept	Mixed potato tissues *Solanum tuberosum* cDNA clone STMCU70, BQ114536	[5]blastn	3e-43	115	43
GH456555	DL2-ssept	*Solanum lycopersicum* chromosome 11 clone, AC171734.2	[1]nr/nt	4e-10	213	43
GH456555	DL2-ssept	*Solanum tuberosum* chromosome 1 clone RH043C23, gb|AC233383.1	[4]blastn	9.5e-15	213	43
GH456556	DL49-ssept	*Triticum monococcum* subsp. aegilopoides clone BAC TbBAC5, DQ904440	[1]nr/nt	4e-66	184	41
GH456557	DL138-ssept	Aquilegia cDNA library *A.formosa* x *A.pubescens* CO1Z974, DT765603.1	[1]est	2e-28	107	47
GH456558	DL17-ssept	*S.lycopersicum* DNA sequence, clone LE_HBa-31H5 on chromosome 4 CT485992.1	[1]nr/nt	6e-13	173	42
GH456573	DL90-ssept	*Capsicum annuum* cv. L11 (under stress) cDNA-AFLP Capsicum FE193189.1	[1]est	1e-30	259	47
GH456587	DL54-ssept	*S.tuberosum* Diploid genotype RH89-039-16 chromosome 1 clone, gb|AC232046.1	[4]tblastn	9e-04	224	37
GH456559	DL10-ssed	Beta-amylase, *Glycine max*, AJ871579.1	[1]nr/nt	3e-04	184	32
GH456559	DL10-ssed	*P. infestans*-challenged potato leaf, incompatible reaction (Kennebec), BQ047506.1	[1]est	4e-10	184	32
GH456560	DL16-ssed	Sweet potato (Ayamurasaki) developing tuberous root cDNA clone, DC880070	[1]est	3e-54	165	48
GH456561	DL39-ssed	*P. infestans*-challenged potato, mixed potato tissues *Solanum tuberosum*, BQ118239	[1]est	6e-70	155	43
GH456562	DL91-ssed	*Solanum chacoense* cDNA, mRNA sequence, DN981828.1 9	[1]est	7e-05	126	42
GH456563	DL123-ssed	*Homo sapiens* BAC clone , AC073475.4|	[1]nr/nt	1e-16	265	44
GH456564	DL144-ssed	Opium poppy root cDNA library *Papaver somniferum* cDNA, FG598894	[1]est	7e-07	198	43
GH456586	DL40-ssed	*Lycopersicon esculentum* clone 132606R, mRNA sequence, BT013743.1	[1]nr/nt	2e-37	177	38
GH456586	DL40-ssed	Cell division control protein CDC6b, putative (CDC6b) -Arabidopsis, NP_172207.2	[1]blastx	8e-06	177	38
GH456565	DL81-rtant	Potato callus cDNA library, POCAZ41, mRNA sequence, CK248238	[5]blastn	8e-36	276	47
GH456581	DL24-avr	Interaction library Potato tuber and *P.infestans* (US8 genotype), ES463440	[1]est	2e-12	103	47
GH456581	DL24-avr	Avr4/Cf-4 and Avr9/Cf-9 cDNA-AFLP *Solanum lycopersicum* cDNA , CK348338	[1]est	3e-16	103	47
GH456582	DL41-avr	*P. infestans* avirulence-associated protein 3.4F-A(3.4F-A) gene, DQ390339.1	[1]nr/nt	1e-14	152	52
GH456582	DL41-avr	*P. infestans* supercont1.40 of Phytophthora infestans [DNA] 1391132-1391178	[2]blastn	2.9e-14	152	52
GH456585	DL22-avr	*Bradyrhizobium japonicum* inoculation, Glycine max cDNA, DY762990	[1]est	3e-05	241	45.3
GH456572	DL145-avr	Supercont1.37 of *Phytophthora infestans*	[2]blastn	2e-04	216	38
GH456576	DL142-ssor	Novel - potential *P. infestans* suppressor gene				55

Table 2.1. Similarities between sequences identified in this study and those available from different databases **(Cont.)**

*Accession No.**	*TDF ***	*Closest similarity*	*BLAST*	*Score*	*Length (pb)*	***% GC***
GH456568	DL127-ssed	Novel - potential potato suppressed gene				50
GH456569	DL129-ssed	Novel - potential potato suppressed gene				53
GH456570	DL124-ssed	Novel - potential potato suppressed gene				43
GH456574	DL126-ssed	Novel - potential potato suppressed gene				51
GH456583	DL146-ssed	Novel - potential potato suppressed gene				48
GH456584	DL122-ssed	Novel - potential potato suppressed gene				53
GH456588	DL31-ssor	*P. infestans* clone PI-BAC-26O7, gnl|ogdg|14 # 67889..76750|, AC147180	[6]tblastn	0.047	232	55
GH456591	DL20-ssor	*Phytophthora infestans* clone PI-BAC-42H10, gnl|ogdg|10#10701..105830|, AC147005	[7]tblastn	0.076	103	43.2
GH456589	DL47-ssept	*S. tuberosum* genotype RH89-039-16 chromosome 5 clone RH204H15, b|AC232127.1|	[4]tblastn	0.0039	247	30
GH456590	DL28-ssept	*S. tuberosum* genotype RH89-039-16 chromosome 5 clone, gb|AC232126.1|	[4]tblastn	0.994	142	48
GH456566	DL120-ssed	*S. tuberosum* chromosome 1 clone RH088J16, gb|AC233448.1|	[4]tblastn	0.9999	129	40
GH456567	DL121-ssed	*S. tuberosum* genotype RH89-039-16 chromosome 5 clone RH130I12,gb|AC232089.1|	[4]tblastn	0.97	115	45

[1] NCBI Database, [2] *Phytophthora infestans* Database (Broad Institute), [3] Virginia Bioinformatics Institute - VBI Microbial Database,
[4] The Solanaceae Genomics Resource at MSU, [5] GabiPD - The Max Planck Institute of Molecular Plant Physiology, [6] Oomycete genomic database, [7] Oomycete genomic database.
* Sequences submitted to the NCBI GenBank database.
** Potential pathogenicity factor (ssor), potentially suppressed potato gene (ssed), potential *P. infestans* avirulenc gene (avr), potential resistance gene (rtant), potential plant disease susceptibility gene (ssept).

2.4.4. Identification of gene origin

To determine potato or pathogen origin of the TDF (DL81, Potential resistance gene) with significant homology to a gene in potato, specific primers were designed and the corresponding probe was prepared for this transcript. Southern blot and northern blot analyses (Figure 2.3 A), confirmed that DL81 was of potato origin. Southern blot analysis was performed with genomic DNA from both potato cultivars, and *P. infestans* strains. Under low stringency conditions, DL81 identified the homologous sequence in the genomic DNA from both potato cultivars. Northern blot was performed at least twice in independent experiments and the differential expression was confirmed for the transcript. In this experiment, the RNA for each plant-pathogen treatment was the same time course mixture (3-144 hpi) as was used for the subtractive hybridization (SH)/cDNA-AFLP protocol. RNAs from RB+US8, RB+US11, DF+US8, DF+US11, RB control and DF control were included. To reconfirm the Southern blot results, the *P. infestans* RNA mixture (sporangia, zoospores, germinating sporangia, germinating zoospores, appressoria and mycelium) was also included. The signal for DL81 was detected again in DF+US8 and DF+US11, the same interactions from which the transcript was excised.

2.4.5. Validation of expression patterns using qRT-PCR

To investigate the reliability of SH/cDNA-AFLP for detecting differentially expressed genes and verify the expression patterns observed, qRT-PCR analyses were carried out for four TDFs. Similarly to results from Southern blot and northern blot analyses, DL81 was detected in DF+US8 and DF+US11 by qRT-PCR (Fig. 2.3B). DL81 was induced in Defender 4- and 2-fold over the control plants (DF+H2O) in response to

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

In order to study the dynamics of gene expression during disease development, qRT-PCR analysis for the TDF DL81 was performed using two treatments and nine different times after inoculation similarly to those used for the SH/cDNA-AFLP analysis (Fig. 2.3C). Treatments RB+US8 and DF+US8 were selected based on their contrasting expression patterns in the mixed RNA that was used in the SH/cDNA-AFLP and qRT-PCR experiments (Fig. 2.3B). DL81 transcript was induced at 12 hpi in DF+US8 (Fig. 2.3C). The highest induction of this transcript occurred at 24 hpi. The transcript increased by 5.35 fold and 6.27 fold, respectively over the potato inoculated

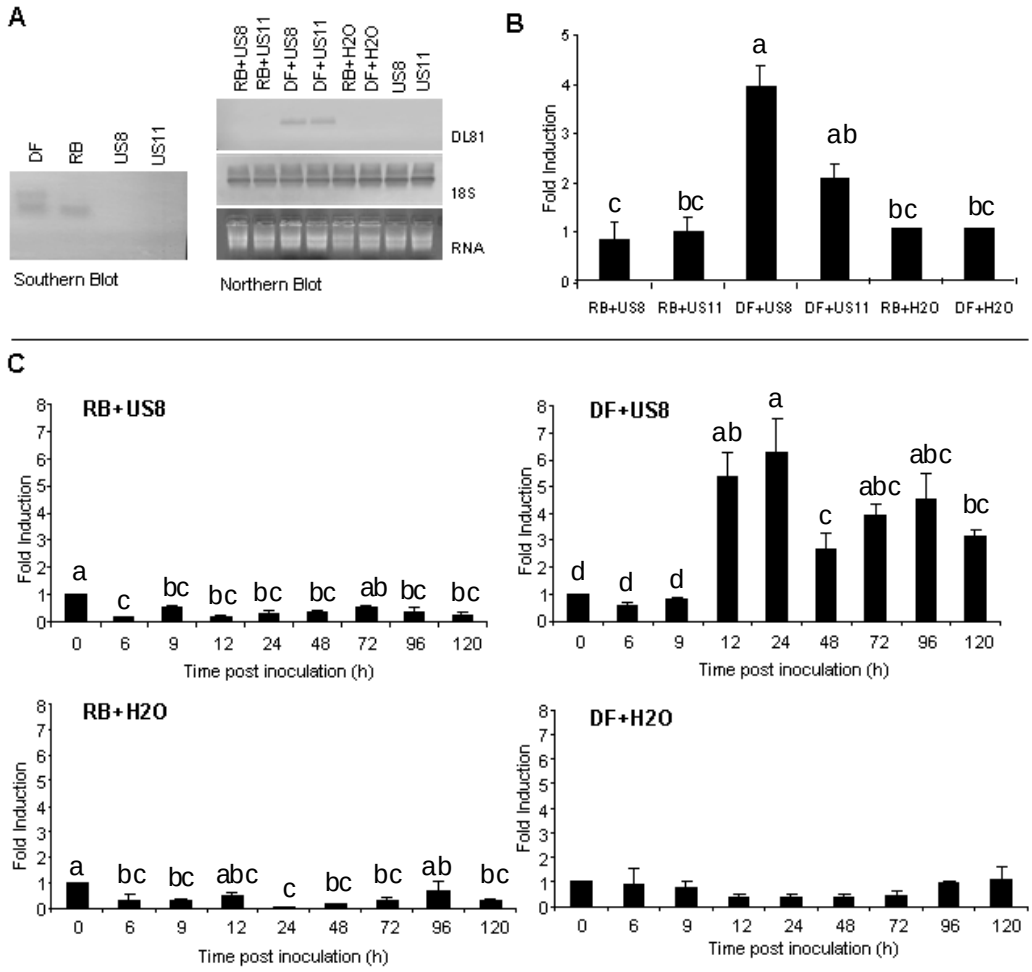

Figure 2.3. Validation of the potential resistance gene transcript DL81. Southern blot and Northern blot (A), qRT-PCR for mixed RNA from each plant-pathogen treatment (3-144 hpi) similar to those used for the (SH)/cDNA-AFLP analysis (B) and different times after inoculation similar to those used for the (SH)/cDNA-AFLP analysis (C). In Southern blot analysis: DF, genomic DNA of cultivars Defender (DF) and Russet Burbank (RB) and of *P. infestans* genotypes US8 and US11 were digested with EcoRI. Northern blot analysis and qRT-PCR show Russet Burbank inoculated with US8 (RB+US8), Russet Burbank inoculated with US11 (RB+US11), Defender inoculated with US8 (DF+US8), Defender inoculated with US11 (DF+US11), Russet Burbank control (RB+H2O), Defender control (DF+H2O), and *P. infestans* genotypes US8 and US11. In Real rime qRT-PCR, all PCR reactions were performed from triplicate biological samples. The $2^{-\Delta\Delta C(T)}$ method (Livak

and Schmittgen 2001) was used to calculate the fold expression relative to the controls. The elongation factor gene was used to normalize small differences in template amounts. plant near 0 hpi. The expression decreased slightly thereafter and remained relatively constant from 48 to 120 hpi. In contrast, DL81 transcript was down-regulated as early as 6 hpi in RB+US8. Means with the same letter are not significantly different according to Tukey's Studentized Range test. A $p < 0.05$ was considered to indicate significant differences.

Parallel inoculation with sdH_2O was included as a control for each time point (RB+H2O and DF+H2O). We compared expression levels of DL81 in control samples across all time points and found that expression levels of DL81 did not change significantly without inoculation of *P. infestans* (Fig. 2.3C). The results from control treatments at all time points support the conclusion that this transcript changed its expression levels in response to inoculation with the pathogen.

Figure 2.4A, also validates the reliability of SH/cDNA-AFLP for detecting differentially expressed genes. DL21, as a potential plant disease susceptibility gene, was excised from the compatible interaction between Defender and US8 and it was not present in the incompatible interaction between Defender and US11. The qRT-PCR analysis using the mixed RNA that was used in the SH/cDNA-AFLP revealed an up-regulation of DL21 in DF+US8 6-fold over the control plant (DF+H2O). Resembling DL81, only the up-regulated transcript DL21 in DF+US8 remaining in the supernatant (SH product selected - transcript of interest) was used for further cDNA-AFLP analysis.

Similar to DL81, qRT-PCR analysis for the TDF DL21 was performed at two treatments and nine different times after inoculation similarly to those used for the SH/cDNA-AFLP analysis (Fig. 2.4A). The treatments DF+US8 and DF+US11 were selected based on their contrasting expression patterns. DL21 transcript was induced at 6 hpi in DF+US8 (Fig. 2.4A). The expression was strongly up-regulated thereafter, with a maximum induction at 96 and 120 hpi. The transcripts increased from 3.38 fold (6 hpi.) to 25.13 fold (120 hpi.) over those from potato inoculated plants near 0 hpi. In contrast, the transcript DL21 was slightly up-regulated in DF+US11 at 9, 12 and 96 hpi., with a 3.75, 3.86 and 3.82-fold increase, respectively, over the potato inoculated plant near 0 hpi. In addition, the expression levels of DL21 in DF control (DF+H2O) across all time points did not change their expression level significantly without inoculation with *P. infestans* (Fig. 2.4A). Therefore, this transcript changed its expression levels in DF+US8 and DF+US11 in response to the pathogen infection.

According to qRT-PCR data, the potentially suppressed plant gene DL39, excised from DF+US8 and DF+US11, was upregulated 5- and 3-fold over the control (DF+H2O), respectively (Fig. 2.4B) and suppressed in RB+US8 and RB+US11. DL28, as a potential plant disease susceptibility gene, was excised from the compatible interaction between Russet Burbank and US8 (RB+US8) and was not expressed in RB+US11, DF+US8 or DF+US11. Consistently, the qRT-PCR analysis revealed an up-regulation of DL28 in RB+US8, 2-fold over the control and no expression in DF+US8 or DF+US11 (Fig. 2.4C). Gene expression of DL28 in RB+US11 was similar to the control RB+H2O. As a consequence, it was hybridized with the control and attached to the magnetic particles.

Only in RB+US8, the DL21 precursor remained in the supernatant and it was used for further cDNA-AFLP analysis and detected in the polyacrylamide gel.

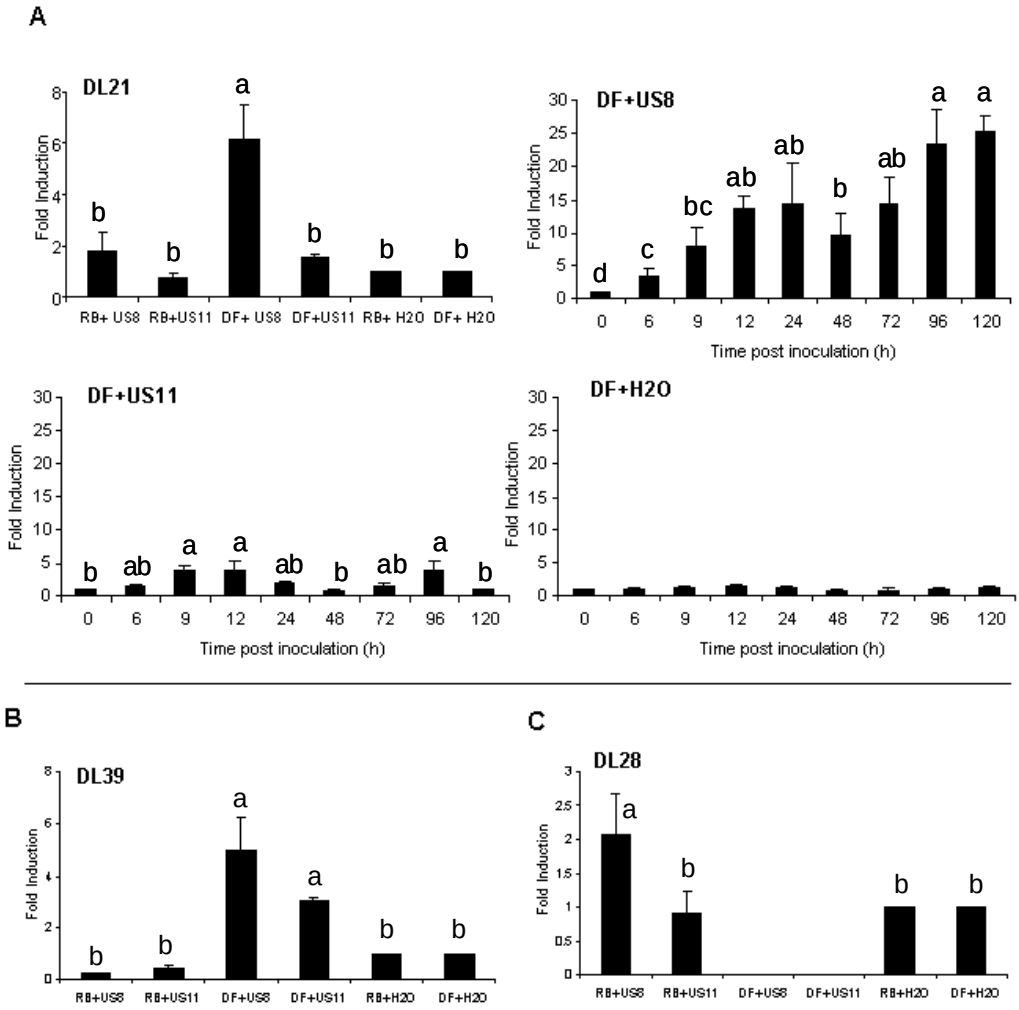

Figure 2.4. Validation of the TDFs DL21, DL39 and DL28 using qRT-PCR. DL21 qRT-PCR for mixed RNA from each plant-pathogen treatment (3-144 hpi) similar to those used for the (SH)/cDNA-AFLP analysis (A) and different times after inoculation similar to those used for the (SH)/cDNA-AFLP analysis (A). DL39 and DL28 qRT-PCR for mixed RNA from each plant-pathogen treatment (3-144 hpi) similar to those used for the (SH)/cDNA-AFLP analysis (B and C, respectively). RB+US8, Russet Burbank inoculated with US8; RB+US11, Russet Burbank inoculated with US11; DF+US8,

Defender inoculated with US8; DF+US11, Defender inoculated with US11; RB+H2O, Russet Burbank control; DF+H2O, Defender control; US8, *P. infestans* genotype US8; US11, *P. infestans* genotype US11. All PCR reactions were performed from triplicate biological samples. The $2^{-\Delta\Delta C(T)}$ method (Livak and Schmittgen 2001) was used to calculate the fold expression relative to the controls. The elongation factor gene was used to normalize small differences in template amounts. Means with the same letter are not significantly different according to Tukey's Studentized Range test. A $p < 0.05$ was considered to indicate significant differences.

2.5. DISCUSSION

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

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

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

50.2 %, cDNAs from potentially suppressed potato genes with an average GC content of 42.2 % and *P. infestans* potential avr genes with an average GC content of 45.5%.

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

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

The *P. infestans* transcript DL119 (ssor) exhibits sequence similarity to N-succinyl diaminopimelate aminotransferase. This is an important enzyme in the lysine

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

The sequences obtained from our TDFs, characterized as potentially suppressed potato genes, as well as the potential resistance gene DL81, did not match with any sequence from microorganisms. These results confirm the effectiveness of the subtractive hybridization (SH)/cDNA-AFLP protocol to separate genes from the pathogen and from the host. DL39 and DL10 matched with EST sequences from the interaction of potato with P. *infestans*. DL10 also showed homology with Beta-amylase, which is an important

protein of tuberous storage root of sweet potato and accumulation of starch in leaves with a possible role in defense (Ohto et al. 1992).

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

Little is known about the plant genes required for susceptibility. In 2002, N.A. Eckardt had reported that in a search of the ISI Web of Science identified, 524 documents related to "plant disease resistance" and just 1 match for the phrase "plant disease susceptibility". Seven years later, our search of the ISI Web of Science identified 1,130 documents related to "plant disease resistance" and just 11 matches for the phrase "plant disease susceptibility". Also, we found 2,254 documents referring to "resistance gene" and 5 documents referring to "susceptibility gene" in Plant Sciences. In our research, we found TDFs classified as potential plant disease susceptibility genes (DL21, DL33, DL2, DL49, DL138, DL17, DL90 and DL54) that showed homology with *Solanum tuberosum*, *Solanum lycopersicum*, *Triticum monococcum* subsp. Aegilopoides, *Capsicum annuum* and *Aquilegia* sp., and none with pathogens. Further studies of these potential susceptibility genes would represent a novel form of disease resistance derived from the failure of a gene that is necessary during a compatible interaction, rather than the activation of known host defense pathways (Vogel et al. 2006).

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

The gel-based subtractive hybridization profiling technique uses the advantages of cDNA-AFLP and subtractive hybridization in order to amplify cDNA products in a polyacrylamide gel and remove the constitutively/commonly expressed sequences. Using subtractive hybridization with paramagnetic particles, we did not have problems with re-annealing processes, because inoculated plant is represented by single stranded cDNAs. In addition, the control plant is also single strand cDNAs that attach to paramagnetic particles in the process. Therefore, there were no problems related to kinetics of hybridization and as a consequence, no need for a double hybridization, making the

process simple and reliable. The present study has shown that using the subtractive hybridization (SH)/cDNA-AFLP approach, it was not necessary to sequence hundreds of samples to detect *P. infestans* genes potentially controlling pathogenesis or avr genes, and potato genes potentially involved in resistance or susceptibility to plant disease.

CHAPTER 3: CLONING AND CHARACTERIZATION OF A NOVEL GENE FROM THE PLASTID-LOCALIZED 2-C-METHYL-D-ERYTHRITOL 4-PHOSPHATE (DOXP-MEP) PATHWAY IN POTATO INFECTED BY *PHYTOPHTHORA INFESTANS*

3.1. ABSTRACT

Isoprenoids are synthesized by two separate routes, the cytoplasmic mevalonate (Ac-MVA) and the novel plastidial 2*C*-methyl-D-erythritol-4-phosphate (DOXP-MEP) pathways. The initial step of the plastidial pathway is catalyzed by 1-deoxy-D-xylulose 5-phosphate synthase (DXS). We have cloned a 2,421 bp cDNA that contains a complete ORF (2,160 bp) from DXS in potato. This *DXS* gene named *StDXS1* encodes a protein that contains 719 amino acid residues, with a predicted molecular mass of 77.8 kDa and a deduced isoelectric point of 6.3. Southern blot analysis indicated that two gene copies of *StDXS* exist in the potato genome. Sequence alignments showed that StDXS1 has high homology to known DXS proteins from other plant species and belonged to the class I plant DXS based on phylogenetic analysis. Using a quadratic system composed of one susceptible and one moderately resistant potato cultivar during their interactions with one weakly- and one highly-aggressive strain of *Phytophthora infestans*, we found that *StDXS1* was up-regulated in the susceptible cultivar and down-regulated in the moderately resistant one, compared to non-inoculated potato plants. When assessing the expression pattern of five other genes controlling steps in the DOXP-MEP pathway, we detected changes in their expression in response to inoculation with the pathogen.

