MOLECULAR BASIS OF *VERTICILLIUM DAHLIAE* PATHOGENESIS ON POTATO

BY

AHMED FARAG A. M. EL-BEBANY

A Thesis Submitted to the Faculty of Graduate Studies
in Partial Fulfillment of the Requirements for the Degree of

DOCTOR OF PHILOSOPHY

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THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

El-Bebany, Ahmed Farag Ahmed Mohamed. Ph.D., University of Manitoba, November, 2010. Molecular Basis of *Verticillium dahliae* Pathogenesis on Potato. Advisor: Dr. Fouad Daayf.

Verticillium wilt disease affects a wide range of economic dicotyledonous crops, including potato, worldwide. Verticillium wilt of potato is caused, primarily, by the soilborne fungus *Verticillium dahliae* Kleb. Management of Verticillium wilt is a challenge due to the persistent nature of the pathogen and the absence of effective control practices including completely resistant potato cultivars. The success of disease control methods relies on understanding the mechanisms of *V. dahliae* pathogenesis and its interactions with potato, the main objective of this study.

A differential potato-*V. dahliae* pathosystem was established where the pathogenicity of four *V. dahliae* isolates (Vd1396-9, Vs04-28, Vs06-13 and Vs06-14) with different levels of aggressiveness was evaluated on two potato cultivars (Kennebec and Ranger Russet) possessing contrasting levels of susceptibility. Disease severity, vascular discoloration and host growth measurements over time from 2 to 7 weeks after inoculation revealed that *V. dahliae* isolates Vd1396-9 and Vs06-14 are highly and weakly aggressive, respectively. These two isolates were selected for further transcriptomics and proteomics investigations in order to identify their pathogenicity-related factors. Transcriptomics analysis was conducted in the highly (Vd1396-9) and weakly (Vs06-14) aggressive isolates after elicitation by root extracts from either susceptible (Kennebec) or moderately resistant (Ranger Russet) potato cultivars using a

combinational approach involving subtractive hybridization (SH) and amplified fragment length polymorphism (AFLP). Five hundred and seventy three differentially expressed transcripts were detected in one or the other isolate in response to the different root extracts. The differentially expressed transcripts were greater in the highly aggressive than the weakly aggressive isolate and in response to the root extract from the susceptible than the moderately resistant cultivar. One hundred eighty five transcripts of interest were recovered, re-amplified, sequenced and queried against the National Center for Biotechnology Information (NCBI) and the Broad Institute *V. dahliae* genome databases using (Basic Local Alignment Search Tool) BLASTn and BLASTx for putative identification.

The highly (Vd1396-9) and weakly (Vs06-14) aggressive isolates of *V. dahliae* were used for a comparative proteomics investigation to explore their differentially expressed proteins. The first proteomic map of *V. dahliae* was established in this part of this thesis. The proteomics analysis was carried out using two-dimensional electrophoresis (2-DE) and mass spectrometry (MS) analyses. Twenty five proteins were differentially expressed in one or the other isolate. Tentative identification of the differentially expressed proteins was conducted using the MASCOT search engine on a database consisting of NCBInr fungal sequences and the Broad Institute *V. dahliae* genome databases.

Many of the identified differentially expressed genes/proteins from transcriptomics and proteomics analyses showed potential involvement in pathogenesis-related functions in *V. dahliae* or other related fungi. These functions include cell-wall degradation, colonization, survival structure formation, stress response tolerance and

suppression of plant defense signaling responses. Among these potential pathogenicity factors, stress response regulator A (oxidative stress tolerance factor), isochorismatase hydrolase (potential plant defense signaling suppressor) and tetrahydroxynaphthalene reductase (involved in melanin biosynthesis and microsclerotia formation) were selected for further analyses. These three genes were isolated from both the highly (Vd1396-9) and the weakly (Vs06-14) aggressive isolates of *V. dahliae* and cloned. Comparative sequence analysis of these genes showed many differences that may explain their differentially expressed pattern in the two differentially aggressive isolates and, consequently, *V. dahliae* pathogenesis.

Production of secondary metabolites is one of the defense mechanisms that are involved in plant disease resistance. Given that some of the identified genes/proteins are potentially involved in overcoming and suppressing plant defense mechanisms, phenolics were extracted from root, stem and leaves of Kennebec- inoculated with either the highly or the weakly aggressive isolate and analyzed using high performance liquid chromatography (HPLC). Among the detected variations in phenolics profiles, caffeic and ferulic acids accumulated in roots and stems after inoculation with *V. dahliae* isolates, especially the highly aggressive one. However, in the stem, the accumulation of rutin in response to inoculation was inhibited by the highly aggressive *V. dahliae* isolate relative to the weakly aggressive one. The results obtained from the three molecular levels will help in understanding *V. dahliae*-potato interactions and could be considered in building an efficient integrated management program to control Verticillium wilt disease.

FOREWORD

This thesis has been written in manuscript style as outlined by the Department of Plant Science and the Faculty of Graduate Studies, University of Manitoba. A general introduction and literature review precede the five main chapters of the thesis. Each chapter consists of its own abstract, introduction, materials and methods, and results and discussion. Part of the first chapter (section 3.0) and the entire third chapter (section 5.0) have been published in Proteomics (2010) 10: 289-303, the version included in the thesis is the pre-peer reviewed one. The second chapter (section 4.0) is submitted to Environmental and Experimental Botany, thus the version included in the thesis may differ from the one that will be published. The five main chapters are followed by a General Discussion and Conclusions section, a list of literature cited and appendices.

1.0 INTRODUCTION

Verticillium wilt is a very serious disease that affects hundreds of dicotyledonous plant species including fruit trees, shrubs, field crops, vegetables, and ornamental plants worldwide (Pegg and Brady 2002). Potato is one of the important economic food crops that are challenged by Verticillium wilt. The disease can cause 30-50 % yield losses, and will affect the crop quality as well. The main causal agent of potato Verticillium wilt is the soilborne Deuteromycete *Verticillium dahliae* Kleb., which sometimes infects potato in association with the nematode *Pratylenchus penetrans* and other pathogens. The disease complex in this case is called potato early dying syndrome (Rowe and Powelson 2002).

The symptoms of potato infection by *Verticillium dahliae* include chlorosis and necrosis, vascular discoloration, stunting, and eventually wilting, which result in decreased potato yield and tuber quality. *Verticillium dahliae* can survive in the soil for 10-15 years as microsclerotia (Wilhelm 1955). The microsclerotia are stimulated to germinate by potato root exudates, and then develop hyphae, penetrate and colonize the roots. The hyphae reach the xylem vessels, and reproduce asexually by forming conidiospores that are able to move upward in the vascular system. As a result, the latter gets plugged by the fungus along with some structural defense components, which are responses to the infection. Water is prevented from reaching the aerial parts of the plant and the disease symptoms start developing. At the end of the disease cycle, the fungus forms new microsclerotia on the dead tissues of the plant (Huisman 1982; Pegg and Brady 2002).

Current control methods are dependent on soil fumigation (Miller et al. 1967), which is not an eco-friendly method; soil solarization (Pullman et al. 1981), which is not applicable in all regions of potato cultivation; crop rotation and soil amendments (Shetty et al. 2000), which are limited by farmers' choices and marketing factors; and potentially biological control (Uppal et al. 2008), which should be optimized for field conditions. Tolerant cultivars are also used in the absence of potato cultivars completely resistant to *V. dahliae*. The main obstacles to managing Verticillium wilt are the persistence of microsclerotia, the primary source of infection, in soils, in addition to the wide genetic diversity of *V. dahliae*, and the wide host range of the pathogen, which allow it to easily find an alternative host (Powelson and Rowe 1993; Klosterman et al. 2009; Johnson and Dung 2010). The host side has been investigated in many studies that focused on plant defense mechanisms such as the production of phytoalexins and pathogenesis-related (PR) proteins (Daayf et al.1997; Maksimov et al. 2009). On the other hand, there is limited knowledge about the pathogen side (Neumann and Dobinson 2003). Development of a successful Verticillium wilt management strategy requires a good understanding of both organisms as well as their interactions in the potato-*V. dahliae* pathosystem.

The pathogenesis mechanisms of *V. dahliae* at the molecular level are still poorly understood due to the complexity of the system. In other words, *V. dahliae* is a hemibiotrophic fungus, survives in the soil environment for many years, lives in the plant vascular system, is distributed in several geographical climatic regions worldwide, and has a broad range of genetic diversity and hosts. Together, all of these factors make the study of this disease a real challenge, but reveal the need for more knowledge in this pathosystem. This important knowledge could be achieved through enhances

understanding of the molecular mechanisms of *V. dahliae* pathogenesis, by integrating information based on genomics, proteomics, and metabolomics investigations.

The main objectives of this study were: **i)** to establish a differential model of the potato-*V. dahliae* pathosystem that consists of contrasting-aggressiveness *V. dahliae* isolates on potato cultivars with different levels of resistance, **ii)** to identify potential pathogenicity-related genes in *V. dahliae* in the selected highly and weakly aggressive isolates using transcriptomics approaches, **iii)** to conduct a comparative proteomics analysis and explore the proteomic map of the *V. dahliae* selected isolates in order to identify potential pathogenicity factors, **iv)** to select and characterize *V. dahliae* genes that are potentially involved in pathogenesis based on integration of results from transcriptomics and proteomics analyses, and **v)** to complement these studies with a screening and profiling of secondary metabolites in potato in response to these *V. dahliae* isolates.

2.0 LITERATURE REVIEW

2.1 The Host, Potato, *Solanum tuberosum* L.

2.1.1 Origin and classification

Potato, *Solanum tuberosum* L., is an important food crop worldwide. The origin of potato is the Andes, South America. It was introduced by the Spanish to Europe and from there, it was distributed to the other parts of the world (Brown, 1993). Potato is an annual dicotyledonous plant that belonging to the Family, *Solanaceae,* Genus: *Solanum*. The latter contains about 1000 species; *S. tuberosum* is the cultivated potato species (Hawkes, 1992).

2.1.2 Geographical distribution

Potatoes are grown in two major zones, the temperate climate zone and the subtropical lowlands, where it is considered as a summer or winter crop, respectively (Hijmans, 2001). This wide distribution of the potato crop makes it a staple food for highly populated areas worldwide. Wild potato species are reportedly found in 16 countries, and concentrated in Argentina, Bolivia, Mexico, and Peru (Hijmans and Spooner, 2001).

2.1.3 Global production

The global production of potato is around 320 million tonnes. The three top producing countries of potato are China, the Russian Federation and India, according to Food and Agriculture Organization (FAO) statistics (http://faostat.fao.org/site/339/default.aspx, Accessed April, 22, 2010). The year 2008 was declared by the FAO as the

international year of the potato, (http://www.potato2008.org/en/index.html, Accessed April, 22, 2010), which reflected the importance of the potato as a worldwide food crop.

2.1.4 Production in Canada and Manitoba

Canada is the 13th country among the top potato producers worldwide, according to FAO statistics (http://faostat.fao.org/site/339/default.aspx, Accessed April, 22, 2010). The total production of potato in Canada was about 5 million tonnes per year for the period 2006-2009. The two major provinces for potato production are Prince Edward Island and Manitoba, with about 50% of the total production. In 2009, the potato production in Manitoba was 1,008,353 tonnes and the average yield was 31.94 tonnes/ hectare, which is similar to the production in 2006-2008, Statistics Canada (http://www.statcan. gc.ca/pub/22-008-x/22-008-x2010001-eng.pdf, Catalogue no. 22-008-X).

2.1.5 Potato diseases

Potato is the host of the most famous and disastrous disease in the history of plant pathology, late blight caused by the Oomycete *Phytophthora infestans* (Mont.) de Bary, which was the reason for the well-known Irish potato famine in the 1840s (Agrios, 2005). Potato is challenged by many biotic and abiotic diseases. These include fungal diseases [e.g. Verticillium wilt caused by *Verticillium dahliae* Kleb.; early blight caused by *Alternaria solani* (Ellis & G. Martin) L.R. Jones and Grout], bacterial diseases [brown rot caused by *Ralstonia solanacearum* (Smith) Yabuuchi et al.); common scab caused by *Streptomyces scabies* (Thaxt.) Lambert and Loria], viral diseases [potato leaf roll virus;

potato virus x], nematodes [potato cyst nematode, *Globodera rostochiensis* (Wollenweber) Behrens], and abiotic diseases such as black spot (Wale et al. 2008). It seems that the high nutritional value of potato makes it an attractive host for so many microorganisms.

Twenty two diseases were diagnosed on potato in 2009 in Manitoba, Canada. These diseases were diagnosed in foliar and subterranean parts of infected potato. The causal agents were fungal, bacterial and physiological disorders (Desjardins 2010). One of the important diagnosed diseases was potato Verticillium wilt caused by the soilborne fungus *V. dahliae*.

2.2 The Pathogen, *Verticillium dahliae* Kleb.

2.2.1 Nomenclature and classification

The genus *Verticillium* was described and named in 1816 by Nees von Essenbeck based on the features of its conidiophores branching in whorls (Isaac 1976). The *Verticillium* contains many species that can affect a wide range of organisms including plants, insects, nematodes, spiders and other fungi (Bidochka et al. 1999). The genus of *Verticillium* is classified (Fradin and Thomma, 2006) as follows:

Kingdom: Fungi

Phylum: Ascomycota

Subphylum: Pezizomycotina

Class: Sordariomycetes

Order: Phyllachorales

Genus: *Verticillium*

There are six plant pathogenic *Verticillium* species; *V. dahliae*, *V. albo-atrum*, *V. nigrescens*, *V. nubilum*, *V. tricorpus* and *V. theobromae*. The two major plant wilt pathogens are *V. dahliae* and *V. albo-atrum* (Barbara and Clewes, 2003). *Verticillium dahliae* was described as a distinct species by Klebahn in 1913 after it was isolated from infected dahlia plants (Isaac 1976). The sexual (teleomorph) state of *V. dahliae* is still unknown, and thus it is classified as a Deuteromycete (Pegg and Brady 2002; Klosterman et al. 2009). However, based on genome sequence analysis, the Broad Institute of Massachusetts Institute of Technology (MIT) and Harvard, Fungal Genome Initiative has classified *V. dahliae* as a member of Ascomycota (http://www.broadinstitute.org/ science/projects/fungal-genome-initiative/ fungal-genome-initiative).

2.2.2 Host range and global distribution

The host range of the *Verticillium* is very broad among plant species. It can infect hundreds of plant species including herbaceous annuals, perennials, and woody plants (Pegg and Brady 2002). *Verticillium dahliae* hosts are mainly dicotyledonous plants (Klosterman et al. 2009). However, *V. dahliae* microsclerotia, resting structures, were observed in the roots of gramineous plants, and could be a way of pathogen survival if a favorable host is absent (Krikun and Bernier 1990).

Verticillium wilt is distributed worldwide and several studies have been conducted on the population structure and vegetative compatibility of *V. dahliae* isolates from many geographical regions and countries such as; Canada, (Uppal et al. 2007); the USA, (Vallad et al. 2006); European countries, (Rataj-Guranowska 2006), and Japan (Wakatabe et al. 1997). In the USA, Atallah et al. (2010) detected genetic recombinations

and transcontinental gene flow in *V. dahliae* isolates using population genetic analyses and suggested that this gene flow could be a reason for Verticillium wilt outbreaks on crops in some areas.

Hiemstra and Rataj-Guranowska (2003) investigated and compared isolates of *V. dahliae* from the Netherlands with tester isolates from Europe and the USA and indicated that there are bridge isolates that could facilitate the continuum of *V. dahliae* genetic variations and explain the worldwide distribution of the disease.

2.2.3 Morphological and molecular characteristics

Verticillium dahliae reproduces asexually by forming conidiospores produced on conidiophores. The conidiophores are branched to phialides in groups along the main branch of the conidiophore. The most important morphological feature of *V. dahliae* is the production of microsclerotia as survival structures, while *V. albo-atrum* survives as melanized hyphae (Klosterman et al. 2009).

The genome size of *V. dahliae* has been investigated by Pantou and Typas (2005) using a pulsed field gel electrophoresis technique. Their studies showed that *V. dahliae* has seven chromosomes and a genome approximately 28.4 Mb in size. The sizes of the chromosomes I, II, III, IV, V, VI, and VII were 2.4, 3.1, 3.1, 3.4, 4.1, 5.6 and 6.7 Mb, respectively, and the total genomic size ranged from 26.6 to 29.1 Mb among the different *V. dahliae* tested isolates. The complete sequence of the mitochondrial genome of *V. dahliae* has been published (Pantou et al. 2006) and the genome sequence has been released by the Broad Institute of MIT and Harvard. The statistics of the *V. dahliae*

isolate VdLs.17 genome showed 8 chromosomes with a genome size of 33.83 Mb (http://www.broadinstitute.org/annotation/genome/verticillium_dahliae/MultiHome.html)

Molecular identification of *V. dahliae* has been investigated in several studies. Nazar et al. (1991) designed and used specific PCR primers to detect, identify and differentiate between *V. dahliae* and *V. albo-atrum* isolates based on small differences in the nucleotide sequence of the internal transcribed spacers (ITS 1 and 2) of the ribosomal genes. Two other primers, Vda1 and Vda2, were designed based on the sequence of the ITS1 region to identify *V. dahliae* in soil and infected tomato tissues (Lievens et al. 2003). In another study, *V. dahliae* was successfully identified in DNA extracted from soil samples using PCR with *V. dahliae* specific primers (Volossiouk et al. 1995). Li et al. (1999) found that a DNA fragment of 567 bp was produced using E20 RAPD primer only in *V. dahliae* isolates and was not found in *V. albo-atrum* and *V. tricorpus* isolates. Based on the sequence of this DNA specific fragment, two specific PCR primers VDS1 and VDS2 were designed and tested for 62 *V. dahliae* isolates from different locations and hosts. The amplified fragment was specific to all *V. dahliae* isolates and was absent in other related fungi.

2.2.4 Survival structures

Verticillium dahliae produces microsclerotia as survival structures. Microsclerotia are a melanized thick-walled mass of cells with globular to spherical shape. Their sizes vary among isolates but are generally 40 to more than 100 μm in diameter (Pegg and Brady 2002). The structure of *V. dahliae* microsclerotia was described in detail using transmission (Nadakavukaren, 1963; Griffiths, 1970) and scanning electron microscopes

(Brown and Wyllie 1970). Microsclerotia can stay viable in the soil as a source of inoculum for more than 10 years (Wilhelm 1955), which is one of the obstacles to managing Verticillium wilt. Plant root exudates stimulate the germination of *V. dahliae* microsclerotia (Schreiber and Green 1963) as an initial step of the infection process and an early interaction between the host plant and *V. dahliae*, even before physical contact occurs.

2.2.5 Vegetative compatibility groups

Vegetative compatibility of plant pathogenic fungi, e.g. *V. dahliae*, is the ability of given isolates to form heterokaryons through the anastomosis process (Leslie 1993). Vegetative compatibility is considered a source of genetic diversity in *V. dahliae* and contributes to many features of the pathogen such as pathogenicity, population structure, and host range (Collado-Romero et al. 2008). To identify the vegetative compatibility groups (VCGs) of *V. dahliae*, specific nitrate non-utilizing (*nit*) mutants are used (Joaquim and Rowe 1990). Recently, Collado-Romero et al. (2009) developed a multiplex-nested PCR-based procedure to detect and identify *V. dahliae* VCGs *in planta* by using specific PCR primers that produce differential PCR marker bands of 334, 688, and 964 bp. Based on absence/presence of these marker bands in a specific manner, VCGs of *V. dahliae* can be detected as VCG1, VCG2, VCG3, VCG4, and VCG6 groups with VCG1A, VCG1B, VCG2A, VCG2B, VCG4A, and VCG4B subgroups.

The majority of *V. dahliae* potato isolates belong to group VCG4; either the VCG4A or VCG4B subgroups. The isolates of subgroup VCG4A were highly virulent as compared to groups VCG4B and VCG2 (Joaquim and Rowe 1991). VCGs of *V. dahliae*

were investigated extensively with isolates from many other hosts such as cotton (Daayf et al. 1995), artichoke and other vegetables (Berbegal et al. 2010), chrysanthemum (Göre 2009), and olive trees (Bellahcene et al. 2005).

Genetic diversity of *V. dahliae* is well known and it might be the reason behind the wide host range of this pathogen. Recently, Collado-Romero et al. (2010) conducted phylogenetic analyses of DNA sequences of some conserved genes of *V. dahliae* isolates representing several geographic regions, VCGs, and hosts. The results suggested that hybridization events between isolates of *V. dahliae* VCG1 and/or VCG4A may have led to the appearance of *V. dahliae* var. *longisporum*.

2.2.6 *Verticillium dahliae* pathogenicity determinants

2.2.6.1 Infectious structures

Production of infectious structures is a principal component that is required for pathogens to attack host plants and establish a successful infection (Mendgen and Deising 1993). There are several types of infectious structures which vary from pathogen to pathogen. They could be sexual spores, asexual spores, hyphal fragments, and resting structures or other structures (Agrios 2005).

Microsclerotia of *V. dahliae* in the soil are the inoculum for initial infection of plant roots. The fungus then colonizes the root cortex. *Verticillium dahliae* produces conidiospores which move upward in the xylem vessels of the plant vascular system and infect new vessels (Fradin and Thomma, 2006). It has been reported that the infection of cotton plants with *Verticillium* was increased with increasing microsclerotial density in

the soil (Ashworth et al. 1972) and the same relationship was reported in the *V. dahliae*-tomato pathosystem (Grogan et al. 1979).

A high level of conidiospore production was associated with a highly virulent *Verticillium* strain on cotton as compared with a weakly virulent one (Schnathorst 1963). The infection and disease severity of *V. dahliae* on tomato plants was linear with the conidiospores concentration at the inoculation time (Visser and Hattingh 1981). Xiao and Subbarao (1998) studied the relationship between the *V. dahliae* inoculum density and Verticillium wilt disease severity on cauliflower in field experiments. They found that 4 and 10 microsclerotia/ g soil caused 16% and 50% wilt incidence, respectively, and more than 20 microsclerotia/ g soil did not result in increased Verticillium wilt disease.

2.2.6.2 Production of cell-wall degrading enzymes

Cell-wall degrading enzymes (CWDEs) have been investigated intensively in the genus *Verticillium*. Cellulases, polygalacturonases, xylanase, and proteases are assumed to play an important role in *Verticillium* sp. pathogenicity (Bidochka et al. 1999). Cell-wall degrading enzymes are one set of the pathogenicity determinants for *V. dahliae* (Pegg and Brady 2002).

Cellulase activity of some *V. dahliae* isolates with different degrees of aggressiveness was examined by Novo et al. (2006). Their results indicated that the two *V. dahliae* isolates, weakly and highly aggressive, were able to degrade the soluble cellulose. However, the activity of cellulose degrading enzymes varied according to the degree of aggressiveness of the isolate. The highly aggressive isolate showed β-1, 4-glucosidase and endo-β-1, 4-glucosidase activity higher than the weak isolate. These

findings may indicate the role of cellulases in the penetration of roots of host plants by *V. dahliae*.

Polygalacturonase isozyme activity has been detected in 11 *V. dahliae* isolates from North America, Australia, and Europe. The expression of the isozymes was not the same in 11 isolates studied by Durrands et al. (1988), and had no correlation with the isolates' pathogenicity or origin. An endopolygalacturonase with an approximate MW of 29.5 kDa has been isolated and purified from *V. dahliae* culture filtrate and the production of this enzyme was inhibited using a polygalacturonase inhibitor protein purified from salicylic acid-induced cotton (James and Dubery 2001). In *V. albo-atrum*, an endopolygalacturonase enzyme has been isolated and purified from the culture filtrates with a MW of 37 kDa. Endopolygalacturonase hydrolyzes pectin, one of the plant cell wall constituents, which suggests a role in *V. albo-atrum* pathogenesis (Huang and Mahoney, 1999)

2.2.6.3 Production of potential phytotoxins and elicitors

It has been reported in several studies that *V. dahliae* produces potential phytotoxic complexes. Buchner et al. (1982) isolated and partially characterized a glycopeptide from a phytotoxic protein-lipopolysaccharaide complex produced by a pathogenic potato isolate of *V. dahliae* and reported that the phytotoxic activity of the complex arises from the glycopeptide portion. Also, a phytotoxicity effect of a peptide isolated from the filtrate of *V. dahliae* culture has been reported on tomato, however, the latter was harboring a (*Ve)* gene for *Verticillium* resistance. In potato tissue culture, 5 µg/ml of *V. dahliae* toxin decreased the viability of a potato cell suspension of a

susceptible cultivar as compared to the cell suspension of a tolerant one (Nachmias et al. 1987; 1990). In another investigation, Meyer et al. (1994) reported that *V. dahliae* produced a phytotoxic protein-lipopolysaccharaide complex in 7-day-old culture filtrates. Activity of polygalacturonase, cellulase, and 1, 3-β-glucanase was detected in that phytotoxic complex. After immersion of cotton seedlings in a solution of 2.5 μg/ml of the complex, wilting and necrosis symptoms were observed and elicitation of phenylalanine ammonia-lyase and pathogenesis-related (PR) proteins were determined in the treated cotton seedlings.

Buchner et al. (1989) compared the characteristics of purified peptides from *V. dahliae* culture filtrates with those purified from the xylem sap of *V. dahliae*-infected potato stem, and reported that both peptides were similar in terms of their chemical and biological properties. Both peptides induced wilt and necrosis symptoms on potato detached leaves at a very low concentration, 20 ng. Also, using an immunofluorescence assay, they detected the *V. dahliae* toxin in the stem of infected potato even before the detection of the fungus itself, which indicates that the fungus may produce this toxin to prepare the internal microenvironment for colonization.

Several studies have investigated the mode of action of *V. dahliae* toxins at the cellular level of the host plant. Using protein radio-labeling and binding assay procedures, Meyer and Dubery (1993) reported that a protein-lipopolysaccharaide phytotoxin complex purified from the culture filtrates of a virulent isolate of *V. dahliae* had a high-affinity binding to the plasma membrane of cotton seedlings. In another study, the *V. dahliae* toxin was purified from 21-day-old culture filtrates and applied to cuttings from cotton root and leaf tissues. Immediately after treatment, losses of electrolytes

occurred. It was suspected that the *V. dahliae* toxin affects the K^+ and Na^+ ion transport systems in the cell plasma membrane, while Ca^{2+} ions were not affected (Gour and Dube 1985).

At the ultrastructural level of the host plant, it has been reported that treatments of cell suspensions with low concentrations of toxins from *V. dahliae* culture filtrate showed damage in the plasma membrane and cytoplasmic components of cotton cells. In *Arabidopsis thaliana*, a toxin from *V. dahliae* altered the cytoskeletons, microfilaments and microtubules, and nucleoli structures of cells, as demonstrated by transmission electron and confocal laser scanning microscopes (Zhen and Li 2004; Yuan et al. 2006).

Qi et al. (2007) found that nitric oxide (NO) and hydrogen peroxide (H_2O_2) and plant defense signaling molecules were induced in cotton seedlings after treatment with a toxin purified from *V. dahliae* culture filtrate. In another study, Davis et al. (1998) reported that a purified glycoprotein elicitor from *V. dahliae* culture fluids activated phytoalexin biosynthesis in cotton cell suspension cultures. The results from Qi et al. (2007) and Davis et al. (1998) are in agreement with the proposed wilt-inducing/defense-eliciting dual functions of VdNEP (Necrosis- and Ethylene-inducing Protein) protein that was purified from the filtrates of a highly aggressive *V. dahliae* isolate from potato (Yao et al. 2010). The purified VdNEP induced wilt symptoms and up-regulated expression of genes in plant defense signaling pathways in sunflower (Yao et al. 2010). VdNEP was initially purified from the culture filtrates of a cotton *V. dahliae* isolate and identified as an elicitor and cotton wilt-inducing factor (Wang et al. 2004).

2.2.6.4 Signaling in *V. dahliae* pathogenicity

Neumann and Dobinson (2003) constructed two cDNA libraries of *V. dahliae* grown in simulated fluid xylem medium or basal agar medium supplemented with some nutrients to induce near-synchronous microsclerotial production. From both libraries, over 2000 expressed sequence tags (ESTs) were detected. The basic local alignment search tool (BLAST) searches in the National Centre for Biotechnology Information (NCBI) database showed homology of some of these ESTs to genes involved in melanin biosynthesis, microsclerotial development, hydrolysis enzymes, and growth of fungi. One of the identified ESTs in *V. dahliae* was a hydrophobin gene (*VDH1*). Using the *Agrobacterium*-mediated transformation gene disruption method, Klimes and Dobinson (2006) and Klimes et al. (2008) have confirmed the critical role of *VDH1* in microsclerotial production and conidiospore vitality of the fungus. The expression of *VDH1* is regulated by the carbon availability in the growth medium, while the limitation of nitrogen does not affect *VDH1* expression.

The role of trypsin protease (*VTP1*) and glyoxalase I (*VdGLOI*) genes have been also studied in *V. dahliae*. The pathogenicity and *in vitro* growth of the *VTP1*-disrupted *V. dahliae* isolate were not affected (Dobinson et al. 2004). In the case of the *VdGLOI* gene, which is involved in methylglyoxal detoxification, the sensitivity to methylglyoxal, mycelial growth and sporulation were affected noticeably in the *V. dahliae VdGLOI* mutant, whereas the pathogenicity was not influenced (Klimes et al. 2006).

Other studies investigated the role of some *V. dahliae* signaling genes in pathogenesis. Rauyaree et al. (2005) reported that the disruption of the signaling pathway *VMK1* [*Verticillium* mitogen-activated protein (MAP) kinase 1] gene in two *V. dahliae* isolates using an *Agrobacterium*-mediated transformation technique resulted in reduction

of virulence, growth, conidiospore production and microsclerotia formation. In *Fusarium oxysporum*, another vascular wilt fungus, *FMK1*, MAP kinase gene was identified as an essential component for successful infection (Di Pietro et al. 2001). Delgado-Jarana et al. (2005) disrupted the *Fgb1* gene, an upstream component of the *FMK1* signaling pathway, and reported that it is important in growth, development and virulence of *F. oxysporum*.

Verticillium dahliae has two cAMP-dependent protein kinase A (PKA) catalytic subunit genes, *VdPKAC1* and *VdPKAC2*, and the latter one was shown to play a minor role in PKA activity. The disruption of the *VdPKAC1* gene reduced the wilt disease severity, ethylene biosynthesis, conidiospore production, and microsclerotial formation, which verified its important role in the pathogenicity signaling pathways of *V. dahliae* (Tzima et al. 2010). Recently, the phylogenetic analyses of the genomes of *V. dahliae* and *V. albo-atrum* showed identification and classification of 13 classes of important signaling proteins, G protein-coupled receptors, that are critical components for regulating morphogenesis, mating, infection, and virulence in fungi (Zheng et al. 2010).

Pathogenesis mechanisms of *V. dahliae* at the molecular level are still not fully understood. *V. dahliae* is a hemibiotrophic fungus, survives in the soil environment for more than a decade, is a plant vascular (another environment) habitant, is distributed worldwide, and has a broad range of genetic variability and host plants. All of the earlier mentioned factors bring up the necessity to build knowledge about the pathogen's functional genomics, proteomics and metabolomics, and integrate such information to better understand the molecular basis of *V. dahliae* pathogenesis, which could contribute to improved Verticillium wilt management strategies.

2.3 *Verticillium dahliae-Solanum tuberosum* interactions

2.3.1 Disease cycle and symptoms

Verticillium wilt of potato is primarily caused by the soilborne fungus *V. dahliae*. The disease affects a broad range of plant hosts (Pegg and Brady, 2002). Microsclerotia are the primary inoculum of *V. dahliae* in the soil (Wilhelm 1955). After stimulation by plant root exudates, the microsclerotia germinate and develop hyphae that penetrate the potato root through root tips and wounds, and colonize the root cortex. The penetration and colonization of potato roots usually occur in the undifferentiated tissues in the elongation zones of root tips. The hyphae grow and concentrate around the vascular cylinder of the root and enter the xylem tissue through pits in the cell wall (Bowers et al. 1996). Colonization of root cortex and vessels may differ among hosts and even cultivars from the same host plant (Bhat and Subbarao 1999). Once the hyphae reach the xylem tissue of the vascular system, asexual reproduction of *V. dahliae* takes place, leading to the formation of conidiospores that are able to move upward, germinate, and penetrate new xylem vessels. The potato vascular system becomes plugged by the fungus and some host defense reactions response to infection. Water is prevented from reaching the aerial parts of the plant and the disease symptoms start to develop. At the end of the disease cycle, the fungus forms microsclerotia in the dead parts of the plant, which return to the soil as a source of infection for other host plants for more than 10 years (Powelson and Rowe 1993; Klosterman et al. 2009).

The disease symptoms can be classified into two types; external and internal symptoms. The external symptoms appear in the form of chlorosis and necrosis of the foliage; sometimes the chlorosis and necrosis start on one side of the symptomatic leaves.

Wilting and stunting are the final external symptoms of the infected plant. The internal symptom is a discoloration of the vascular system (xylem tissue) as a brown ring in the infected root, stem and tuber. The yield of the infected plant is significantly reduced (Pegg and Brady 2002; Uppal et al. 2007; Alkher et al. 2009)

2.3.2 Economic significance

Verticillium wilt disease causes economic losses in potato production quantitatively and qualitatively (Powelson and Rowe 1993; Fradin and Thomma 2006). Prediction models for potato yield loss due to *V. dahliae* alone or in the presence of *P. penetrans* were developed and potato yield loss was predicted as a function of *V. dahliae* and *P. penetrans* densities in soil using linear regression models (Francel et al. 1987). Another study considered the yield loss as a function only to *V. dahliae* concentration in soil with or without *P. penetrans* (Wheeler et al. 1992). The effect of *V. dahliae* infection rate and temperature was studied in relation to leaf area and tuber yields (Johnson 1988). The losses in potato yield can reach to 30-50 % (Rowe and Powelson 2002).

In Canada, *V. dahliae* was diagnosed on potato plants in the two major potato producing provinces, Prince Edward Island (Clark 2010) and Manitoba (Desjardins 2010), in addition to British Columbia (Joshi and Jeffries 2010). In Manitoba, Verticillium wilt is a serious disease, especially because it was diagnosed on the sunflower crops surveyed in 2009 (Rashid and Desjardins 2010).

2.3.3 Early dying syndrome

Potato early dying syndrome is caused mainly by *V. dahliae* in association with infection by the nematode *Pratylenchus penetrans*. In a field study, Martin et al. (1982) found that several combinations of *V. dahliae* and *P. penetrans* inocula densities caused a significant reduction in potato growth associated with early dying severity. There are other microorganisms that contribute to early dying syndrome, such as *Colletotrichum coccodes* (Johnson and Miliczky 1993).

Kotcon and Rouse (1984) used a split-root culture system to study the effects of co-inoculation of potato with *V. dahliae* and other early dying associated pathogens and concluded that *V. dahliae* is the primary pathogen in the early dying syndrome. However, other pathogens may contribute to the disease either directly by degradation of root tissues or indirectly by changing the physiology of potato plants and increasing its susceptibility to *V. dahliae*. Scrutinizing the crop rotation and evaluating soil inocula can be used for prediction of early dying syndrome in a specific area (Rouse 1985). Powelson and Rowe (1993) provided a detailed explanation of the biology, effects of environmental conditions and potential management strategies of potato early dying syndrome.

2.3.4 Mechanisms of host defense

The majority of studies in plant diseases are focused on the host side rather the pathogen side. Host defenses against pathogens could be constitutive and/or induced (Agrios 2005). There are several molecules that are responsible for signaling plant defense responses to pathogen attack. These include reactive oxygen species (ROS) such as nitric oxide (NO) and hydrogen peroxide (H_2O_2), and plant hormones such as salicylic acid (SA), jasmonic acid (JA), ethylene (ET), and abscisic acid (ABA). Plant defense

signaling molecules trigger and activate many defense mechanisms (e.g., phytoalexins and pathogenesis-related proteins) to resist pathogen infection (Hammond-Kosack and Jones 1996). The interactions among the signaling molecules in plant defense is a very complex process (Kunkel and Brooks 2002), and is not largely investigated in soilborne diseases, especially in Verticillium wilt (Shi et al. 2009).

Ratzinger et al. (2009) detected an increase in SA and SA glucoside concentrations in xylem sap of the *Brassica napus* root, hypocotyl and extracts of shoots above hypocotyl after infection with *V. longisporum*, whereas JA and ABA concentrations did not change. This suggests a potential role of SA and its glucoside in *B. napus- V. longisporum* interactions. Recently, Pantelides et al. (2010) investigated the role of ET signaling using the *A. thaliana* ethylene receptor mutant (*etr1-1*) in *A. thaliana- V. dahliae* interactions. They found that the impaired perception of ET in *A. thaliana via* ETR1 results in activation of resistance. Up-regulation of several PR genes was noticed as well during the interaction, which means that a group of defense genes were activated after infection.

Nitric oxide signal molecule was reported to be involved in signaling defense responses in the root of *A. thaliana* in response to *V. dahliae* toxin treatment. The proposed role is that NO triggers depolymerization of the root cortical microtubule and the latter is working as a mediator for the activation of the PR-1 defense gene (Shi et al. 2009).

The cotton-*V. dahliae* pathosystem was extensively studied to uncover the mechanisms of defense mechanisms against *V. dahliae*. Using histochemical procedures, Mace (1983) detected an accumulation of terpenoid aldehyde phytoalexins around

colonized xylem vessel of infected cotton. Moreover, Mace et al. (1989) reported that desoxyhemigossypol, a terpenoid aldehyde derivative, was localized in the parenchyma of the xylem of infected cotton and on *V. dahliae* mycelium during the interactions.

Daayf et al (1997) described ultrastructural modifications of the xylem parenchyma of *V. dahliae*-resistant and susceptible cotton lines upon infection. The modifications consisted of the deposition of callose and cellulose in addition to the high production of terpenoids and phenolic compounds. The detected defense reactions were observed in the resistant line earlier than the susceptible one, which suggests their essential role in *V. dahliae* resistance. Smit and Dubery (1997) studied the responses of cotton cultivars to a protein-lipopolysaccharide elicitor from *V. dahliae.* They found an increase in lignin and lignin-like phenolics' deposition, and the activity of phenylalanine ammonia-lyase, cinnamyl alcohol dehydrogenase and peroxidase, as defense reactions in cotton hypocotyls. Such reactions were stronger and earlier in a *V. dahliae*-resistant cultivar than in a susceptible one. Hemigossypol, desoxyhemigossypol induction and δ-cadinene synthase, a key enzyme in terpenoids biosynthesis, high activity were noticed in cotton after infection with *V. dahliae* (Bianchini et al. 1999).

Pathogenesis-related (PR) proteins are induced in host plants upon infection. PR proteins contribute to host resistance against a wide range of pathogens. PR protein families include many proteins such as chitinases, peroxidases, glucanases and anti-fungal proteins. PRs have been detected in several plant families (van Loon et al. 2006). An accumulation of PRs, with increasing chitinase activity, was noticed in cotton after inoculation with *V. dahliae*. The accumulation was enhanced when the cotton plants were inoculated with both *V. dahliae* and vesicular-arbuscular mycorrhizal fungi (Liu et al.

1995). Accumulation of PR proteins, chitinases and 1, 3-β-glucanases was associated with phytoalexin production in cotton leaf tissues exposed to cell wall fragments from *V. dahliae* (Dubery and Slater 1997). Recently, chitinase, catalase, and three isoforms of 1, 3-β-glucanase were identified in leaves of *V. dahliae*-infected cotton (Maksimov et al. 2009)

2.3.5 Disease management

Management of Verticillium wilt is a challenge because of the persistent nature of the primary inoculum source (microsclerotia in soil), the fungus habitats and its interactions with a wide range of hosts and other microorganisms. However, there are several potential strategies to diminish the effect of Verticillium wilt disease. These include:

2.3.5.1 Cultural practices

Generally, optimal cultural practices such as crop rotation, fertilization, and irrigation promote plant health and decrease the chances of disease occurrence. Crop rotation is one of the most eco-friendly strategies to manage Verticillium wilt on vegetables. Using broccoli residues as a soil amendment resulted in a reduced pathogen population, viability of microsclerotia, root colonization and disease severity of *V. dahliae* on cauliflower and broccoli crops (Shetty et al. 2000). The effectiveness of broccoli rotation for Verticillium wilt management on strawberry has been recently confirmed in conventional and organic agricultural systems (Njoroge et al. 2009).

The soil solarization procedures may be useful in the pasteurization of infested soils with soilborne plant pathogens. It is based on using plastic sheets for covering the soil during the summer months to capture the sun's heat that can kill the pathogen in the top soil (Pullman et al. 1981). The soil solarization method has been used effectively in warm climate regions and recommended for controlling *V. dahliae* under greenhouse and open field conditions (Bourbos and Skoudridakis, 1996; Berbegal et al. 2008).

Using environmentally safe products such as liquid swine manure was shown to kill *V. dahliae* microsclerotia and the nematode *P. penetrans*. The volatile fatty acids, nitrous acid, and ammonia in liquid swine manure were proposed as the active compounds that affect the causal agents of Verticillium wilt (Tenuta et al. 2002; Conn et al. 2005; Mahran et al. 2008).

2.3.5.2 Chemical control

Soil fumigation is one of the methods to control soilborne plant pathogens in general and Verticillium wilt in particular (Miller et al. 1967). Methyl bromide, aldicarb, and benomyl soil fumigants were used to control *V. dahliae* and the nematode *Heterodera rostochiensis* in potato fields (Hide and Corbett 1974). A lignin derivative, ammonium lignosulfonate, as a soil amendment treatment, demonstrated an efficacy in reducing Verticillium wilt and common scab of potato in fields (Soltani et al. 2002). Using chemicals for soil treatment to control soilborne diseases is a well known strategy. However, there are many health and environmental safety issues with this strategy, such as soil and ground water contamination.

2.3.5.3 Biological control

Biological agents can be used for the management of Verticillium wilt with consideration of field conditions. Uppal et al. (2008) examined soil amendments and seed coating procedures with bacterial agents and plant extracts to manage *V. dahliae* on potato under greenhouse and field conditions. Their results showed that an isolate of *Pseudomonas fluorescens* and Canada milkvetch plant extract were effective in reducing Verticillium wilt. In another study, Berg et al. (2001) used *Pseudomonas putida*, *Pseudomonas chlororaphis* and *Serratia plymuthica* isolated from the rhizosphere of strawberry, potato, and oilseed rape, respectively, as biocontrol agents against *V. dahliae*. The fungus *Trichoderma virens* has been suggested for controlling Verticillium wilt of cotton by seed treatment (Hanson 2000). The suggested roles of these biological agents included enhancing systemic acquired resistance and promoting the growth of cotton (Hanson 2000).

2.3.5.4 Host resistance

Host resistance against Verticillium wilt has been proposed in few crops. In tomato, Diwan et al. (1999) mapped a *Verticillium* resistance gene (*Ve*) and reported its position on chromosome 9. The molecular characterization of tomato *Ve* genes showed that they encode for cell-surface like receptors. The *Ve1* gene, and not *Ve2*, was confirmed to play a role in defense signaling for *Verticillium* resistance (Kawchuk, et al. 2001; Fradin et al. 2009). Similarly, a *V. dahliae*-tolerance (*VET1*) gene was identified in the model plant *Arabidopsis thaliana* (Veronese et al. 2003). In potato, resistance to Verticillium wilt is proposed through the *StVe1* resistance gene (Simko et al. 2004).

Although there are several molecular genetic studies regarding *V. dahliae* resistance genes, no completely resistant potato cultivar is commercially available, but partially resistant or tolerant ones can be found.