3.2. INTRODUCTION

Potato (*Solanum tuberosum* L.) is the most important food crop from the *Solanaceae* family (Friedman and McDonald 1997) and according to FAO (2007), it is the fourth most important crop after maize, wheat and rice, with an annual production of more than 323 million tonnes. Late blight of potato, caused by *Phytophthora infestans* (Mont) de Barry is the most important disease of this crop worldwide (Hardy et al. 1995). This disease can be initiated by sporangia or zoospores, affecting leaves, stems, as well as tubers (Judelson and Blanco 2005).

Several approaches have been used in disease management of fungal and oomycete pathogens (Daayf et al. 2003; El Hassni et al. 2004). In the case of late blight, the most appropriate and practical method would be the use of resistant varieties. However, developing stable resistance to late blight in potato is complex. While specific resistance genes are known, they are rapidly overcome by mutants in local populations of *P. infestans* (Fry and Goodwin 1997). Therefore, a better understanding of the mechanisms governing the interaction between potato and *P. infestans* is a requirement for the further development of durable resistance to late blight. In this context, the most aggressive strains of *P. infestans* (Daayf et al. 2000) suppress potato defense mechanisms, through transcriptional inhibition of phenylpropanoid (PAL) and acetate-mevalonate (Ac-MVA) pathways (Wang et al. 2004b; 2008).

Isoprenoids are all derived from two common precursors, isopentenyl diphosphate (IPP) and its isomer, dimethylallyl diphosphate (DMAPP) (McGarvey and Croteau 1995).

Until recently, it was considered that the mevalonate (Ac-MVA) pathway was the only route for the synthesis of isoprenoid precursors in all organisms (Chappell 1995). However, the 2*C*-methyl-D-erythritol-4-phosphate (MEP) pathway, also called the non-mevalonate route or DOXP-MEP pathway was discovered as an alternative terpenoid biosynthetic route, first in eubacteria (Flesch and Rohmer 1988) and soon after in photosynthetic organisms such as higher plants, algae as well as in cyanobacteria and diatoms (Cvejic and Rohmer 2000). This novel pathway also occurs in the malaria parasite *Plasmodium falciparum* (Jomaa et al. 1999) but not in humans, animals or archaebacteria. Therefore, this DOXP-MEP pathway was suggested as a perfect target to develop herbicides and antibacterial drugs (Lichtenthaler et al. 1997).

The DOXP-MEP pathway consists of eight reactions catalyzed by eight enzymes, seven of which are characterized structurally (Hunter et al. 2003). Briefly, 1-deoxy-D-xylulose 5-phosphate obtained by condensation of pyruvate and D-glyceraldehyde 3-phosphate goes through a reorganization associated with a reduction step. The resulting 2*C*-methyl-D-erythritol 4-phosphate is transformed into its cyclic diphosphate by the action of three enzymes. Then, 2*C*-Methyl-D-erythritol 2,4-diphosphate is transformed into IPP and DMAPP via 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate (Eisenreich et al. 2004).

Analysis of differentially expressed genes in the interaction between potato and *Phytophthora infestans* using the SH/cDNA-AFLP approach (Henriquez and Daayf 2010) revealed a fragment differentially expressed in potato and encoding 1-deoxy-D-xylulose

5-phosphate synthase (DXS), which catalyzes the first step of the DOXP-MEP pathway. To date, none of the DOXP-MEP pathway genes have been identified in potato. Therefore, our objectives were to (i) clone and characterize a 2,421 bp cDNA that contains a complete ORF (2,160 bp) from DXS, and (ii) analyze the expression of this gene and of five others from the DOXP-MEP pathway using real-time qPCR in two potato cultivars (i.e., susceptible vs. resistant) inoculated with two strains of *P. infestans* (i.e., weakly vs. highly aggressive).

3.3. MATERIALS AND METHODS

3.3.1. Plant material and treatments using *Phytophthora infestans* strains and inoculation

Potato cultivars, Russet Burbank (RB, susceptible) and Defender (DF, moderately resistant) were planted in clay pots containing soil-sand-peat-perlite mixture (4:4:4:1) and kept in a growth room at 20±2°C and 16 h photoperiod. Two *Phytophthora infestans* strains were used in this study. The isolate D-03 (lineage US11, weakly aggressive) and D1901 (lineage US8, highly aggressive) were grown on rye agar supplemented with 2% sucrose at 18°C. Potato plants were inoculated with a sporangia suspension (4 X 10^4 sporangia/ml), and with water for non-inoculated control plants following the method described by Wang et al. (2004b). This resulted into four inoculation treatments; (A) RB+US8, (B) RB+US11, (C) DF+US8, (D) DF+US11, plus two controls; non-inoculated Russet Burbank (RB+H2O) and non-inoculated Defender (DF+H2O).

3.3.2. Cloning of DXS cDNA

Using the SH/cDNA-AFLP approach (Henriquez and Daayf 2010) in the interaction between potato and *Phytophthora infestans* we identified a fragment that exhibited sequence similarity to 1-deoxy-D-xylulose 5-phosphate synthase (DXS) in *Lycopersicum esculentum* (AF143812), which catalyzes the first step of the DOXP- MEP pathway. This approach is a gel-based subtractive hybridization profiling technique that uses the advantages of cDNA-AFLP and subtractive hybridization in order to amplify cDNA products in a polyacrylamide gel and remove the constitutively/commonly expressed sequences. Subsequently, a 2,421-bp cDNA (Accession No. GU936657 See Appendix 2) that contains a complete ORF (2,160-bp) DXS was cloned from Russet Burbank using the primer pair P-NMVP1-FULLCDS-F (5`- CACCAACACACCCCACTAGA -3`) and P-NMVP1-FULLCDS-R (5`-AAGAGGAGGGCTTGAACTCAG -3`) designed through the conserved sequence of the known DXS in *Lycopersicum esculentum* (AF143812). RNA from Russet Burbank was extracted with TRIZOL (Invitrogen) and treated with Deoxyribonuclease I (Invitrogen) in order to preserve the integrity of RNA by degrading any possible residual genomic DNA. The DNase-treated RNA was reverse transcribed following the M-MLV (RT) enzyme (Invitrogen) manufacturer's recommendations. The PCR was performed in 25 µl reaction volumes containing 1 µl of potato Russet Burbank cDNA, 1X Buffer (500 mM KCl, 100 mM Tris-Hcl, 1% Triton), 0.1 mM of dNTP mix, 2 mM of $MgCl_2$, 0.25 µM of each primer and 1 unit of Taq DNA polymerase (Invitrogen) in a programmed thermocycler for 35 cycles of 30 s at 94°C, 30 s at the annealing of 55°C and 60 s at 72°C, followed by a final extension for 10 min at 72°C. To confirm the correct amplification of the gene, the PCR product was cloned into the bacterial plasmid

pGEM-T Easy Vector (Promega, Madison, WI, USA) following the manufacturer's directions. The plasmid was then transformed into *E. coli* DH5α, and sequenced using the universal primers T7 and SP6 primers from pGEM-T Easy Vector and the internal primers for the gene P-NMVP1-FULLCDS-F (5`- CACCAACACACCCCACTAGA -3`), P-NMVP1-FULLCDS-R (5`-AAGAGGAGGGCTTGAACTCAG -3`) and P-2NMVP1-F (5`- GCAGATGGTCCAACACATTG -3`). Sequences were analyzed with Seqman within the DNAStar program (DNAStar, Madison, WI, USA) and analyzed using BLASTn algorithm in GenBank.

3.3.3. Southern blotting

Potato DNA was extracted from leaves by the procedure of Mahuku (2004). The DIG–labeled DNA probe synthesis and southern blot were performed using the DIG DNA Labeling and Detection Kit (Roche) following the manufacturer's recommendations. Fifteen µg of genomic DNA from potato DF and RB was digested separately with EcoRI and HindIII and separated by gel electrophoresis. Hybridization of the DIG-labeled DNA probe was performed at 50°C for 24 h. DIG-labeled hybrids were detected with an anti-DIG-alkaline phosphatase conjugate and the substrates NBT and BCIP, which result in a light-blue precipitate. The primers NMVP1-FULLCDS-F (5`-CACCAACACACCCCACTAGA -3`) and P-3NMVP1-R (5'-TACACCGATCCCATTTCCTC-3') were used to prepare a 1813 bp probe from the cDNA fragment.

3.3.4. Bioinformatic analyses

Deduced amino acid sequence alignments were performed using the multiple sequence comparison by log-expectation program (MUSCLE 3.7) (http://www.phylogeny.fr/version2_cgi/one_task.cgi?task_type=muscle) (Edgar 2004; Dereeper et al. 2008). The theoretical molecular weight and pI value were calculated using the Compute pI/Mw tool (http://ca.expasy.org/tools/pi_tool.html) and a phylogenetic tree was constructed using Phylogeny.fr (http://www.phylogeny.fr/version2_cgi/simple_phylogeny.cgi) (Dereeper et al. 2008) at default setting and radial (By Drawtree) option.

3.3.5. qRT-PCR and data analyses

For measurement of transcript abundance, five micrograms of RNA at nine different times (0, 6, 9, 12, 24, 48, 72, 96, 120 hpi) after infection and inoculation with sterile water used as a control were treated with Deoxyribonuclease I (DNase) (Invitrogen) in order to remove DNA contamination. The DNase-treated RNA was converted to cDNA in a 50 µl reaction by reverse transcription modifying the M-MLV (RT) enzyme (Invitrogen) manufacturer's recommendations, and then diluted 1:3 with sterile water. Quantitative Real time PCR (qRT-PCR) was performed using the IQ SYB Green Supermix (Biorad), in 20 µl containing 2 µl diluted template, 6.5 µL of IQ SYB Green Supermix, and 0.375 µM of each primer. All primers used for qRT-PCR are shown in Table 3.1. Amplicons from each primer pair were cloned and sequenced to confirm primer specificity. Gene expression was quantified using a Stratagene Mx3005p cycler, and the following qPCR cycling program was used for all sets of primers: The

thermocycle program included 95 °C (2 min), followed by 40 cycles of 95 °C (15 s), 50 °C (45 s) and 72 °C (45 s). Melt-curve analysis was performed to observe primer-dimer formation and to check amplification of gene-specific products. All PCR reactions were performed from triplicate biological samples. The $2^{-\Delta\Delta C(T)}$ method (Livak and Schmittgen 2001) was used to calculate the fold expression relative to the controls. In addition to non-template (water controls), non-RT controls were used as templates to detect the presence of genomic DNA contamination. The reference gene elongation factor primers; Efactor-F 5'- GATGGTCAGACCCGTGAACAT -3' and Efactor-R 5'-GGGGATTTTGTCAGGGTTGT-3' (180 bp) (Genbank accession number; AB061263) were used to normalize small differences in template amounts.

3.3.6 Statistical analysis

The statistical analyses were performed with the Statistical Analysis Software (SAS) (SAS Institute, Cary, NC; release 9.1 for Windows). Prior to analysis, expression levels of DOXP-MEP pathway gene data sets were checked for normality (PROC Univariate). Thus, data were normalized by log+0.5 transformations for analysis. ANOVA analysis was performed using PROC GLM. Treatment means were separated using the Tukey's Studentized Range test. A $p < 0.05$ was considered to indicate significant differences.

Table 3.1. Primers used for the synthesis of the Southern blot probe, amplification and sequencing of the *StDXS1* gene, and for Quantitative Real time PCR (qRT-PCR) of *StDXS1, DXR* , *MCT* , *CMK* , *MDS* , *HDS* genes.

Primer name	Gene	Sequence 5'- 3'	GenBank Acc #
P-NMVP1-FULLCDS-F	*LeDXS*	CACCAACACACCCCACTAGA	AF143812
P-NMVP1-FULLCDS-R		AAGAGGAGGGCTTGAACTCAG	
NMVP1-FULLCDS-F	*StDXS1*	TACCAACACACCCCACTAGA	GU936657
P-NMVP1-FULLCDS-R		AAGAGGAGGGCTTGAACTCAG	
P-2NMVP1-F		GCAGATGGTCCAACACATTG	
Efactor-F	*Efactor*	GATGGTCAGACCCGTGAACAT	AB061263
Efactor-R		GGGGATTTTGTCAGGGTTGT	
P-3NMVP1-R	*StDXS1*	TACACCTATCCCATTTCCTC	GU936657
dxs-F	*StDXS1*	GCATTTCCTGGGATTTTGAA	GU936657
dxs-R		TTGGCGGTCTCTGTGTGTAG	
IspC-F	*DXR*	CCATCCTGATGCTGTCACTG	AF331705.2
IspC-R		CAAGCCTTGTATGCACTGGA	
IspD-F	*MCT*	CTTTCTCCAGGATGCCTCAG	EF636807.1
IspD-R		TCTTGCAGAGTCATGGATGC	
ispE-F	*CMK*	TGCCTACTGGAGCTGGTCTT	AF263101.1
ispE-R		CCTGAACAACCTCACCCCTA	
ISPF-F	*MDS*	TCTGGGGCTTCCTGATATTG	AK246318.1
ISPF-R		GCACCAAGCAGCTTACACAA	
ISPG-F	*HDS*	CAGCATTTGAGTTTGCCAGA	AF435086.1
ISPG-R		CCGATTGCAGACTTCATCCT	

3.4. RESULTS

3.4.1. Inoculation results

No symptoms were visible within the first 48 hpi, on either cultivar and only small lesions became noticeable at 72 hpi. Disease progress at 120 hpi, is shown in Figure 3.1. The US8 isolate caused spreading disease lesions and extensive tissue damage in Russet Burbank (Fig. 3.1A). By contrast, it only caused limited disease lesions in Defender (Fig. 3.1B). The US11 isolate caused small lesions in Russet Burbank (Fig. 3.1C) and failed to cause disease in Defender (Fig. 3.1D).

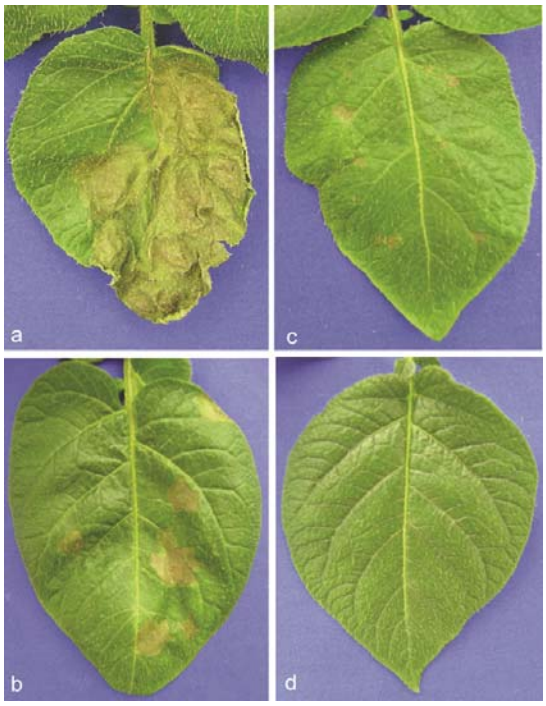

Figure 3.1. Infection of potato by *Phytophthora infestans. a) Russet Burbank* inoculated with US8 (RB+US8), b) Defender inoculated with US8, c) *Russet Burbank* inoculated with US11, d) Defender inoculated with US11 at 120 hpi.

3.4.2. Cloning and sequence analysis

Using the SH/cDNA-AFLP combinational approach (Henriquez and Daayf 2010) in the potato-*Phytophthora infestans* interaction, we identified a cDNA fragment in potato encoding 1-deoxy-D-xylulose 5-phosphate synthase (DXS), which catalyzes the first step of the DOXP-MEP pathway. Using cDNA from the potato cultivar Russet Burbank as template and primer pairs flanking the ORF region from the conserved sequence of the *DXS* gene from *Lycopersicum esculentum* (AF143812), we amplified a product of 2,421-bp in length, which we named *StDXS1*. The nucleotide sequence of

StDXS1 has been submitted to the GenBank database and assigned the accession No. GU936657 (See Appendix 2)

StDXS1 had an open reading frame of 2,160-bp starting with an initiation codon ATG at the position 133 and ending with a termination codon TAA at position 2,289. The protein encoded by this cDNA has 719 amino acid residues with a predicted molecular mass of 77.8 kDa and a deduced isoelectric point of 6.3. The amino acid sequence of StDXS1 is presented in Figure 3.2 along with published sequences of other plant DXS proteins. Sequence alignment (Fig. 3.2) revealed that StDXS1 had high similarity with other published DXS proteins from higher plants *Lycopersicon esculentum* (LeDXS, AF143812, 99% identity), *Nicotiana tabacum* (NtDXS, FN429979, 93% identity), *Medicago truncatula* (MtDXS1, AJ430047, 83% identity, and MtDXS2, AJ430048, 66% identity), *Ricinus communis* (RcDXS, XM_002516797, 85% identity), *Hevea brasiliensis* (HbDXS, AY502939, 84% identity), *Zea mays* (ZmDXS, EF507248, 81% identity), *Oryza sativa* (OsDXS, NM_001062059, 81% identity), *Catharanthus roseus* (CrDXS, AJ011840, 67% identity) and *Mentha piperita* (MpDXS, AF019383, 64% identity).

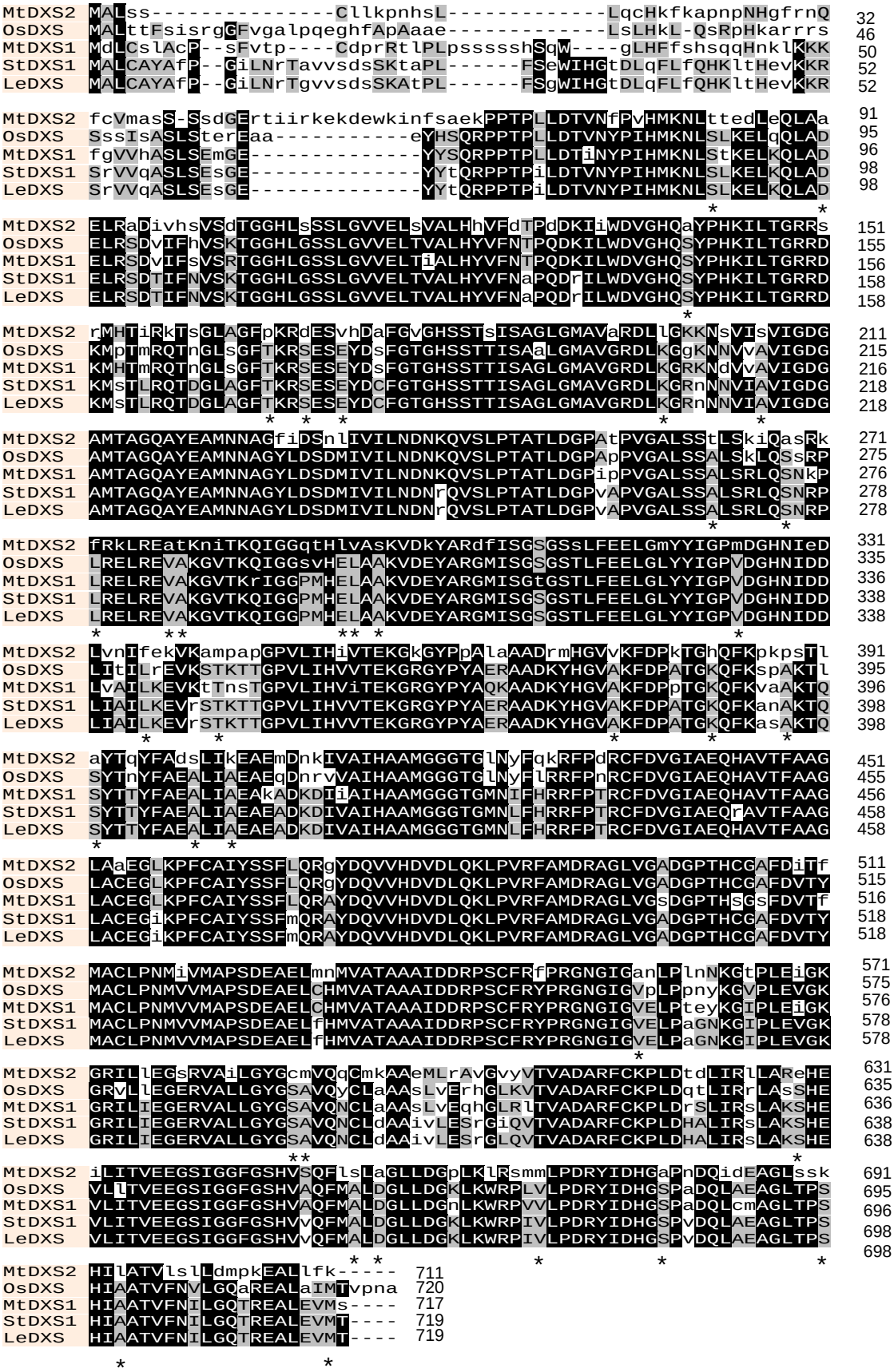
MtDXS2
OsDXS
MtDXS1
StDXS1
LeDXS

Figure 3.2. Alignment of deduced amino acid sequences of *Lycopersicon esculentum* (LeDXS; AF143812), *Medicago truncatula* (MtDXS1; AJ430047), *Medicago truncatula* (MtDXS2; AJ430048), *Oryza sativa* (OsDXS; NM_001062059), and *Solanum tuberosum* (StDXS1; GU936657). Dashes indicate gaps introduced to amino acid sequences to maximize alignment. Residues conserved among all five genes are shown in black, while residues shaded in grey are identical in at least two of five sequences shown. Dark-shaded asterisks indicate novel sites of conserved differences between class I and class II.

The blast searches and multiple alignments strongly suggested that StDXS1 has high homology with other DXSs and should be a functional plant DXS protein. In addition, plant DXS proteins contain an N-terminal domain which is not present in bacteria and feature a plastid targeting sequence (Lois et al. 2000). Our *StDXS1* clone is predicted to encode a 1-deoxy-D-xylulose 5-phosphate synthase (DXS) enzyme, based on the heterologous functional expression and the very high score of 99% identity of a 719 amino-acid sequence from *Lycopersicon esculentum* (LeDXS; AF143812). LeDXS have demonstrated *in vivo* activity and plastid targeting of plant DXS (Lois et al. 2000) (Fig. 3.2)

3.4.3. Southern blot

In order to investigate the genomic organization of *StDXS1*, genomic DNA from potato cultivars Russet Burbank and Defender were digested with EcoRI and HindIII, and subjected to Southern blot analysis in a triplicate experiment. Only two hybridization signals were present, indicating that StDXS1 belongs to a low-copy gene family (Appendix 3). The use of the two enzymes does not give conclusive results, but only an indication on the number of copies present.

3.4.4. StDXS1 is a member of Class I plant DXS

Based on their different expression patterns and amino acid sequences, DXS1 and DXS2 have been grouped in two classes (Class I and Class II) of plant DXS (Walter et al. 2002). To investigate the evolutionary relationships of StDXS1 and other DXS proteins, a phylogenetic tree was created based on the deduced amino acid sequences of DXS enzymes from different plant species (Fig. 3.3).

Phylogenetic analysis revealed that StDXS1 was most closely related to that of *Lycopersicon esculentum* (LeDXS; AF143812), *Nicotiana tabacum* (NtDXS, FN429979), *Medicago truncatula* (MtDXS1; AJ430047), *Ricinus communis* (RcDXS; XM_002516797), *Hevea brasiliensis* (HbDXS; AY502939), *Zea mays* (ZmDXS; EF507248) and *Oryza sativa* (OsDXS; NM_001062059), which were previously represented as plant DXS class I (Walter et al. 2002; Phillips et al. 2007; Seetang-Nun et al. 2008; Zhang et al. 2009), whereas *Catharanthus roseus* (CrDXS; AJ011840), *Mentha piperita* (MpDXS; AF019383Y), *Medicago truncatula* (MtDXS2; AJ430048), and the amino acid sequence deduced from assembled EST (tentative consensus-TC) of *Solanum tuberosum* (TC-StDXS2, TC17144) originated from the TIGR gene indices are grouped in the plant DXS class II.

A total of 37 amino acids (marked by asterisks in Figure 3.2) are conserved in plant DXS class I. It is also noteworthy that the members of DXS class I show high sequence identity (81-99%) with the exception of ZmDXS with RcDXS (63%), than they do to the DXS class II in the same species (63-82%) (Table 3.2).

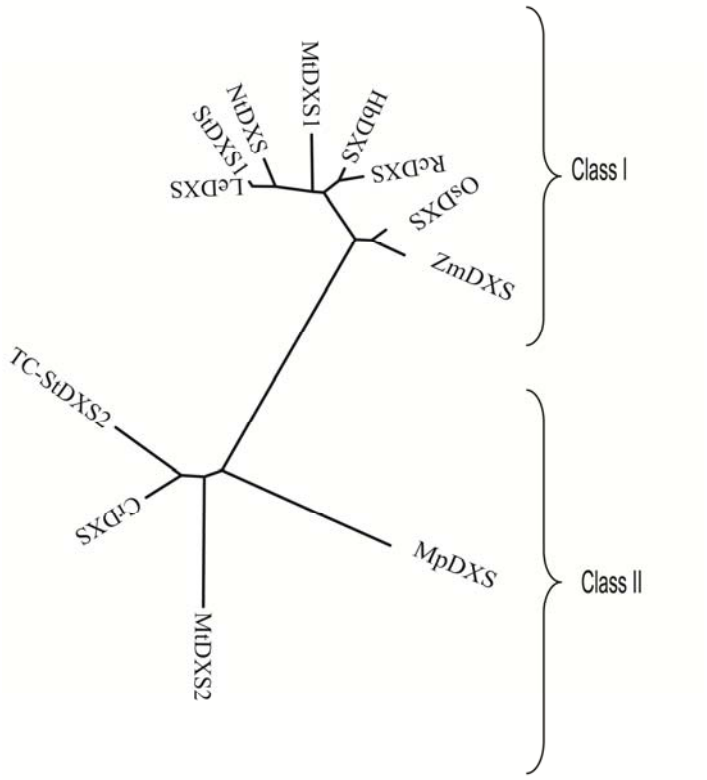

Figure 3.3. Phylogenetic tree of DXS proteins from different plant species. The tree was constructed using Phylogeny.fr (http://www.phylogeny.fr/version2_cgi/simple_phylogeny.cgi) (Dereeper et al. 2008) and the following sequences: *Lycopersicon esculentum* (LeDXS; AF143812), *Nicotiana tabacum* (NtDXS, FN429979), *Medicago truncatula* (MtDXS1; AJ430047), *Medicago truncatula* (MtDXS2; AJ430048), *Ricinus communis* (RcDXS; XM_002516797), *Hevea brasiliensis* (HbDXS; AY502939), *Zea mays* (ZmDXS; EF507248), *Oryza sativa* (OsDXS; NM_001062059), *Catharanthus roseus* (CrDXS; AJ011840) and *Mentha piperita* (MpDXS; AF019383) and *Solanum tuberosum* (*StDXS1*; GU936657) from GenBank database.

Table 3.2. Amino acid sequence identity between DXS class I and II

	StDXS1	LeDXS	NtDXS	MtDXS1	RcDXS	HbDXS	ZmDXS	OsDXS	MtDXS2	CrDXS	MpDXS	TC-StDXS2
	Class I								Class II			
StDXS1	100	99	93	83	85	84	81	81	66	67	64	64
LeDXS		100	93	83	85	85	82	81	66	67	64	64
NtDXS			100	84	86	85	81	82	67	68	65	64
MtDXS1				100	84	83	80	81	66	66	64	63
RcDXS					100	92	63	83	65	67	63	63
HbDXS						100	82	82	66	67	65	63
ZmDXS							100	89	67	68	66	65
OsDXS								100	68	68	64	65
MtDXS2									100	79	71	74
CrDXS										100	72	82
MpDXS											100	69
TC-StDXS2												100

Genbank accession numbers of these amino acid sequences: *Solanum tuberosum* (StDXS1; GU936657) *Lycopersicon esculentum* (LeDXS; AF143812), *Nicotiana tabacum* (NtDXS, FN429979), *Medicago truncatula* (MtDXS1; AJ430047), *Medicago truncatula* (MtDXS2; AJ430048), *Ricinus communis* (RcDXS; XM_002516797), *Hevea brasiliensis* (HbDXS; AY502939), *Zea mays* (ZmDXS; EF507248), *Oryza sativa* (OsDXS; NM_001062059), *Catharanthus roseus* (CrDXS; AJ011840), *Mentha piperita* (MpDXS; AF019383), and *Solanum tuberosum* (TC-StDXS2, TC17144).

3.4.5. *StDXS1* expression under pathogen inoculation in potato

Using four inoculation treatments (A) RB+US8, (B) RB+US11, (C) DF+US8, and (D) DF+US11, sampled nine times post-inoculation to represent all key time points of late blight infection in potato, we have performed a qRT-PCR analysis for our *StDXS1* gene encoding 1-deoxy-D-xylulose 5-phosphate synthase (DXS), which catalyzes the first step of the DOXP-MEP pathway. In order to eliminate possible variations due to experimental conditions, inoculation, plant development, or other factors, a parallel inoculation with sterile water was also evaluated as control for each time-point from non-inoculated Russet Burbank (RB+H2O) and non-inoculated Defender (DF+H2O).

StDXS1 transcripts in RB+US8 were induced at 12 hpi (Fig. 3.4). The transcript increased by 4.95 fold at 12 hpi compared to non-inoculated (control) potato plants. In RB+US11, there were no significant differences compared to non-inoculated potato plants. In RB+US8, the expression decreased drastically thereafter from 48 to 120 hpi. In contrast, *StDXS1* in DF+US8 and DF+US11 was down-regulated as early as 6 hpi. Since the $2^{-\Delta\Delta C(T)}$ method (Livak and Schmittgen 2001) was used to calculate the fold expression relative to the controls, the results at all time points support the conclusion that the expression level of this transcript changed in response to inoculation with the pathogen.

3.4.6. Transcriptional hierarchy of genes in the MEP Pathway

In an effort to deduce the hierarchical sequence of the enzymes involved in the DOXP-MEP pathway, in addition to the *StDXS1* transcript levels study, we assessed the expression pattern of five genes controlling steps in the DOXP-MEP pathway using qPCR. Primers were designed based on conserved sequences of the known genes (*DXR, MCT, CMK, MDS* and *HDS*) (Table 3.1).

The susceptible cultivar Russet Burbank, inoculated with US8 (RB+US8) or US11 (RB+US11) showed a downregulation trend of all five genes analyzed, which act downstream of DXS (*DXR, MCT, CMK, MDS* and *HDS*) (Fig. 3.4). However, the downregulation of *MDS* and *HDS* was less than that of *DXR, MCT* and *CMK*. On the other hand, in the partially resistant cultivar Defender inoculated with US8 (DF+US8) or US11 (DF+US11), *DXR* as well as *CMK* and *MDS* exhibited expression levels similar to

those observed in control plants. However, *HDS* transcript levels were drastically down-regulated as early as 6 hpi with *MCT* transcripts to a minor extent.

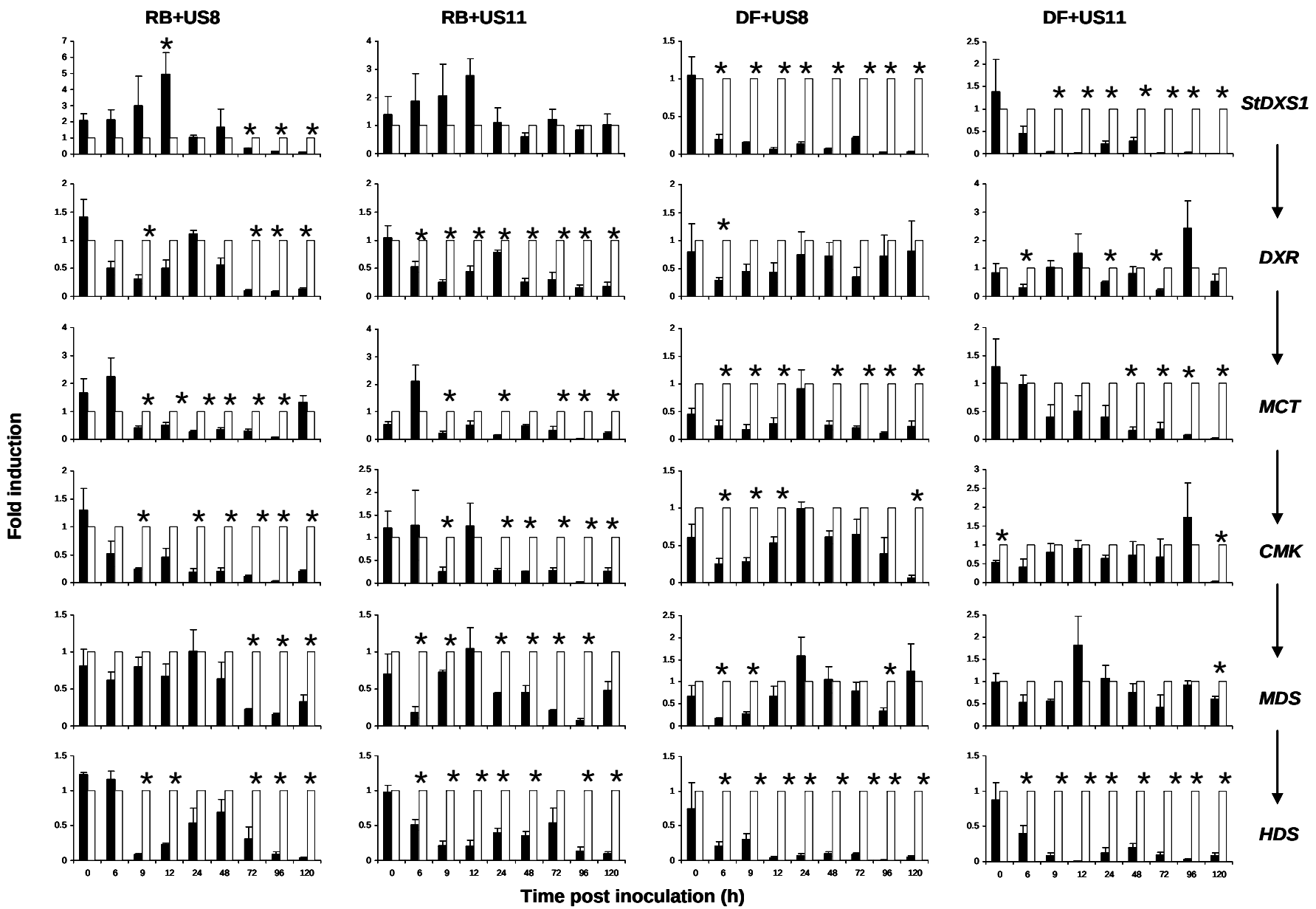
RB+US8
RB+US11
DF+US8
DF+US11
StDXS1
DXR
MCT
CMK
MDS
HDS
Fold induction
Time post inoculation (h)
0 6 9 12 24 48 72 96 120

Figure 3.4. Transcript profiles of genes encoding enzymes in the DOXP-MEP pathway. RB+US8: Russet Burbank inoculated with US8; RB+US11: Russet Burbank inoculated with US11; DF+US8: Defender inoculated with US8; DF+US11: Defender inoculated with US11. White bars represent the control. All qRT-PCR reactions were performed from triplicate biological samples. The $2^{-\Delta\Delta C(T)}$ method (Livak and Schmittgen 2001) was used to calculate the fold expression relative to the controls. The elongation factor gene was used to normalize small differences in template amounts. Mean of three replicates ±1 standard error are shown. Within individual time (h), means with asterisk (*) are significantly ($p < 0.05$) different compared to non-inoculated potato plants.

3.5. DISCUSSION

The DOXP-MEP pathway is an alternative isoprenoid biosynthetic route in plants (Lichtenthaler 2000). We isolated an open reading frame of 2,160-bp in length, from potato, encoding 1-deoxy-D-xylulose 5-phosphate synthase (DXS), which catalyzes the first step in the pathway. The first report of DOXP-MEP pathway regulation in a plant-pathogen interaction was reported by Walter et al. (2000). They reported root colonization of wheat, maize, rice and barley by mycorrhizal fungi to involve the induction of 1-deoxy-D-xylulose 5-phosphate synthase (DXS) and 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR). Subsequently, Walter et al. (2002) studied the expression of *MtDXS1* and *MtDXS2* in *Medicago truncatula* upon colonization of arbuscular mycorrhizas and Gil et al. (2005) analyzed resistance of the Arabidopsis *cbs3* mutant to biotrophic pathogens. The presence of the StDXS1 gene from potato was reported in 2002 by Walter et al. (2002). However, they used only virtual transcripts or

tentative consensus-TC of *Solanum tuberosum* originated from the TIGR gene indices for a tree and alignment data. Therefore, the expression of those virtual transcripts were not confirmed or cloned in potato.

Transcriptional analysis of StDXS1 and five genes controlling steps in the DOXP-MEP pathway in potato plants inoculated with two isolates of *P. infestans* revealed downregulation of five genes (*DXR, MCT, CMK, MDS* and *HDS*) in the susceptible cultivar Russet Burbank. This cultivar showed spreading disease lesions and extensive tissue damage when inoculated with the highly aggressive isolate US8 and small lesions when inoculated with the weakly aggressive US11. This DOXP-MEP pathway gene suppression in Russet Burbank, might lead to the reduction in the accumulation of isoprenoids that participate in essential plant processes such as respiration, photosynthesis, regulation of growth and development and plant protection against pathogens (Lois et al. 2000). The isoprenoids rishitinol, rishitin, lubimin, phytuberin, α-solanine, α-chaconine, solanidine, abscisic acid, cedrol and farnesol (Engstrom et al. 1999; Dixon 2001) have been previously linked to potato resistance against *P. infestans*. In addition, it has been reported transcriptional inhibition by aggressive strains of *P. infestans* of the Mevalonate (Ac-MVA) pathway and further rishitin accumulation in potato (Wang et al. 2004b).

Potato cultivar Defender inoculated with US8 (DF+US8) or US11 (DF+US11) exhibited expression levels of *DXR, CMK* and *MDS* similar to control plants, and down-regulation of *StDXS1*, *HDS* and *MCT* transcripts, in parallel with limited or no disease lesions by the *P. infestans* US8 or US11 isolates, respectively. These results suggested

that there might be another compensation mechanism to deal with the upstream suppression of the MEP pathway in the *DXS* gene expression. Southern blot analysis in this study revealed two gene copies of DXS in potato and it might be possible that the other potato DXS isoform is playing a role in the interaction between Defender and *P. infestans* isolates. The existence of more than one DXS isoform in the plant suggests that biosynthesis of isoprenoids may be dependent on particular DXS activities (Lichtenthaler 2000; Walter et al. 2002). For example, the albino genotype of *cla1* mutants (DXS mutant) in Arabidopsis was not rescued by the other two putative DXS isoforms (Rodriguez-Concepcion and Boronat 2002). In addition, DXS and DXR are both potential contributers to the metabolic flux of plastid isoprenoid precursors. However, when fosmidomycin, which has been shown to inhibit DXR activity in plants (Schwender et al. 1999; Mueller et al. 2000) was applied to tomato plants, it reduced seed dormancy, inhibited carotenoid synthesis, but did not influence other features of ripening. Therefore, at least in tomato fruits, DXR is a non-limiting factor in isoprenoid biosynthesis.

In Arabidopsis, the At4g15560 gene has been demonstrated to encode functional DXS enzyme (Estevez et al. 2000), but two additional genes with homology to DXS sequences are present in Arabidopsis (Rodriguez-Concepcion and Boronat 2002). In *Ginkgo biloba*, two *DXS* genes have been identified, *GbDXS1* (Kim et al. 2005b) and *GbDXS2* (Kim et al. 2006). Three paralogs for Maize DXS were identified (Walter et al. 2002; Vallabhaneni and Wurtzel 2009) and the recently discovered paralog DXS3 in maize is also present in sorghum and rice species (Vallabhaneni and Wurtzel 2009). In

Hevea brasiliensis (HbDXS) two *DXS* genes have been reported (Seetang-Nun et al. 2008).

The regulation of DXS genes have been reported in different plants, under different organs and stress conditions (Rodriguez-Concepcion and Boronat 2002; Walter et al. 2002; Kim et al. 2005a; Seetang-Nun et al. 2008; Vallabhaneni and Wurtzel 2009). However, based on their different expression patterns and amino acid sequences, DXS1 and DXS2 have been grouped in two classes (Class I and Class II) of plant DXS (Walter et al. 2002). Phylogenetic analysis revealed that StDXS1 was most closely grouped with previously represented class I plant DXS (Walter et al. 2002; Phillips et al. 2007; Seetang-Nun et al. 2008; Zhang et al. 2009). In fact, StDXS1 was grouped with MtDXS1 (AJ430047), previously reported to be abundant in all above-ground tissues including leaves, stems, and flowers in *Medicago truncatula,* with minor levels in roots and mycorrhizal roots. MtDXS2 (AJ430048) transcript accumulation was stimulated in mycorrizhal roots compared to non-mycorrizhal control roots. Phillips et al. (2007) investigated the role of three *Picea abies DXS* genes in a time-course analysis of plants treated by mechanical wounding or MeJA. *PaDXS1* transcript responded only slightly to these treatments, but *PaDXS2 and PaDXS3* were highly up-regulated.

The drastic suppression of the *HDS* gene in Defender inoculated with US8 and US11 is probably an unpredicted link between the DOXP-MEP pathway and plant defense, similar to the response in Arabidopsis mutant *cbs3* with reduced HDS activity, which showed a strong resistance to biotrophic pathogens (Gil et al. 2005). Whereas wild-type plants were heavily colonized by the oomycete *Hyaloperonospora parasitica*,

and allowed it to complete its life cycle, Arabidopsis plants with reduced HDS activity significantly repressed the growth of this pathogen. *Csb3* plants also showed enhanced accumulation of SA and the activation of the defense-related marker genes PR1 and PR2, which are ultimately controlled by the SA-mediated signalling pathway (Durrant and Dong 2004). Further experiments directed towards understanding the effects of the DOXP-MEP pathway on the accumulation and SA signalling in potato will help elucidate the role of this pathway in disease resistance.

Preliminary data from a mevalonate (Ac-MVA) pathway study (See Appendix 3) by qRT-PCR analysis, showed up-regulation of *HMGR2* transcripts in RB+US8, RB+US11 and DF+US8 using the same treatments and time post-inoculation analyzed in this research. Based on these data, a perspective study of a possible "compensation" route from the mevalonate (Ac-MVA) pathway in the accumulation of the plastid isoprenoid precursors, isopentenyl diphosphate (IPP) and its isomer, dimethylallyl diphosphate (DMAPP), should be further carried out. Recent studies have established that, in plants, minor amounts of metabolites common to the cytoplasmic mevalonate (Ac-MVA) and plastidial non-mevalonate pathway (DOXP-MEP) can be exchanged in both directions via the plastid membranes (Eisenreich et al. 2004). Some exchange of isopentenyl diphosphate (IPP) or common down-stream intermediates have also been reported to take place between the plastids and the cytoplasm (Lichtenthaler et al. 1997; Eisenreich et al. 1998). However, the dynamics and regulation of compensation routes might differ considerably in different cell types, species, and/or developmental stages (Rodriguez-Concepcion and Boronat 2002).

This work opens up new perspectives to direct further research into (i) novel functions of the DOXP-MEP pathway in plants in response to pathogens, (ii) DOXP-MEP regulation of defense signalling, and (iii) the part of this pathway, versus the mevalonic pathway, in terpenoid-related mechanisms of defense. Transgenic plants expressing DXS and the other DOXP-MEP genes should provide further evidence to determine the role of each enzyme, and their combination as a pathway, in plant-pathogen interactions.

CHAPTER 4: *PHYTOPHTHORA INFESTANS* EFFECTORS SUPPRESS SECONDARY METABOLISM PATHWAYS IN POTATO

4.1 ABSTRACT

The effects of *P. infestans* glucans and eicosapentanoic acid (EPA), as well as isolates of this pathogen, on the differential expression of 14 genes from the phenylpropanoid, the mevalonate (Ac-MVA), and the DOXP-MEP pathways were analyzed in potato by RT-PCR and qRT-PCR. The expression ratios of phenylalanine ammonia-lyase *PAL-1*, 4-coumarate:coenzyme A ligase *(4CL*), 3-hydroxy-3-methylglutaryl coenzyme A reductase *HMGR, HMGR3* and squalene synthase (*SQS*) in potato were not affected by *P. infestans*, its tested effectors or the combination of both. Sesquiterpene cyclase (*SC*) transcripts were down-regulated in the treatments showing most symptoms (RB+US8 and RB+GL+US8). The function of EPA eliciting defense responses in Defender inoculated with US8 could be related to the up-regulation of phenylalanine ammonia-lyase *PAL-2*. Down-regulation of (E)-4-hydroxy-3-methylbut-2-enyl diphosphate synthase (*HDS*) transcrips in the moderately resistant cultivar Defender inoculated with US8 and US11 is probably an unpredicted link between the DOXP-MEP pathway and plant defense. These finding suggests that genetic resistance in potato against *P. infestans* is not the result of isolated reactions against the pathogen, rather the combination of different factors that suggest a polygenic trait or horizontal resistance.

4.2 INTRODUCTION

Plant pathogens have the ability to manipulate biochemical, physiological and morphological processes in their host plants through a diverse array of extracellular effector molecules that can either promote the infection or trigger defense responses (Torto et al. 2006b). Many of these effectors are soluble glucans, glycoproteins or proteinaceous compounds synthesized constitutively by the fungal pathogen. However, many can be surface components released from the cell wall of the microbe or the host (Cote et al. 1998; Radman et al. 2003; El Modafar et al. 2006; Wolski et al. 2006).

The oomycete *Phytophthora infestans*, which causes potato late blight disease, remains a subject of study by many researchers, because of its great economic importance and the genotypic changes in its population structure during the last two decades (Daayf et al. 2001). The success of the new *P. infestans* genotypes was suggested to be due at least partially to their differential ability to suppress plant defenses through more efficient effectors (Wang et al. 2008).

Effectors that suppress host defense responses have been described in several pathosystems involving the genus *Phytophthora*. Sanchez et al. (1994) showed that water-soluble glucans (WSG) from *Phytophthora capsici* suppress the elicitor-induced death of suspension culture cells of susceptible sweet pepper and tomato, but not that of resistant pepper and tobacco. Ozeretskovskaya et al. (2001) also isolated potato immunosuppressors ß-1,3-ß-1,6-glucans from both mycelium and liquid culture of *P. infestans*. Glucans isolated from *P. infestans'* cell walls suppressed the accumulation of

phytoalexins and reduced ß-1,3-glucanase activity in potato tubers (Andreu et al. 1998). All these glucan-suppressors from *Phytophthora* showed race specificity because they suppressed potato defenses only when they were obtained from the strain that was compatible with the potato cultivar tested (Andreu et al. 1998; Ozeretskovskaya et al. 2001; Vasyukova et al. 2003).

Phytophthora infestans also produces elicitors, such as arachidonic acid (AA) and eicosapentaenoic acid (EPA), which elicit phytoalexin accumulation (Andreu et al. 1998; Coquoz et al. 1998). During plant-pathogen interactions, cell wall reinforcement through lignin synthesis, production and accumulation of secondary metabolites from the phenylpropanoid and octadecanoid pathways, and the accumulation of phytoalexins are well-known plant defense responses (Kombrink and Somssich 1995; Hammerschmidt 1999; Hückelhoven 2007). Phenylpropanoids exhibit a broad-spectrum antimicrobial activity and are therefore believed to help the plant fight microbial diseases (Dixon et al. 2002).

Terpenes and related secondary metabolites (sesquiterpenoids and sterols) are important factors in plant resistance to several pests and pathogens (Harborne 1988). The exposure of potato tubers to *P. infestans* or to a crude elicitor (hyphal wall components, HWC) induces the accumulation of phenylpropanoid compounds and sesquiterpenoid phytoalexins (i.e., lubimin, rishitin) (Nakane et al. 2003). The enzyme 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) catalyzes the first step specific to isoprenoid biosynthesis. Downstream, squalene synthase and sesquiterpene cyclase

catalyze the first steps in the branches leading to sterols and steroidal glycoalkaloids (SGA), and sesquiterpenoid phytoalexins, respectively (Krits et al. 2007). Yoshioka et al. (2001) have shown that HWC induced gene expression of HMGR and sesquiterpene cyclase, key enzymes of rishitin biosynthesis in the isopropenoid pathway.

Many studies reported the effect of different glucan suppressors on defense responses during the potato-*P. infestans* interaction (Andreu et al. 1998; Ozeretskovskaya et al. 2001; Vasyukova et al. 2003). However, they were all carried out "*in vitro*" or using potato tubers, and none have shown these effects in whole plants.

The objective of this study was to investigate the effects of (i) glucans (suppressors), (ii) the eicosapentanoic acid (EPA, elicitor), and (iii) *P. infestans* isolates on gene expression of two potato cultivars with different levels of resistance to late blight. We proposed to carry out these studies through the differential expression of genes from the DOXP-MEP pathway as an extension of our previous work (See Chapter 3), assessing the expression pattern of the six genes controlling steps in this pathway (*STDXS1, DXR, MCT, CMK, MDS* and *HDS*). The expression of known genes from the phenylpropanoid and mevalonate (Ac-MVA) pathways was also assessed.

4.3 MATERIALS AND METHODS

4.3.1 Plant, fungal races and growth conditions

The highly susceptible cultivar "Russet Burbank" (RB) and the moderately resistant cultivar "Defender" (DF) were used. Plants were produced from high quality tubers and planted in clay pots containing soil-sand-peat-perlite mixture (4:4:4:1) and kept in a growth chamber at 20 ± 2 °C and 16 hours photoperiod (Bowyer et al. 1995). The *P. infestans* strains D-03 (lineage US11, A1 mating type, weakly aggressive) and D1901 (lineage US8, A2 mating type, highly aggressive) were grown on rye agar medium supplemented with 2 % sucrose at 18°C (Wang et al. 2004b).