2.3.5.5 Integrated disease management

The main factors for successful Verticillium wilt management include reducing the microsclerotial level in the infected soils and enhancing the plant resistance to vascular infection. These could be achieved by applying an integrated disease management program that considers the best combination of disease control elements, in the right time, crop and climatic conditions (Powelson and Rowe 1993; Johnson and Dung 2010). The application of a combination of cauliflower residue amendments, low concentrations of metham sodium, and soil solarization helped for Verticillium wilt management on artichoke (Berbegal et al. 2008). Development of Verticillium wilt integrated disease management programs should be based on a good understanding of the host defense mechanisms, fungal pathogenesis, and the interactions between both with consideration to the effects of environmental conditions.

[*]3.0 ESTABLISHMENT OF A DIFFERENTIAL POTATO-*VERTICILLIUM DAHLIAE* PATHOSYSTEM

3.1 Abstract

Verticillium wilt disease affects a broad range of economic crops, including potato, in several regions of the world. In the present chapter, out of 60 pre-screened *V. dahliae* isolates, a pathogenicity evaluation was carried out for four selected *V. dahliae* isolates (Vd1396-9, Vs04-28, Vs06-13, and Vs06-14) on two potato cultivars (Kennebec; susceptible and Ranger Russet; moderately resistant) in order to confirm their aggressiveness and establish a differential model pathosystem of potato-*V. dahliae*. Microsclerotia were observed microscopically and their size ranged from 40 to 50 µm in diameter. Mycelial growth and production of conidiospores were assessed for the selected *V. dahliae* isolates. There was no difference in the growth. However, the conidiospore count of Vd1396-9 was significantly higher than that of Vs06-14 isolate. Four-week-old potato seedlings were inoculated by root dipping in a 10^6 conidiospores/ml suspension. Disease severity, as measured by evaluating the external and internal symptoms based on a pre-established Verticillium wilt symptoms' scale, was recorded weekly from 2 to 7 weeks after inoculation (w.a.i.). Vascular discoloration and host growth measurements were analyzed at 7 w.a.i. Increase in disease severity was observed over time for all isolates, with higher levels on cultivar Kennebec. Vascular discoloration was higher in

[*] **This chapter *(the data of V. dahliae Vs04-28 and Vs06-13 isolates were included here)* is part of the pre-peer reviewed version of the following article:**
El-Bebany, A. F., Rampitsch, C., and Daayf, F. 2010. Proteomic analysis of the phytopathogenic soilborne fungus *Verticillium dahliae* reveals differential protein expression in isolates that differ in aggressiveness. Proteomics 10: 289-303. DOI 10.1002/pmic.200900426

the lower stem sections as compared with middle and higher ones in Kennebec. However, no vascular discoloration was seen in the higher sections of Ranger Russet. Growth of the inoculated plants was significantly more affected in Kennebec than in Ranger Russet. We confirmed that Vd1396-9 and Vs06-14 had the highest and lowest levels of aggressiveness, respectively. Vd1396-9 and Vs06-14 isolates were chosen for further investigation of potential pathogenicity factors using transcriptomics and proteomics approaches.

3.2 Introduction

Verticillium dahliae Kleb. is a soilborne pathogen and one of the causal agents of vascular wilt on hundreds of plant species worldwide (Pegg and Brady 2002). *Verticillium dahliae* is a deuteromycete with high variability in aggressiveness towards its hosts. It causes both external (leaf chlorosis, necrosis) and internal (vascular discoloration) symptoms on the infected plants. The characteristic leaf wilting symptoms are often associated with stunting and eventually result in reduction of yield and quality (Rowe et al. 1987). *Verticillium dahliae* forms microsclerotia as survival structures that can stay viable in the soil for more than 10 years (Wilhelm 1955). Germination of microsclerotia is stimulated by plant root exudates released in the soil (Mol 1995). The germinated hyphae penetrate host roots, colonize the cortex, and enter xylem vessels. In the vascular system, *V. dahliae* reproduces asexually by conidiospores, which move upward, germinate, and infect new xylem vessels. Hyphae and plant responses to infection eventually plug the vessels and prevent water from reaching the aerial parts of the plant, resulting in the characteristic wilt symptoms. The fungus forms microsclerotia

on dead or dying plant tissues. A detailed explanation of *V. dahliae* diversity, taxonomic controversy of *Verticillium* spp. and new insights on Verticillium wilt disease cycle has recently been published (Klosterman et al. 2009). Due to the persistent nature of *V. dahliae*, its control requires integrated disease management programs (Fradin and Thomma 2006). However, in the absence of complete host resistance, the success of Verticillium wilt control will also rely on a better understanding of this host-pathogen interaction. The pathogenicity factors of *V. dahliae* compose an important component of the latter, because they are potential targets for future control methods. Unfortunately, their structure(s) as well as their mechanisms of action are not fully established. Once a pathogen is recognized by its host, a cascade of signaling reactions controlled by enzymes in several pathways are activated, leading to defense responses and, ultimately, to resistance (Hammond-Kosack and Jones 1996).

Pathogenic variability and aggressiveness of *V. dahliae* isolates have been evaluated on several crops including trees (Bellahcene et al. 2005), ornamental flowering plants (Göre 2009), and vegetables (Berbegal et al. 2010). The collection of *V. dahliae* isolates in our laboratory was evaluated on potato and sunflower plants and was tested for cross-pathogenicity on the two hosts (Uppal et al. 2007; Alkher et al. 2009).

The main objective of this chapter was to establish a differential model of *V. dahliae*-potato that consists of highly and weakly aggressive isolates of the fungus and potato cultivars with susceptible and moderately resistant responses to the pathogen. This model is to be used for further studies of the molecular mechanisms of *V. dahliae* pathogenesis on potato.

3.3 Materials and Methods

3.3.1 *Verticillium dahliae* isolates

Four single-spore isolates of *V. dahliae* (Vd1396-9, Vs04-28, Vs06-13, and Vs06-14) were selected from 60 pre-screened isolates, based on the aggressiveness on Kennebec and Ranger Russet differential potato cultivars. All isolates were recovered from diseased plant tissues collected from commercial and experimental fields in Manitoba, Canada. The original host of isolates Vd1396-9 and Vs04-28 was potato and of isolates Vs06-13 and Vs06-14 was sunflower. All the 60 *V. dahliae* isolates were identified through their morphological and molecular characteristics, and the pathogenicity of all the 60 isolates were previously assessed (Uppal et al. 2007; Alkher et al. 2009).

Production of microsclerotia by the four selected isolates was observed on potato dextrose agar (PDA) and Czapek-Dox broth (CDB) media. Microscopic analysis of microsclerotia produced by two isolates (Vd1396-9 and Vs06-14) grown on PDA medium was carried out using a light microscope (Leitz Wetzlar/E. Leitz Wetzlar microscope, Germany) equipped with a digital camera (Hitachi KP-D50, Hitachi Kokusai Electric Canada, Ltd., Canada). Microsclerotia were observed at 125X and 400X magnification.

Fungal growth and sporulation assessments were carried out for Vd1396-9 and Vs06-14 isolates in liquid media. For each flask, one 0.9 mm-diameter plug was chosen from the edge of an actively growing culture and transferred into 50 ml of autoclaved Czapek-Dox Broth (CDB) liquid medium (Difco Laboratories, MD, USA). All flasks were incubated in the dark at room temperature (22.5 ± 0.5°C) on a shaker at 120 rpm

(C2 Platform Shaker, Edison, NJ, USA). For each *V. dahliae* isolate, fungal growth and sporulation rates were evaluated at 3, 6, 9, 12, and 15 days-old-culture. An average of 3 replicates (flasks)/time point for fresh weight of the fungal biomass was recorded; fresh weights of the pre-cultured plugs (average of 3) were subtracted to calculate the net fungal biomass. Sporulation was measured by counts of conidiospores using a haemocytometer (Hausser Scientific, Horsham, PA, USA). Three independent biological replicates/time point, with an average of four squares counted per replicate, were used per time-point for each isolate.

3.3.2 Potato cultivars

The four *V. dahliae* isolates were used to re-assess and confirm their levels of pathogenicity on two differential potato cultivars. Two potato cultivars (Kennebec and Ranger Russet) with differential levels of resistance to *V. dahliae* were used in this study. Kennebec and Ranger Russet were obtained from Prairie Dome Enterprises, Yorkton, Saskatchewan and Almasippi Irrigation Farms Ltd, Portage la Prairie, Manitoba, Canada, respectively. Kennebec is highly susceptible while Ranger Russet is moderately resistant to *V. dahliae* (Alkher et al. 2009). Seed pieces of the two cultivars were planted in plastic trays containing an autoclaved mixture of sand and soil (1:1, v/v) supplemented with 50 gm of nitrogen: phosphorus: potassium fertilizer granules (16:20:16). The plants were grown in a growth room for 4 weeks at conditions of 20/16 ± 2°C day/night, 16 hr photoperiod, with a light intensity of 350 μmol m^{-2} s^{-1}.

3.3.3 Inoculum preparation, inoculation procedure, and experimental design

Two-week-old cultures of Vd1396-9, Vs04-28, Vs06-13, and Vs06-14 *V. dahliae* isolates grown on PDA medium were used for inoculum preparation. Conidiospores were collected by scraping the surface of the cultures into 10 ml of sterilized distilled water. The inoculum concentration was adjusted to 10^6 conidiospores/ml with sterilized distilled water. Four-week-old potato plants were inoculated by the root dip method as described previously (Alkher et al. 2009). In parallel, potato plants were dipped in sterilized distilled water as non-inoculated controls. The inoculated and control plants were transplanted into 16 cm-diameter clay pots filled with an autoclaved mixture of soil, sand, peat and perlite (4:4:4:1, v/v/v/v) and received 5 g/l NPK (20:20:20) fertilizer. All plants were incubated in a growth room under the same conditions mentioned above for 7 weeks. The plants were distributed in a randomized complete block design. For each cultivar, 8 replicates of 1 plant each were inoculated with each isolate. The same number of plants was used as control.

3.3.4 Disease assessment and statistical analysis

Disease assessment was carried out through weekly analysis of disease severity from 2 to 7 weeks, whereas vascular discoloration and growth measurements were assessed 7 weeks after inoculation due to the need for destructive sampling. Vascular discoloration was evaluated at three levels of stem sections (lower, right above soil surface; middle; and upper, right below the apical meristem). Pre-established rating scales for disease severity and vascular discoloration were used as described previously (Alkher et al. 2009). Plant height, root and shoot fresh and dry weights were also recorded. *Verticillium dahliae* isolates were successfully re-isolated on PDA medium

from root and stem sections of the inoculated plants only. Data were analyzed statistically using SAS 9.1 software (SAS Institute Inc., Cary, NC, USA). Differences among the means were compared using the least significant difference (LSD) test at $p<0.05$.

3.4 Results and Discussion

3.4.1 Microscopic analysis, growth, and sporulation of *V. dahliae* isolates

Production of microsclerotia, which are survival structures, is a characteristic taxonomic feature of *V. dahliae* (Klosterman et al. 2009). Microsclerotia production was observed in all four isolates. The production of microsclerotia of isolates Vd1396-9 and Vs06-14 was compared on PDA medium (Fig. 3.1. A and B). The highly aggressive isolate Vd1396-9 produced microsclerotia within 2-3 weeks, whereas Vs06-14, the weakly aggressive one, produced microsclerotia only after two months. Vd1396-9 produced a higher quantity of microsclerotia as compared to Vs06-14.

Microscopic analysis of microsclerotia from the two isolates on PDA is shown with magnification 125X (Fig. 3.1 C and D) and 400X (Fig. 3.1 E and F). Microsclerotia were 40-50 μm in diameter in both isolates (Fig. 3.1 E and F). After 2-3 weeks incubation in CDB liquid medium, Vd1396-9 produced microsclerotia, whereas Vs06-14 did not.

Fungal growth and conidiospore production in CDB liquid medium of both isolates Vd1396-9 and Vs06-14 were measured 3, 6, 9, 12, and 15 days after incubation. The fungal fresh weight of both isolates increased significantly with the culture age. In contrast, conidiospore production in Vd1396-9 was greater than in Vs06-14 (Fig. 3.2).

It has been reported that a more virulent *V. dahliae* pathotype on cotton was associated with higher levels of conidiospore production in contrast with a less virulent

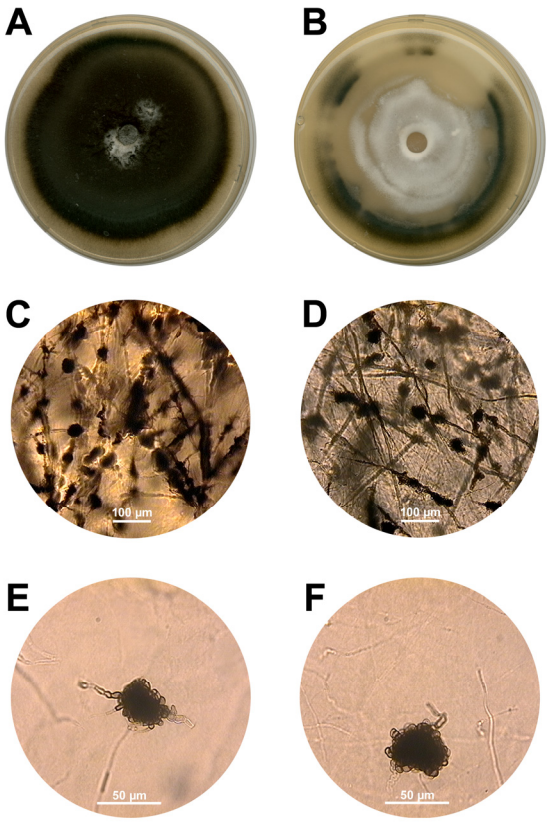

Figure 3.1. *Verticillium dahliae* microsclerotia formation on PDA by isolates Vd1396-9 (A), the highly aggressive isolate after 3 weeks and Vs06-14 (B), the weakly aggressive isolate after 2 months. Microscopic views of Vd1396-9 and Vs06-14 microsclerotia are at 125 X (C and D) and 400 X (E and F), respectively.

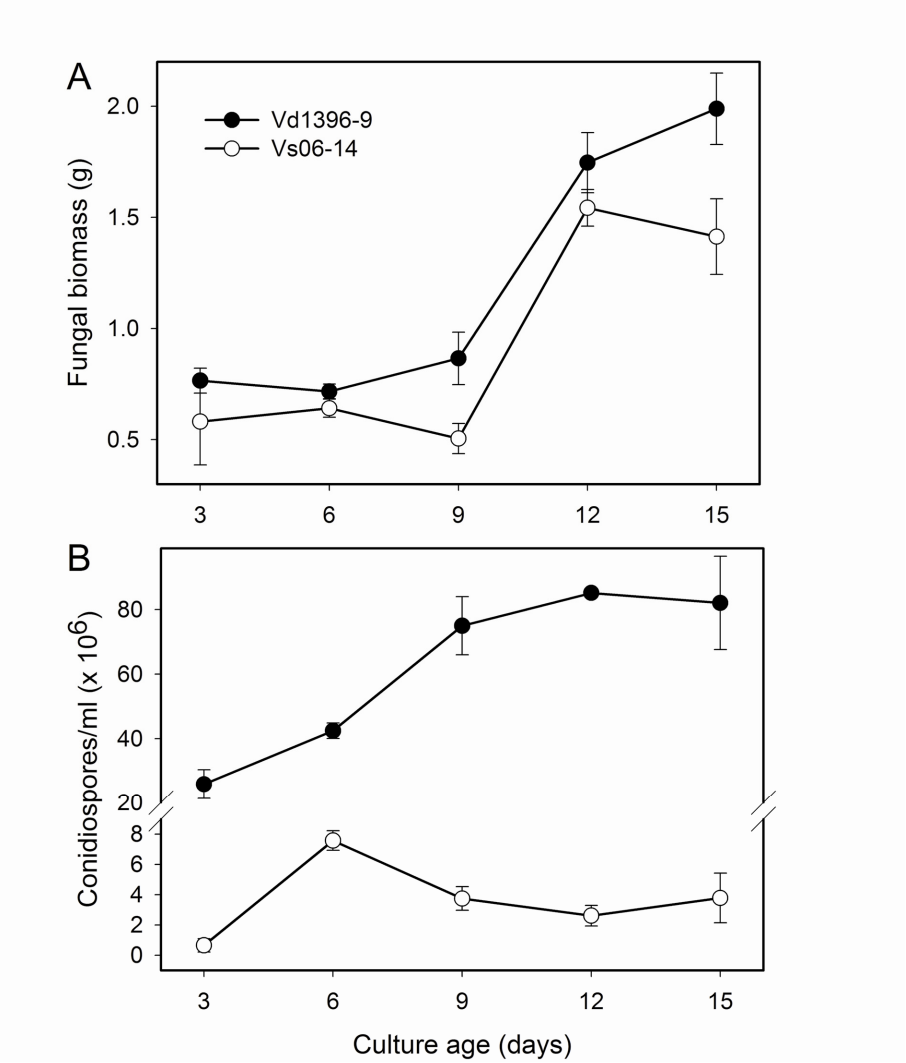

Figure 3.2. Fungal biomass (A) and conidiospore counts (B) of Vd1396-9, the highly aggressive, and Vs06-14, the weakly aggressive, *V. dahliae* isolates. Each data point represents the average of three independent biological replicates ± standard error.

one (Schnathorst 1963). Therefore, high rates of microsclerotia and conidiospore production could contribute to survival longevity and aggressiveness of Vd1396-9 as compared to Vs06-14.

3.4.2 Pathogenicity evaluation of the *V. dahliae* isolates

Disease severity was assessed weekly, and stem vascular discoloration, and growth measurements of the two cultivars were assessed 7 weeks after inoculation with the *V. dahliae* isolates to confirm their aggressiveness levels. The external symptoms ranged from chlorosis to necrosis which started from the margin of the leaf into the main vein and wilt occurred on both cultivars. Disease symptoms appeared earlier on cv. Kennebec (2 w.a.i.) as compared with cv. Ranger Russet (3 w.a.i.) (Fig. 3.3). Disease severity increased gradually until 7 w.a.i., and was higher on Kennebec than on Ranger Russet. Vd1396-9 had the highest statistically significant aggressiveness level on both cultivars, with a disease severity index of 3.61 on Kennebec and 2.55 on ranger Russet at 7 w.a.i., whereas Vs06-13 and Vs06-14 were weakly aggressive (Fig. 3.3; Table 3.1).

The vascular discoloration in the lower, middle, and upper stem sections of potato cultivar Kennebec was greater than in cultivar Ranger Russet. There was no noticeable vascular discoloration in the upper section of Ranger Russet inoculated with any isolate. However, vascular discoloration of the upper stem sections was noticed only in cultivar Kennebec inoculated with Vs04-28 or Vd1396-9 with a significant level of discoloration in the case of isolate Vd1396-9 (Table 3.1). It is possible that, the high rate of conidiospore production in Vd1396-9 (Fig. 3.2) facilitated spreading of the infection and colonization of the upper part of stems (Schnathorst 1963) or that Ranger Russet

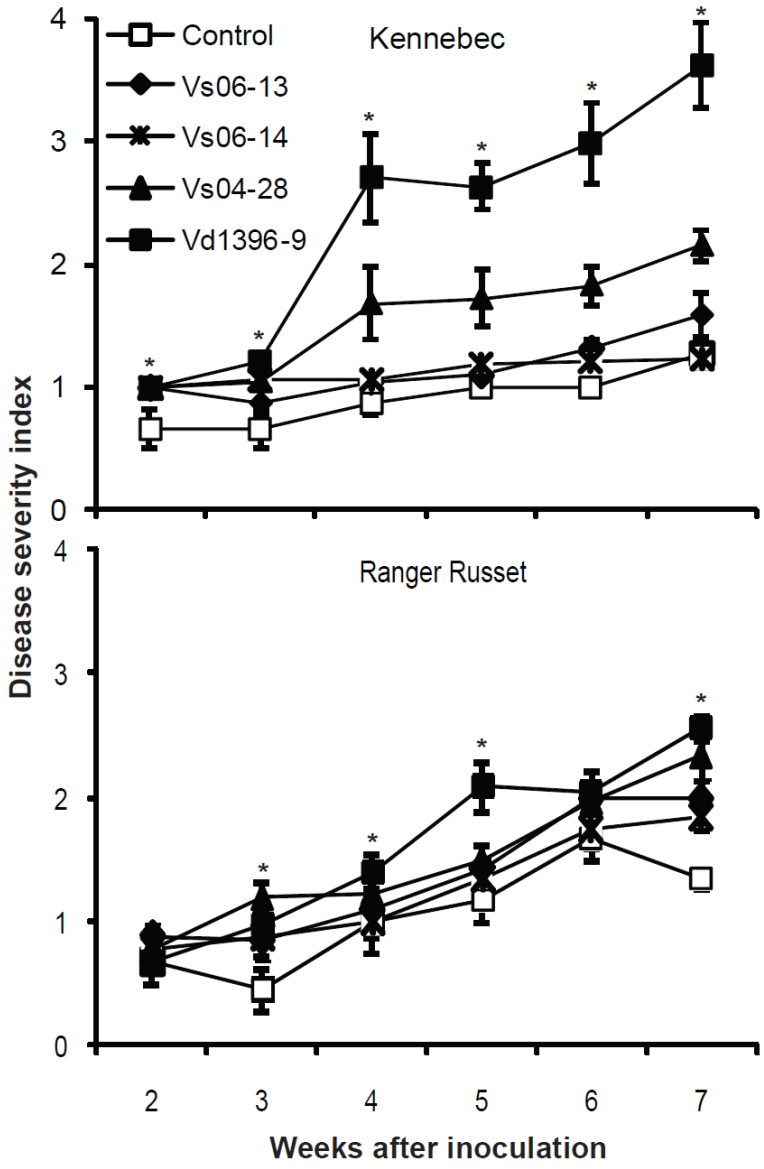

Figure 3.3. Disease severity of *V. dahliae* isolates Vd1396-9, Vs04-28, Vs06-13, and Vs06-14 on Kennebec and Ranger Russet potato cultivars. Each data point represents the average of 8 independent replicates ± standard error. The disease severity index is based on a 0 to 5 scale where; 0 = no chlorosis or necrosis, 1 = visible chlorosis with < 1% necrosis, 2 = up to 40% chlorosis and 1–20% necrosis, 3 = up to 65% chlorosis and 20–35% necrosis, 4 = 100% chlorosis and 35–70% necrosis, 5 = 100% chlorosis and 70–100% necrosis. The asterisks represent statistically significant differences between the isolates at a given time on a given cultivar according to the LSD test at (P<0.05).

responded earlier to the infection and developed physical and/or chemical defense mechanisms such as tyloses and phenolic compounds, respectively. The latter reactions are known to restrict further movement of the fungus in the vascular system (Daayf et al. 1997).