4.3.2 Experimental design

A full 2x3x4 factorial design, completely randomized and replicated, was used. This 2x3x4 factorial arrangement consists of the following treatments: two cultivars: Russet Burbank and Defender; three inoculums: H_2O (control), *P. infestans* strains D1901 (lineage US8, A2 mating type, highly aggressive) and D-03 (lineage US11, A1 mating type, weakly aggressive); and four effectors: eicosapentanoic acid (EPA), Glucans (GL), EPA+GL, and H_2O. Twenty four treatment combinations in total were used (Table 4.1). The primary leaflet of the fourth fully-grown potato leaf in each stem of 8-wk-old Russet Burbank and Defender plants, were treated as follows: 1) 100 µl of 0.2 µM eicosapentanoic acid (EPA) applied on the leaflet (as multiple tiny droplets using a micropipette to prevent run off) 2) 100 µl of glucans from race C (1, 4, 10, 11); Mating

type A2) (kindly provided by Dr. Adriana Andreu, Universidad Nacional de Mar del Plata - Argentina) (200 µg in 100 µl of water as emulsion), 6 hours after EPA treatment, 3) Ten 10-µl-droplets of *P. infestans* spore suspension (4X 10^4 sporangia /ml) 12 h after EPA application. Control plants were treated with water. The plants were placed in a moist chamber (20 ± 2 °C, 16 photoperiod, 100% relative humidity) for 48 h and then, put in a growth chamber at 20 °C ± 2 and 16 h photoperiod. We used three replicates per treatment.

Table 4.1. Treatments from the 2x3x4 factorial design.

1	RB+ H_2O
2	RB+ US8
3	RB+EPA
4	RB+GL
5	RB+EPA+GL
6	RB+EPA+US8
7	RB+ GL+US8
8	RB+EPA+GL+US8
9	DF+H_2O
10	DF+US8
11	DF+EPA
12	DF+GL
13	DF+EPA+GL
14	DF+EPA+US8
15	DF+ GL+US8
16	DF+EPA+GL+US8
17	RB-US11
18	RB+EPA+US11
19	RB+ GL+US11
20	RB+EPA+GL+US11
21	DF-US11
22	DF+EPA+US11
23	DF+ GL+US11
24	DF+EPA+GL+US11

RB: Russet Burbank, DF: Defender, H_20: sterile water, US8: *P. infestans* strains D1901 (lineage US8, A2 mating type, highly aggressive), US11: *P. infestans* strains D-03 (lineage US11, A1 mating type, weakly aggressive), EPA: eicosapentanoic acid (EPA), Gl: Glucan race C.

4.3.3 RT-PCR analysis and Sequence analysis

Five micrograms of total RNA extracted with Trizol (Invitrogen) from three replicates of each treatment (Table 4.1) were used to obtain the first–strand synthesis following the M-MLV (RT) enzyme (Invitrogen) manufacturer's recommendations. The RT-PCR was performed in 25 µl reaction volumes containing 1 µl of the first–strand

synthesis pool from each treatment, 1X Buffer (500 mM KCl, 100 mM Tris-Hcl, 1% Triton), 0.1 mM of dNTP mix, 2 mM of $MgCl_2$, 0.25 μM of each primer and 1 unit of Taq DNA polymerase (Invitrogen) in a thermocycler programmed for 35 cycles of 30 s at 94°C, 30 s at 55°C for all the primers with the exception of *SQS* which has annealing temperature of 45°C, and 60 s at 72°C, followed by a final extension for 10 min at 72°C. The sequences of primers used are listed in Table 4.2. Potato elongation factor gene was included in the RT-PCR assay as a constitutively expressed internal control. In addition, to verify the correct amplification of each gene, the PCR product from each one was isolated with the Qiaex II gel-extraction kit (Qiagen Inc., Alameda, CA, USA) following the manufacturer's instructions. The isolated fragments were cloned into the bacterial plasmid pGEM-T Easy Vector (Promega, Madison, WI, USA) following the manufacturer's instructions. The plasmids were then transformed into *E. coli* DH5α, sequenced and analyzed with Seqman within the DNAStar program (DNAStar, Madison, WI, USA). Sequences were examined with Seqman within the DNAStar program (DNAStar, Madison, WI, USA) and analyzed using BLASTn algorithm in GenBank.

The PCR products were resolved by electrophoresis on ethidium bromide stained gels, photographed utilizing an Alpha Imager (Alpha Innotech Corporation, San Leandro, Calif.), and the intensity of the bands was evaluated using the densitometry function and numerically expressed as the relative density in comparison to the optical density of the background. Furthermore, all results were normalized to the expression of the elongation factor housekeeping gene. Each sample was assayed in triplicate.

Table 4.2. Primers used for the RT-PCR analysis

Primer Name	Gene	Primer Sequence 5' – 3'	GenBank Accession Number
QRT-chalc-synth-F	*CHS*	TGGTGGTTGAAGTACCAAAACTTG	X14599
QRT-chalc-synth-R3		AGTACAGTGCCACCAGCAAA	
QRT-4CL-F	*4CL*	TCGTAGCGCTGCCGTATTC	AF150686
QRT-4CL-R2		AAATTGCTGCTCCGACTCTC	
PAL1-F	*PAL1*	TGCACAAGTTGCATCCATT	X63103
PAL1-R		AAGAGCACCACCATTTTTGG	
PAL2-F	*PAL2*	GCACCATCAATTGCACAAAA	X63104
PAL2-R		TGCAACTTGTGCAACAGTCA	
QRT-hmg-F	*HMGR*	CGTTCTGGATTACCTTCAGAGTGA	L01400
QRT-hmg-R2		CACAAGAGCAGCAACCTCAG	
QRT-hmg3-F	*HMGR3*	TCATCGGCATATCTGGGAACT	U51986
QRT-hmg3-R2		GAGACAATATTGCTGGCATGG	
SqualeneSyn-F	*SQS*	ATGGCACTTTGCATGTGGTA	AB022599
SqualeneSyn-R		CCAGAGGCATGGAACAGTTT	
SesqCyclas-F	*SC*	TCCAATTCCGATTGCTTAGG	gi|3108342
SesqCyclas-F		AGTGGAGAAAGCGAGTGCAT	
QRT-EF-1-alpha-F	*Efactor-F*	GATGGTCAGACCCGTGAACAT	AB061263
QRT-EF-1-alpha-R2		GGGGATTTTGTCAGGGTTGT	

Gene: Phenylalanine ammonia-lyase (*PAL-1* and *PAL-2*), 4-coumarate:coenzyme A ligase *(4CL*) and chalcone synthase (*CHS*), 3-hydroxy-3-methylglutaryl coenzyme A reductase (*HMGR, HMGR3*), squalene synthase (*SQS*), sesquiterpen cyclase (*SC*), *Elongation factor* (*Efactor*).

4.3.4. qRT-PCR and data analyses

Five micrograms of total RNA from three replicates of each treatment (Table 4.1) were used to obtain the first-strand synthesis following the M-MLV (RT) enzyme (Invitrogen) manufacturer's recommendations. Quantitative Real time PCR (qRT-PCR) was performed using the IQ SYB Green Supermix (Biorad), in 20 µl containing 2 µl diluted template, 6.5 µL of IQ SYB Green Supermix, and 0.375 µM of each primer. All primers used for qRT-PCR are shown in Table 4.3. Amplicons from each primer pair were cloned and sequenced to confirm primer specificity. Gene expression was quantified

using a Stratagene Mx3005p cycler, and the following qPCR cycling program was used for all sets of primers: The thermocycle program included 95 °C (2 min), followed by 40 cycles of 95 °C (15 s), 50 °C (45 s) and 72 °C (45 s). Melt-curve analysis was performed to observe primer-dimer formation and to check amplification of gene-specific products. All PCR reactions were performed from triplicate biological samples. The $2^{-\Delta\Delta C(T)}$ method (Livak and Schmittgen 2001) was used to calculate the fold expression relative to the control inoculated with water. The reference gene elongation factor was used to normalize small differences in template amounts.

Table 4.3. Primers used for Quantitative Real time PCR (qRT-PCR) of *StDXS1, DXR*, *MCT*, *CMK*, *MDS and HDS* genes.

Primer name	Gene	Sequence	GenBank Acc #
Efactor-F	*Efactor*	GATGGTCAGACCCGTGAACAT	AB061263
Efactor-R		GGGGATTTTGTCAGGGTTGT	
dxs-F	*StDXS1*	GCATTTCCTGGGATTTTGAA	GU936657
dxs-R		TTGGCGGTCTCTGTGTGTAG	
IspC-F	*DXR*	CCATCCTGATGCTGTCACTG	AF331705.2
IspC-R		CAAGCCTTGTATGCACTGGA	
IspD-F	*MCT*	CTTTCTCCAGGATGCCTCAG	EF636807.1
IspD-R		TCTTGCAGAGTCATGGATGC	
ispE-F	*CMK*	TGCCTACTGGAGCTGGTCTT	AF263101.1
ispE-R		CCTGAACAACCTCACCCCTA	
ISPF-F	*MDS*	TCTGGGGCTTCCTGATATTG	AK246318.1
ISPF-R		GCACCAAGCAGCTTACACAA	
ISPG-F	*HDS*	CAGCATTTGAGTTTGCCAGA	AF435086.1
ISPG-R		CCGATTGCAGACTTCATCCT	

Gene: 1-deoxy-D-xylulose 5-phosphate synthase (*StDXS1*), 1-deoxy-D-xylulose 5-phosphate reductoisomerase (*DXR*), 2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase (*MCT*), 4-(cytidine 50-diphospho)-2-Cmethyl-D-erythritol kinase (*CMK*), 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase (*MDS*) and (E)-4-hydroxy-3-methylbut-2-enyl diphosphate synthase (*HDS*)

4.3.5 Statistical analysis

All statistical analyses were performed with the Statistical Analysis Software (SAS) (SAS Institute, Cary, NC; release 9.1 for Windows). Prior to analysis, expression levels of phenylalanine ammonia-lyase *PAL-1* and *PAL-2*, 4-coumarate: coenzyme A ligase *(4CL*) and chalcone synthase (*CHS*) gene data sets were checked for normality (PROC Univariate). Thus, data were normalized by log+0.5 transformations for analysis. Data collected from the 2x3x4 factorial arrangement with two materials, three levels of inoculums and four levels of effectors were analyzed by analysis of variance (ANOVA-PROC GLM). Comparisons between grouped treatments (Table 5) within materials were made when a significant *F*-test ($P < 0.05$) for the material*inoculum, material*effector or material*inoculum*effector interaction were found (Table 4.4). One-way ANOVA analysis for each individual group was performed using PROC GLM. Treatment means were separated using the Tukey's Studentized Range test. A $p < 0.05$ was considered to indicate significant differences.

4.4 RESULTS

4.4.1 Disease development

The susceptible cultivar RB inoculated with US8 (RB+US8) showed typical late blight lesions, with a brown necrotic spot surrounded by a chlorotic ring (Fig. 4.1A). The leaves from potato plants pretreated with EPA and then inoculated with US8 (RB+EPA+US8) showed a lower number of lesions, with a reduced size, than leaves from plants inoculated only with US8 (RB+US8). However, when RB plants were treated

with the glucan fraction and then infected with US8 (RB+GL+US8), the number and size of lesions was higher than in plants only infected with US8. In the RB+EPA+GL+US8 treatment, showed a higher disease symptoms than RB plants pretreated with water and then infected with US8 (Fig. 4.1A).

Defender inoculated with US8 (DF+US8) showed typical late blight lesions. The number and size of the lesions in Defender plants inoculated with US8 were smaller than in the susceptible cultivar RB (Fig. 4.1A). In addition, no visible symptoms were identified in Defender inoculated with US8 after EPA (DF+EPA+US8), or EPA with the glucan (DF+EPA+GL+US8) treatments. However, a hypersensitive-like reaction (HR) was detected in (DF+GL+US8) (Fig. 4.1A)

Russet Burbank inoculated with the US11 isolate after treatment with EPA, glucan, or EPA and glucan, showed typical late blight lesions. In RB+US11, the number and size of lesions were higher than DF+US8. However, in RB+EPA+US11, RB+GL+US11 and RB+EPA+GL+US11, lesions were reduced significantly (Fig. 4.1B). In addition, late blight lesions were not detected in DF+US11, DF+EPA+US11 or DF+GL+US11. A small hypersensitive-like reaction (HR) was detected in the DF+EPA+GL+US11 (Fig. 4.1B). Finally, late blight symptoms were not detected in the treatments without *P. infestans* inoculation, RB+H_2O, RB+EPA, RB+GL, RB+EPA+GL, DF+H_2O, DF+EPA, DF+GL, and DF+EPA+GL (data not shown).

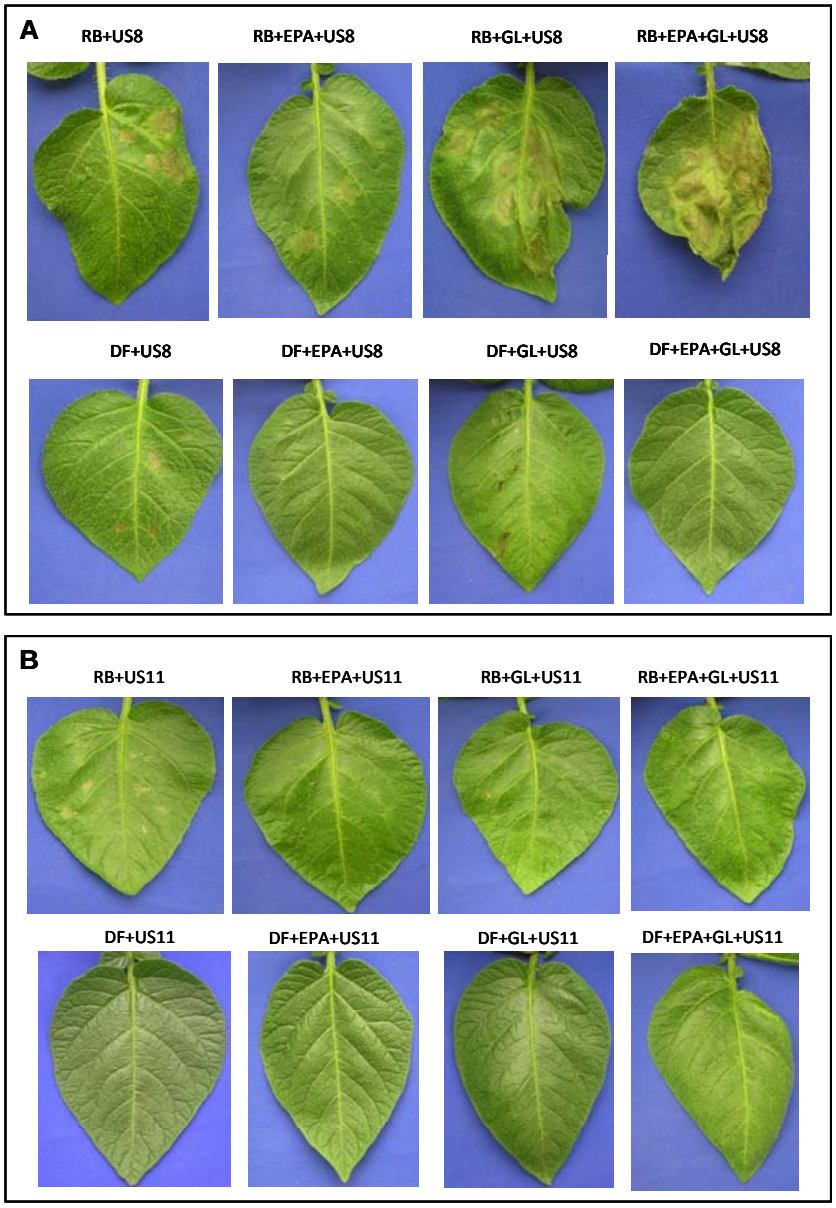

Figure 4.1. Effect of EPA elicitor and Glucan suppressor on the disease development caused by *P. infestans* in Russet Burbank and Defender cultivars. Potato plants from Russet Burbank (RB) and Defender (DF) were pre-treated with eicosapentanoic acid (EPA), Glucan race C (GL) or both as was described in Materials and Methods. Then potato plants were infected with a spores suspension of *P. infestans* ($4x10^4$ sporangia /ml.), US-8 (Highly aggressive) or US-11 (Weakly aggressive). Control plants were treated with water. The plants were placed in a growth chamber (20 °C, 16h photoperiod, 100% relative humidity) during 48 hours. The pictures are from one replicate representative out of three.

4.4.2. Analysis of variance

Data collected from the 2x3x4 factorial arrangement with two cultivars, three inoculums and four effectors from the expression levels were analyzed by analysis of variance (ANOVA-PROC GLM) (Table 4.4). Based on these data, comparisons between grouped treatments (Table 4.5, 4.6 and Fig. 4.2- 6), for all primers was performed using PROC GLM, due to significant variations for the material*inoculum, material*effector or material*inoculum*effector interaction, with the exception of *HMGR3* and *SQS*.

Table 4.4. Analysis of variance (ANOVA) *P*- values.

	PAL1	*PAL2*	*4CL*	*CHS*	*HMGR1*	*HMGR3*	*SQS*	*SC*	*StDXS1*	*DXR*	*MCT*	*CMK*	*MDS*	*HDS*
Cultivar	0.9263	0.5744	0.4856	0.422	0.7139	0.9274	0.9039	0.1991	<0.0001	0.0003	0.9405	0.0041	0.6328	0.0159
Fungus	0.2313	0.0003	0.9631	<0.0001	0.4395	0.143	0.4554	0.4353	<0.0001	0.3407	0.8712	0.0001	0.1171	<0.0001
Elicitor	0.1227	<0.0001	0.5458	0.4813	0.3845	0.1688	0.2153	0.3661	<0.0001	0.4572	0.0328	0.0108	0.3328	0.2639
Cultivar *Inoculum	0.0117	<0.0001	0.6227	0.0874	0.0187	0.1412	0.1812	0.0826	0.0016	0.0678	0.0509	0.0066	<0.0001	0.4888
Cultivar *Effector	0.0777	<0.0001	0.0468	0.0044	0.0131	0.6554	0.1398	0.4544	0.0322	0.1751	0.5037	0.0045	<0.0001	0.3071
Fungi*Effector	0.3153	<0.0001	0.148	<0.0001	0.0299	0.1861	0.8984	0.0373	<0.0001	0.0007	0.0484	<0.0001	0.0006	<0.0001
Cultivar*Effector*Inoculum	0.2798	0.0004	0.4921	0.0251	0.9612	0.6002	0.8913	0.0172	<0.0001	0.4982	0.3189	0.0053	0.0009	0.0014

Cultivar: Russet Burbank, Defender; Inoculum: H_20 (control), *P. infestans* strain D1901 (lineage US8, A2 mating type, highly aggressive), *P. infestans* strain D-03 (lineage US11, A1 mating type, weakly aggressive); Effector: eicosapentanoic acid (EPA), Glucan race C (GL), EPA+GL and H_2O.

4.4.3 Gene expression analysis in the phenylpropanoid pathway

Expression levels of phenylalanine ammonia-lyase *PAL-1* and *PAL-2*, 4-coumarate:coenzyme A ligase *(4CL*) and chalcone synthase (*CHS*) genes were assessed by semi-quantitative RT-PCR analysis. Table 4.5 shows no significant difference among treatments in expression levels of *PAL-1* and *4CL* in all groups. On the other hand, *PAL-2* was down-regulated in RB+EPA and RB+EPA+GL+US8 when compared with RB+H_2O (Figure 4.2 group 1). However, there were no significant differences between RB+H_2O and the other treatments. In addition, *CHS* was down-regulated in all treatments compared with RB+H_2O, with the exception of RB+US8 and RB+GL (Figure 4.2).

Table 4.5. RT-PCR analysis of the relative expression of *PAL-1* and *4CL* transcripts.

Group	Treatment	Phenylpropanoid Pathway	
		PAL 1	***4CL***
Group 1	RB+ H_2O	1.19 ± 0.07	0.61 ± 0.029
	RB+ US8	1.05 ± 0.11	0.35 ± 0.074
	RB+EPA	1.11 ± 0.08	0.38 ± 0.064
	RB+GL	0.96 ± 0.13	0.5 ± 0.072
	RB+EPA+GL	0.9 ± 0.09	0.28 ± 0.018
	RB+EPA+US8	1.08 ± 0.13	0.52 ± 0.118
	RB+ GL+US8	1.11 ± 0.06	0.38 ± 0.044
	RB+EPA+GL+US8	1.14 ± 0.14	0.43 ± 0.084
	P > F	NS	NS
Group 2	D+ H_2O	0.99 ± 0.14	0.45 ± 0.105
	D+ US8	0.71 ± 0.12	0.31 ± 0.02
	D+EPA	0.96 ± 0.05	0.47 ± 0.099
	D+GL	1 ± 0.08	0.32 ± 0.081
	D+EPA+GL	1.13 ± 0.14	0.48 ± 0.079
	D+EPA+US8	1.07 ± 0.13	0.54 ± 0.102
	D+ GL+US8	0.99 ± 0.19	0.37 ± 0.153
	D+EPA+GL+US8	0.9 ± 0.21	0.5 ± 0.097
	P > F	NS	NS
Group 3	RB+ H_2O	1.19 ± 0.07	0.61 ± 0.029
	RB+US11	0.78 ± 0.09	0.54 ± 0.101
	RB+EPA	1.11 ± 0.08	0.38 ± 0.064
	RB+GL	0.96 ± 0.13	0.5 ± 0.072
	RB+EPA+GL	0.9 ± 0.09	0.28 ± 0.018
	RB+EPA+US11	0.84 ± 0.19	0.4 ± 0.092
	RB+ GL+US11	0.82 ± 0.1	0.31 ± 0.055
	RB+EPA+GL+US11	0.93 ± 0.06	0.3 ± 0.024
	P > F	NS	NS
Group 4	D+ H_2O	0.99 ± 0.14	0.45 ± 0.105
	D+US11	0.72 ± 0.07	0.37 ± 0.069
	D+EPA	0.96 ± 0.05	0.47 ± 0.099
	D+GL	1 ± 0.08	0.32 ± 0.081
	D+EPA+GL	1.13 ± 0.14	0.48 ± 0.079
	D+EPA+US11	1.5 ± 0.11	0.49 ± 0.098
	D+ GL+US11	1.17 ± 0.18	0.51 ± 0.026
	D+EPA+GL+US11	0.9 ± 0.22	0.47 ± 0.09
	P > F	NS	NS

RB: Russet Burbank, DF: Defender, H_20: sterile water, US8: *P. infestans* strains D1901, US11: *P. infestans* strains D-03, EPA: eicosapentanoic acid (EPA), GL: Glucan race C. NS: no significant differences according to Tukey's Studentized Range test.

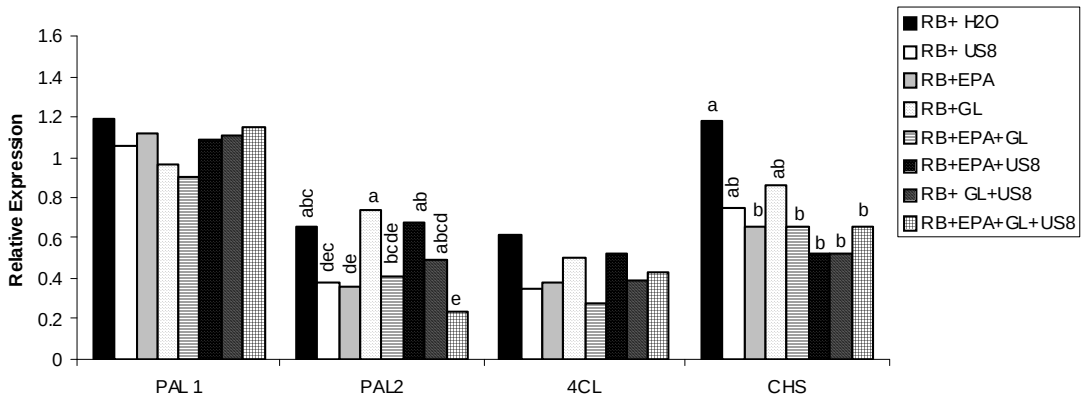

Figure 4.2. RT-PCR analysis of the relative expression of *PAL-1, PAL-2, 4CL and CHS* in group 1. RB: Russet Burbank, DF: Defender, H_20: sterile water, US8: *P. infestans* strain D1901 (lineage US8, A2 mating type, highly aggressive), US11: *P. infestans* strain D-03 (lineage US11, A1 mating type, weakly aggressive), EPA: eicosapentanoic acid (EPA), GL: Glucans race C. Means with the same letter are not significantly different, according to Tukey's Studentized Range test. A $p < 0.05$ was considered to indicate significant differences.

PAL-2 was up-regulated in both DF+EPA+US8 and DF+GL+US8, whereas no differences were found among the other treatments (Figure 4.3 group 2). *CHS* was down-regulated in DF+GL+US8 as compared with the DF+H_2O treatment, with no other treatment significantly different from the DF+H_2O treatment.

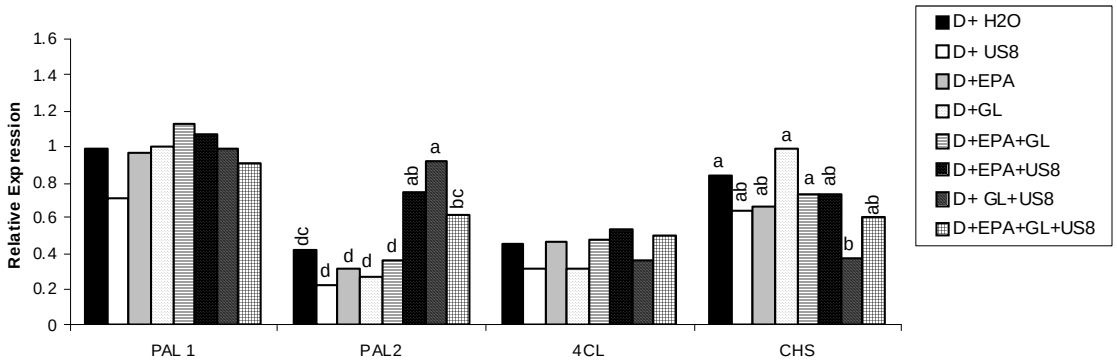

Figure 4.3. RT-PCR analysis of the relative expression of *PAL-1, PAL-2, 4CL and CHS* in group 2. RB: Russet Burbank, DF: Defender, H20: sterile water, US8: *P. infestans* strains D1901 (lineage US8, A2 mating type, highly aggressive), US11: *P. infestans* strains D-03 (lineage US11, A1 mating type, weakly aggressive), EPA: eicosapentanoic acid (EPA), GL: Glucans race C. Means with the same letter are not significantly different according to Tukey's Studentized Range test. A $p < 0.05$ was considered to indicate significant differences.

Compared with RB+H_2O, *PAL-2* was down-regulated in RB+US11, RB+EPA, RB+GL+US11 and RB+EPA+GL+US11 (Figure 4.4, group 3). However, there were no significant differences among RB+H_2O and RB+GL, RB+EPA+GL and RB+EPA+US11. In addition, *CHS* was down-regulated in all treatments compared with RB+H2O, with the exception of RB+GL (Figure 4.4).

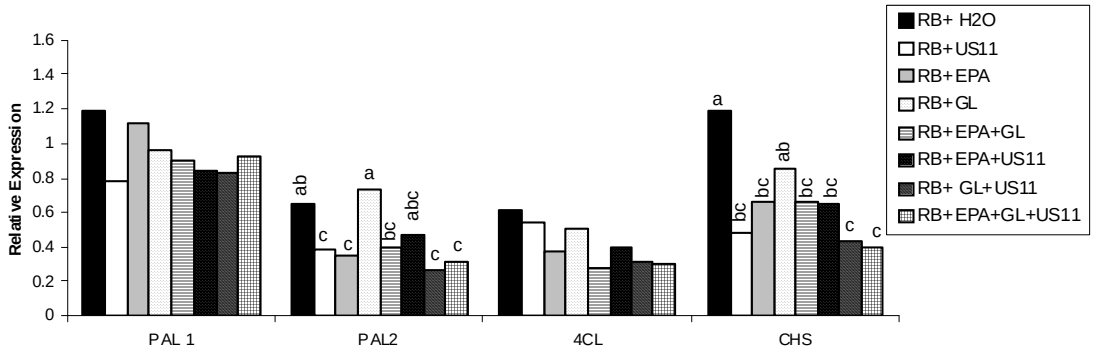

Figure 4.4. RT-PCR analysis of the relative expression of *PAL-1, PAL-2, 4CL and CHS* in group 3. RB: Russet Burbank, DF: Defender, H_20: sterile water, US8: *P. infestans* strains D1901 (lineage US8, A2 mating type, highly aggressive), US11: *P. infestans*

strains D-03 (lineage US11, A1 mating type, weakly aggressive), EPA: eicosapentanoic acid (EPA), GL: Glucans race C. Means with the same letter are not significantly different according to Tukey's Studentized Range test. A $p < 0.05$ was considered to indicate significant differences.

With the exception of down-regulation of *CHS* in DF+US11, there were no significant differences in *PAL-2* or *CHS* transcripts among treatment, compared with DF+H_2O (Figure 4.5, group 4).

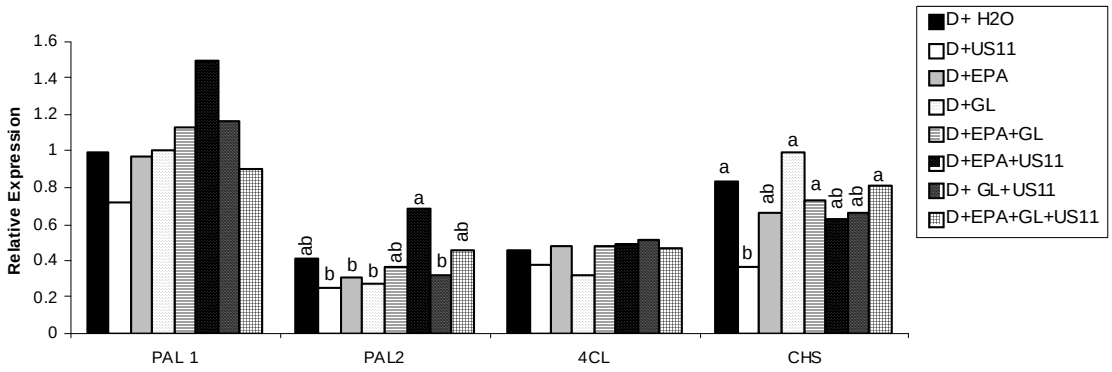

Figure 4.5. RT-PCR analysis of the relative expression of *PAL-1, PAL-2, 4CL and CHS* in group 4. RB: Russet Burbank, DF: Defender, H_20: sterile water, US8: *P. infestans* strains D1901 (lineage US8, A2 mating type, highly aggressive), US11: *P. infestans* strains D-03 (lineage US11, A1 mating type, weakly aggressive), EPA: eicosapentanoic acid (EPA), GL: Glucans race C. Means with the same letter are not significantly different according to Tukey's Studentized Range test. A $p < 0.05$ was considered to indicate significant differences.

4.4.4 Analysis of gene expression in the mevalonate (Ac-MVA) pathway

Expression levels of the mevalonate pathway 3-hydroxy-3-methylglutaryl coenzyme A reductase *HMGR, HMGR3,* squalene synthase (*SQS***)** and sesquiterpene cyclase (*SC*) genes were assessed by semi-quantitative RT-PCR analysis. Table 4.6 and appendix 3 shows no significant difference among treatments in expression levels of *HMGR1, HMGR3, SQS* or *SC* in all groups. However, for *SC*, transcripts were down-regulated in the group 1 for RB+US8, RB+EPA+GL, and RB+GL+US8 compared with RB+US8.

Table 4.6. RT-PCR analysis of the relative expression of mevalonate pathway transcripts.

		Mevalonate Pathway	
		HMGR1	*SC*
Group 1	RB+ H_2O	1.48 ± 0.21	0.49 ± 0.014 a
	RB+ US8	1.13 ± 0.11	0.17 ± 0.02 b
	RB+EPA	0.81 ± 0.19	0.35 ± 0.113 ab
	RB+GL	0.99 ± 0.22	0.32 ± 0.029 ab
	RB+EPA+GL	0.98 ± 0.21	0.3 ± 0.044 b
	RB+EPA+US8	1.15 ± 0.14	0.33 ± 0.03 ab
	RB+ GL+US8	1 ± 0.12	0.18 ± 0.028 b
	RB+EPA+GL+US8	0.95 ± 0.23	0.3 ± 0.053 ab
	P > F	NS	0.0272
Group 2	D+ H_2O	1.15 ± 0.05	0.36 ± 0.105
	D+ US8	0.74 ± 0.12	0.23 ± 0.014
	D+EPA	0.85 ± 0.08	0.19 ± 0.034
	D+GL	0.97 ± 0.04	0.17 ± 0.005
	D+EPA+GL	1.27 ± 0.13	0.51 ± 0.048
	D+EPA+US8	1.13 ± 0.19	0.42 ± 0.089
	D+ GL+US8	0.64 ± 0.16	0.44 ± 0.12
	D+EPA+GL+US8	1.03 ± 0.22	0.28 ± 0.021
	P > F	NS	NS
Group 3	RB+ H_2O	1.48 ± 0.21	0.49 ± 0.014
	RB+US11	1 ± 0.06	0.24 ± 0.019
	RB+EPA	0.81 ± 0.19	0.35 ± 0.113
	RB+GL	0.99 ± 0.22	0.32 ± 0.029
	RB+EPA+GL	0.98 ± 0.21	0.3 ± 0.044
	RB+EPA+US11	0.9 ± 0.1	0.25 ± 0.072
	RB+ GL+US11	1.01 ± 0.14	0.24 ± 0.037
	RB+EPA+GL+US11	0.72 ± 0.22	0.28 ± 0.028
	P > F	NS	NS
Group 4	D+ H_2O	1.15 ± 0.05	0.36 ± 0.105
	D+US11	0.99 ± 0.08	0.16 ± 0.019
	D+EPA	0.85 ± 0.08	0.19 ± 0.034
	D+GL	0.97 ± 0.04	0.17 ± 0.005
	D+EPA+GL	1.27 ± 0.13	0.51 ± 0.048
	D+EPA+US11	1.47 ± 0.23	0.54 ± 0.145
	D+ GL+US11	1.04 ± 0.2	0.37 ± 0.096
	D+EPA+GL+US11	1.14 ± 0.1	0.3 ± 0.088
	P > F	NS	NS

RB: Russet Burbank, DF: Defender, H_20: sterile water, US8: *P. infestans* strains D1901, US11: *P. infestans* strains D-03, EPA: eicosapentanoic acid (EPA), GL: Glucan race C. NS: not significant differences according to Tukey's Studentized Range test.

4.4.5 Gene expression analysis in the DOXP-MEP pathway

There was a down-regulation of *StDXS1* transcripts in all group 1 treatments when compared with RB+H_2O, with the exception of RB+GL which showed no significant differences with RB+H_2O (Fig 4.6). On the other hand, with the exception of the down-regulation of *CMK* in RB+ GL+US8, no significant difference was obseved in *DXR*, *MCT*, *CMK*, *MDS* or *HDS* in all treatments, compared with RB+H_2O.

There was a down-regulation of *StDXS1* transcripts in all group 2 treatments, compared with DF+H_2O (Fig. 4.6). *DXR*, *MCT*, *MDS* and *HDS* transcripts showed no significant difference in all treatments compared with DF+H_2O, with the exception of down-regulation of *DXR* and *HDS* in DF+EPA+GL and of *HDS* in DF+US8 and DF+GL+US8. *CMK* was down-regulated in all treatments compared with DF+H_2O, with the exception of DF+GL and DF+EPA+GL+US8, which showed no significant differences with DF+H_2O.

There was a down-regulation of *StDXS1* transcripts in group 3 RB+EPA, RB+EPA+US11, RB+GL+US11 and RB+EPA+GL+US11, compared with RB+H_2O (Fig. 4.6). *DXR*, *MCT*, *MDS* and *HDS* transcripts showed no significant difference in all treatments compared with RB+H2O, with the exception of up-regulation of *DXR* and *HDS* in RB+GL and down-regulation of *HDS* in RB+GL+US11. On the other hand, *CMK* was down-regulated in RB+US11, RB+EPA+GL, RB+GL+US11 and RB+EPA+GL+US11 compared with RB+H_2O.

There was a down-regulation of *StDXS1* transcripts in all group 4 treatments, compared with DF+H2O (Fig. 4.6). *DXR*, *MCT*, *MDS* and *HDS* transcripts showed no significant difference in all treatments, compared with DF+H_2O, with the exception of down-regulation of *DXR* in DF+US11 and DF+EPA+GL, *HDS* in DF+US11, DF+EPA+GL and DF+GL+US11, and up-regulation of *MDS* in DF+US11. *CMK* was down-regulated in DF+EPA, DF+EPA+GL, DF+EPA+US11, and DF+EPA+GL+US11, compared with DF+H_2O.

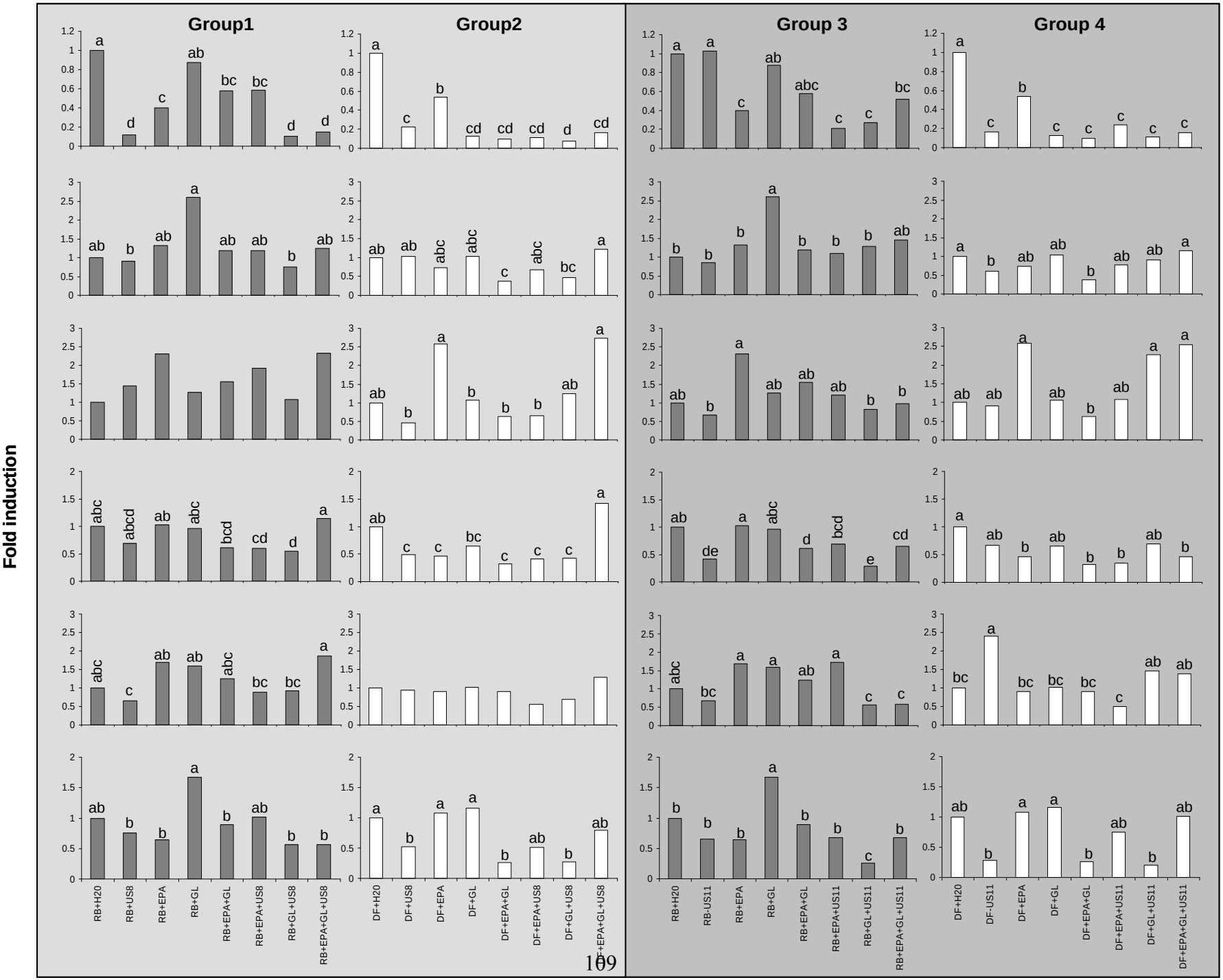
Group1
Group2
Group 3
Group 4
Fold induction
StDXS1
DXR
MCT
CMK
MDS
HDS
RB+H20
RB+US8
RB+EPA
RB+GL
RB+EPA+GL
RB+EPA+US8
RB+GL+US8
RB+EPA+GL+US8
DF+H20
DF+US8
DF+EPA
DF+GL
DF+EPA+GL
DF+EPA+US8
DF+GL+US8
DF+EPA+GL+US8
RB-US11
RB+EPA+US11
RB+GL+US11
RB+EPA+GL+US11
DF-US11
DF+EPA+US11
DF+GL+US11
DF+EPA+GL+US11

Figure 4.6. Transcript profiles of genes encoding enzymes in the DOXP-MEP pathway. RB: Russet Burbank, DF: Defender, H_20: sterile water, US8: *P. infestans* strains D1901 (lineage US8, A2 mating type, highly aggressive), US11: *P. infestans* strains D-03 (lineage US11, A1 mating type, weakly aggressive), EPA: eicosapentanoic acid (EPA), GL: Glucan race C. RB+US8: Russet Burbank inoculated with US8; RB+US11: Russet Burbank inoculated with US11; DF+US8: Defender inoculated with US8; DF+US11: Defender inoculated with US11. All qRT-PCR reactions were performed from triplicate biological samples. The $2^{-\Delta\Delta C(T)}$ method (Livak and Schmittgen 2001) was used to calculate the fold expression relative to the control ($RB+H_2O$ or $DF+H_2O$). The elongation factor gene was used to normalize small differences in template amounts. Means with the same letter are not significantly different according to Tukey's Studentized Range test. A $p < 0.05$ was considered to indicate significant differences.

4.5. DISCUSSION

4.5.1 Disease symptoms and potato defense responses

Russet Burbank plants treated with the glucan fraction and then infected with US8 (RB+GL+US8), had dramatically higher number and size of lesions than plants only infected with US8. On the other hand, lesions were reduced significantly in RB+GL+US11, when compared with RB+US11. The reason why in Russet Burbank, the glucan fraction either suppressed or elicited defense responses remains to be investigated. However, it has been shown that glucan oligosaccharides, which are released from pathogen cell walls, are effectors that either induce a wide range of plant defense responses (Cote and Hahn 1994) or suppress them (Garas et al. 1979; Doke et al. 1980; Currier 1981; Andreu et al. 1998; Ozeretskovskaya et al. 2001).

EPA elicited potato defense responses in Russet Burbank and Defender plants inoculated with US8 or US11, thereby reducing symptoms of late blight. Cohen et al. (1991), had reported that eicosapentaenoic acid (EPA) induces systemic resistance in potato against *P. infestans.* Therefore, disease symptoms are associated with the suppression or elicitation of potato defense responses.

No effects of defense suppression by the glucan fraction related with disease symptoms in the moderately resistant potato cultivar Defender inoculated with US8 were identified. A hypersensitive-like reaction (HR) was detected in the moderately resistant

cultivar Defender (DF+GL+US8). Explanations of this glucan fraction outcome in Defender are provided in section 4.5.3.

4.5.2 Effects of secondary metabolic pathways in potato defense responses to *P. infestans*

In order to identify the metabolic pathways being affected in the potato cultivars inoculated with the *P. infestans* isolates US8 and US11, or pre-treated with either eicosapentanoic acid (EPA), glucans or their combination before the inoculation, we carried out studies through the differential expression of genes from the phenylpropanoid, mevalonate (Ac-MVA) and DOXP-MEP pathways in potato during its interaction *in vivo* with *P. infestans*. Several previous studies have demonstrated transcriptional activation of specific genes in potato in response to *P. infestans* or an elicitor (Yoshioka et al. 2001; Wang et al. 2005c; Wang et al. 2006; Wang et al. 2008), but most were conducted *in vitro*, and none have considered an integrated view of more than one of the pathways potentially involved.

4.5.2.1 Defender defense responses to *P. infestans*

Defender originated from a cross between KSA195-90 and Ranger Russet, where KSA195-90 is the source of resistance to late blight. However, the original source of late blight resistance found in KSA195-90 is unclear, because many Mexican and South American wild and cultivated species are in its background (Novy et al. 2006). Corsini et

al. (1999) suggested that R-genes from *Solanum demissum* are likely present in KSA195-90, conferring resistance to *P. infestans*. We can suggest that elicitors or avirulence determinants from *P. infestans* could be recognized by Defender receptors or R proteins from one or more of theses R-genes before initiating signaling pathways, which lead to defense reactions such as synthesis of pathogenesis-related proteins, or defense secondary metabolites. Therefore, the moderate resistance of Defender to the highly aggressive *P. infestans* isolate US8 and the low aggressive *P. infestans* isolate US11, should also be attributed to primary or secondary phenolic and isoprenoid compounds, based on our results from the tested phenylpropanoid and mevalonate (Ac-MVA) pathways genes.

In Defender inoculated with US8 or US11, the expression of phenylpropanoid pathway gene phenylalanine ammonia-lyase *PAL-1, PAL-2*, and 4-coumarate:coenzyme A ligase *(4CL*) was not affected when compared with control plants. Therefore, preformed secondary metabolites, phytoalexins, signal molecules (salicylic acid) and lignin synthesis (Hahlbrock and Scheel 1989) may have been unaffected by inoculation of this cultivar. Despite a down-regulation of *CHS* in Defender inoculated with US11, late blight lesions were not detected, indicating that *CHS* gene homologs or downstream genes for flavonoid synthesis may have been unaffected by inoculation of this cultivar.

In Defender the expression levels of the mevalonate (Ac-MVA) pathway genes 3-hydroxy-3-methylglutaryl coenzyme A reductase *HMGR, HMGR3,* squalene synthase (*SQS***)** and sesquiterpene cyclase (*SC*) genes were also not affected by isolates US8 and US11. It is important to have in consideration that terpenoids produced in the mevalonate

(Ac-MVA) pathway, participate in essential plant processes such as respiration (ubiquinone), regulation of growth and development (cytokinins) and plant protection against pathogens (rishitin). Without them plants would not be able to function (Wanke et al. 2001; Rodriguez-Concepcion and Boronat 2002).

Down-regulation of *StDXS1* transcripts in Defender suggesting a possible cultivar-specific effect on the expression level of *StDXS1* in potato might be the result of *P. infestans* effectors that are blocking the signal transduction pathway during the elicitor-mediated activation of this gene or affecting the formation of binding complexes in its promoter region suppressing its expression (Shiraishi et al. 1994). It might be a compensation mechanism from the second *DXS* gene in potato (*StDXS2*) as shown in chapter 3, playing a role in the activation of the enzyme 1-deoxy-D-xylulose 5-phosphate synthase in Defender. However, a strong down-regulation of *HDS* transcrips in Defender inoculated with US8 and US11, where late blight lesions were small or not detected respectively, are probably an unpredicted link between the DOXP-MEP pathway and plant defense, similar to the response in Arabidopsis mutant *cbs3* with reduced HDS activity. Arabidopsis mutant *cbs3* with reduced HDS activity significantly repressed the growth of the oomycete *Hyaloperonospora parasitica* and showed enhanced accumulation of SA and the activation of the defense-related genes PR1 and PR2 (Durrant and Dong 2004; Gil et al. 2005). Therefore, *HDS* could be a potential susceptibility gene induced in potato by *P. infestans* playing a role as an endogenous plant defense suppressor.

The function of elicitation by EPA of defense responses in Defender inoculated with US8, as suggested in section 4.5.1, could be explained by the up-regulation of phenylalanine ammonia-lyase *PAL-2* in DF+EPA+US8, when compared with the control plant, Defender inoculated with US8 (DF+US8) and DF+EPA. In addition, the expression levels of *PAL-1*, *4CL* and *CHS* were not affected when compared with the control plant and DF+US8. On the other hand, up-regulation of *PAL-2* in DF+EPA+US8 could lead to the synthesis of salicylic acid, because induction of *PAL-2* may translate into an increase in abundance of phenolic compounds, including salicylic acid (Ludwikow et al. 2004), while the PAL inhibitor 2-aminoindan-2-phosphonic acid reduces salicylic acid accumulation in potato (Coquoz et al. 1998).

Up-regulation of *PAL-2,* in the (DF+GL+US8), when compared with the control plant, Defender inoculated with US8 (DF+US8) and DF+GL, could also be correlated with salicylic acid accumulation in and around the hypersensitive-like (HR) lesions, as reported in tobacco inoculated with the mosaic virus (TMV) (Enyedi et al. 1992). Finally, in DF+EPA+GL+US8, DF+GL+US11, DF+EPA+US11 and DF+EPA+GL+US11, the expression level of *PAL-1*, *PAL-2, 4CL*, *CHS, HMGR1, HMGR3, SQS* and *SC* were not affected when compared with control plants and DF+US8 or DF+US11. This confirms that defense response in Defender against *P. infestans* should also be attributed to products of the phenylpropanoid and mevalonate (Ac-MVA) pathways. The reason why both glucan and EPA up-regulated *PAL-2* in Defender remains to be investigated.