Based on stem vascular discoloration, isolate Vd1396-9 showed a high level of aggressiveness and an ability to colonize and spread through the vascular system more efficiently, as compared to the other tested isolates. The current results on disease severity and vascular discoloration are in agreement with previous pathogenicity assessments of these isolates on the same potato cultivars (Alkher et al. 2009).

The effect of *V. dahliae* isolates on potato growth was also analyzed. Stunting symptoms were noticed on both cultivars. Plant height, root and shoot fresh and dry weights were measured at 7 w.a.i. (Table 3.1). There was a significant reduction in plant height of both cultivars in response to the inoculation with Vd1396-9 and Vs04-28 isolates. The reduction in Kennebec height was significantly different from the control in the case of inoculation with isolates Vs04-28 or Vd1396-9. The latter caused about a 42.5% reduction of plant height as compared to the control plants. In the cultivar Ranger Russet, a significant plant height reduction was noticed with both isolates, (24%) as well as with Vs06-13. While there was a significant reduction of the root and shoot fresh weight in Kennebec inoculated with isolates Vd1396-9 or Vs04-28, Ranger Russet shoot fresh weight was not significantly affected. The highest reduction in root and shoot fresh, and root and shoot dry weight was detected in the case of Kennebec inoculated with isolate Vd1396-9 by 67.2%, 55.7%, 73.4%, and 43.6% reduction, respectively, as compared to the control. Data from root and shoot dry weights showed a significant

Table 3.1. Disease assessment of Verticillium wilt on Kennebec and Ranger Russet potato cultivars after 7 weeks of inoculation with isolates Vd1396-9, Vs04-28, Vs06-13, and Vs06-14 of *V. dahliae*

Cultivar	Isolate	Disease severity index[x)]	Vascular discoloration of stem sections[y)]			Plant height (cm)	Fresh weight (g)		Dry weight (g)	
			Lower	Middle	Upper		Root	Shoot	Root	Shoot
Kennebec	Vd1396-9	3.61 a[z)]	4.25 a	3.00 a	1.87 a	14.87 b	10.33 b	32.22 c	0.88 b	4.60 c
	Vs04-28	2.15 b	3.12 b	2.12 b	0.37 b	16.25 b	15.31 b	54.92 b	1.47 b	6.13 b
	Vs06-13	1.59 c	1.87 c	0.50 c	0.00 b	22.87 a	29.46 a	60.98 b	3.10 a	7.75 a
	Vs06-14	1.22 c	1.62 c	0.50 c	0.00 b	25.50 a	32.12 a	65.13 ab	3.20 a	8.27 a
	Control	1.27 c	0.00 d	0.00 c	0.00 b	25.93 a	31.53 a	72.87 a	3.31 a	8.17 a
Ranger Russet	Vd1396-9	2.55 a	1.50 a	0.62 a	0.00 a	10.37 c	14.91 b	37.45 a	1.35 a	5.00 a
	Vs04-28	2.33 ab	1.12 ab	0.12 b	0.00 a	10.37 c	16.31 ab	41.97 a	1.47 a	5.63 a
	Vs06-13	1.85 c	0.75 bc	0.12 b	0.00 a	12.25 b	19.43 ab	44.45 a	1.60 a	5.83 a
	Vs06-14	1.98 bc	0.37 cd	0.00 b	0.00 a	11.37 bc	17.67 ab	37.52 a	1.62 a	5.12 a
	Control	1.33 d	0.00 d	0.00 b	0.00 a	13.68 a	20.63 a	42.90 a	1.58 a	4.93 a

x) Severity index based on 0 to 5 scale, 0 = no chlorosis or necrosis, 1 = visible chlorosis with < 1% necrosis, 2 = up to 40% chlorosis and 1–20% necrosis, 3 = up to 65% chlorosis and 20–35% necrosis, 4 = 100% chlorosis and 35–70% necrosis, 5 = 100% chlorosis and 70–100% necrosis.

y) Vascular discoloration of stem sections based on 0 to 5 scale, 0 = no vascular discoloration, 1 = traces to < 9% of the section area with vascular discoloration symptom, 2 = 10–24%, 3 = 25–49%, 4 = 50–74%, and 5 = 75–100%.

z) Each value in the table is an average of 8 replicates. For each cultivar, values within a column followed by the same letter are not significantly different according to LSD test at $P < 0.05$.

effect of inoculation with Vd1396-9 or Vs04-28 on Kennebec. There were no significant effects with any isolate on root or shoot dry weight of cultivar Ranger Russet (Table 3.1).

As a result of the severe external and internal symptom development on Kennebec, leaf expansion, photosynthesis (Sadras et al. 2000) and nutrient translocation might be affected and, subsequently, result in a reduction of plant growth. It has been reported that *V. dahliae* affected potassium leaf content in cotton (DeVay et al. 1997), carbon assimilation in potato (Saeed et al. 1997) and growth of tomato and eggplants (Karagiannidis et al. 2002). The effects of *V. dahliae* on growth were greater when potato was inoculated with a combination of *V. dahliae*, *Fusarium oxysporum* f. sp. *tuberosi* and *Meloidogyne javanica* (Daami-Remadi et al. 2009).

Combining the data on disease severity, vascular discoloration, and growth assessment is a powerful tool for pathogenicity evaluation of *V. dahliae* (Bhat and Subbarao 1999). The current results showed and confirmed that *V. dahliae* isolates Vd1396-9 and Vs06-14 are highly and weakly aggressive on potato, respectively. Both isolates were selected for further investigation on molecular analysis of their potential pathogenicity factors.

[†]4.0 TRANSCRIPTOMIC ANALYSIS TO IDENTIFY CANDIDATE PATHOGENICITY-RELATED GENES IN *VERTICILLIUM DAHLIAE* AFTER ELICITATION WITH POTATO ROOT EXTRACTS

4.1 Abstract

Verticillium dahliae is the main pathogen causing Verticillium wilt in potato. Management of this vascular disease is very challenging due to the soilborne nature of the pathogen. A better understanding of the molecular host-pathogen interactions is a requirement towards the development of novel strategies to control Verticillium wilt. In this pathosystem, such interactions are initiated before any physical contact between potato and *V. dahliae*, through root exudates, which stimulate germination of the pathogen. The present chapter reports on the use of potato root extracts derived from susceptible (Kennebec) and moderately resistant (Ranger Russet) cultivars to elicit pathogenicity-related genes in highly- (Vd1396-9) and weakly aggressive (Vs06-14) isolates of *V. dahliae*. Using a combinational approach of subtractive hybridization and cDNA-AFLP, 573 transcripts differentially expressed in one or the other isolate in response to treatment with root extracts were detected. Sixteen primer combinations representing *Eco*RI/*Mse*I AFLP primers +A/T/C/ or G were used to provide full coverage of the subtractive hybridization products. The number of differentially expressed genes in the highly aggressive isolate was higher than in the weakly aggressive one. Among the amplified transcripts, 185 were recovered from the PAGE gel then re-amplified by PCR

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El-Bebany, A. F., Henriquez, M.A. Badawi, M., Adam, L.A., El Hadrami, A., and Daayf, F. 2010. Induction of pathogenicity-related candidate genes in *Verticillium dahliae* in response to elicitation with potato root extracts.

and further sequenced. BLAST search against the NCBI and the Broad Institute databases showed that 41 transcripts matched with known sequences, all with assigned functions in *V. dahliae* such as polygalacturonases or with conserved hypothetical proteins. The remaining 144 had no matches in these two databases. The results are discussed based on the potential involvement of these genes in the pathogenesis of *V. dahliae* on potato.

4.2 Introduction

Verticillium wilt is mainly caused by the soilborne pathogen *Verticillium dahliae* Kleb. The disease affects a wide range of plant hosts including herbaceous annuals, perennials, and woody plants (Pegg and Brady 2002; Fradin and Thomma 2006) causing chlorosis and necrosis of the foliage, discoloration of the vascular system, and often wilting and stunting (Alkher et al. 2009). In potato, besides a lower tuber quality, yield losses due to this disease may reach 30-50% (Rowe and Powelson 2002).

Verticillium dahliae typically survives in the soil for many years as microsclerotia. Upon stimulation by plant root exudates, the microsclerotia germinate and develop hyphae that penetrate and colonize the root cortex. Once the hyphae reach the xylem, asexual reproduction starts, leading to the formation of conidiospores that are able to move upward, germinate, and penetrate new vessels. At the end of the disease cycle, the fungus forms microsclerotia in the dead parts of the plant (Fradin and Thomma 2006; Klosterman et al. 2009).

Current integrated disease management strategies to control Verticillium wilt in potato include fumigation (Powelson and Rowe 1993), soil solarization (Katan 1981), crop rotation (Stevens et al. 2003), biological control (Tjamos et al. 2004; Lopez-

Escudero et al. 2007; Ochiai et al. 2007; Uppal et al. 2008), and the use of tolerant varieties in the absence of completely resistant ones. Deployment of tolerant/resistant varieties will require a better understanding of the host-pathogen interaction. Very little is known about potato defense mechanisms against *V. dahliae*, and most information available is from other pathosystems involving *V. dahliae* (Daayf et al. 1995; 1997; Fradin and Thomma 2006). Pathogenicity factors such as extracellularly secreted cell-wall degrading enzymes (CWDEs) and phytotoxic peptides have been characterized using either *V. dahliae* or *V. albo-atrum* isolates. A 29.5 kDa endo-polygalacturonase has been isolated and partially purified from *V. dahliae* culture filtrates (James and Dubery 2001) while another one from *V. albo-atrum* filtrate was of a higher molecular mass (37 kDa) (Huang and Mahoney 1999). These enzymes are thought to play a major role in pathogenesis (Di Pietro and Roncero 1998; ten Have et al. 1998; Isshiki et al. 2001) and to work together with xylanases, cellulases, pectinases, β-1,3-glucanases and proteases during the root penetration process (Bidochka et al. 1999; Sattarova 2001). Jointly with this enzymatic cocktail, activities of cellulases and β-1,4-glucosidases seemed to correlate with the level of aggressiveness of isolates (Novo et al. 2006). In addition, several authors have suggested that the pathogenesis of *V. dahliae* involves toxins (Buchner et al. 1982; Pu et al. 2007). *VdNEP*, a necrosis and ethylene-producing factor, was suggested to be responsible for wilting in cotton (Wang et al. 2004) while other genes such as *VMK1* and *VDH1*, encoding for a MAP kinase and a hydrophobin, respectively, have been ascribed a role in growth, development and pathogenicity of *V. dahliae* or microsclerotia development and persistence in the soil (Rauyaree et al. 2005; Klimes and Dobinson 2006; Klimes et al. 2006).

Knowledge about the pathogen's functional genomics and understanding the molecular basis of its pathogenesis will greatly contribute to the adjustment of Verticillium wilt management practices. To date, little work has been done on the functional genomics of *V. dahliae* (Neumann and Dobinson 2003). Pulsed field gel electrophoresis revealed that the genome of *V. dahliae* is on seven chromosomes with an estimated total genomic size ranging from 26.6 to 29.1 Mb, and an average of 28.4 Mb (Pantou and Typas 2005; Usami et al. 2008). Genetic and physical mapping of the entire genome is ongoing to increase the coverage of the existing draft sequence of a reference isolate from *V. dahliae* along with another one from *V. albo-atrum*. Updated annotated genes and transcripts are available on the Broad Institute of MIT and Harvard (http://www.broad.mit.edu/annotation/genome/verticillium_dahliae/Info.html). The present study is a contribution to the knowledge about *V. dahliae* genomics through the identification of candidate pathogenicity-related genes. Based on the results from the previous chapter, the established differential model involving one weakly and one highly aggressive isolate of *V. dahliae* and one highly susceptible and one moderately resistant cultivar of potato was used in this study. Because interactions in this pathosystem are initiated before any physical contact between potato and *V. dahliae*, through root exudates, the first phase in this interaction is the stimulation of the pathogen by the plant root exudates. Therefore, plant root extracts from susceptible and moderately resistant potato cultivars were used to elicit the expression of pathogenicity-related genes in the selected isolates *in vitro*. This presents the advantage of eliminating interference with accumulated transcripts from the host.

The objective of this investigation was to identify *V. dahliae* genes involved in the earliest stages of its interaction with potato. A combinational approach of subtractive hybridization and cDNA-AFLP (Henriquez and Daayf 2010) was used to selectively amplify only the genes differentially expressed in one or the other isolate, and only after exposure to root extracts. This way, candidate pathogenicity-related genes that differentially express in each tested isolate in response to extracts from either a susceptible or a moderately resistant potato cultivar would be identified.

4.3 Materials and methods

4.3.1 *Verticillium dahliae* isolates

Two of the four *V. dahliae* isolates used in the pathogenicity evaluation (Chapter 3.0) were selected to carry out transcriptomics analysis of the genes differentially elicited by root-extracts. These two isolates, Vd1396-9 (highly aggressive) and Vs06-14 (weakly aggressive), were selected after their aggressiveness was confirmed on two potato cultivars, Kennebec (susceptible) and Ranger Russet (moderately resistant). The two *V. dahliae* isolates were grown in 50 ml Czapek-Dox Broth (CDB) liquid medium. The liquid cultures were incubated for two weeks in the dark at 22.5 ± 1 °C on a shaker set at 120 r.p.m. (C2 Platform Shaker, Edison, NJ, USA).

4.3.2 Potato root extract preparation and elicitation treatments

Five grams of roots from healthy potato plants (cultivars Kennebec and Ranger Russet) were reduced to a powder using a mortar and a pestle pre-cooled in liquid nitrogen then suspended in 25 ml of sterilized distilled water (SDW) and agitated for 4

hrs. After centrifugation at 2,000 *g* for 5 min, the supernatants were filter-sterilized through a 0.2 μm microfilter and transferred into new tubes. One milliliter of root extract from the susceptible or moderately resistant cultivar was added to each actively growing isolate. For each isolate, three flasks were included and the controls were amended with one milliliter of SDW. Fungal mycelium from each treatment and controls were harvested at 2, 4, 8, 12, 24, 72 hours and two weeks after treatment, and immediately reduced to powder using liquid nitrogen in a mortar and pestle. The mycelial powder was then stored at – 80 °C until required.

4.3.3 Total RNA extraction and mRNA isolation

Total RNA was extracted with TRIzol® Reagent (Invitrogen Inc., ON, Canada) using 200-300 mg of fungal biomass from each treatment at 2, 4, 8, 12, 24, 72 hours and 2 weeks after treatment. The RNA quality was assessed by standard agarose gel electrophoresis and OD 260/280 measurement (Ultrospec 3100, Biochrom Ltd., Cambridge, UK). Total RNA was extracted from the same time-point experiments in control treatments (amended with SDW). These controls are necessary for further subtraction of genes that are constitutively expressed. Messenger RNAs were isolated from 100 μg of total RNA of the time-points mixture (the time points represented equally in the 100 μg total RNA, i.e., 14.28 μg RNA per time point) of each treatment, using the Novagen straight A's mRNA isolation system (EMD Chemicals Inc., NJ, USA) following the manufacturer's instructions.

4.3.4 Analysis of differentially expressed transcripts in *V. dahliae* isolates using SH/cDNA-AFLP

In order to identify *V. dahliae* genes that are differentially expressed in response to potato root extracts, and consequently, potentially involved in the pathogenicity of *V. dahliae*, a subtractive hybridization (SH)/cDNA-AFLP combinational approach was used as described by Henriquez and Daayf (2010). Briefly, synthesis of cDNA was performed from transcripts of interest obtained from SH between root extract and control treatments. The resulting double-strand DNA (SH-second strand) was used for further cDNA-AFLP analysis using *Eco*RI/*Mse*I for SH-digestion, followed by SH-ligation, pre-amplification (SH+0) and SH-amplification (SH+1). The digestion of the SH products was conducted at 37 ^{o}C for 2h and the ligation at 20 ^{o}C for another 2h. The cDNA-AFLP was performed using the AFLP® Analysis System for Microorganisms (Invitrogen Inc. ON, Canada), starting with 10 µL of SH-second strand for digestion. PCR reactions were performed in a Techne TC-412 thermal cycler (Barloworld Scientific US Ltd., Burlington, N.J., USA). The SH+0 pre-amplification PCR program was as follows: initial denaturation at 94 °C for 5 min, 25 cycles of 30 sec at 94 °C, 60 sec at 55 °C, and 60 sec at 72 °C, and 10 min of final extension at 72 °C. However, SH+1 amplifications were performed using a touchdown PCR program consisting of one cycle at 94 °C for 30 sec, 60 °C for 30 sec and 72 °C for 60 sec, followed by 11 cycles under the same conditions except that the annealing temperature was decreased by 0.7 °C per cycle. The last 22 cycles of the program were carried out at 94 °C for 30 sec, followed by annealing at 56 °C for 30 sec, and extension at 72 °C for 60 sec with a final extension at 72 °C for 10 min. Combinations of 16 primer sets (*Eco*RI/*Mse*I +A/T/C or G; 2^4=16; Tables 4.1; 4.2) were

used, allowing full coverage of all possible SH products. The SH+1 products were separated on 5% PAGE and bands detected by $AgNO_3$ staining. Primer combinations involving +3 nucleotides were also assayed during the selective amplification of SH products, but with little success as compared to SH+1. Therefore, only SH+1 was used.

Fragments corresponding to differentially expressed transcripts on the PAGE were scored, and these of interest were excised from the dried gel to be further re-amplified according to the following PCR program: initial denaturation at 94 °C for 5 min, followed by 35 cycles at 94 °C for 30 sec, 50 °C for 30 sec, and 72 °C for 60 sec, and a final extension at 72 °C for 10 min. PCR products were analyzed on 1% agarose gels and visualized using an AlphaImager HP gel documentation system (Alpha Innotech Corporation, San Leandro, CA, USA) equipped with a P93D thermoprinter (Mitsubishi Electric Co., Tokyo, Japan). The size of each amplified fragment was compared with its original counterpart detected on the polyacrylamide gel. The PCR products were further purified using the Qiaex II gel-extraction kit (Qiagen Inc., Alameda, CA, USA) following the manufacturer's instructions, then sent for sequencing (Macrogen Co., USA).

4.3.5 Experimental design and data analysis

All flasks were distributed in a random design. Three flasks (replicates) were included in each time data-point and the whole experiment was repeated once. Among the amplified transcripts using the SH/cDNA-AFLP approach, a total of 185 differentially expressed transcripts were sequenced and subjected to multi-alignment using WU-Blast 2.0 program on Do-It-Yourself Sequence Comparison website (http://www.proweb.org/Tools/WU-blast.html) to eliminate redundancies. A check for the presence of primers and

Table 4.1. Sequences of the AFLP primers

Primer name	Sequence (5' to 3')[a]
*Eco*RI-A	GACTGCGTACCAATTC**A**
*Eco*RI-T	GACTGCGTACCAATTC**T**
*Eco*RI-C	GACTGCGTACCAATTC**C**
*Eco*RI-G	GACTGCGTACCAATTC**G**
*Mse*I-A	GATGAGTCCTGAGTAA**A**
*Mse*I-T	GATGAGTCCTGAGTAA**T**
*Mse*I-C	GATGAGTCCTGAGTAA**C**
*Mse*I-G	GATGAGTCCTGAGTAA**G**

a: The selective additional nucleotide of the primer sequence is indicated in bold

Table 4.2. Primer combinations used for the SH/cDNA-AFLP

Primers combination		*Eco*RI + 1			
		A	T	C	G
*Mse*I + 1	A	1	5	9	13
	T	2	6	10	14
	C	3	7	11	15
	G	4	8	12	16

adaptors was conducted across all obtained sequences and trimmed-off when necessary. The sequences were further queried using BLASTN and BLASTX algorithms (Altschul et al. 1997) against the NCBI GenBank databases (http://www.ncbi. nlm.nih.gov/blast) and against the draft genome of the reference *V. dahliae* VdLs.17 isolate from the Broad Institute of MIT and Harvard (MA, USA) (http://www.broad.mit.edu/annotation/genome/verticillium_dahliae/Blast.html). All the sequences of the amplified differentially expressed transcripts are shown in Appendix 10.1.

4.4 Results

4.4.1 Differential expression of root extract-elicited transcripts in *V. dahliae* and their classification

A total of 573 differentially expressed transcripts were detected in the weakly (Vs06-14) and highly aggressive (Vd1396-9) isolates in response to induction by root extracts from either the susceptible cv. Kennebec or the moderately resistant cv. Ranger Russet. These transcripts were found in four unique tested situations after subtraction of control transcripts representing each isolate treated with SDW, followed by the AFLP step. These situations are referred to in Fig. 4.1 as (A) resulting from the subtraction: Vs06-14 treated with Kennebec root extract minus Vs06-14 treated with SDW, (B) resulting from the subtraction: Vs06-14 treated with Ranger Russet root extract minus Vs06-14 treated with SDW, (C) resulting from the subtraction: Vd1396-9 treated with Kennebec root extract minus Vd1396-9 treated with SDW, and (D) resulting from the subtraction: Vd1396-9 treated with Ranger Russet root extract minus Vd1396-9 treated with SDW.

Amplified transcripts were classified into two main groups. The first one contained 301 genes, which were expressed only in the highly aggressive isolate Vd1396-9. This group was subdivided into sub-groups 1-1 and 1-2, composed of 165 and 136 differential Vd1396-9 transcripts unique to exposure to Kennebec and Ranger Russet root extracts, respectively. The second group consisted of 272 transcripts differentially expressed in the weakly-aggressive isolate Vs06-14. This group was subdivided into sub-groups 2-1 and 2-2, with 150 and 122 genes expressed after exposure to Kennebec or Ranger Russet root extract, respectively.

A total number of 185 transcripts of interest from the tested situations were selected for further sequencing and characterization. The relative distribution of the differentially expressed genes in each specific case is illustrated in Fig. 4.2. Seventy six genes (representing 41% of the total sequenced transcripts) were induced in both tested isolates after exposure to root extracts from both potato cultivars. In terms of their isolate-specific patterns, 45 genes (24%) were differentially expressed only in isolate Vd1396-9 in response to both potato cultivars. Thirty three genes (18%) were differentially expressed only in Vs06-14 isolate in response to both cultivars. Eleven genes (6%) were differentially expressed in all cases except in Vs06-14 treated with Ranger Russet root extract. Another three genes (about 2%) were differentially expressed in all situations except Vd1396-9 treated with Ranger Russet root extract. Some detected genes were differentially expressed only in one case out of the four tested situations, 12 genes (6%) only in Vd1396-9 challenged with Kennebec root extract, and five genes (3%) only in Vs06-14 in response to Kennebec root extract.

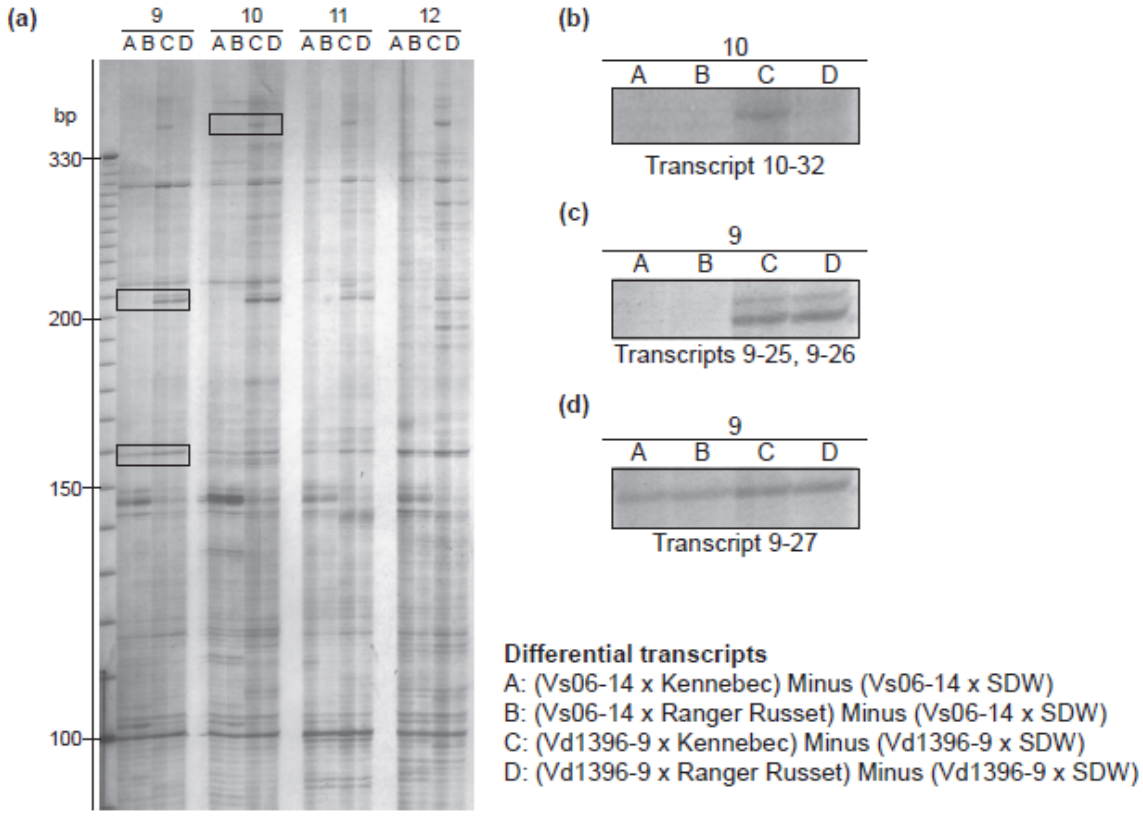

Figure 4.1. (a) Polyacrylamide gel electrophoresis (PAGE) of SH/cDNA-AFLP (primers combinations 9 to 12) to amplify differentially expressed transcripts in *V. dahliae* isolates Vs06-14 (weakly aggressive) and Vd1396-9 (highly aggressive) in response to elicitation with root extracts from susceptible potato cultivar Kennebec and moderately resistant cultivar Ranger Russet. The bands represent transcripts of genes expressed in the fungus treated with potato root extracts but not in the fungus treated with sterilized distilled water (SDW). **(b)** Magnification of transcript 10-32 (differentially expressed in Vd1396-9 after elicitation by Kennebec root extract, primer combination is *Eco*RI+C and *Mse*I+T). **(c)** Magnification of transcripts 9-25 and 9-26 (differentially expressed in Vd1396-9 after elicitation by Kennebec or Ranger Russet root extracts, primer combination is *Eco*RI+C and *Mse*I+A). **(d)** Magnification of transcripts 9-27 (differentially expressed in both isolates after elicitation by either Kennebec or Ranger Russet root extract, primer combination is *Eco*RI+C and *Mse*I+A).

4.4.2 Amplification of the differentially expressed transcripts and functional assignment

Among all the amplified transcripts on the initial PAGE, a set of selected transcripts representing the above-mentioned situations were excised and re-amplified by PCR (Fig. 4.3). In all cases, the size estimated on the agarose gel for each amplified transcript was comparable to its original counterpart detected on the PAGE. Over all of the analyzed transcripts, the size ranged from 50 to 400 bp. Figure 4.3 shows examples of transcripts amplified by PCR (e.g. 7-195, 11-81), identified by two numbers (the primer combination number – the transcript number), and sequenced.

The size (bp) of all amplified transcripts further sequenced, their differential expression in the four SH-cDNA-AFLP situations on the PAGE, their homology with sequences in GenBank, and *E*-values are listed in Table 4.3. BLAST search analysis indicated that 144 transcripts out of the initial 185 had no match, whereas 41 transcripts displayed homology with known sequences in the NCBI database (Table 4.3). In the case of genes that were differentially expressed under all treatments, transcript 13-125 (247 bp) showed homology with an EST from *V. dahliae* expressed during its pathogenic growth and microsclerotial development (E=1x10^{-86}). Transcript 7-192 (254 bp) is homologous to a sequence from mature appressoria of *Colletotrichum higginsianum* (E=8x10^{-45}). Two other transcripts, 12-102 (E=2x10^{-13}) and 14-144 (E=0.029), exhibited homology with sequences of hypothetical proteins from *Gibberella moniliformis* and *Aspergillus terreus*, respectively. One transcript, 6-185, showed homology with a gene that is up-regulated by thermal stress in *Schistosoma mansoni* (E=1x10^{-06}). Transcripts 2-13 and 5-183 exhibited similarities with a sequence from *Terfezia boudieri*, expressed at

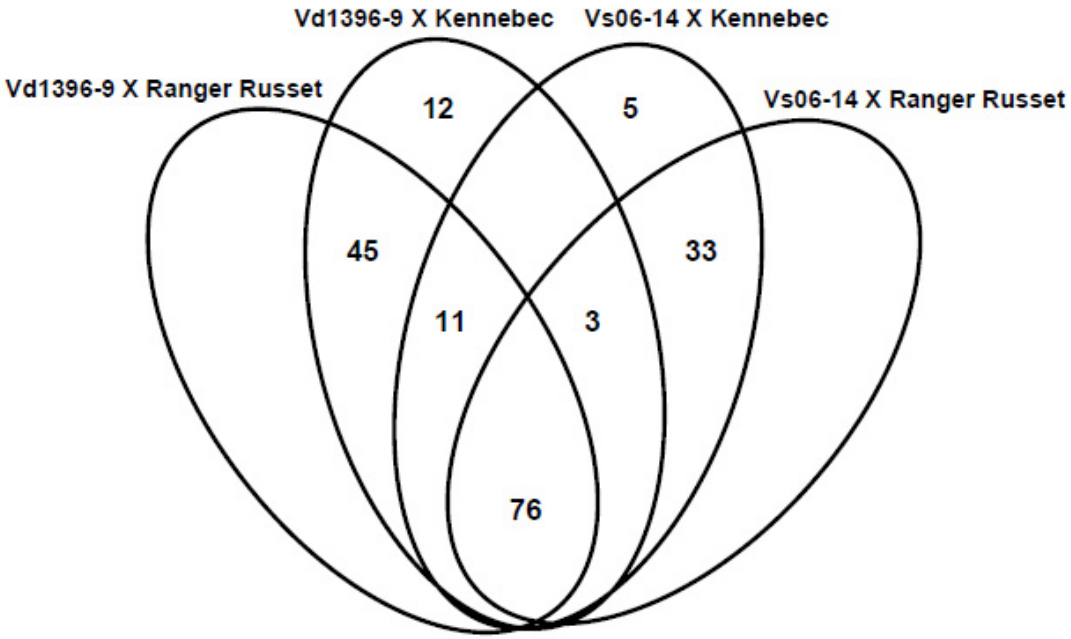

Figure 4.2. Numbers and distribution of the 185 sequenced differentially expressed transcripts in two *V. dahliae* isolates Vd1396-9 and Vs06-14, highly and weakly aggressive, respectively, in response to elicitation with root extracts from Ranger Russet and Kennebec, moderately resistant- and susceptible potato cultivars, respectively.

the pre-infection stage of *Citus incanus*, and a putative secreted protein from *Xanthomonas campestris* pv. *campestris*, respectively. Transcript 14-140 had homology with *Thielavia terrestris* pooled RNA from cells grown in medium containing cellulose and transcript 4-173 exhibited homology with a sequence from *V. albo-atrum.* Six transcripts shared homology with rRNA from *V. dahliae* and other microorganisms. Among the interesting cases, transcript 12-96, which was expressed in response to root extracts except in the weakly aggressive isolate Vs06-14 treated with the moderately resistant Ranger Russet root extracts, had high homology with a *Neisseria meningitidis* TspB protein, which is a virulence factor. Even more interesting are the cases of transcripts 4-174, 12-106, and 10-32, which were differentially expressed only in the highly aggressive isolate Vd1396-9, and had homology with a *C. higginsianum* mature appressorial protein (E=6x10^{-9}), *V. dahliae* genes expressed during pathogenic growth and microsclerotial development (E=7x10^{-11}), and one *Neurospora crassa* exo-polygalacturonase (E=5x10^{-14}), respectively.

All the sequenced transcripts were further searched against the genome of the *V. dahliae* isolate VdLs.17 from the Broad Institute (Table 4.4). Transcripts 10-32, 10-55 and 10-56 showed homology with the exo-polygalacturonase and polygalacturonase located on chromosome 6 (E=0.0, 8.3x10^{-12}, and 6x10^{-16}, respectively). Transcript 2-14 exhibited similarity to a DNA-(apurinic or apyrimidinic site) lyase (E=8.3x10^{-11}). Transcripts 10-62 and 10-63 shared homology with a glutaminyl-tRNA synthetase. Transcripts 12-93 and 13-124, which were differentially expressed in the isolate Vd1396-9 only in response to the susceptible Kennebec root extract, had homology with a myo-inositol 2-dehydrogenase and a xanthine dehydrogenase, with E=1x10^{-32} and 9.8x10^{-5},

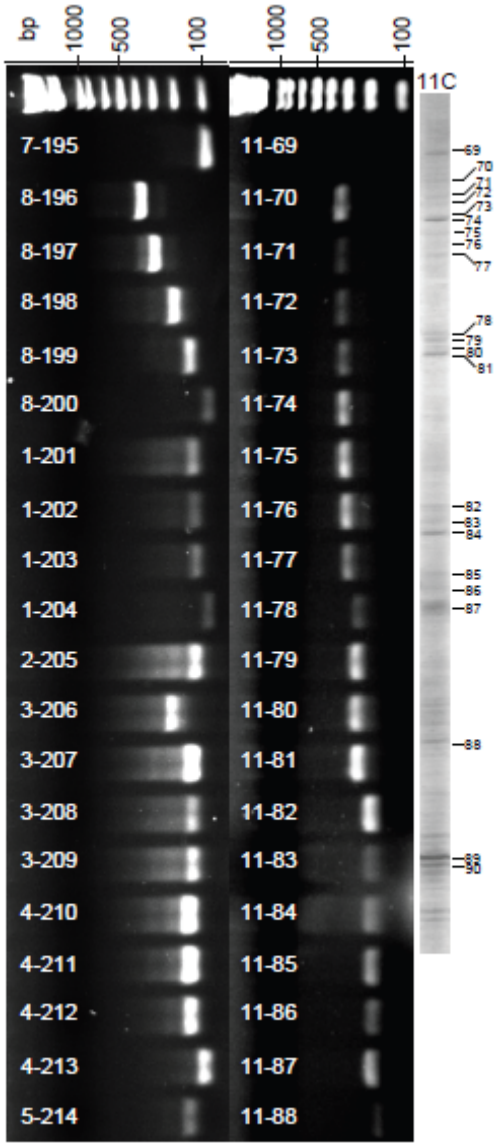

Figure 4.3. PCR re-amplification, after recovery from the PAGE (e.g. primer combination 11C extracted from Fig. 4.1) of differentially expressed transcripts in two *V. dahliae* isolates Vd1396-9 (highly aggressive) and Vs06-14 (weakly aggressive) in response to elicitation with root extracts from susceptible potato cultivar Kennebec and moderately resistant Ranger Russet. The transcripts are identified as follows: primer combination number – PAGE fragment number. The percentage of recovery from the PAGE was about 73% (185/252) and some of the transcripts were not successfully re-amplified (e.g., 11-69)

Table 4.3. BLAST search against NCBI GenBank database of *Verticillium dahliae* transcripts differentially expressed in isolates Vd1396-9 (highly aggressive) and Vs06-14 (weakly aggressive) in response to elicitation with root extracts from the susceptible potato cultivar Kennebec and the moderately resistant Ranger Russet. The transcripts are identified as follows: primer combination number – PAGE transcript fragment number, followed by their size after sequencing and their presence or absence after subtractive hybridization (SH). Homologous sequences from the GenBank database are listed along with the corresponding *E*- values.

Transcript ID	Size (bp)	Presence / Absence after SH				Homology	*E* value
		1	2	3	4		
2-13	101	+	+	+	+	*Terfezia boudieri* at preinfection of *Cistus incanus* hairy root clones gb|DV205800.1	0.002
7-20	46	+	+	+	+	*V. dahliae* isolate cotton001 rRNA gb|EU835817.1	$6e^{-04}$
12-102	178	+	+	+	+	*Gibberella moniliformis* hypothetical protein during infection to maize gb|DR631451.1|	$2e^{-13}$
12-113	113	+	+	+	+	Uncultured ascomycete clone bt7g1 28S rRNA gene gb|AY273325.1	$2e^{-04}$
13-125	247	+	+	+	+	*V. dahliae* cDNA VD0109E06 VD01. ESTs during pathogenic growth and microsclerotia development gb|BQ110502.1	$1e^{-86}$
14-140	265	+	+	+	+	*Thielavia terrestris* pooled RNA from cells grown in MY50 (rich medium) and minimal medium with Arbocel (cellulose) gb|GR261402.1|	$2e^{-34}$
14-144	60	+	+	+	+	*Aspergillus terreus* NIH2624 conserved hypothetical protein CYCLIN Domain (ATEG_02641) partial mRNA ref|XM_001211819.1	0.029
2-158	195	+	+	+	+	*V. dahliae* isolate 111 rRNA, partial sequence, Phylogenetic Analyses of Phytopathogenic Isolates gb|DQ165241.1	$2e^{-51}$
2-161	186	+	+	+	+	*V. dahliae* crucifer isolate Variation in *V.dahliae* isolate, MD73 small subunit rRNA group I intron, partial sequence gb|AY056821.1	$1e^{-10}$
4-173	101	+	+	+	+	*V. albo-atrum* strain ATCC MYA-4576 18S ribosomal RNA gene, partial sequence gb|GU291258.1|	$6e^{-35}$
5-183	62	+	+	+	+	*Xanthomonas campestris* pv. *Campestris* complete genome, strainB100 Features in this part of subject sequence: Putative secreted protein	0.11
6-185	276	+	+	+	+	*Schistosoma mansoni* genome sequence supercontig Smp_scaff005928 ref|NW_003031502.1|	$1e^{-06}$
7-192	254	+	+	+	+	*Colletotrichum higginsianum* AM496479 mature appressoria IMI 349063A *C. higginsianum* cDNA clone b6-SP6, mRNA sequence emb|AM496479.1	$8e^{-45}$
7-195	50	+	+	+	+	Uncultured ectomycorrhiza (Rhizopogonaceae) isolate 18_ENYO_lp_320 internal transcribed spacer 1, partial sequence; 5.8S gb|DQ351517.1|	$2e^{-07}$
8-200	56	+	+	+	+	*Ovis aries* cDNA, mRNA sequence gb|EE874202.1	$6e^{-04}$
9-229	93	+	+	+	+	*Oryza sativa* (japonica cultivar-group) cv. Azucena cDNA-AFLP fragment cDNA clone 20_3c, mRNA sequence gb|DQ883903.1	$9e^{-25}$
16-249	59	+	+	+	+	*V. dahliae* isolate 45 rRNA partial seq. gb|DQ165200.1	$1e^{-13}$
a		+	+	+	+	No match	nd
12-96	244	+	+	+	-	*Anopheles gambiae* str. PEST, AGAP000417-PA. Conserved domain in *Neisseria meningitidis* TspB protein. This family consists of several *Neisseria meningitidis* TspB virulence factor proteins	0.008
12-100	226	+	+	+	-	*Penicillium marneffei* ATCC 18224 conserved hypothetical protein, ref|XP_002148360.1|	2.8
13-126	243	+	+	+	-	*Neosartorya fischeri* NRRL 181 hypothetical protein	$1e^{-27}$

						NFIA_061320, ref|XP_001267665.1|	
7-194	121	+	+	+	-	*V. tricorpus* gene for 5.8S r RNA emb|Z29524.1	$1e^{-05}$
8-197	314	+	+	+	-	*Neosartorya fischeri* NRRL 181 hypothetical protein NFIA_061320, ref|XP_001267665.1	$3e^{-19}$
b		+	+	+	-	No match	nd
10-32	380	+	+	-	-	*Neurospora crassa* OR74A hypothetical protein NCU06961 similar to exo-polygalacturonase gb|EAA33365.2	$5e^{-14}$
12-101	195	+	+	-	-	*Magnaporthe grisea* 70-15 hypothetical protein (MGG_02688) partial mRNA ref|XM_366612.2	0.046
12-106	156	+	+	-	-	*V. dahliae* VD0106H01 VD01 cDNA. ESTs during pathogenic growth and microsclerotia development gb|BQ110278.1	$7e^{-11}$
14-142	202	+	+	-	-	*V. dahliae* isolate 147 small subunit rRNA group I intron, partial sequence gb|AY056822.1	$1e^{-08}$
2-159	155	+	+	-	-	*V. dahliae* crucifer isolates, Variation in *V. dahliae* isolates small subunit rRNA group I intron,gb|AY056822.1	$2e^{-69}$
4-174	109	+	+	-	-	*Colletotrichum higginsianum* AM496673 mature appressoria IMI 349063A clone 1-h12-T7 emb|AM496673.1	$6e^{-09}$
5-178	269	+	+	-	-	*Neurospora crassa* G688P572RB3.T0 cDNA - 4 hours Vegetative Growth in constant light *Neurospora crassa* cDNA, mRNA sequence gb|GH277745.1|	$3e^{-18}$
c		+	+	-	-	No match	nd
11-79	141	+	-	-	-	*Phillyrea latifolia* ,hypothetical protein precursor emb|CAK18872.1	$2e^{-15}$
10-35	311	+	-	-	-	*Callithrix jacchus* chromosome UNK clone CH259-98H12, complete gb|AC187129.1	$5e^{-04}$
12-93	338	+	-	-	-	*Sclerotinia sclerotiorum* 1980 hypothetical protein SS1G_03763 gb|EDO01289.1	1.0
5-176	235	+	-	-	-	*Neosartorya fischeri* NRRL hypothetical protein NFIA_061320 181 ref|XP_001267665.1	$5e^{-31}$
d		+	-	-	-	No match	nd
1-204	50	-	-	+	+	*V. dahliae* mitochondrion, complete genome gb|DQ351941.1	$7e^{-07}$
4-210	136	-	-	+	+	*V. dahliae* var. *longisporum* strain K2 rRNA complete sequence gb|AY555951.1	$3e^{-09}$
5-215	117	-	-	+	+	*Botrytis cinerea* expressed sequence tags *Botryotinia fuckeliana* cDNA 5', mRNA sequence gb|EB803893.1	0.007
8-225	85	-	-	+	+	*V. dahliae* mitochondrion, complete genome gb|DQ351941.1	$1e^{-23}$
7-221	103	-	-	+	+	*V. dahliae* mitochondrion, complete genome gb|DQ351941.1|	0.071
f		-	-	+	+	No match	nd
g		+	-	+	+	No match	nd
3-206	205	-	-	+	-	*V. dahliae* rRNA gene, complete sequence gb|AF104926.1	$4e^{-22}$
6-218	93	-	-	+	-	*V. dahliae* cytochrome oxidase subunit I ref|YP_667836.1	$7e^{-04}$
6-220	106	-	-	+	-	*V. dahliae* mitochondrion, complete genome, gb|DQ351941.1	$1e^{-24}$
h		-	-	+	-	No match	nd