4.5.2.2 Russet Burbank defense responses to *P. infestans*

PAL-1 PAL-2, 4CL and *CHS* expression ratios were not affected in Russet Burbank inoculated with US8, when compared with the control plants. However, in Russet Burbank inoculated with US11, *PAL-2* and *CHS* were the only tested genes that were down-regulated, when compared with the control plants. In RB+US11, the number and size of late blight lesions were lower than RB+US8. The amount of *CHS* mRNA was reported to increase significantly in roots inoculated with zoospores of either an avirulent or virulent race of *Phytophthora megasperma* f. sp. *glycinea* (Pmg) (Dhawale et al. 1989). However, inoculation of bean with *P. syringae* pv. *phaseolicola (Pph)* inhibited the activity of Phenylalanine ammonia-lyase (PAL), Chalcone synthase (CHS) and Chalcone isomerase (CHI) (Jakobek et al. 1993). In fact, the defense-suppressing effect of glucans suggested in section 4.5.1, can not be explained by a suppression of the *PAL-1*, *PAL-2* and *4CL* genes, because their expression ratios were not affected when compared with the control plant and RB+US8.

Similar results to the defense-suppressing effect of glucans were found in the defense elicitor effect of EPA in RB+EPA+US8 and RB+EPA+US11 as suggested in the section 4.5.1. Similarly as in the defense elicitor effect of glucans in RB+GL+US11 and in the additive defense-suppressing effect in Russet Burbank. These results were evident when EPA was applied with glucans before the inoculation with the *P. infestans* isolates US8 (RB+EPA+GL+US8), that showed higher disease symptoms than Russet Burbank plants pre-treated with water and then infected with US8 and RB+GL+US8. *PAL*

expression ratios not affected by the pathogen were previously reported from soybean inoculated by *R. solani* (Chen et al. 2009), but transcriptional activation of *4CL* has been reported in potato infected with *P. infestans* (Schmelzer et al. 1989; Becker-Andre et al. 1991; Uhlmann and Ebel 1993). It is not clear why we did not have a transcriptional activation of *4CL*, but this could be attributed to the experimental setup, type of tissue analyzed or the pathosystem studied.

Expression levels of the mevalonate pathway 3-hydroxy-3-methylglutaryl coenzyme A reductase *HMGR, HMGR3,* squalene synthase (*SQS***)** and sesquiterpene cyclase (*SC*), were not affected in any of the tested treatments, except for *SC* transcripts which where down-regulated in RB+US8 and RB+GL+US8. This specific down-regulation of sesquiterpene cyclase in the treatments showing most symptoms would be associated with a significant role of sesquiterpenoid compounds in the potato defense to *P. infestans*. Sesquiterpene cyclase (*SC*) catalyzes the first steps in the branch leading to sesquiterpenoid phytoalexins, such as rishitin, lubimin and phytuberin, which have been previously linked to potato resistance against *P. infestans* (Engstrom et al. 1999; Dixon 2001; Krits et al. 2007). A possible suppression or reduction in the accumulation of these phytoalexins in RB+US8 and RB+GL+US8 would be associated with their late blight disease. In fact, Wang et al., (2008) showed that the highly aggressive *P. infestans* genotypes (US8) led to a reduced accumulation of rishitin at the inoculation site in potato.

Our study of the induction of genes encoding key checkpoint enzymes from the DOXP-MEP pathway showed that *StDXS1* expression ratios were down-regulated in

Russet Burbank. With the exception of RB+GL and RB+US11, all treatments showed down-regulation of *StDXS1*. These results would be associated with the spreading disease lesions and extensive tissue damage in Russet Burbank as shown in Figure 4.1. However, without the further analysis of *STDXS2*, we do not know if *StDXS1* is associated with susceptibility or it is specifically expressed in one variety. Silencing *StDXS1*, *StDXS2*, would help elucidate potential gene function of the DOXP-MEP pathway in potato-pathogen interactions. In addition, despite the effect of down-regulation of *CMK* and *StDXS1* in different treatments, other key genes from the DOXP-MEP pathway (*DXR*, *MCT*, *MDS* and *HDS*) seem to be not affected by the majority of treatments. Therefore, it is possible that the DOXP-MEP pathway is not playing a major role in defense responses in potato at a late stage of the interaction.

To our knowledge, no other reports have used whole plant systems (*in planta*) to integrate analyses of the effects of suppressors (glucans from *P. infestans*), elicitors (eicosapentanoic acid, EPA) and of *P. infestans* on the differential expression of genes encoding key checkpoint enzymes from the phenylpropanoid, mevalonate (Ac-MVA) and DOXP-MEP pathways in potato. Here we provide experimental evidence for one particular case, but the latter presents indications to explain additional mechanisms of resistance in potato. The current findings imply that genetic resistance in potato against *P. infestans* is not the result of isolated reactions against the pathogen, other than the combination of different factors that suggest a polygenic trait or horizontal resistance. The result in this study highlights the necessity of investigating molecular responses of potato to *P. infestans* not only of individual genes from the phenylpropanoid, mevalonate

(Ac-MVA) or DOXP-MEP pathways but several genes and their gene homologs, as well as how metabolic profiles of secondary metabolites are altered in potato tissues after inoculation with *Phytophthora infestans.*

CHAPTER 5: ALTERED METABOLIC PROFILE OF SECONDARY METABOLITES IN POTATO LEAVES AFTER INOCULATION WITH *PHYTOPHTHORA INFESTANS*

5.1 ABSTRACT

Phytophthora infestans is the cause of late blight, a devastating disease in potato. Many of the mechanisms underlying *P. infestans* pathogenesis and defense responses in potato are still unknown. We investigated the effects of *P. infestans* on the time course of changes in the accumulation of secondary metabolites in potato cultivars. The experiments were carried out using the whole plant. Four preformed flavonoids and one terpenoid compounds in potato were affected by the inoculation with *P. infestans.* In Russet Burbank the accumulation of catechin and rutin were suppressed by the *P. infestans* isolates US11 and US8, while the flavonoid P3 was associated with the susceptibility to *P. infestans*. On the other hand, catechin, flavonone (P2), rutin and the terpenoid (T1), played a potential role in the defense of Defender to the *P. infestans* isolates US8 and US11. The present study provides evidence that different flavonoids accumulate in potato leaves depending on the *P. infestans* isolate and the potato cultivar involved. The present study provides new evidence that different preformed flavonoids and terpenoids in potato may play important roles in its defense or susceptibility to *P. infestans.* This study is a contribution to the knowledge about the potato - *P. infestans* interaction that may help to develop novel management strategies to control late blight.

5.2 INTRODUCTION

Late blight caused by the oomycete *Phytophthora infestans* (Mont.) de Bary is the most devastating disease of potato (*Solanum tuberosum*) and was responsible for the Irish potato famine in 1845 (Fry and Goodwin 1997). This pathogen infects directly from sporangia or through zoospores, affecting leaves, stems, and potato tubers (Goodwin et al. 1998; Vleeshouwers et al. 2000; Judelson and Blanco 2005). Zoospores and sporangia penetrate the leaf surface either through stomata or directly through the epidermal cell wall. Germination of sporangia occurs mainly at temperatures above 12 °C. The germ tube is able to differentiate into an appressorium and a penetration peg is formed to aid the passage of the pathogen through the host cell wall. After three or four days, secondary sporulation and infection of leaves occur to initiate once more the disease cycle of *P. infestans* (van West and Vleeshouwers 2004).

Wang et al., (2005a) studied the expression patterns of potato genes associated with quantitative resistance to late blight during *P. infestans* infection using cDNA microarrays and showed that approximately 37.2% of all the known *P. infestans*-responsive genes were identified with a general or secondary metabolism function. Many genes that encode enzymes participating in the defense-related metabolic pathways such as the biosynthesis of phenylpropanoids and alkaloids were activated by *P. infestans* (Wang et al. 2005a).

The majority of antimicrobial secondary metabolites are derived from the phenylpropanoid, isoprenoid, alkaloid or fatty acid pathways (Dixon 2001). During plant-

pathogen interactions, the cell wall reinforcement through lignin synthesis, production and accumulation of secondary metabolites from the phenylpropanoid and mevalonate pathways, and the accumulation of phytoalexins (Kombrink and Somssich 1995; Hammerschmidt 1999; Hückelhoven 2007) are well-known plant defense responses. Phenylpropanoids exhibit a broad-spectrum antimicrobial activity and are therefore believed to help the plant fight microbial disease (Dixon et al. 2002). Cell wall-bound phenolics also accumulate locally to restrict fungal penetration. However, the functions of phenolics are still poorly understood (Hahlbrock et al. 1995; Von Ropenack et al. 1998).

Chlorogenic acid, caffeic acid, scopoletin, scopolin (Bryant et al. 2006) and p-coumaroyloctopamine (Mittelstra et al. 2006), are phenolic compounds from the phenylpropanoid pathway linked to potato resistance against *P. infestans*. Compounds from the isoprenoid pathway or mevalonate pathway linked to potato resistance to *P. infestans* include rishitin, phytuberin and lubimin (Mittelstra et al. 2006). A previous study (Chapter 4), demonstrated that the differential expression of some genes from the phenylpropanoid, mevalonate (Ac-MVA) and DOXP-MEP pathways in potato were affected by *P. infestans.* Therefore, as an extension of that work, the objective of this study was to investigate the accumulation of secondary metabolites in potato after inoculation with *P. infestans.* The experiments were carried out *in vivo* using whole plants of the susceptible cultivar "Russet Burbank", and the moderately resistant cultivar "Defender".

5.3 MATERIALS AND METHODS

5.3.1 Plant, fungal races and growth conditions

The highly susceptible cultivar "Russet Burbank" (RB) and the moderately resistant cultivar "Defender" (DF) were used. Plants were grown in a chamber at 20 ± 2 °C and 16 hours photoperiod (Bowyer et al. 1995). The *P. infestans* strains D-03 (lineage US11, A1 mating type, weakly aggressive) and D1901 (lineage US8, A2 mating type, highly aggressive) were grown on rye agar medium supplemented with 2 % sucrose at 18°C

as described by Wang et al. (2004b).

5.3.2 Experimental design

A 2 x 3 factorial arrangement of treatments was used in a randomized complete block design. Factors consisted of cultivars (Russet Burbank and Defender) and inoculum (H_2O, US8 and US11). Thus, the resulting six treatment combinations consisted of 1) Russet Burbank inoculated with H_2O; 2) Russet Burbank inoculated with US8; 3) Russet Burbank inoculated with US11; 4) Defender inoculated with H_2O; 5) Defender inoculated with US8; and 6) Defender inoculated with US11. The primary leaflet of the fourth fully-grown potato leaf in each stem of 8-wk-old Russet Burbank and Defender plants, were treated, with 10 drops of 10 µl of *P. infestans* spore suspension (4X 10^4 sporangia/ml) or H_2O for the controls. The plants were placed in a moist chamber (20 ± 2 °C, 16 photoperiod, 100% relative humidity) for 48 h and then, incubated in a growth chamber at 20°C ± 2 and 16h photoperiod. Samples from three biological replicates were collected

at 0, 12, 72, and 120 hours post inoculation. The samples were stored at -80ºC until used for secondary metabolite extraction. Visualisation of the *P. infestans* structures from 3 to 24 hpi were done by staining leaf tissue with tryphan blue (0.05% tryphan blue, 70% lactic acid, 6% glycerol) and examined by light microscopy.

5.3.3 Extraction of secondary metabolites

One gram of tissue from each biological replicate was ground in a mortar with liquid nitrogen and extracted with 1 ml of methanol 80 % under vigorous vortexing. The homogenates were centrifuged 3 minutes at 7000 rpm. The methanol extraction was repeated three times and the methanol extracts were evaporated under nitrogen to about 600 µl. Then samples were depigmented with petroleum ether and phenolics were extracted with ethyl acetate, evaporated to dryness and finally dissolved in 500 µl of HPLC-grade methanol.

5.3.4 Analysis of secondary metabolites by high performance liquid chromatography (HPLC)

Extracts from potato leaves were analyzed by reverse phase HPLC using a Water 2695 separation module coupled with a Water 996 photodiode array detector. Fifty microlitres of each phenolic extract were injected in a RP-18 (5 um) lichrospher 100 column and eluted with a gradient of acetonitrile/0.1%H_3PO_4 in water as follows: time in min/ %acetonitrile/flow rate in mL min^{-1}: 0/0/1, 5/5/1, 10/5/1, 14/10/1, 20/20/1, 23/20/1, 30/35/1, 35/35/1, 43/50/1, 48/75/1, 55/100/1, 60/100/1, 62/0/1, 65/0/1, 90/0/1. Data were analyzed using the Empower Pro program (Waters, Ville-Saint-Laurent PQ, Canada). In

all cases, the software was programmed to show peaks at their maximum absorbance (max plot). Secondary metabolites were identified by co-elution and by comparing the UV-spectra and retention times with those of HPLC standards from our database. Area under the peak was then converted to µg equivalents of the identified peak per gram of fresh weight, by reference to a pre-established standard curve using commercial compounds.

5.3.5 Statistical analysis

All statistical analyses were performed with the Statistical Analysis Software (SAS) (SAS Institute, Cary, NC; release 9.1 for Windows). The residuals for each parameter were examined for normality and homogeneity of variances. Plant secondary metabolite concentration values were log-transformed (log+0.5) to normalize the data. Data were analyzed as a 2 x 3 factorial in a completely randomized design with three replicates and repeated measures over time. In each cultivar, a completely randomized design was employed to account for the repeated measures using the MIXED model procedure of SAS. When interactions were detected ($P < 0.05$, unless otherwise noted), a mixed-model analysis for each individual time (hpi) was performed using PROC MIXED. Inoculum was considered a fixed effect and rep (inoculum) a random effect. Inoculum means were separated using the Bonferronni's procedure at a probability level of $P < 0.05$, where inoculum effects were significant.

5.4 RESULTS

5.4.1 Disease development

We used two isolates of *P. infestans,* D-03 (US8) and D1901 (US11) to inoculate the cultivar Russet Burbank (highly susceptible) and Defender (moderately resistant). No symptoms were visible within the first 48 hpi on either cultivar and only small lesions became noticeable at 72 hpi in Russet Burbank and Defender inoculated with US8. Late blight symptoms were visible in Russet Burbank inoculated with US11 at 120 hpi. Disease progress observations from 72 to 144 hpi are shown in Figure 5.1. The US8 isolate caused spreading disease lesions and extensive tissue damage in Russet Burbank, whereas it only caused limited disease lesions in Defender. The US11 isolate caused small lesions in Russet Burbank and failed to cause disease in Defender.

5.4.2 Analysis of secondary metabolites

The time points selected for the profiling analysis of secondary metabolites accumulated in potato by inoculation with *P. infestans*, were determined by the disease development of late blight. Seventy hours post inoculation was selected as a time point, because as this time small lesions became noticeable in RB and DF. Then, 120 hpi was selected because Russet Burbank inoculated with US8 showed extensive tissue damage, whereas in Defender spreading lesion were detected. In addition, at 120 hpi small lesions became noticeable in Russet Burbank inoculated with US11 (Fig. 5.1).

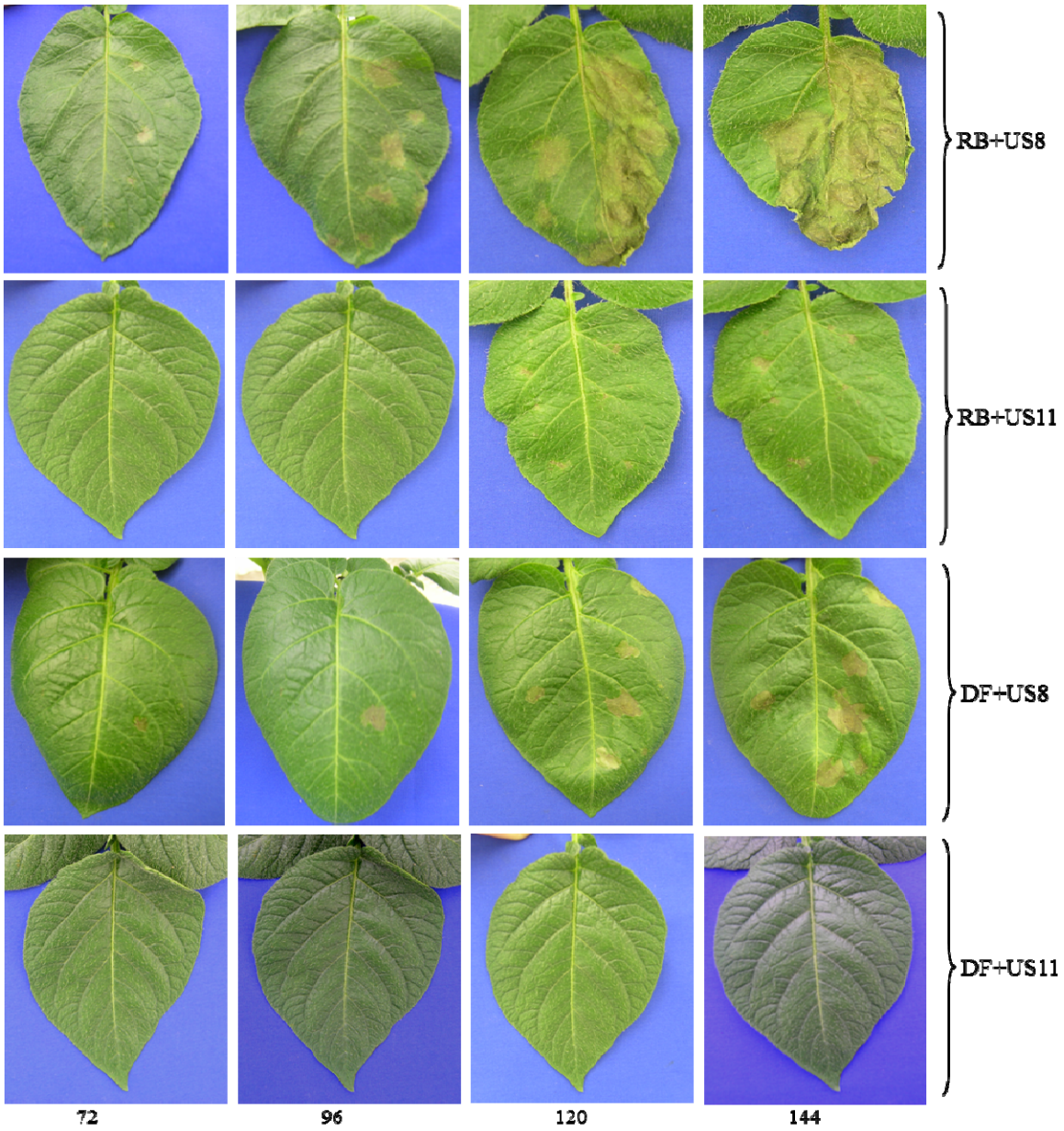

Figure 5.1. Infection development by *Phytophthora infestans* in potato. Russet Burbank inoculated with US8 (RB+US8), Russet Burbank inoculated with US11 (RB+US11), Defender inoculated with US8 (DF+US8), Defender inoculated with US11 (DF+US11).

Finally, an early stage of the interaction was selected, based on a preliminary microscopy analysis for germination and penetration of *P. infestans* in potato (Fig. 5.2). Figure 5.2 shows that in Russet Burbank inoculated with US8, at 9 hpi, sporangia started to germinate, and at 12 hpi appressoria were formed, whereas in the moderately resistant potato cultivar Defender inoculated with US8, there is no appressorium formation. Therefore, 12 hpi was selected as an early stage of *P. infestans* - potato interaction for the HPLC analysis.

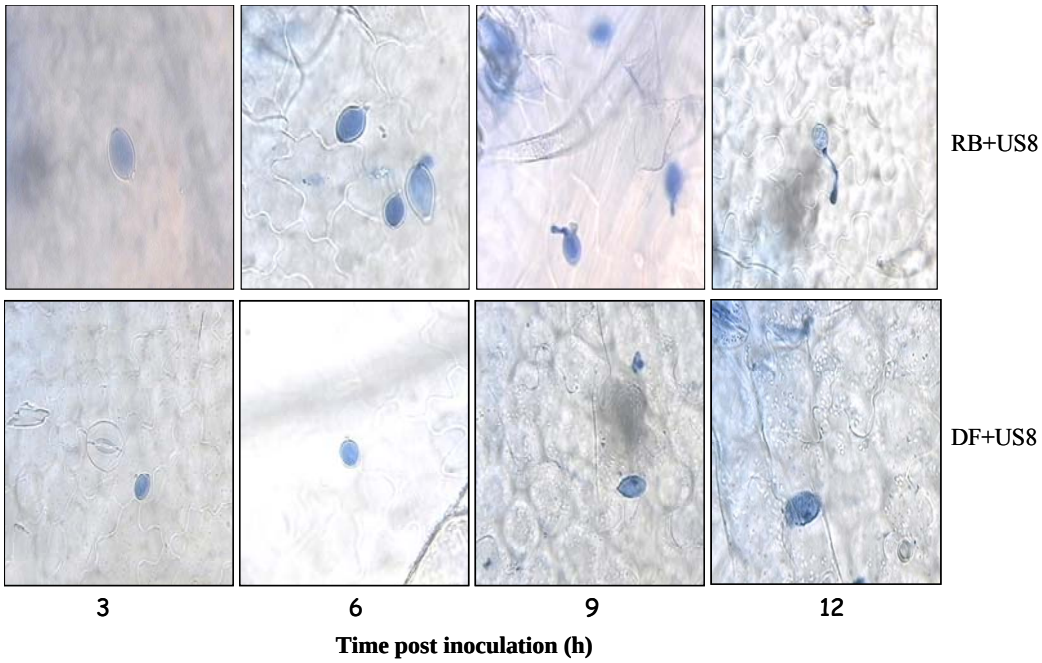

Figure 5.2. Microscopic comparison of *P. infestans* development in Russet Burbank and Defender. Russet Burbank inoculated with US8 (RB+US8), Defender inoculated with US8 (DF+US8).

5.4.2.1. Secondary metabolites identified in potato leaves after inoculation with *P. infestans* isolates

Secondary metabolites in potato leaves at 0, 12, 72 and 120 hpi after treatment with sterile distilled water (control) or inoculated with *P. infestans* were analyzed by HPLC (Fig. 5, 6–8). The chromatographic profiles of potato plants RB and DF inoculated with the highly aggressive *P. infestans* isolate (US8) and the weakly aggressive isolate (US11) showed a significant variation in the accumulation of secondary metabolites eluded during 18 to 28 min of retention time (Fig. 5.3). Four putatively identified flavonoid compounds and one putatively identified terpenoid presented variations in potato leaves in response to inoculation. Four peaks observed and referred to as P1- P4, as indicated in Figure 5.3, were identified as the flavonoid compounds catechin (P1), flavonone (P2), flavonoid (P3) and rutin (P4). The peak observed and referred to as T1, was identified as terpenoid (Table 5.1).

The selected accumulated compounds were putatively identified (Table 5.1) based on their retention time, typical UV spectra, and comparison with commercial standards. Co-elution of the samples with commercial standards was also conducted when necessary to confirm putative identifications. The absorption spectra of the putatively identified compounds are shown in appendix 4.