1= (Vd1396-9 x Kennebec) Minus (Vd1396-9 x SDW), 2= (Vd1396-9 x Ranger Russet) Minus (Vd1396-9 x SDW), 3= (Vs06-14 x Kennebec) Minus (Vs06-14 x SDW), 4= (Vs06-14 x Ranger Russet) Minus (Vs06-14 x SDW); +/- = Presence/Absence after subtractive hybridization step (SH). (a) includes transcripts 1-2, 1-3, 1-5, 1-6, 5-19, 10-33, 10-42, 10-47, 10-55, 10-56, 10-57, 10-58, 10-59, 10-60, 10-61, 10-62, 10-63, 10-64, 10-65, 10-66, 10-68, 11-74, 11-82, 11-88, 12-94, 12-103, 12-109, 12-114, 12-115, 12-116, 12-117, 12-118, 12-119, 12-120, 12-122, 13-128, 13-132, 13-133, 13-134, 13-137, 15-150, 15-151, 16-153, 16-155, 16-156, 2-162, 3-164, 3-167, 3-168, 3-169, 4-171, 4-172, 4-175, 6-190, 8-199, 9-228, 9-230, 16-251, 16-252; (b) encompasses transcripts 11-85, 5-181, 5-182, 6-186, 6-188, 6-189; (c) regroups transcripts 1-4, 2-14, 10-34, 10-37, 10-38, 10-39, 10-40, 10-41, 10-43, 10-44, 10-45, 10-46, 10-48, 10-49, 10-50, 10-51, 10-52, 10-53, 10-54, 11-71, 11-75, 12-95, 12-98, 12-99, 12-104, 12-105, 12-107, 12-110, 12-111, 13-129, 13-130, 16-154, 2-160, 3-165, 5-179, 5-180, 6-187, 8-198; (d) transcripts 1-1, 12-112, 13-124, 16-152, 3-163, 4-170, 6-184, 9-21; (f) transcripts 1-201, 1-202, 1-203, 2-205, 3-207, 3-208, 3-209, 4-213, 5-214, 5-216, 5-217, 7-222, 7-223, 8-224, 10-231, 13-232, 14-233, 14-234, 14-235, 14-236, 15-240, 15-241, 15-242, 15-243, 15-244, 16-246, 16-247, 16-248; (g) transcripts 4-211, 4-212, 14-141; (h) transcripts 15-237, 15-238.

respectively. Several transcripts differentially expressed in all of the four tested cases (i.e. 12-102, 13-128, 13-132, 14-140, and 14-144) exhibited homology with transport protein SEC31, glucan endo-1,3 α-glucosidase agn1, serine/threonine protein kinase, DNA repair protein RAD51, and nuc-1 negative regulatory protein preg, with *E* values of 0.0, 0.0, $5.6x10^{-44}$, 0.0, and $1.7x10^{-8}$, respectively. Transcripts 12-106 and 4-174, expressed only in the isolate Vd1396-9 after exposure to both root extracts, demonstrated similarity to stress response regulator SrrA and RasGAP-like protein, with E=$4.2x10^{-24}$ and $5.4x10^{-4}$, respectively. Transcript 14-141 had homology to LysM domain-containing protein (E=$6.8x10^{-17}$), whereas transcript 15-237, expressed only in isolate Vs06-14 after treatment with Kennebec root extract, showed homology to an ATP-dependent RNA helicase SUV3 (E=0.0). Another eleven transcripts exhibited similarity with conserved hypothetical proteins with unknown functions.

4.5 Discussion

Understanding the molecular basis of the *V. dahliae*-potato interaction is a mean to discerning the pathogen's weaknesses and strengths, and may lead to successful knowledge-based disease control strategies. In the present study, we used a differential model relying on two *V. dahliae* isolates with contrasting levels of aggressiveness and two potato cultivars with divergent responses to Verticillium wilt (Section 3.0). Given that the interaction between potato and *V. dahliae* starts through signal cues contained in root exudates before any physical contact, root extracts of both cultivars were used to induce potential pathogenicity-related genes in the two selected isolates. This represents the first essential step in understanding the intricacies of this host-pathogen interaction.

Table 4.4. BLAST search against the Broad Institute of MIT and Harvard genome database of *V. dahliae* transcripts differentially expressed in isolates Vd1396-9 (highly aggressive) and Vs06-14 (weakly aggressive) in response to elicitation with root extracts from the susceptible potato cultivar Kennebec and the moderately resistant Ranger Russet. The transcripts are identified as follows: primer combination number – PAGE transcript fragment number. Homologous sequences from the Broad Institute VdLs.17 *V. dahliae* genome database are listed along with the corresponding *E*- value. Chr Chromosome; U= Unpositioned scaffolds.

Transcript ID	Homologous sequences and function if any	Full Length Gene 'nt' (Potein 'aa')	Location	*E*-value
2-14	VDAG_02445 - DNA-(apurinic or apyrimidinic site) lyase	2300 (550)	Chr3	$8.3e^{-11}$
10-32	VDAG_03463 - exopolygalacturonase	1836 (461)	Chr6	0.0
10-55	VDAG_08098 – polygalacturonase	1549 (440)	Chr6	$8.3e^{-12}$
10-56	VDAG_08098 – polygalacturonase	1549 (440)	Chr6	$6e^{-16}$
10-62	VDAG_08134 - glutaminyl-tRNA synthetase	2216 (634)	Chr6	$3.6e^{-16}$
10-63	VDAG_08134 - glutaminyl-tRNA synthetase	2216 (634)	Chr6	$2e^{-11}$
11-82	VDAG_10098 - conserved hypothetical protein	3942 (1008)	Chr1	$4.1e^{-4}$
12-93	VDAG_08205 - myo-inositol 2-dehydrogenase	1802 (306)	Chr6	$1e^{-32}$
12-96	VDAG_08192 - conserved hypothetical protein	5495 (1805)	Chr6	0.0
12-98	VDAG_08192 - conserved hypothetical protein	5495 (1805)	Chr6	$7e^{-18}$
12-101	VDAG_04524 - conserved hypothetical protein	1994 (602)	Chr1	0.0
12-102	VDAG_08922 - transport protein SEC31	709 (188)	Chr5	0.0
12-105	VDAG_06127 - conserved hypothetical protein	2949 (650)	Chr8	$9.8e^{-4}$
12-106	VDAG_02250 - stress response regulator SrrA	2245 (638)	Chr7	$4.2e^{-24}$
12-117	VDAG_02637 - conserved hypothetical protein	1055 (311)	Chr3	$5.7e^{-28}$
12-122	VDAG_10325 - conserved hypothetical protein	2021 (573)	U	$8.4e^{-7}$
13-124	VDAG_07735 - xanthine dehydrogenase	4220 (1291)	Chr5	$9.8e^{-5}$
13-125	VDAG_09518 - conserved hypothetical protein	1390 (273)	Chr7	0.0
13-128	VDAG_04101 - glucan endo-1,3-alpha-glucosidase agn1	4261 (1171)	Chr1	0.0
13-132	VDAG_04632 - serine/threonine-protein kinase	2127 (691)	Chr3	$5.6e^{-44}$
13-134	VDAG_04616 - conserved hypothetical protein	5675 (1875)	Chr3	$1.4e^{-4}$
14-140	VDAG_08796 - DNA repair protein RAD51	1194 (354)	Chr1	0.0
14-141	VDAG_00902 - LysM domain-containing protein	1182 (375)	Chr1	$6.8e^{-17}$
14-144	VDAG_06766 - nuc-1 negative regulatory protein preg	1366 (433)	Chr8	$1.7e^{-8}$
16-155	VDAG_05363 - conserved hypothetical protein	2766 (921)	Chr2	$4.2e^{-32}$
2-162	VDAG_07925 - predicted protein	1974 (657)	Chr2	$1.7e^{-8}$
4-174	VDAG_01012 - RasGAP-like protein	5175 (1701)	Chr1	$5.4e^{-4}$
5-181	VDAG_01757 - conserved hypothetical protein	1957 (568)	Chr7	$1.2e^{-4}$
15-237	VDAG_10122 - ATP-dependent RNA helicase SUV3	2301 (748)	U	0.0

In the 573 differentially expressed genes detected on the PAGE gel, in both highly- (Vd1396-9) and weakly aggressive (Vs06-14) isolates, the number of differentially expressed genes in response to susceptible Kennebec root extract was higher (165 and 150, respectively) than in response to moderately resistant Ranger Russet root extract (136 and 122, respectively), with more genes induced in the highly aggressive isolate. This suggests that many of the induced genes are pathogenicity-related, especially in isolate Vd1396-9 after exposure to Kennebec (susceptible) root extract. Ranger Russet, as a moderately resistant cultivar may have released several antimicrobial compounds (Heinz et al. 1998; van Loon et al. 2006; Wang et al. 2008), phytoalexins, or other defense molecules (Daayf et al. 1997; Arfaoui et al. 2007) that could have inhibited some of the pathogenicity-related genes in *V. dahliae* isolates (El Hadrami et al. 2009a,b).

Potato root exudates play a role in stimulating primary inoculum of the *V. dahliae*. However, the type of genes involved in this early process of *V. dahliae* pathogenesis and their variation in expression according to the aggressiveness of the isolates were unknown. In order to address this issue, a SH/cDNA-AFLP approach (Henriquez and Daayf 2010) was used to simultaneously analyze different treatments and remove constitutively and commonly expressed genes. Using this system, 90 transcripts that were differentially expressed both in the highly- and weakly aggressive isolates and another 45 only in the highly aggressive isolate were identified.

The GenBank database search showed that a high number of sequences (144 out of the initial 185 sequenced) had no match. This may be due to the fact that these transcripts were amplified after an initial subtractive screening that had eliminated

commonly expressed genes and/or to the fact that the majority of sequences in the GenBank are from pathogens grown under other conditions not necessarily related to pathogenesis. Conceptually, once *V. dahliae* is stimulated by the host root exudates, the machinery of protein synthesis starts to produce the required tools (El Hadrami et al. 2009a,b), such as pathogenicity factors, to attack and penetrate the host roots. We found numerous sequences showing similarities with rRNA and proteins involved in amino-acid and protein transport from either *V. dahliae* or related fungi (Tables 4.3; 4.4).

Transcripts such as 13-125, 12-106, 7-192 and 4-174 were revealed to be associated with *V. dahliae* pathogenicity since they all had homology with sequences isolated during the pathogenic growth of *V. dahliae* or from mature appressoria from *C. higginsianum* (Neumann and Dobinson 2003; Kleemann et al. 2008). Also, transcript 10-32, detected only in the highly aggressive isolate, as a homologue of a *V. dahliae* exo-polygalacturonase and the model fungus *N. crassa* indicates its potential role as a pathogenicity-related gene. Polygalacturonases are known as pathogenicity factors in many fungi because they are responsible for the degradation of pectin, one of the main components of the host cell-wall (Di Pietro and Roncero 1998; ten Have et al. 1998; Huang and Mahoney 1999; Isshiki et al. 2001; James and Dubery 2001). Likewise, transcript 2-13 could play a role *prior to* infection since it exhibited similarities with *Terfezia boudieri* transcripts expressed at the pre-infection stage of *Cistus incanus* (Zaretsky et al. 2006). Meanwhile, the homology of transcript 12-106 with the stress response regulator SrrA suggests the activation of signal transduction pathway(s) for stress tolerance in the pathogen after sensing plant roots extract. In *Aspergillus nidulans*, two response regulators SrrA and SskA, are known to be essential for stress signal

transduction and asexual sporulation and perhaps even in fungicide sensitivity and spore viability (Vargas-Pérez et al. 2007). Activation of signaling cascades in *V. dahliae* upon elicitation by root extracts is also supported by the homology of transcript 13-132 with a serine/threonine protein kinase responsible for the phosphorylation/dephosphorylation of many proteins including the ones that are pathogenicity-related or involved in fungicide resistance (Orth et al. 1995; Dufresne et al. 1998). Transcript 13-124, on the other hand, has been revealed to be a homologue to a xanthine dehydrogenase, which may play a role in overcoming the action of certain host defense molecules using mechanisms such as the one described by El Hadrami et al. (2009b). Transcript 4-174, which had homology with RasGAP-like protein, would contribute to regulating *V. dahliae* growth and pathogenesis as it was hypothesized to play the same role in *Fusarium graminearum* (Bluhm et al. 2007).

The putatively identified differentially expressed transcripts indicate potential involvement in several pathogenicity-related functions such as cell-wall degradation (polygalacturonases and exo- polygalacturonases), protein secretion (transport protein SEC31), signal transduction (serine/threonine protein kinase) and stress responses regulation (stress response regulator SrrA). Many transcripts matched with sequences from a collection of *V. dahliae* ESTs during pathogenic growth and microsclerotia formation. These data support the role of these candidate genes in *V. dahliae* pathogenesis.

Many of the unmatched *V. dahliae* sequences could also be pathogenicity-related transcripts yet to be identified or candidates involved in the signaling pathways directly involved during pathogenesis or indirectly regulating the process as transcriptional

factors. Extensive functional genomics analysis of those sequences is still needed to understand their role. Further in-depth functional genomic analysis of selected differentially expressed genes needs to be investigated to explain, in detail, their role in *V. dahliae* pathogenesis.

‡ 5.0 COMPARATIVE PROTEOMIC ANALYSIS OF THE PHYTO-PATHOGENIC SOILBORNE FUNGUS *VERTICILLIUM DAHLIAE* REVEALS POTENTIAL PATHOGENICITY FACTORS IN AGGRESSIVE ISOLATES

5.1 Abstract

Verticillium dahliae is a phytopathogenic soilborne fungus causing vascular wilt and losses in a broad range of economic crops worldwide. We used the highly (Vd1396-9) and weakly (Vs06-14) aggressive isolates of *V. dahliae* to conduct a comparative proteomics investigation, as a means to identify pathogenicity factors of this pathogen. Twenty five protein spots were consistently produced by only one or the other isolate (18 from Vd1396-9, 7 from Vs06-14). Following 2-DE, the 25 differential spots identified using LC-ESI-MS/MS and MASCOT database search engine analysis of fungal sequences predicted pathogenesis-related functions such as colonization, melanin biosynthesis, survival structure formation, antibiotic resistance, and fungal penetration. These are important functions for infection of the host and extension of the pathogen's longevity in soil. One protein was identified as isochorismatase hydrolase, a potential plant-defense suppressor, only in the highly aggressive isolate. This enzyme may inhibit the production of salicylic acid, known in plant-defense signaling. Other proteins were related to protein degradation, phosphonate hydrolysis and phosphoryl groups transfer. The majority of these potential pathogenicity factors were identified in the highly

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‡ **This is the pre-peer reviewed version of the following article:**
El-Bebany, A. F., Rampitsch, C., and Daayf, F. 2010. Proteomic analysis of the phytopathogenic soilborne fungus *Verticillium dahliae* reveals differential protein expression in isolates that differ in aggressiveness. Proteomics 10: 289 – 303. DOI 10.1002/pmic.200900426

aggressive isolate. In combination with functional genomics, proteomic analysis will help understand the molecular basis of *V. dahliae* pathogenesis and help further develop management strategies for this disease. This is the first comparative proteomics-based investigation of *V. dahliae*.

5.2 Introduction

Verticillium dahliae Kleb. is a phytopathogenic soilborne fungus and one of the main causal agents of vascular wilt in a wide range of plant species worldwide. *Verticillium dahliae* is known for its high pathogenic variability and induces both external (leaf chlorosis, necrosis) and internal (vascular discoloration) symptoms in its hosts. Wilting is the ultimate symptom and is often accompanied by stunting, eventually resulting in reduced yield and quality. *Verticillium dahliae* produces microsclerotia as survival structures that can remain in the soil for more than a decade. Their germination is stimulated by plant root exudates released in the soil. The germinated hyphae penetrate host roots, colonize the cortex, and enter the xylem. There, the fungus reproduces asexually by forming conidiospores, which move upward in the xylem, germinate, and penetrate new vessels. The hyphae, along with some products of the plant defense reactions, eventually plug the vessels and prevent water from reaching the plant's aerial parts, resulting in wilt. At the end of the disease cycle, the fungus forms microsclerotia on the plant's dead tissues. Due to the soilborne nature of Verticillium wilt, its control necessitates integrated disease management programs (Pegg and Brady 2002; Fradin and Thomma 2006). In the absence of true host resistance, the success of such strategies relies

on understanding *V. dahliae*'s pathogenicity factors and ultimately how to manipulate them for reducing the impact of the disease.

Proteomics, based on a combination of two-dimensional gel electrophoresis (2-DE), followed by mass spectrometry (MS) analysis, is a key research tool to study microbial pathogens in terms of their proteome maps, stage-specific proteomics, and pathogenicity factors. Such information is reliable and relevant to host-pathogen interactions, because of the biological functions these proteins hold (Bhadauria et al. 2007). Integration of knowledge from genomic studies, such as gene expression and function with proteomics-based studies is an important combination in biology in general and plant disease resistance improvement in particular (Jordan et al. 2006).

Functional genomics of *V. dahliae* have been investigated in a few studies (Klimes and Dobinson 2006). *Verticillium dahliae* has been reported to produce phytotoxic protein-lipopolysaccharides (Buchner et al. 1982) and cell wall degrading enzymes such as polygalacturonases and cellulases (Pegg and Brady 2002). However, to our knowledge, comprehensive proteomics analysis of *V. dahliae* has not been reported so far. Complementation between functional genomics and proteomics will be a corner-stone in systems biology of *V. dahliae* since the generated information will rely on several biological levels and will help understand the molecular basis of its interaction with host plants.

The objectives of this chapter were to optimize a proteomic procedure for *V. dahliae* and then conduct a comparative proteomic analysis between a highly and a weakly aggressive isolate of this pathogen. Since proteins are related directly to functions in biological systems and because of the differences that we found in 2-DE gels of the

two aggressiveness-contrasting isolates, and considering the advantage of *V. dahliae* genome draft sequence availability at the Broad Institute of MIT and Harvard, our results will help identify pathogenicity factors that are used by this important pathogen to attack and/or suppress plant defense mechanisms. Identifying pathogenicity factors through proteomics will help identify pathogen-specific targets that could be used for disease control and avoid affecting non-target microorganisms in soil.

5.3 Materials and Methods

5.3.1 Fungal isolates and culture conditions

Two single-spore derived *V. dahliae* isolates were selected, from 60 pre-screened isolates, based on the aggressiveness of their pathogenicity on differential potato cultivars. Disease severity, percent of infection, and vascular discoloration for all the 60 *V. dahliae* isolates were assessed (Uppal et al. 2007; Alkher et al. 2009). Vd1396-9, a highly aggressive, and Vs06-14, a weakly aggressive, isolates were re-assessed to confirm their levels of pathogenicity on two differential potato cultivars (Section 3.0). *Verticillium dahliae* isolates were grown on Potato Dextrose Agar (PDA) medium. Fungal growth on 0.9 mm-diameter plugs were chosen from the edge of the growing culture and transferred into 50 ml of autoclaved Czapek-Dox Broth (CDB) liquid medium (Difco Laboratories, MD, USA). Six independent biological replicates were used per isolate. The cultures were incubated in the dark at room temperature (22.5 ± 0.5°C) on a shaker at 120 rpm (C2 Platform Shaker, Edison, NJ, USA). After 2 weeks of incubation, the fungal biomass was harvested, ground in liquid nitrogen and stored at – 80°C until used for proteomic analysis.

5.3.2 Protein extraction and quantification

Total protein was extracted from the fungal mycelium using an acetone/trichloroacetic acid (TCA) precipitation method (Damerval et al. 1986). Briefly, 0.75 to 1.0 gm of ground and – 80°C stored fungal mycelium was used for protein extraction. *Verticillium dahliae* samples were transferred from – 80°C to siliconized glass centrifuge tubes with the addition of 10 ml of pre-chilled acetone containing 10% (w/v) TCA and 0.07% (w/v) dithiotheritol (DTT), and kept at – 20°C for 2 to 3 h. The samples were centrifuged at 9,680 g for 20 min, at – 5°C. The supernatants were discarded and the pellets were washed with pre-chilled acetone/DTT 0.07% (w/v) solution and stored overnight at – 20°C. The acetone/DTT 0.07% (w/v) washing step was repeated seven times under the same centrifugation conditions. The pellets were dried carefully under a gentle stream of nitrogen gas to remove all acetone. The dried samples were stored at – 80°C. Samples were dissolved in 1.5 to 2 ml of isoelectric focusing (IEF) solution consisting of 7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 20 mM DTT and 0.5 % (v/v) of pH 3 – 10 ampholyte (Bio-Rad Laboratories, Hercules CA, USA). The IEF dissolving step was carried out on a shaker for 2 hours. The samples were sonicated (Misonix, Farmingdale NY, USA) five times for 5 sec. each time with immersion of the sample tube in water during sonication to maintain a constant temperature of 22°C. The samples were then centrifuged at 9,000 *g* for 20 min, at 22°C. The supernatants were transferred into microfuge tubes and re-centrifuged at 16,100 *g* for 20 min, at 22°C, and the supernatants stored at – 80°C. The supernatants were concentrated and purified by IEF buffer exchange through centrifugation at 3,800 *g* for 30 min, at 22°C in spin tubes

(Vivaspin, Viva Scientific, UK) with a 5 kDa cut-off. The supernatants were centrifuged at 90,000 *g* (Airfuge, Beckman Coulter, USA) for 30 min to eliminate fine particulate matter. The protein was quantified using a 2-D Quant Kit (Amersham Biosciences Corp. NJ, USA) according to the manufacturer's instructions and the volume adjusted to contain 500 µg protein/450 µl of IEF buffer for the next step.

5.3.3 2-DE (IEF/ SDS-PAGE) and gel analysis

Immobile pH gradient (IPG) strips (24 cm, pH 4-7, Immobiline DryStrip, GE Healthcare Bio-Sciences AB, Uppsala, Sweden) were rehydrated with 500 µg protein/450 µl for each sample in a reswelling tray at 22°C. Strips in the tray were covered by fluid oil (Dry Strip Fluid, Amersham Biosciences), balanced horizontally and left overnight at 22°C. Rehydrated IPG strips were transferred for isoelectric focusing (Multiphor II, Amersham Biosciences) at 20°C. The IEF voltage program was applied for 24 hrs for a total of 58.2 kvhr. IPG focused strips were transferred into an equilibration tray and covered with 10 ml of equilibration buffer (50 mM Tris.HCl pH 8.8, 6 M urea, 30% (v/v) glycerol, 20% (w/v) SDS, and a trace of bromophenol blue in addition to 1% (w/v) DTT) with gentle shaking for 8 min and this step was repeated once. The same steps were repeated twice with 2.5 % (w/v) idoacetamide in the equilibration buffer instead of 1 % (w/v) DTT.

The equilibrated IPG strips were subjected to 12 % SDS-PAGE for the second dimension (Ettan Dalt-6, Amersham Biosciences). Electrophoresis was carried out with 0.5 W/gel for the first hour followed by a 17 W/gel until the bromophenol blue dye marker reached the bottom of the gel. The gels were fixed in 12.5% (w/v) TCA for 30

min. and stained overnight with Coomassie brilliant blue (CBB) G-250 on a shaker, then destained with nanopure water several times until clean. The gels were scanned and the images saved as TIF files and printed for visual analysis. The 2-DE gels were prepared from six independent biological replicates for each isolate. Only consistently differentially expressed protein spots were processed for identification using LC-ESI-MS/MS.

5.3.4 In-gel digestion

Twenty five protein spots were excised from the 2-DE gels, cut to pieces of 1 mm^2 and digested in-gel by trypsin (Sequencing grade modified trypsin, Promega, Madison, WI, USA). Briefly, in ice, trypsin digestion buffer (100 mM NH_4HCO_3, 10 % (v/v) acetonitrile (ACN), 2.5 mM $CaCl_2$) was added to cover the gel pieces and left for 30 min. Trypsin concentration in the digestion buffer was adjusted to 12 ng/µl. Samples were incubated at 37°C overnight. Peptides were extracted from the gel through vortexing and centrifugation in a series of formic acid (FA)/acetonitrile (CAN) (v/v) extraction solutions as follows: 5% FA, 1% FA/ 5% ACN, 1% FA/ 60% ACN, and 1% FA/ 99% ACN. The collected supernatants which contained the peptide mixtures were dried in a Speed Vac and peptide mixtures were taken for MS analysis.

5.3.5 Mass spectrometry and database searches

Mass spectrometric analysis by LC-ESI-MS/MS for the peptide mixtures was conducted using a nanoflow HPLC system (Ultimate 3000: Dionex, Germany) to separate peptides on an in-house packed C_{18} column (5 µm particle size, 300 Å pores, 10 cm)

eluting directly into a linear ion trap mass spectrometer (LTQ: Thermo Fisher, San Jose CA) at 250 nl/min *via* nano electrospray ionization. A 2% (v/v) ACN to 80% ACN (v/v) gradient in 1% (v/v) FA and 0.5% (v/v) acetic acid was used for HPLC, with a total run time of 65 minutes. A "Big 5" programme was used for MS/MS analysis: the five most abundant peaks in each survey scan were used as precursor ions for an MS^2 spectrum, with dynamic exclusion for 30 sec. The instrument was tuned on α[glu]fibrinopeptide, with the collision energy in CID set to 30. The MASCOT search engine (v2.2) was used for database searching. A created database consisted of the NCBInr fungal entries (http://www.ncbi.nlm.nih.gov/) (536620 sequences), common mammalian contaminants, and the Broad Institute of MIT and Harvard *V. dahliae* VdLs.17 genome (http://www.broad.mit.edu/annotation/genome/verticillium_dahliae/MultiHome.html), which was queried to assign tentative identities to the proteins. MASCOT searches were performed in May 2009 with the following parameters: trypsin with one missed cleavage permitted, fixed modification of C (cam), variable modifications of M (ox) and NQ (deam); the precursor tolerance was ±1Da, MS/MS tolerance was ±0.8 Da. Automatic decoy searches were included with each query.

5.4 Results and Discussion

5.4.1 Biological differences and 2-DE

The pathogenicity of *V. dahliae* isolates, Vd1396-9 and Vs06-14, was confirmed on two potato cultivars, Ranger Russet (moderately resistant), and Kennebec (susceptible). Disease severity, and vascular discoloration caused by the two isolates were assessed (Section 3.0). *Verticillium dahliae* isolates Vd1396-9 and Vs06-14 are

highly and weakly aggressive, respectively. A proteomic map (pH 4-7) of samples from the two isolates was achieved with comparative analysis of high quality 2-DE gels. Meticulous inspection and comparison of the proteome profiles from the two isolates revealed several qualitative variations when considering spots consistently produced only by one or the other isolate. Twenty five protein spots were consistently produced either by Vd1396-9 or Vs06-14. Protein spots 1 to 18 were differentially expressed in Vd1396-9, the highly aggressive isolate (Fig. 5.1A), whereas spots 19 to 25 were differentially expressed in Vs06-14, the weakly aggressive isolate (Fig. 5.1B).

5.4.2 MS analysis and database search

Tentative identification of differentially expressed protein spots was carried out using LC-ESI-MS/MS-MASCOT. All the 25 protein spots were identified using MASCOT search, except the protein spot 6. Although all fungal sequences from NCBI were included in the created searchable database used in this study, all of the protein spots matched with sequences from *V. dahliae* VdLs.17 (Table 5.1), a highly aggressive isolate from lettuce (Vallad et al. 2006).

The calculated molecular mass of the identified proteins ranged from approximately 13 to 63 kDa. The coverage percent of amino acid sequence with significantly matched peptides, high scoring peptides, number of peptides, and the total score for the identified protein are presented in Table 5.1. For example, spot 18 was found only in Vd1396-9, the highly aggressive isolate, and identified as a tetrahydroxynaphthalene reductase with 97% coverage, 20 significantly matched peptides with 2012 total ions score.

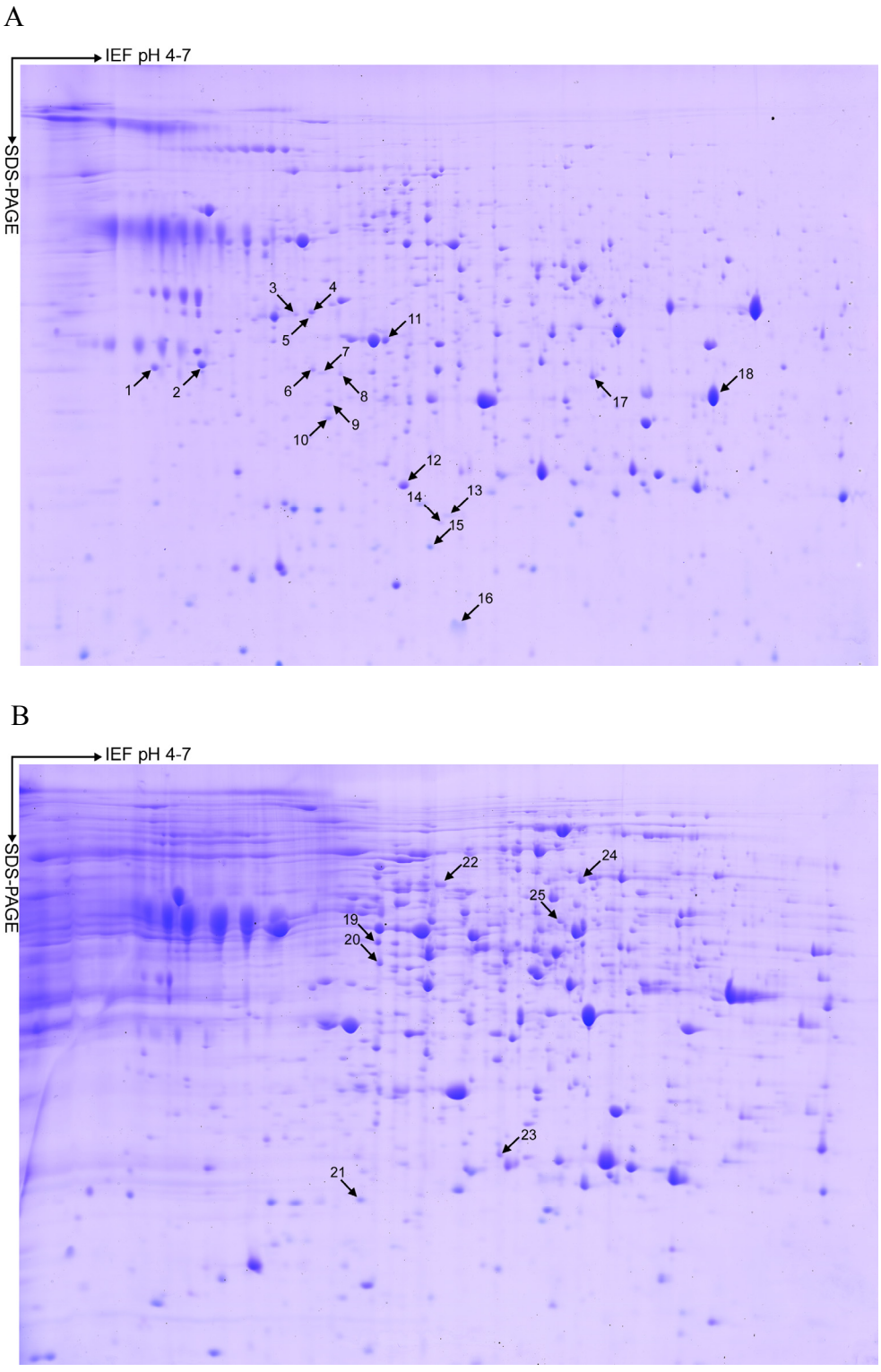


Figure 5.1. 2-D gels of proteins extracted from *V. dahliae* isolates Vd1396-9, the highly aggressive, (A) and Vs06-14, the weakly aggressive, (B). Differentially expressed proteins, indicated by arrows, were subjected to digestion and LC-ESI-MS/MS analysis.

Table 5.1. Identification of differentially expressed proteins in Vd1396-9, highly aggressive, and Vs06-14, weakly aggressive, *V. dahliae* isolates by LC-ESI-MS/MS and MASCOT

No.[a)]	Protein putative identity	ID	Organism	MS/MS-MASCOT High scoring peptides [b)]	Cov.[c)] %	Pept.[d)] No.	Total score[e)]
1	wos2	VDAG_08865	*Verticillium dahliae* VdLs.17	KNFVYLTITVPDVPASSLKL (104) KLDLKPTGLTFDGHSDTLK KD (66)	28	4	406
2	Elongation factor 1-beta	VDAG_04372	*V. dahliae* VdLs.17	KAPAAEEDEDDVDLFGSD DEEEDAEAARI (103) KSVVTLDVKPWDDETDMA ALEAAVRG (95)	54	5	557
3	Conserved hypothetical protein	VDAG_06120	*V. dahliae* VdLs.17	KLMVYDTEDLGALDLEKV (110) RGAEGTLGLSAIQGAELSG GLKV (98)	37	2	403
	Thioredoxin	VDAG_04529	*V. dahliae* VdLs.17	KQIAPLFQQLSETAAEIESV KF (84) RGYQDITDQIEPKG (58)	16	2	207
4	14-3-3 protein epsilon	VDAG_03126	*V. dahliae* VdLs.17	KQAFDDAIAELDSLSEESY RD (138) KSATDVAQTELTPTHPIRL (86)	68	10	941
5	F-actin-capping protein subunit beta	VDAG_04499	*V. dahliae* VdLs.17	RQVEQELAVEGDESHIANI GRL (106) KANEAFDVYRE (55)	29	3	396
	Allergen Asp f2-like protein	AAS45249.1 GI:42742377	*V. dahliae*	RTQLEAALAETMTIAAHAR D (97) RRPLAALCGGGYTVAESKL (68)	22	3	287
6	Conserved hypothetical Protein	VDAG_00509	*V. dahliae* VdLs.17	RLNDFDVLVVVGGGTDAV IKS (108) KSEAEPLQLIDAYSELQQK D (86)	44	3	437
7	Alpha-soluble NSF attachment protein	VDAG_01963	*V. dahliae* VdLs.17	KEALGELYETELGDTQKA (71) RCLDVAINQYCTKG (68)	69	7	727
8	Proteasome subunit alpha type-1	VDAG_08391	*V. dahliae* VdLs.17	KQLEPFQVLDGQDVKV (86) RIFQIEYAAEAVKQ (66)	56	6	615
9	NADH-ubiquinone oxidoreductase 29.9 kDa subunit	VDAG_09026	*V. dahliae* VdLs.17	RSALLYLYGSTLDRL (62) RLALVEAAVPPGYEEWNA ENKD (58)	35	2	305
10	HAD-superfamily hydrolase	VDAG_08490	*V. dahliae* VdLs.17	KASLVACPGVDEQLEALQ AGGKY (133) KLYNFTMEQEELETYVRR (93)	64	7	816
	Conserved hypothetical protein	VDAG_06867	*V. dahliae* VdLs.17	KDTQGGFLALAMAEASKL (102) RASVQQQGLDEDDVLAKY (70)	37	4	378

Table 5.1. Continued

No.[a)]	Protein putative identity	ID	Organism	MS/MS-MASCOT High scoring peptides [b)]	Cov.[c)] %	Pept.[d)] No.	Total score[e)]
11	Pyruvate dehydrogenase E1 component subunit beta	VDAG_01642	*V. dahliae* VdLs.17	KTLYMSGGIQPCNITFRG (104) REALNEALAEELESNEKV (84)	52	8	822
12	Isochorismatase hydrolase	VDAG_05103	*V. dahliae* VdLs.17	KVALAEIADVFGTLVSSKD (100) KISNIEASRPNISSLLEKY (76)	65	5	520
13	Glycine-rich RNA-binding protein	VDAG_03252	*V. dahliae* VdLs.17	KAIDAMNNVEFDGRT (85)	16	1	111
14	1,2-dihydroxy-3-keto-5-methyl-thiopentene dioxygenase	VDAG_06607	*V. dahliae* VdLs.17	KNRDEIIVSPEKM (59) RDEIIVSPEKM (56)	44	3	363
15	RPEL repeat protein	VDAG_06025	*V. dahliae* VdLs.17	RSADEKYEEAIEEEYAKR (68)	33	1	132
16	Ubiquitin-conjugating enzyme variant MMS2	VDAG_05365	*V. dahliae* VdLs.17	RNGVVDPQQLPCLAQWKR (67)	41	1	193
17	Serine 3-dehydrogenase	VDAG_09532	*V. dahliae* VdLs.17	KLDILVNNAGYILSGGVEE CSRA (138) RAATVIDDLAAGVDGTITA LDAYDRQ (126)	85	10	1287
	Uricase	VDAG_05692	*V. dahliae* VdLs.17	KNTGADAEVYAPQSGPNG LIKC (97) KMCEQILDAAPETKT (56)	35	3	381
18	Tetrahydroxynaph-thalene reductase	VDAG_03665	*V. dahliae* VdLs.17	RIPGPLGLASASLEGKVAL VTGAGRG (129) KVIVNYANSSESAEEVVQA IKKS (127)	97	20	2012
19	Argininosuccinate synthase	VDAG_10449	*V. dahliae* VdLs.17	RGCYDSPAMTLLRL (60)	35	1	462
	ATP-dependent RNA helicase eIF4A	VDAG_04288	*V. dahliae* VdLs.17	KGNDVIAQAQSGTGKT (97) KMFVLDEADEMLSRG (75)	8	2	178
20	Beta-lactamase family protein	VDAG_03942	*V. dahliae* VdLs.17	RLTAIVDNACADAKS (102) KVLAVLLNDGTCPRS (84)	44	7	806
21	CipC protein	VDAG_09386	*V. dahliae* VdLs.17	KQAESLYENQYGDLEQYD PRE (110) KELLAGFAGAEVDKLVET KG (102)	50	4	382
22	Inositol-3-phosphate synthase	VDAG_00041	*V. dahliae* VdLs.17	RELFTVNSSNVTYTDNEILS KY (87) RAANYYGSVIMSSTIKL (74)	47	8	821

Table 5.1. Continued

No.[a)]	Protein putative identity	ID	Organism	MS/MS-MASCOT High scoring peptides [b)]	Cov.[c)] %	Pept.[d)] No.	Total score[e)]
23	ATP synthase subunit 4	VDAG_04250	*V. dahliae* VdLs.17	KALQQVLAQSITDVEKL (90) KAQSLIDSLPGNSLLSKT (85)	46	5	552
	grpE	VDAG_07064	*V. dahliae* VdLs.17	RMTETVLMNTLEKH (81) KEGDAAAPESANPELDALK KQ (76)	30	5	391
24	Cyclopentanone 1,2-mono-oxygenase	VDAG_03943	*V. dahliae* VdLs.17	RCAVIGTGASGVQIVQNW GPKA (117) KDNLFNPEANEASYAFWA EKT (99)	45	7	1070
	Pyruvate decarboxylase	VDAG_09443	*V. dahliae* VdLs.17	KWVGSVNELNAGYAADG YARV (120) KEKIESADLILSIGTLKS (98)	16	3	375
25	Alanine aminotransferase	VDAG_05538	*V. dahliae* VdLs.17	KIYLSGGASSGVNTLLNVIC AGPKT (105) RALVVINPGNPTGASLPED NIRA (105)	39	7	786

a) Spot numbers correspond to the 2-D gels. Spots 1 to18 were present only in Vd1396-9, the highly aggressive isolate, and spots 19 to 25 were present only in Vs06-14, the weakly aggressive isolate.

b) The two highest scoring peptides with peptide ions score indicated between parentheses.

c) Coverage percentage of amino acid sequence with the statistically significant matched peptides.

d) Number of statistically significant matched peptides.

e) MASCOT total ions score of the statistically significant matched peptides higher than 48 indicate identity or extensive homology ($p < 0.05$).

5.4.3 Differential proteins in the highly aggressive *V. dahliae* isolate

Verticillium dahliae pathogenicity is associated with several factors including hydrolytic enzymes, toxins, and infectious and survival structures. These components are required to establish infection, colonization and disease development. The protein spots tentatively identified in the highly aggressive isolate, Vd1396-9, suggests various predicted functions related to pathogenicity and survival. Spot 1 exhibited similarity with the *wos2* gene, which encodes p23 protein, a co-chaperone for heat shock protein Hsp90 (Munoz et al. 2002). Also, *wos2* was identified through EST analysis of *Schizophyllum commune* grown under nitrogen starvation conditions (Guettler et al. 2003). Spot 3 (thioredoxin) is involved in penicillin biosynthesis in *Penicillium chrysogenum* and was found in *Cryphonectria parasitica*, the causal agent of chestnut blight disease (Cohen et al. 1994; Kim and Kim 2006). Thioredoxin was expressed in *Alternaria brassicicola* after exposure to brassicaceous defense metabolites and classified as an oxidative burst, stress and defense gene (Sellam et al. 2007). Since these two tentatively identified proteins were found in the highly aggressive isolate Vd1396-9 and under stress conditions in other fungi, both wos2 and thioredoxin may play a role in *V. dahliae* stress tolerance and survival.

Spot 5 was identified either as an F-actin-capping protein subunit beta or allergen Asp f2-like protein. Actin has been found in three protein spots in *Blumeria graminis* f.sp. *hordei* conidiospores (Noir et al. 2008), and the penetration peg of *Magnaporthe grisea* (Bourett and Howard 1992). The capability of pathogens to produce infectious structures efficiently is considered as a pathogenicity factor. Actin may be involved in *V. dahliae* conidiosporogenesis. Allergen Asp f2-like protein EST sequence was generated

from a *V. dahliae* pathogenic cotton isolate and functionally classified in the group of cell rescue, defense, and virulence sequences. It was studied as a potential wilt-inducing and virulence factor in *V. dahliae* (Wang et al. 2004). Spots 8 and 10 exhibited homology to enzymes involved in protein degradation and hydrolysis, respectively. Proteasome, tentatively identified in spot 8, is an enzyme complex that plays a role in protein processing and degradation activity (Coux et al. 1996). Its activity was detected in the mycelial extract of lignin-degrading white rot fungi (Staszczak, 2002) and in *Colletotrichum acutatum* during the appressorium development stage and under nitrogen deficiency conditions (Brown et al. 2008). Spot 10 had homology to two proteins, HAD-(haloacid dehalogenase) superfamily hydrolase and a conserved hypothetical protein. However, the highest total score (816) and the greater amino acids coverage percent and peptide number (64% and 7) to the HAD-superfamily hydrolase. This superfamily is conserved among a wide range of living organisms. It includes several phosphate- carbon bond hydrolases (i.e. phosphoesterases and phosphatases) (Burroughs et al. 2006), which may play a role in *V. dahliae* pathogenicity.

Interestingly, the protein from spot 12 showed homology with isochorismatase hydrolase, which is related to suppression of plant defenses. Isochorismate is known as a precursor for salicylic acid biosynthesis, which is an important signaling molecule that is required to activate plant defense mechanisms against a broad spectrum of pathogens (Wildermuth et al. 2001). Isochorismatase converts Isochorismate, in the presence of water, to 2, 3-dihydroxybenzoate and pyruvate. It was found in the secretome of five phytopathogenic fungi (Soanes et al. 2008). The importance of plant-defense suppression has been emphasized in several studies recently (Wang et al. 2008; El Hadrami et al.

2009a). We identified isochorismatase hydrolase in the highly aggressive isolate in protein spot 12 with 65% coverage, 5 peptides and total ion score of 520. Isochorismatase hydrolase may be acting as a plant-defense suppressor produced by the highly aggressive isolate of *V. dahliae* to overcome host resistance. This protein could be a target for disease control through inhibition of its activity in the pathogen or by supplying the host with plant defense activators to compensate for salicylic acid shortage caused by pathogen's isochorismatase hydrolase.