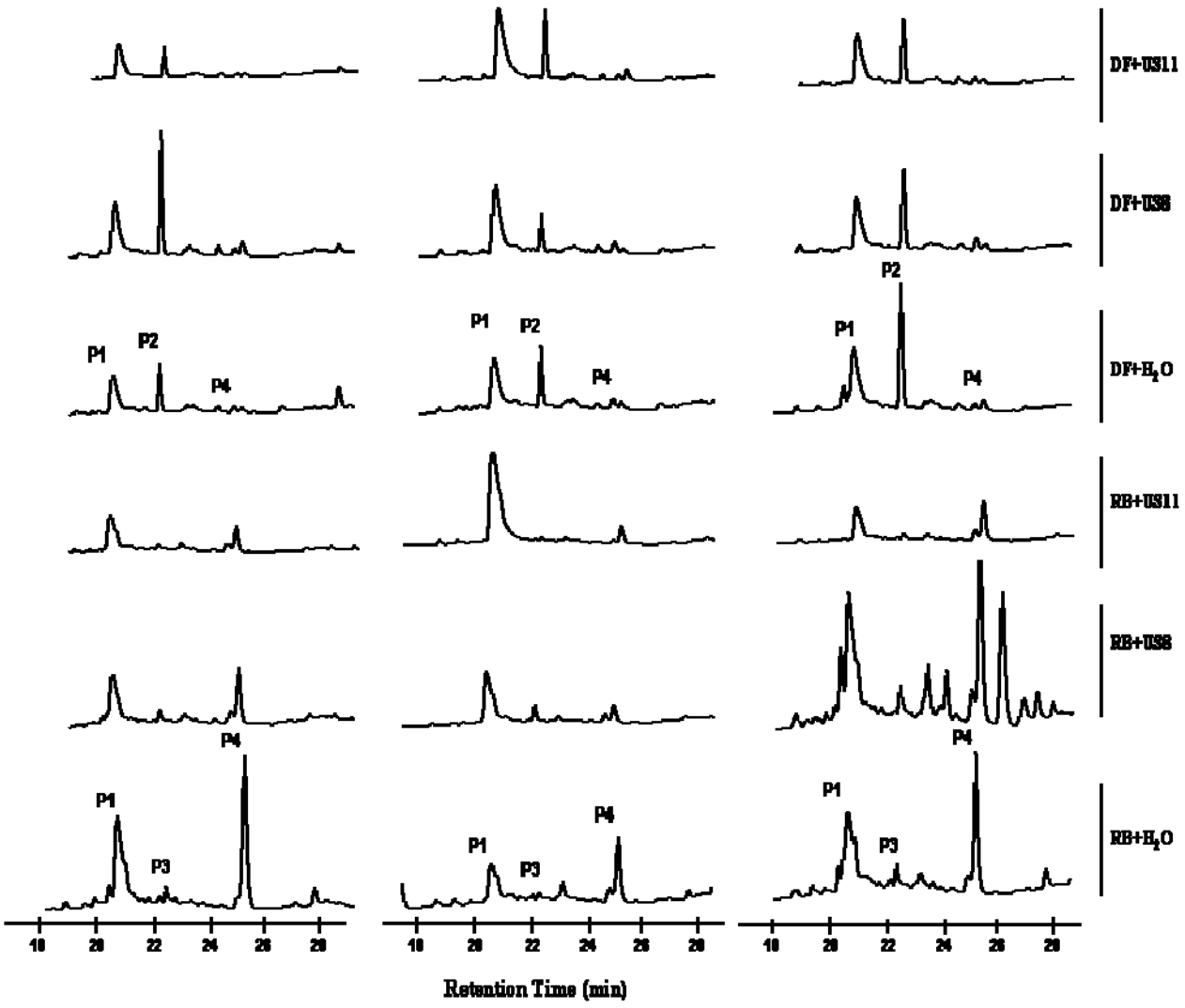

Figure 5.3. Chromatograpic profiles of secondary metabolites in potato plants inoculated with *P. infestans*. RB: Russet Burbank, DF: Defender, H20: sterile water, US8: *P. infestans* strains D1901 (lineage US8, A2 mating type, highly aggressive), US11: *P. infestans* strains D-03 (lineage US11, A1 mating type, weakly aggressive), RB+US8: Russet Burbank inoculated with US8; RB+US11: Russet Burbank inoculated with US11; DF+US8: Defender inoculated with US8; DF+US11: Defender inoculated with US11. isolates US8 and US11. (P1): cathechin (P2): flavonone (P3): flavonoid (P4): rutin. These results are representative of one of three replicates.

Table 5.1. Secondary metabolites putatively identified in potato leaves after inoculation with *P. infestans* strains D1901 (lineage US8, A2 mating type, highly aggressive) and *P. infestans* strains D-03 (lineage US11, A1 mating type, weakly aggressive) isolates.

Secondary metabolite	Putative identified compound	Potato Cultivar *	Retention time (min.)	Maximum absorbance (nm)
P1	Catechin	RB ; DF	20.8	219.1-279.2
P2	Flavonone	DF	22.45	205-255.6-354
P3	Flavonoid	RB	22.52	193.4-226.1-283.9
P4	Rutin	RB ; DF	25.30	205-255.6-356.4
T1	Terpenoid	RB ; DF	47.44	196.9

* RB:Russet Burbank; DF:Defender

Catechin (P1) was detected in Russet Burbank and Defender leaves inoculated with the *P. infestans* isolates US8 and US11 and control plants in concentrations ranging from 49.9 to 406.8 $\mu g.g^{-1}$ fresh weight (Figure 5.4). In Defender, the concentration of catechin increased in a time-dependent manner in inoculated plants as well as in control tissues to 72 hours. After this, cathechin concentrations leveled off or declined slightly. On the other hand, in Russet Burbank, the concentration of catechin decreased at 12 hpi from 365.6 $\mu g.g^{-1}$ fresh weight in the control to 147.6 and 83.5 $\mu g.g^{-1}$ fresh weight in RB+11 and RB+US8, respectively. Thereafter, a further gradual increase was observed in RB+US8 until 120 hpi, whereas in RB+US11 the level of catechin at 72 hpi was about double those detected in the control and in RB+US8. However, the level of catechin decreased significantly from 406.7 to 210.8 $\mu g.g^{-1}$ fresh weight at 72 and 120 hpi, respectively. Overall, the concentration of catechin was higher in RB and DF inoculated with US11, than in plants inoculated with US8.

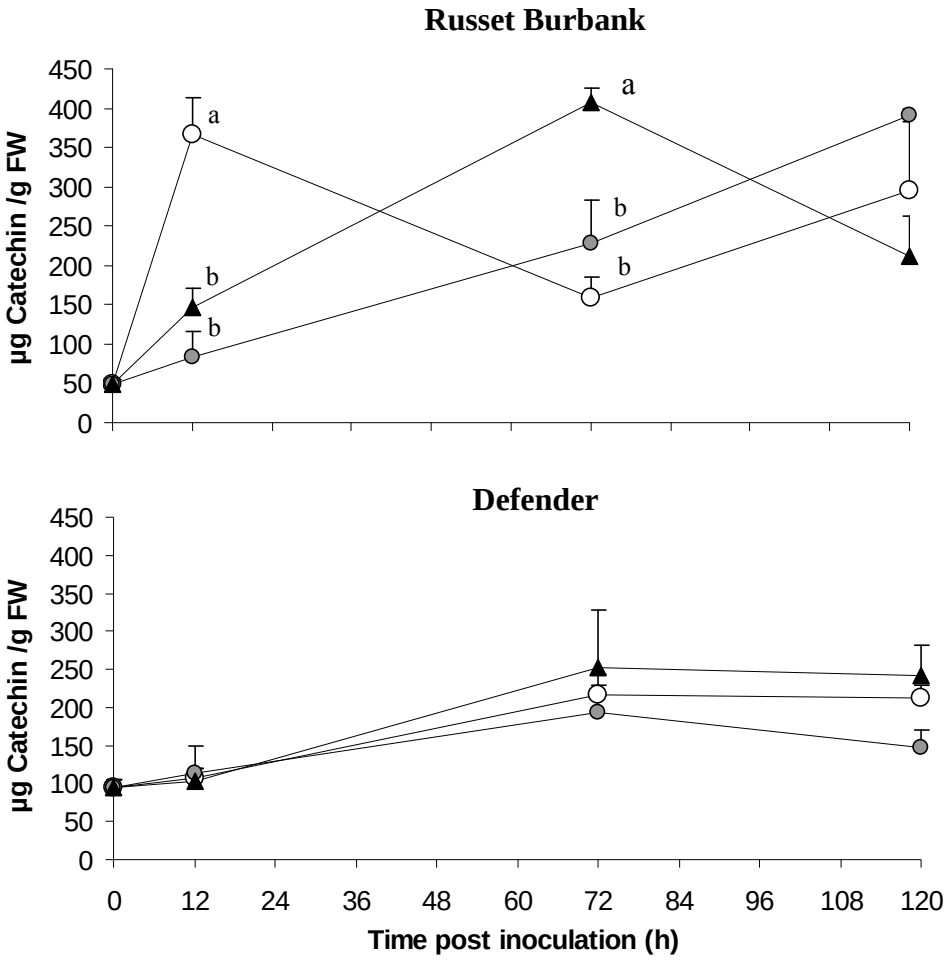

Figure 5.4. Cathechin content (µg.g^{-1} fresh weight) in potato leaves inoculated with *P. infestans*. Potato cultivar Russet Burbank and Defender inoculated with water (○), US8 (●) and US11 (▲). Within individual time (hpi), means with different letter are significantly ($p < 0.05$) different.

Flavonone (P2) was only detected in the moderately resistant cultivar Defender in concentrations ranging from 13.5 to 86.7 $\mu g.g^{-1}$ fresh weight (Figure 5.5). The concentration of flavonone (P2) increased in a time-dependent manner in DF+US11 plants as well as in control tissues. However, in DF+US8 the levels of flavonone (P2) at 72 and 120 hpi were about the half of those detected in the control plant.

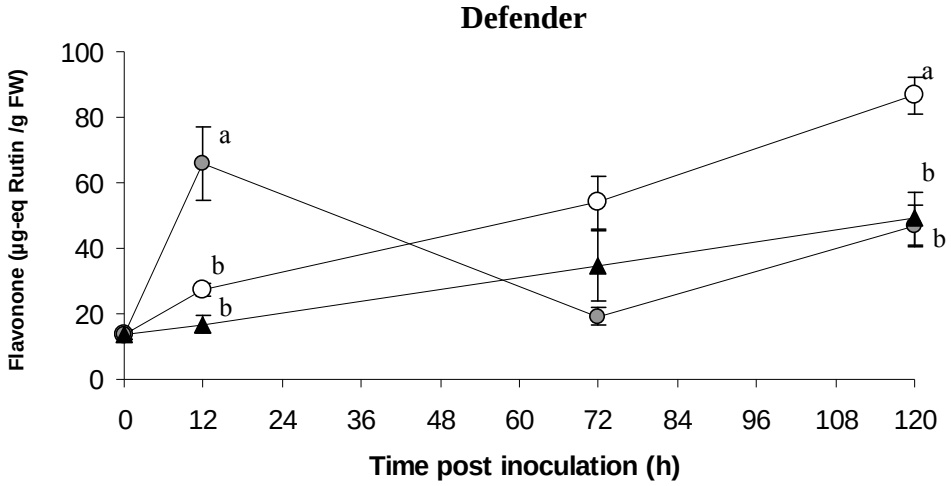

Figure 5.5. Flavonone P2 content (µg-eq Rutin/g fresh weight) in potato leaves inoculated with *P. infestans*. Potato cultivar Defender inoculated with water (○), US8 (●) and US11 (▲). Within individual time (hpi), means with different letter are significantly ($p < 0.05$) different.

Flavonoid (P3) was only detected in the susceptible cultivar Russet Burbank, in concentrations ranging from 2.07 to 18.9 $\mu g.g^{-1}$ fresh weight (Figure 5.6). The levels of flavonoid (P3) increased in a time-dependent manner in RB+US8, with levels about double of those detected in the control plant at 72 and 120 hpi. On the other hand, the

levels of flavonoid (P3) in RB+US11 were almost constant until 120 hpi in lowest concentration than the control.

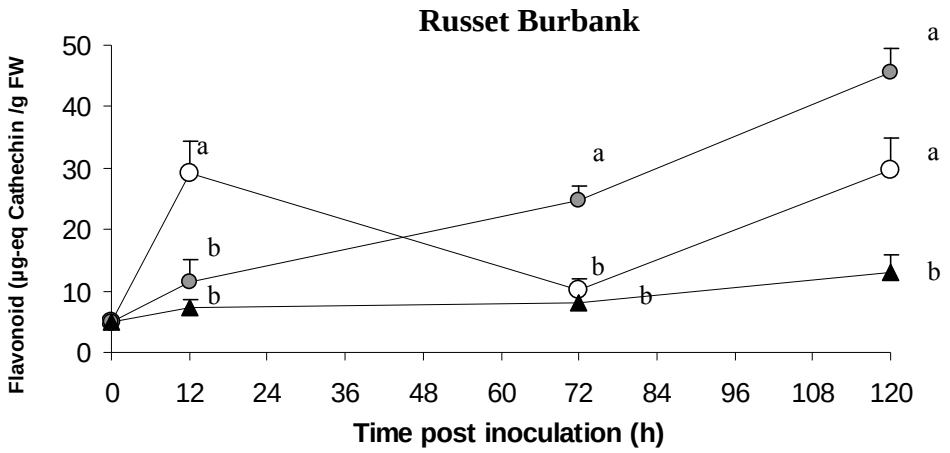

Figure 5.6. Flavonoid P3 content (µg-eq Cathechin/g fresh weight) in potato leaves inoculated with *P. infestans*. Potato cultivar Russet Burbank inoculated with water (○), US8 (●) and US11 (▲). Within individual time (hpi), means with different letter are significantly ($p < 0.05$) different.

Rutin (P4) was detected in Russet Burbank and Defender leaves inoculated with the *P. infestans* isolates US8 and US11 and control plants in concentrations ranging from 1.56 to 162.9 $\mu g.g^{-1}$ fresh weights (Figure 5.7). In Russet Burbank inoculated with US8 the concentration of rutin was considerably lower than in the control at 12 to 72 hpi. Thereafter, an increase was observed in RB+US8 until 120 hpi to the level of the control. The levels of rutin in RB+US11 were almost constant until 120 hpi and considerably

lower than in the control. On the other hand, the concentration of rutin increased in a time-dependent manner in DF+US11 plants as well as in control tissues. However, in DF+US8 the levels of rutin significantly increased at 12 hpi compared to the other two treatments and then did not differ from those treatments at 72 and 120 hpi.

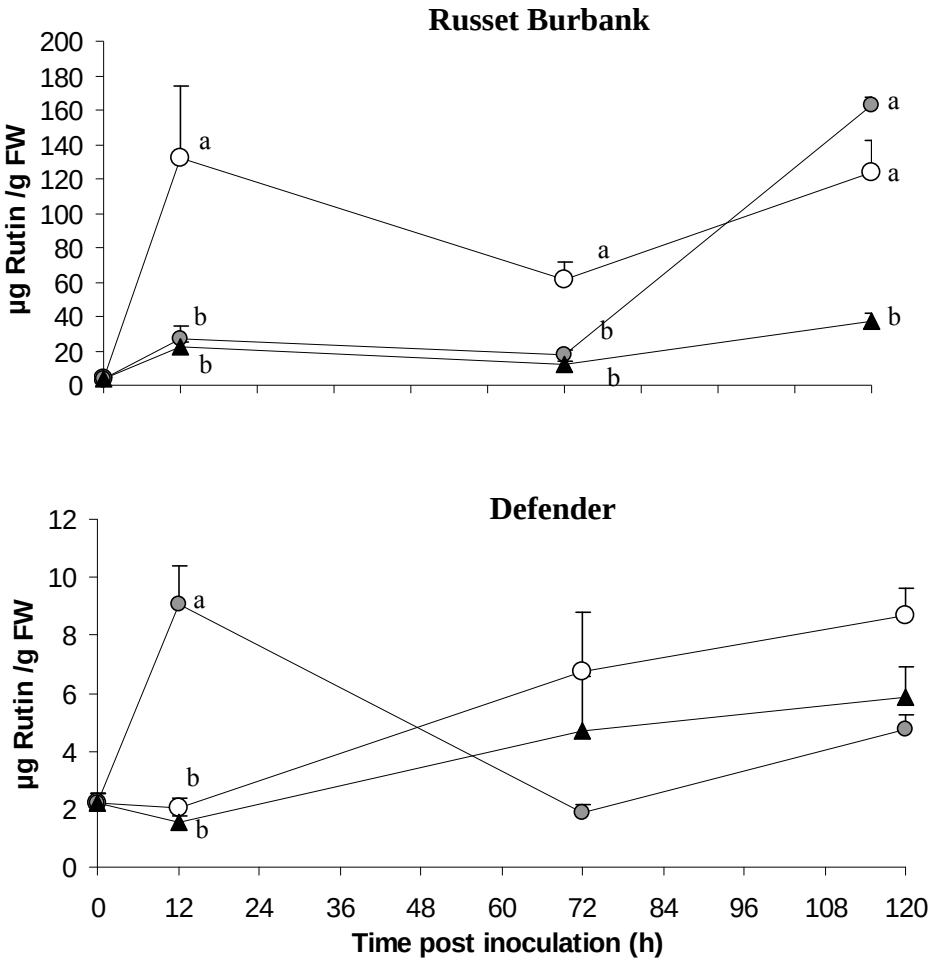

Figure 5.7. Rutin content (µg.g^{-1} fresh weight) in potato leaves inoculated with *P. infestans*. Potato cultivar Russet Burbank and Defender inoculated with water (○), US8 (●) and US11 (▲). Within individual time (hpi), means with different letter are significantly ($p < 0.05$) different.

Terpenoid (T1) was detected in Russet Burbank and Defender leaves inoculated with the *P. infestans* isolates US8 and US11 and control plants in concentrations ranging from 3.74 to 69.07 $\mu g.g^{-1}$ fresh weights (Figure 5.8). The levels of terpenoid (T1) in Russet Burbank and Defender decreased in a time-dependent manner in inoculated plants as well as in control tissues. In Defender, no significant differences were identified among treatments within individual time (hpi), whereas in Russet Burbank the level of terpenoid (T1) was higher in RB+US8 than in RB+US11 and the control (RB+H_2O) at 120 hoi, and at 12 hpi, RB+US8 differed from RB+US11, but neither treatment was singnificantly different from the control.

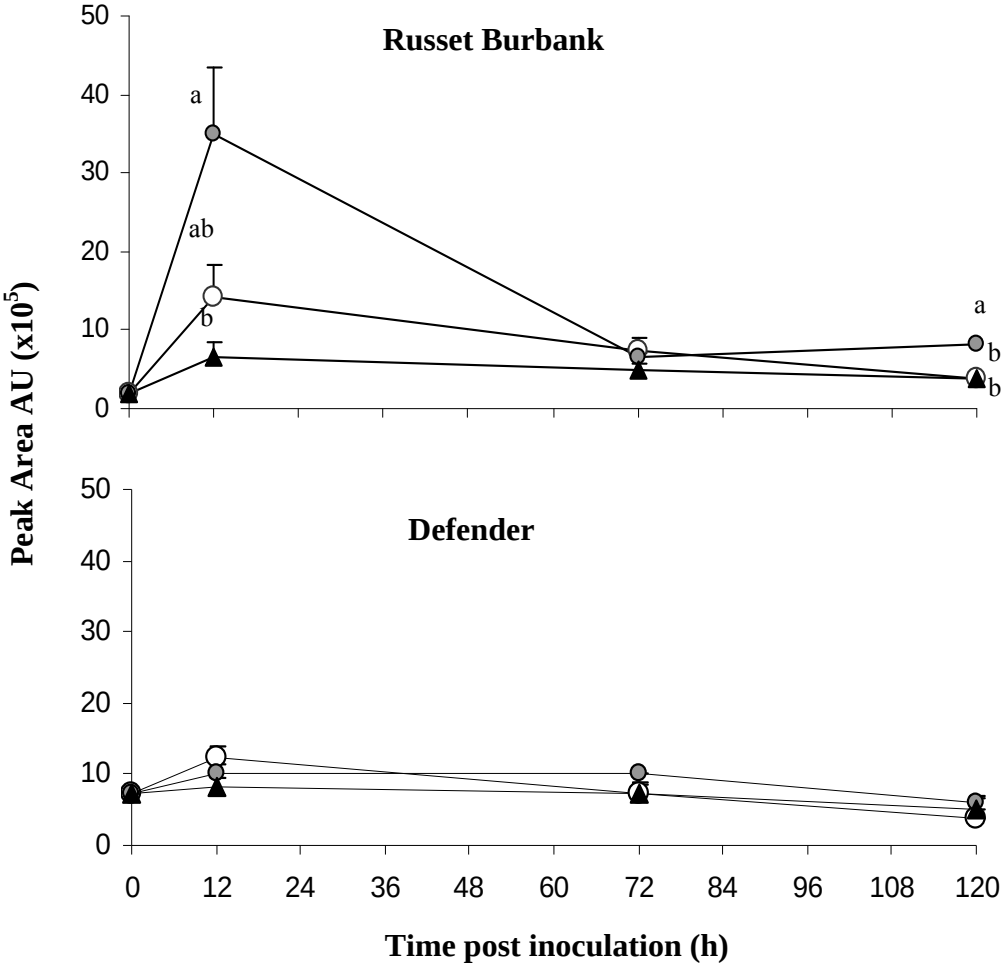

Figure 5.8. Terpenoid (T1) content (Absorbance units) in potato leaves inoculated with *P. infestans*. Potato cultivar Russet Burbank and Defender inoculated with water (○), US8 (●) and US11 (▲). Within individual time (hpi), means with different letter are significantly ($p < 0.05$) different. A.U: Absorbance Units.

5.5 DISCUSSION

In this research, we investigated the effects of *P. infestans* on the accumulation of secondary metabolites in the susceptible and moderately resistant potato cultivars "Russet Burbank" (RB) and "Defender" (DF), respectively. Our results suggest a relationship between the disease level and the accumulation of some secondary metabolites. We identified four preformed flavonoids and one terpenoid that were affected in potato by the inoculation with *P. infestans.* The flavonoid compounds catechin and rutin, and the terpenoid (T1) were detected in Russet Burbank and Defender inoculated with *P. infestans* isolates US8 and US11 and control plants. On the other hand, a flavonoid (P3) was only detected in Russet Burbank, whereas a flavonove (P2) was identified only in Defender. According to Schlosser (1994), preformed flavonoids are innately synthesized during the normal development of the plant tissue and they are also involved in several host-pathogen interactions. These preformed compounds are frequently stored in the plant later, playing a role in signalling and/or a direct function in defense.

The reduction in the accumulation of catechin in the susceptible potato cultivar Russet Burbank inoculated with US8 at 12 hpi compared with the level of catechin in the

control plant, would be associated with the reduced ability of this cultivar to inhibit the formation of appressoria and successful penetration of the fungus in the host tissue. Chen et al. (2006) reported that catechin significantly inhibited conidial germination and appressorial formation of *C. gloeosporioides*. On the other hand, in the moderately resistant cultivar Defender, the concentration of catechin increased in a time-dependent manner in inoculated plants as well as in control tissues. Interestingly, in Russet Burbank inoculated with US11, where late blight symptoms were visible only at 120 hpi, the level of catechin at 72 hpi was about double those detected in the control and decreased significantly at 120 hpi. A delay in the appearance of typical disease lesions might be due to a reduced number of appresoria that maintain viability (Chen et al. 2006). The reduction in the accumulation of catechin in Russet Burbank by US8 could be related to its suppression by this *P. infestans* isolate. However, more studies are needed to understand the molecular mechanism of this suppression at the gene and biochemistry level.

We suggest a possible implication in resistance against *P. infestans* by the flavonone (P2) accumulated only in the resistant cultivar Defender. Our results showed that the defense response by the flavonone (P2) in Defender inoculated with the highly aggressive isolate US8 was activated at 12 hpi, with levels about double those detected in the control plant. On the other hand, similar concentrations of flavonone (P2) in the control plant in Defender inoculated with the low aggressive isolate US11 might be sufficient to control the sporangia germination and appressorium formation in *P. infestans*. The role of flavonoids in resistance has been related to the mechanisms of

crosslinking pathogen enzymes, inhibition of pathogen cellulases, xylanases, pectinases, enzyme chelation, and formation of crystalline structures as a physical barrier against pathogen attack (Skadhauge et al. 1997).

A positive influence of flavonoids in plant susceptibility to the arbuscular mycorrhizal (AM) fungi has been described by Scervino et al. (2009), where the flavonoid 3-methoxi-5,6,7,8-hydroxy-4'hydroxy flavone (NMHTV) increased the percentage of tomato root colonized by *Giaspora margarita.* Our research suggests the possible implication of the flavonoid (P3) identified in this work and susceptibility of Russet Burbank to *P. infestans*. The levels of the flavonoid P3 increased in a time-dependent manner in RB+US8, with levels about double those detected in the control plant, whereas its levels in RB+US11 were almost constant until 120 hpi. In fact, this compound was only identified in the susceptible potato cultivar Russet Burbank and not in the moderately potato cultivar Defender. It is possible that this compound, only present in Russet Burbank, is essential for the survival and development of infection by *P. infestans* in potato, similar to bacterial soft roft disease in potatoes caused by *Erwinia carotovora* var. *atroseptica,* which has been shown to be less severe on potatoes with low-reducing sugar content than in potatoes high in reducing sugars (Otazu and Secor 1981). Also, in order to infect a plant, *Rhizoctonia* which causes damping off in many plants, requires compounds from susceptible plants for the formation of a hyphal cushion to penetrate the host (Agrios 2005).

Flavonoids possess a wide range of biological activities in plants. They are beneficial for the plant itself as physiologically active compounds, and environmental stress agents, protecting the plant against UV radiation, pathogens and herbivores (Treutter 2006). In our research, in the susceptible cultivar Russet Burbank inoculated with US8 and US11, the levels of rutin were about half those detected in the control plant at 12 and 72 hpi. The suppression in the accumulation of rutin suggests that *P. infestans* was able to manipulate this compound in Russet Burbank, thereby resulting in susceptibility to both *P. infestans* isolates. Two studies have reported that *P. infestans* is capable of producing suppressors such as water-soluble glucans that suppress plant defense responses (Andreu et al. 1998; Ozeretskovskaya et al. 2001). One explanation of such a specific suppression, attributed this phenomenon to a pathogen suppressor which may affect the formation of binding complexes in the promoter region and suppress the expression of specific genes (Shiraishi et al. 1994), such as key genes from the phenylpropanoid pathway. This suppression of rutin did not occur in the cultivar Defender where the accumulation of rutin is likely should be associated with its moderate resistance to *P. infestans*. Our results showed that the defense response by rutin in Defender inoculated with the highly aggressive isolate US8 was activated at 12 hpi, with levels about double those detected in the control plant. On the other hand, there was a similar concentration of rutin to the control plant in Defender inoculated with the low aggressive isolate US11. These results are in agreement with previous reports suggesting that rutin plays a role in potato defense response against *Verticillium dahliae* (El Hadrami et al. 2008).

Andreu et al. (1998) showed that the addition of EPA and glucan to potato tubers reduced the accumulation of the terpenoid rishitin, phytuberim and lubimin, compared with the potato tuber treated only with EPA. Similar results were described by Garas et al. (1979), who reported that suppressors from the virulent races of *P. infestans* inhibited the accumulation of rishitin and lubimin. Our results did not show a suppression of a terpenoid (T1) identified in Russet Burbank and Defender. In fact, no significant differences were identified in the levels of the terpenoid (T1) among treatments within individual times (hpi) in Defender, suggesting that this compound may help this cultivar in its moderate resistance to *P. infestans*. Conversely, in Russet Burbank inoculated with the highly aggressive isolate US8, the high accumulation of terpenoid (T1) at 12 and 120 hpi, with levels about double those detected in the control plant, is not helping the plant to stop the development of the infection process.

In chapter 4, we suggested that moderate resistance of Defender to both isolates should also be attributed to products of the phenylpropanoid and mevalonate (Ac-MVA) pathways. Our results in this investigation confirm that hypothesis, because the preformed flavonoids identified in this investigation; catechin, flavonone (P2), rutin and the terpenoid (T1), played a potential role in the defense of Defender to the *P. infestans* isolates US8 and US11. On the other hand, in chapter 4, we also suggested that in Russet Burbank the phenylpropanoid pathway is not playing a potential role in the possible synthesis of phenolic compounds that participate in defense responses. Our results in this investigation also support that hypothesis, because the accumulation of catechin and rutin were suppressed by the *P. infestans* isolates US11 and US8. As we suggested above, one

explanation of such a specific suppression, is that *P. infestans* suppressors may affect the formation of binding complexes in the promoter region and suppress the expression of specific genes from the phenylpropanoid pathway or by blocking the signal transduction pathway during the elicitor-mediated activation of the defense responses (Shiraishi et al. 1994). In addition, the only phenylpropanoid compound up-regulated in Russet Burbank (flavonoid P3) was associated in this investigation with the susceptibility of Russet Burbank to the *P. infestans* isolate US8.

The present study provides new evidence that different preformed flavonoids and terpenoids in potato may play important roles in its defense or susceptibility to *P. infestans.* This study is a contribution to the knowledge about the *potato - P. infestans* interaction that may help to develop novel management strategies to control late blight. This research also gives a basis to future studies of genetic manipulation of flavonoid biosynthesis in potato by metabolic engineering that could contribute to a better appreciation of the role of flavonoids in the potato – *P. infestans* interaction. Also, results in this study highlight the necessity of chemical identification of several compounds including flavonone (P2) and the flavonoid (P3).

CHAPTER 6: SUMMARY AND CONCLUSION

Manitoba is the second largest potato producer in Canada after Prince Edward Island (Statistics Canada 2006). This crop faces many disease problems including the most famous and devastating one, late blight, caused by the oomycete pathogen *Phytophthora infestans* (Daayf et al. 2001; Kamoun 2003). The hypothesis tested in this investigation is that *Phytophthora infestans* suppresses defense responses in potato (*Solanum tuberosum*) by alterating the expression of defense-related genes, and the biosynthesis and accumulation of secondary metabolites and secondary metabolism pathway genes. This study was carried out using one susceptible (Russet Burbank) and one moderately resistant (Defender) potato cultivars during their interactions with one weakly- (US11) and one highly-aggressive (US8) strain of *P. infestans.*

The first step was to identify both host and pathogen genes differentially stimulated during the potato-*P. infestans* interaction. This step was achieved by developing a SH/cDNA-AFLP approach (Henriquez and Daayf 2010) (Chapter 2) that uses the advantages of cDNA-AFLP and subtractive hybridization in order to amplify cDNA products in a polyacrylamide gel and remove the constitutively/commonly expressed sequences. Genes potentially controlling pathogenesis or avr genes in *P. infestans*, as well as those potentially involved in potato resistance or susceptibility to this pathogen were identified.

- Two transcripts (DL119 and DL95) are *P. infestans* TDFs potentially involved in pathogenicity. DL119 exhibits sequence similarity with N-succinyl

diaminopimelate aminotransferase which is an important enzyme in the lysine biosynthesis via the diamino-pimelate pathway (Tyler 2001). DL95 showed a similar expression pattern to the TetR Family of Transcriptional Repressors, which controls genes involved in pathogenicity of gram-negative and gram-positive bacteria (Ramos et al. 2005).

- The potato transcript DL39 was exclusively up-regulated in the moderately resistant potato cultivar (Defender), while it was suppressed in the susceptible cultivar Russet Burbank, during the intercation with US8 and US11, which made this transcript a good candidate for future studies of gene resistance.
- A potential resistance gene DL81 was induced at 12 hpi in the moderately resistant cultivar Defender, whereas it was down-regulated as early as 6 hpi in the susceptible cultivar Russet Burbank.
- DL21 and DL28 have potential involvement as potato susceptibility genes. DL21 transcripts were up-regulated in the compatible interation of Defender inoculated with US8, while DL28 transcripts were up-regulated exclusively in the susceptible cultivar Russet Burbank.
- DL41 and DL 24 are potential *P. infestans* avirulence-associated genes.

Identifying differentially expressed genes is one of the most useful approaches to understand the molecular mechanisms underlying biological systems. Their screening has been explored through different molecular techniques, such as; cDNA-AFLP, conventional cDNA library construction and screening, suppression subtractive hybridization (SSH) and microarray. Every technique has its advantages and

disadvantages. However, the main inconvenience in most methodologies lies with their ineptitude to simultaneously evaluate gene expression from different treatments. In the case of host-pathogen interactions, such inconvenience extends to the inability to integrate different forms of the associated pathogen in the same analysis. Evaluating different treatments at the same time would contribute to the identification of common genes that do not play a major role in the biological system. In addition, there are several affordability limitations for many academic laboratories around the word, especially in developing countries. High throughput techniques, such us microarrays, are associated with equipment and facilities that are costly and beyond access for most academic laboratories, and also only feasible when wide sequence information and/or cDNA and subtractive libraries are available.

In Chapter 3, we revealed the first identification of DOXP-MEP pathway genes in potato and its regulation in response to the oomycete *P. infestans.* We cloned a 2,421 bp cDNA from a fragment differentially expressed in potato using the SH/cDNA-AFLP approach (Henriquez and Daayf 2010). The cloned cDNA contains a complete ORF (2,160 bp) from the enzyme 1-deoxy-D-xylulose 5-phosphate synthase (DXS) in potato. DXS catalyzes the first step in the DOXP-MEP pathway, which is an alternative isoprenoid biosynthetic route in plants (Lichtenthaler 2000). Based only on the expression of *StDXS1*, we may speculate that this gene is associated with susceptibility, because we found that *StDXS1* was up-regulated in the susceptible cultivar and down-regulated in the moderately resistant one, compared to non-inoculated potato plants. However, when assessing the expression pattern of five other genes controlling steps in

the DOXP-MEP pathway, we detected changes in their expression in response to inoculation with the pathogen, showing the high complexity of this pathway. It is possible that downstream pathway members (gene families) are functioning, leading to the induction of gene products in this pathway.

Glucans isolated from *P. infestans* cell walls suppressed the accumulation of isoprenoid phytoalexins and reduced ß-1,3-glucanase activity in potato tubers (Andreu et al. 1998). On the other hand, it has been shown that *P. infestans* produces elicitors, such as arachidonic acid (AA) and eicosapentaenoic acid (EPA), which are able to elicit the accumulation of phytoalexins (Andreu et al. 1998; Coquoz et al. 1998) inducing defense responses. In Chapter 4, we investigated the effects of (i) glucans (suppressors), (ii) the eicosapentanoic acid (elicitor) (EPA) and (iii) *P. infestans* isolates in the differential expression of genes from the isoprenoid DOXP-MEP pathway as an extension of our previous work (Chapert 3), assessing the expression pattern of the six genes controlling steps in this pathway (*STDXS1, DXR, MCT, CMK, MDS* and *HDS*). In addition, known genes from the phenylpropanoid and mevalonate (Ac-MVA) pathways were evaluated. Here are some important findings from this chapter:

- EPA treatment reduced the symptoms of late blight in Russet Burbank and Defender inoculated with US8 and US11, indicating a posible role in eliciting potato defense responses. The function of EPA eliciting defense responses in Defender inoculated with US8, could be related to the up-regulation of

phenylalanine ammonia-lyase *PAL-2* gene, which would lead to the synthesis of salicylic acid (Ludwikow et al. 2004).

- Glucans displayed a defense-suppressing effect in Russet Burbank when the the *P. infestans* isolate used in the inoculation was as aggressive as the ones from which the glucans applied to the plant before the inoculation were extracted.
- Glucans showed a defense elicitor effect in Russet Burbank when the *P. infestans* isolate used in the inoculation had a different level of agressiveness than the one from which glucans applied to the plant before the inoculation, were extracted.
- In addition to the R-genes from *Solanum demissum* likely present in the background of Defender (Corsini et al. 1999), its moderate resistance to *P. infestans* isolates US8 and US11, should also be attributed to products of the phenylpropanoid and mevalonate (Ac-MVA) pathways.
- Similar to the time series experiment showed in Chapter 3, down-regulation of (E)-4-hydroxy-3-methylbut-2-enyl diphosphate synthase (*HDS*) transcrips in the moderately resistant cultivar Defender inoculated with US8 and US11, is probably an unpredicted link between the DOXP-MEP pathway and plant defense (Durrant and Dong 2004; Gil et al. 2005).
- In the susceptible potato cultivar Russet Burbank, glucans and EPA did not induce suppression or up-regulation of *PAL-1 PAL-2* or *4CL* genes, because their expression ratios were not affected when compared with the control plant and RB+US8.

- Sesquiterpene cyclase (*SC*) gene that catalyzes the first steps in the branch leading to sesquiterpenoid phytoalexins, such as rishitin, lubimin and phytuberin (Engstrom et al. 1999; Dixon 2001; Krits et al. 2007) was the only gene down-regulated in this pathway in RB+US8 and RB+GL+US8. A possible suppression or reduction in the accumulation of these phytoalexins could be associated with their late blight disease symptoms.

The results of this study suggest the metabolic pathways affected in potato by *P. infestans*, which play a role in potato defense to late blight. The current findings imply that genetic resistance in potato against *P. infestans* is not the result of isolated reactions against the pathogen, other than the combination of different factors that suggest a polygenic trait or horizontal resistance. It has been previously shown that loci corresponding to genes related to the phenylpropanoid pathway (*PAL*, *CHI*, and *CHS*) are significantly associated with quantitative disease resistance in potato to *P. infestans* and *PAL* has been localized with QTLs for resistance to other diseases (Faris et al. 1999; Geffroy et al. 2000; Wang et al. 2001). Our results highlight the necessity to investigate the molecular response of potato to *P. infestans* by integrating not only individual genes from the phenylpropanoid, mevalonate (Ac-MVA) or DOXP-MEP pathways but several genes and their gene homologs.

The majority of secondary metabolites playing a role in plant protection against pathogens are derived from the phenylpropanoid, mevalonate, alkaloid or fatty acid pathways (Dixon 2001). In Chapter 5, we identified four preformed flavonoids and one

terpenoid compound in potato that were affected by the inoculation with *P. infestans*. Our results in this investigation confirm our hypothesis that the moderate resistance of Defender to the highly aggressive *P. infestans* isolate US8 and the weakly aggressive *P. infestans* isolate US11, should also be attributed to products of the phenylpropanoid and mevalonate (Ac-MVA) pathways. Preformed flavonoids identified in this investigation; catechin, flavonone (P2), rutin and the terpenoid (T1), played an important role in the defense of Defender to the *P. infestans* isolates US8 and US11. On the other hand, in chapter 4, we also suggested that the phenylpropanoid pathway is not playing an active role in defense responses in Russet Burbank. Our results also confirm that hypothesis, because the accumulation of catechin and rutin were suppressed by the *P. infestans* isolates US11 and US8, which may have affected the formation of binding complexes in the promoter region and suppressed the expression of specific genes from the phenylpropanoid pathway (Shiraishi et al. 1994). In addition, the only phenylpropanoid compound up-regulated in Russet Burbank (flavonoid P3) was associated in this investigation with the susceptibility of Russet Burbank to the *P. infestans* isolate US8.

In conclusion, the original hypothesis was confirmed; *P. infestans* suppresses defense responses in potato (*Solanum tuberosum*) by alterating the expression of defense-related genes, accumulation of secondary metabolites and secondary metabolism pathway genes. This study is a contribution to the knowledge about the *potato - P. infestans* interaction that may help develop novel management strategies to control late blight. However, future work is necessary to determine the biological functions of the transcripts identified in chapter 2. Since plants and many fungi have different lysine biosynthesis pathways (Walters et al. 1997), the inhibition of lysine formation in the fungi might be a

good disease control strategy. For that reason, the inhibition of DL119, could be explored. It has been shown that lysine is an important component in the *P. infestans* pathogenicity, because the inhibition of the lysine biosynthetic enzyme dihydrodipicolinate synthase (DHDPS) reduces mycelial growth of *P. infestans* and completely inhibits blight infection of leaf discs (Walters et al. 1997). In addition, based on the results, we can suggest that DL39, DL81 are good candidates for future studies of gene resistance by gene silencing and Marker Assisted Selection (MAS) studies, as well as DL21 and DL28 as potential plant disease susceptibility genes. Further studies of these potential susceptibility genes would represent a novel form of disease resistance derived from the failure of a gene that is necessary during a compatible interaction, rather than the activation of known host defense pathways (Vogel et al. 2006).

Without the analysis of *STDXS2*, we do not know if *StDXS1* (Chapter 3) is associated with susceptibility or if it is expressed in a specific plant variety. Silencing *StDXS1*, *StDXS2*, *HDS* and its gene homologs, would help to elucidate potential gene function of the DOXP-MEP pathway in potato-pathogen interactions. Special focus shoud be given to the *HDS* gene, due to its drastic suppression in Defender inoculated with US8 and US11 (Chapter 3 and 4), and a probable link between the DOXP-MEP pathway and plant defense, similar to the response in Arabidopsis mutant *cbs3* with reduced HDS activity. Gil et al. (2005) found that Arabidopsis plants with reduced HDS activity significantly repressed the growth of the oomycete *Hyaloperonospora parasitica*, and showed enhanced accumulation of Salycilic Acid and the activation of the defense-related marker genes PR1 and PR2. The *HDS* full length cDNA can be obtained by

designing primers through the conserved sequence of the known *HDS* in *Lycopersicum esculentum* (AF435086.1) and RACE. Afterwards, in order to investigate the genomic organization of the *HDS* gene in potato, genomic DNA from the potato cultivars can be subjected to Southern blot analysis. To attempt the potato *HDS* gene silencing, the RNA interference approach should be used. The RNAi occurs when long dsRNA and miRNA precursors are processed to small interfering RNA (siRNA) or micro RNA (miRNA) duplexes by the RNase-III-like enzyme Dicer. The siRNAs / miRNAs bind to a nuclease complex to form the RNA-inducing silencing complex (RISC) and the active RISC then targets the homologous transcript by base pairing interaction and cleaves the specific mRNA (Tuschl 2001). In addition, the *HDS* deficient plants can be evaluated by phenotypic and microscopy techniques. Salicylic Acid accumulation can be analyzed by HPLC and the study of activation of the defense-related marker genes controlled by the SA-mediated signalling pathway can be evaluated by Quantitative Real Time PCR.

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APPENDICES

Appendix 1 (Chapter 2):

EST#: DL12

Sequence

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EST#: DL119

Sequence

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EST#: DL142

Sequence

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EST#: DL21

Sequence

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EST#: DL32

Sequence

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EST#: DL33

Sequence

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EST#: DL95

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EST#: DL2

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EST#: DL49

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EST#: DL138

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EST#: DL16

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EST#: DL39

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EST#: DL91

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EST#: DL123

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EST#: DL144

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EST#: DL81

Sequence

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EST#: DL24

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EST#: DL41

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EST#: DL40

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EST#: DL54

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EST#: DL31

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EST#: DL47

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EST#: DL28

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EST#: DL120

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EST#: DL129

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EST#: DL145

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EST#: DL146

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Appendix 2 (Chapter 3):

Sequence analysis of *StDXS1*

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CCTCTTA

Deduced protein sequence of *StDXS1*

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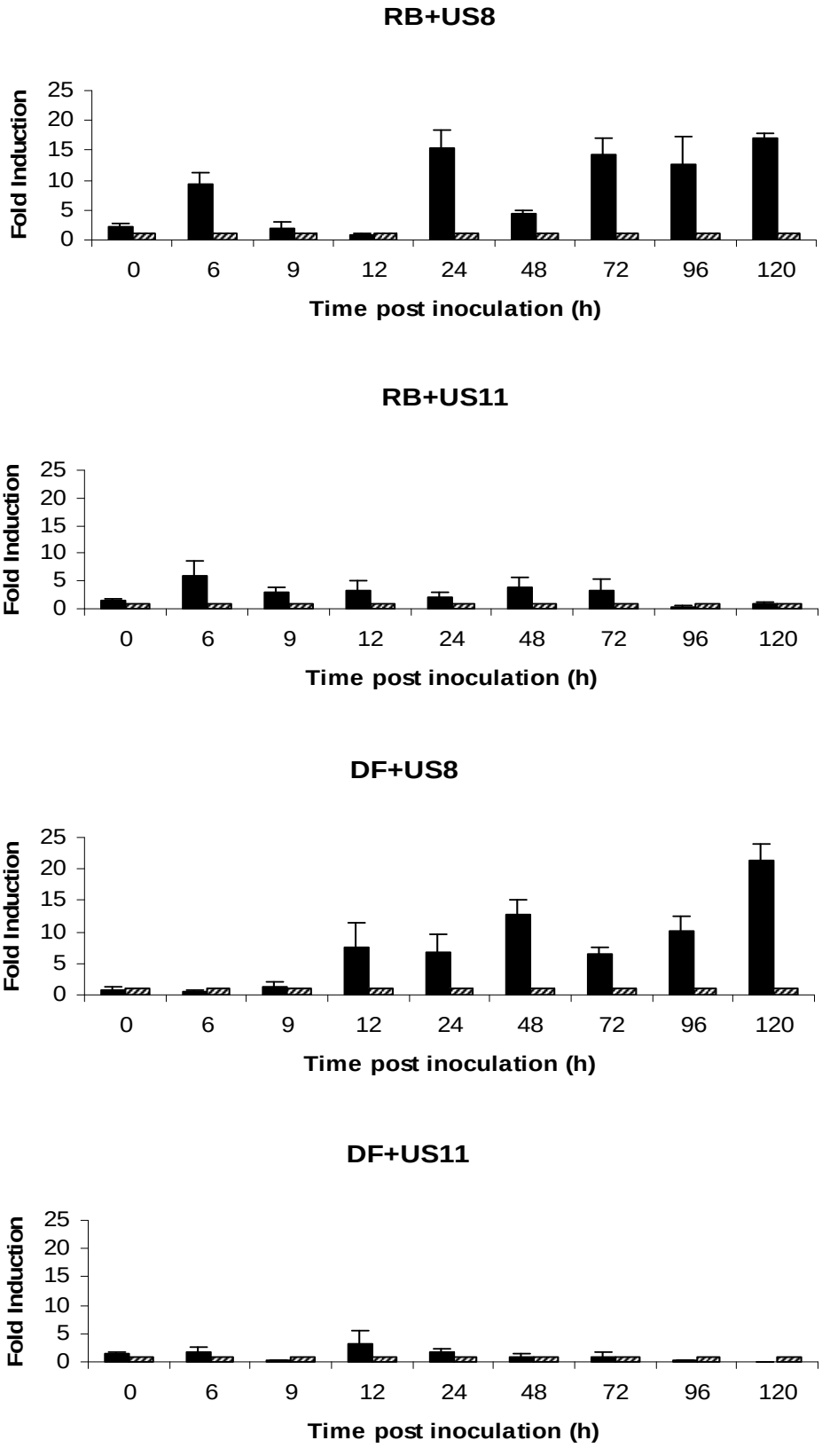
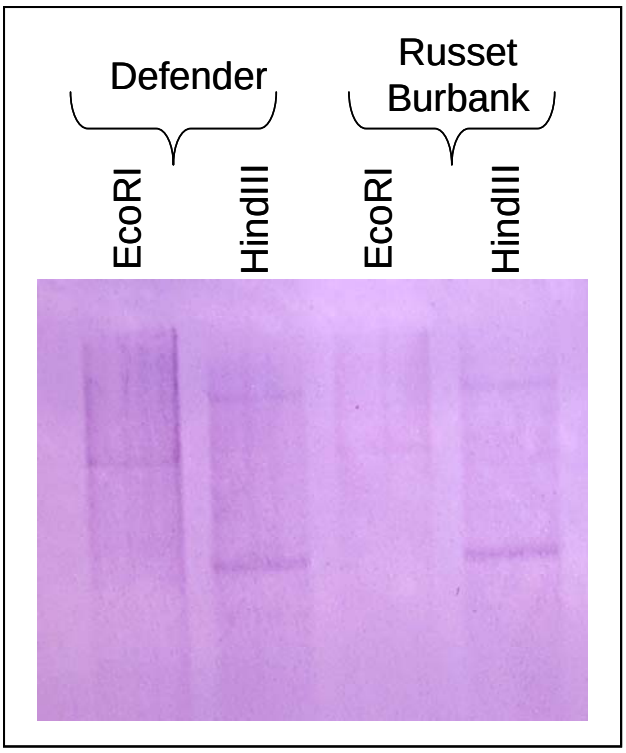
TREALEVMT

Appendix 3 (Chapter 3)

HMGR2

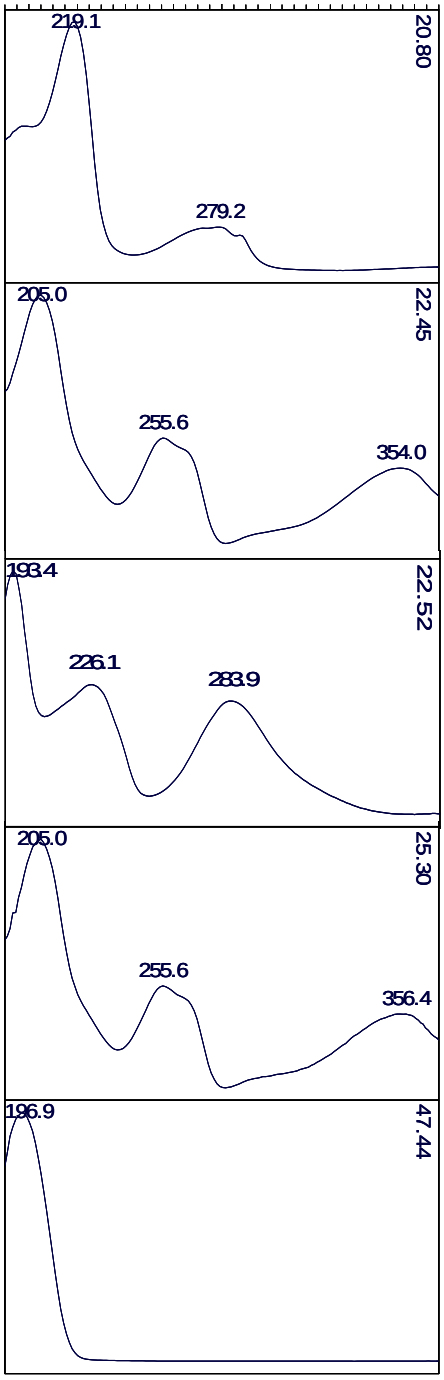


Appendix 3 (Chapter 3)



Southern blot analysis to investigate the genomic organization of *StDXS1*, genomic DNA from potato cultivars Russet Burbank and Defender were digested with EcoRI and HindIII, and subjected to in a triplicate experiment.

Appendix 4 (Chapter 5)



Absorption spectra of putatively identified compounds. Catechin (P1), flavonone (P2), flavonoid (P3), rutin (P4) and terpenoid (T1).