An ubiquitin conjugating enzyme was putatively identified in spot 16. Using Differential Display, an ubiquitin conjugating enzyme was reported in *Colletotrichum gloeosporioides* conidia after their contact with a hard surface, suggesting a role in conidial germination and appressoria differentiation (Liu and Kolattukudy 1998). Also, through expressed sequence tag analysis and suppression subtractive hybridization techniques, an ubiquitin-conjugating enzyme was identified during disease development and host-pathogen interactions in several pathosystems such as rice-*Magnaporthe grisea* (Kim et al. 2001a), and tomato-*Phytophthora parasitica* (Le Berre et al. 2008). Therefore, ubiquitin-conjugating enzymes may play a role in colonization and sporulation of *V. dahliae*.

Melanin pigment has been shown to be important component in appressorium maturation, facilitation of penetration, pathogenesis, and survival of many fungal pathogens (Howard and Ferrari 1989; Nosanchuk and Casadevall 2003). Protein spot 18 was identified, with 97% coverage, 20 peptides, and total ions score of 2012, as tetrahydroxynaphthalene (THN) reductase. THN is a component in melanin biosynthesis

via the polyketide pathway and melanin was reported to be involved in *V. dahliae* microsclerotia formation (Bell and Wheeler 1986).

The biological function of tetrahydroxynaphthalene reductase as an important enzyme in the melanin biosynthesis pathway matched with the phenotype of the highly aggressive isolate Vd1396-9 (Fig. 5.2). Pathogenicity of *Magnaporthe grisea* albino mutants was restored by gene transformation of the THN melanin pathway from *Alternaria alternata* (Kawamura et al. 1997). This protein may facilitate microsclerotia production and survival of *V. dahliae*. The highly aggressive isolate Vd1396-9 was also noticed to produce microsclerotia during its growth in CDB liquid medium.

Spot 4 was identified as a 14-3-3 protein, which is a multifunctional regulatory protein (Fu et al. 2000). A 14-3-3 protein was identified through proteomics studies of *Puccinia triticina* interactions with wheat (Rampitsch et al. 2006). Genetic analysis of a 14-3-3 protein revealed its role in the cell cycle and morphogenesis of *Ustilago maydis* (Mielnichuk and Pérez-Martín 2008) and signal transduction in the wheat-*Gaeumannomyces graminis* interaction (Guilleroux and Osbourn 2004). A 14-3-3 protein was also found in an EST analysis of mycelia and appressoria of *Magnaporthe grisea* (Soanes and Talbot 2005), which would suggest a role in morphogenesis and sporulation of *V. dahliae.* Other identified proteins are involved in cellular metabolic activities: elongation factor 1-beta (spot 2), NADH-ubiquinone oxidoreductase 29.9 kDa subunit (spot 9), Pyruvate dehydrogenase E1 component subunit beta (spot 11), Glycine-rich RNA-binding protein (spot 13), and serine 3- dehydrogenase (spot 17).

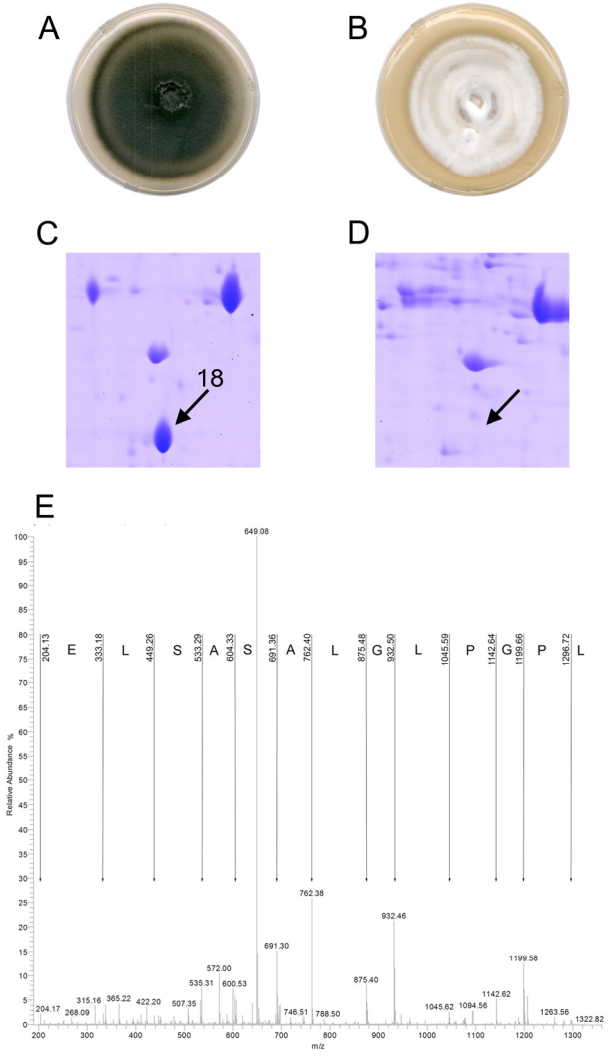


Figure 5.2. *V. dahliae* isolates Vd1396-9 (A), highly aggressive, and Vs06-14 (B), weakly aggressive, on PDA medium, and magnification of their 2-DE gels, (C) and (D), respectively, showing spot 18, (E) MS^2 spectrum and deduced amino acid sequence of the peptide (I^{16} to K^{30}) tentatively identified as tetrahydroxynaphthalene reductase (a key enzyme in melanin biosynthesis). Sequence coverage of VDAG_03665 was 97% with only a tri- and tetrapeptide undetected.

5.4.4 Differential proteins in the weakly aggressive *V. dahliae* isolate

In the weakly aggressive isolate, Vs06-14, a Beta-lactamase family protein was identified in spot 20. Beta-lactams are antibiotics produced by several microorganisms including fungi. There are five classes of beta-lactams, all of which have a beta-lactam ring (e.g. penicillins and cephalosporins), and it is well known that antibiotics produced by microorganisms can inhibit other microorganisms (Brakhage 1998). Beta-lactams have antifungal activity against some phytopathogenic fungi (Arnoldi et al. 1990). The production of beta lactamase is one of the strategies in some bacteria to resist beta-lactam antibiotics by hydrolyzing the beta-lactam ring and inactivating the antibiotic (Majiduddin et al. 2002). Beta-lactamase production in *V. dahliae* isolate Vs06-14 could help its survival in soil by inactivating antibiotics produced by other soil microorganisms. However, it is not clear why this protein was not detected in the highly aggressive isolate. It may be speculated that through co-evolution with host plants, some pathogens lose some of their pathogenicity factors, which became recognizable by the host plant as elicitors.

Protein spots 21 and 24 were putatively identified as concanamycin-induced protein C (CipC) and cyclopentanone 1, 2-monooxygenase, respectively. Concanamycin-induced protein C (CipC) (spot 21) was previously identified through proteomic analysis of *Aspergillus nidulans* treated with the antibiotic concanamycin A (Melin et al. 2002) and in *Stagonospora nodorum* (Tan et al. 2008). Cyclopentanone 1, 2- monooxygenase (spot 24) was identified originally in *Pseudomonas* sp. NCIMB 9872 and induced in *Comamonas* sp. after exposure to cyclopentanol or cyclopentanone (Griffin and Trudgill 1976; Iwaki et al. 2002). Most of the identified proteins in the weakly aggressive isolate

Vs06-14 were related to regular metabolism. Proteins from spots 19, 22, 23, and 25 had similarities with enzymes involved in amino acid metabolism and energy production.

5.5 Concluding Remarks

There are several applications of proteomics in plant pathology. From a host perspective, it could be used to compare resistant and susceptible cultivars in terms of resistance/susceptibility elements, and to identify resistance components that can be used to improve plant resistance through breeding programs. In the pathogen, it could be applied to compare virulent and avirulent isolates, different infectious stages, and to explore pathogenicity determinants. In host-pathogen interactions, proteomics could provide information about establishment of infection and colonization, and the effect of environmental conditions on disease development. In the present investigation, we report the first proteomic map of *V. dahliae* and demonstrate that comparative proteomics, including 2- DE and MS analyses, is an informative approach to discover isolate-specific proteins in *V. dahliae*. The tentatively identified proteins are involved in various pathogenesis-related functions, including infection, penetration, colonization, melanin biosynthesis, survival structure formation, antibiotic resistance, morphogenesis, conidiospore formation, and suppression of plant defense mechanisms. Most of the potential pathogenicity factors were differentially expressed in the highly aggressive isolate, which supports their role in *V. dahliae* pathogenesis.

Detecting pathogenicity factors specific to the highly aggressive isolate may in the future help design target-directed pesticides for disease control and explore the factors that are a challenge to the host plant. These potential pathogenicity factors, especially the

plant-defense suppressor, should be considered in plant resistance improvement programs. Its possible effect on plant defense suppression could be manipulated through application of plant defense activators like salicylic acid derivatives for disease control. More comprehensive proteomics of *V. dahliae*-host interactions combined with genomics studies using the same model will be useful in elucidating the basis of the molecular interactions in this pathosystem and assist towards a more successful disease management program.

6.0 ISOLATION, CLONING AND COMPARATIVE SEQUENCE ANALYSIS OF STRESS RESPONSE REGULATOR A, ISOCHORISMATASE HYDROLASE AND TETRAHYDROXYNAPHTHALENE REDUCTASE GENES FROM THE HIGHLY AND WEAKLY AGGRESSIVE *VERTICILLIUM DAHLIAE* ISOLATES

6.1 Abstract

Verticillium dahilae is a soilborne fungus that causes wilt in a wide range of economic crops. Using comparative transcriptomics and proteomics analyses, 185 and 25 differentially expressed genes and proteins, respectively, have been putatively identified in *V. dahliae* isolates Vd1396-9 and Vs06-14 (Sections 4.0 and 5.0). Many of the genes/proteins were identified as potential pathogenicity factors that could play a role in *V. dahliae* pathogenesis. In the present chapter, stress response regulator A (*VdSrrA*), identified through the transcriptomics analysis (section 4.0), and isochorismatase hydrolase (*VdIsoch*) and tetrahydroxynaphthalene reductase (*VdThnr*), both identified through a proteomics analysis (section 5.0), were selected based on their differential expression pattern in the highly (Vd1396-9) and weakly aggressive (Vs06-14) *V. dahliae* isolates. The three genes are putatively involved in pathogenesis-related functions: oxidative stress tolerance (*VdSrrA*), suppression of signaling plant defense (*VdIsoch*), and survival (*VdThnr*) of *V. dahliae*. The three genes were isolated, amplified from both *V. dahliae* isolates and cloned in pGEM®-T Easy vector. Comparative sequence analyses of the three genes from the two isolates and VdLs.17 *V. dahliae* isolate (The Broad Institute) revealed many differences that might explain the differential expression of these genes in

the two, highly and weakly aggressive, isolates and, consequently, *V. dahliae* pathogenesis.

6.2 Introduction

Verticillium dahilae Kleb. is a soilborne fungus causing wilt on hundreds of dicotyledonous plants. It causes economic yield losses in many crops worldwide (Pegg and Brady, 2002). *Verticillium dahliae* survives in soil as microsclerotia, which germinate after being stimulated by root exudates from host plants. The germinated hyphae penetrate and colonize the roots, then spread to the vascular system of the infected plant by forming conidiospores (Pegg 1985). Understanding the pathogenesis of *V. dahliae* is critical for future development of effective Verticillium wilt management strategies. Pathogenicity factors could be involved directly in fungal pathogenesis such as penetration and colonization of the host plant or indirectly such as through suppression of plant defense mechanisms and pathogen survival.

Production of reactive oxygen species (ROS), such as H_2O_2 and NO, is one of the first reactions during plant resistance to pathogen invasion. The role of ROS in disease resistance could be direct through damage to the pathogen's cell components (e.g. membranes), or indirect through activation of several plant defense mechanisms in a very complex cross-talk network of signaling (Hammond-Kosack and Jones 1996). The activated defense mechanisms include lignin biosynthesis, pathogenesis-related proteins and production of phytoalexins. It has been reported that stress response regulators are required components for oxidative stress tolerance in fungi (Moye-Rowley 2003). Stress response regulators are involved in osmotic stress response, melanin formation and

pathogenicity of the model fungus *Magnaporthe oryzae*, the causal agent of rice blast (Motoyama et al. 2008). However, in *V. dahliae*, information about oxidative stress tolerance is not available.

Plant defense signaling is controlled by several molecules including ROS, salicylic, jasmonic, and abscisic acids, as well as ethylene (Hammond-Kosack and Jones 1996). The role of salicylic acid (SA) in plant disease resistance was shown in many pathosystems, especially the activation of plant systemic acquired resistance (Delaney et al. 1994). Salicylic acid is biosynthesized in plants *via* two pathways, the phenylpropanoid and the isochorismate pathways (Wildermuth et al. 2001). Disturbance of signaling molecules (i.e. SA) biosynthesis may lead to interference with plant resistance. The role of the isochorismatase family in suppression of plant defenses by hydrolyzing isochorismate and interfering with SA biosynthesis was hypothesized for several pathogenic fungi (Soanes et al. 2008). In *V. dahliae*-host interactions, signalling plant defense responses is not fully understood. Moreover, the mechanism of how the fungus interacts with plant signalling molecules/pathways has not been fully investigated.

Survival of plant pathogens is another factor that is indirectly related to pathogenicity. In *V. dahliae*, microsclerotia ensure survival for more than 10 years. The level of microsclerotia in soil is a determinant of Verticillium wilt disease severity (Xiao and Subbarao 1998). Melanin, one of the main components of microsclerotia, is a major player in fungal pathogenesis. It is involved in direct mechanical penetration in several foliar fungal pathogens (Schäfer 1994) and in sclerotia and microsclerotia formation in many soilborne fungi including *V. dahliae* (Wheeler et al.1976).

In the previous two chapters, comparative transcriptomics and proteomics analyses of the two *V. dahliae* isolates differing in their level of aggressiveness revealed differential expression of 185 transcripts and 25 proteins. Many of these genes/proteins are potential pathogenicity-related factors. Three genes potentially involved in overcoming and suppressing plant defense mechanisms, and survival of *V. dahliae* were selected for further analysis.

The main objective of the current chapter was to isolate, clone and analyze the sequences of stress response regulator A, isochorismatase hydrolase, and tetrahydroxynaphthalene reductase genes in the two *V. dahliae* Vd1396-9 (highly aggressive) and Vs06-14 (weakly aggressive) isolates as a preliminary step for further molecular and functional characterization of these genes in *V. dahliae*- host interactions.

6.3 Materials and Methods

One gene was selected based on the transcriptomics analysis, stress response regulator A, transcript number 12-106 (Section 4.0; Table 4.4) and the other two genes were selected based on the proteomics analysis, isochorismatase hydrolase and tetrahydroxynaphthalene reductase, proteins number 12 and 18, respectively (Section 5.0; Table 5.1).

6.3.1 *Verticillium dahliae* isolates

The two *V. dahliae* model isolates, Vd1396-9 (highly aggressive) and Vs06-14 (weakly aggressive), were used in this study. *Verticillium dahliae* isolates were grown in CDB liquid medium for two weeks at room temperature on a shaker at 120 rpm (C2

Platform Shaker, Edison, NJ, USA) in the dark. Mycelia were collected and ground in liquid nitrogen and kept at - 80 ºC until used.

6.3.2 Genomic DNA extraction

The total genomic DNA was extracted using a cetyl trimethyl ammonium bromide (CTAB) extraction method as described by Wally et al. (2008). Briefly, 200-300 mg the ground fungal mycelium was removed from storage at -80 ºC and mixed with a 60ºC CTAB buffer (2% CTAB, 100 mM Tris–HCl pH 8.0, 20 mM ethylenediaminetetra-acetic acid (EDTA), and 1.4 M NaCl). Tubes were vortexed and incubated at 60ºC for 30 min., then cold chloroform: isoamyl alcohol (24:1) solution was added, mixed and centrifuged at 9,000 rpm for 15 min. Cold isopropanol was added and mixed with the supernatant in a new tube and kept at -20ºC for 30 min to precipitate the DNA. Tubes were centrifuged at 14,000 rpm for 15 min. DNA precipitate was washed with 70% ethanol and left to air dry. DNA was re-suspended in 20 µl of TE buffer (10 mM Tris-HCl pH 8.0 and 1 mM EDTA).

6.3.3 PCR amplification

The full length sequences of the three genes were obtained from the Broad Institute *V. dahliae* genome database of VdLs.17, a highly virulent isolate, (http://www.broadinstitute.org/annotation/genome/verticillium_dahliae/MultiHome.html) (Appendix 10.2). Forward and reverse primers were designed for each gene in order to isolate and amplify the full length gene from our two *V. dahliae* isolates, Vd1396-9 (highly aggressive) and Vs06-14 (weakly aggressive) (Table 6.1). For PCR amplification

of the stress response regulator A gene, 1 µl of genomic DNA was added to a 20 µl PCR mixture (1X iProof HF buffer, 200 µM of each dNTP, 1 µM of each primer, 0.4 U iProof™ High-Fidelity DNA polymerase (Bio-Rad) and H_2O). In the case of the isochorismatase hydrolase and tetrahydroxynaphthalene reductase genes, the PCR mixture consisted of 1X PCR buffer, 1 mM dNTPs, 5 mM $MgCl_2$, 0.25 U Taq DNA polymerase, 1 µM of each primer and H_2O to a volume of 20 µl. PCR amplification was carried out in a Bio-Rad thermocycler (MyCycler™ thermal cycler, Bio-Rad Laboratories, Inc.). PCR amplification conditions and product sizes are shown in Table 6.2. PCR products were run on 1% agarose gel containing ethidium bromide and visualized using an AlphaImager HP gel documentation system (Alpha Innotech Corporation, San Leandro, CA, USA) equipped with a P93D thermoprinter (Mitsubishi Electric Co., Tokyo, Japan).

6.3.4 Cloning, sequencing and comparative sequence analysis

Polymerase chain reaction products of each gene were excised, extracted and purified from the agarose gel using MinElute Gel Extraction Kit (Qiagen Inc., USA) and then cloned into pGEM®-T Easy Vector System (Promega Corp., WI, USA) according to the manufacturer's instructions. Three positive bacterial clones were picked up from the LB agar plates with 100 mg/ml ampicillin and transferred into LB liquid media containing the same antibiotic and incubated over night on a shaker at 37°C to reproduce the transformed bacteria. Plasmids were extracted using QIAprep Spin Miniprep Kit (Qiagen Inc., USA) according to the manufacturer's instructions. PCR re-amplification was carried out for each gene using the same primer pairs (Table 6.1) and thermocycling

Table 6.1. Sequences of primers used for amplification of stress response regulator A (*VdSrrA*), isochorismatase hydrolase (*VdIsoch*) and tetrahydroxynaphthalene reductase (*VdThnr*) genes from Vd1396-9 (highly aggressive) and Vs06-14 (weakly aggressive) *V. dahliae* isolates

Gene	Primer	Sequence (5' to 3')
VdSrrA	Forward	ATGCCTCCCGAAGGTGATGG
	Reverse	TTATTGAGCGTAGCCTCCCTG
VdIsoch	Forward	ATGTCCTCATTCCGCTCCAT
	Reverse	CTAGTTGATATCCTTGCTCGAGA
VdThnr	Forward	CCTTGACGCATCAGCACCACCA
	Reverse	TTACATGCAAGCGGCACCGTCA

Table 6.2. PCR conditions used for amplification of stress response regulator A (*VdSrrA*), isochorismatase hydrolase (*VdIsoch*) and tetrahydroxynaphthalene reductase (*VdThnr*) genes from Vd1396-9 (highly aggressive) and Vs06-14 (weakly aggressive) *V. dahliae* isolates

Gene	Initial denaturation	Denaturation	Annealing	Extension	Number of cycles	Final extension	PCR product size (bp)
VdSrrA	98.0 ºC 30 sec	98.0 ºC 10 sec	62.5 ºC 30 sec	72.0 ºC 90 sec	15	72.0 ºC 10 min	2245
VdIsoch	94.0 ºC 5 min	94.0 ºC 30 sec	55 ºC 45 sec	72.0 ºC 60 sec	30	72.0 ºC 10 min	629
VdThnr	95.0 ºC 5 min	95.0 ºC 30 sec	60 ºC 30 sec	72.0 ºC 60 sec	30	72.0 ºC 10 min	1056

conditions (Table 6.2) to confirm the presence of the insert (gene) in the extracted plasmid. For each gene, the extracted plasmids from three separate clones were sent for sequencing (Macrogen Co., USA). For the isochorismatase and tetrahydroxynaphthalene reductase genes, M13 forward and M13 reverse universal primers were used for the sequencing. In the case of stress response regulator A, the gene which is 2245 bp, in addition to M13 forward and M13 reverse primers, internal primers were designed and used for sequencing in order to cover the full length of the gene.

The obtained sequences were checked out, and the plasmid DNA contamination and overlapping sequences (in case *VdSrrA* gene) were removed. Multiple alignment sequence analysis for each gene from our *V. dahliae* isolates Vd1396-9 (highly aggressive), Vs06-14 (weakly aggressive) and the Board Institute VdLs.17 isolate was carried out using CLUSTAL W software (Thompson et al. 1994) (http://www.ebi.ac.uk/Tools/clustalw2/index.html) and GeneDoc. software (http://www.nrbsc.org/gfx/genedoc/).

6.4 Results and Discussion

Plant defense mechanisms (e.g. production of secondary metabolites and PR proteins) are controlled by signaling molecules such as ROS and SA. Pathogens developed strategies to counter-defend the plant defense mechanisms such as detoxification of plant secondary metabolites and suppression of plant defenses (Daayf et al. 2010). Stress response regulator A, isochorismatase hydrolase and tetrahydroxynaphthalene reductase genes were isolated, amplified (Fig. 6.1) and sequenced from the two isolates Vd1396-9 (highly aggressive) and Vs06-14 (weakly

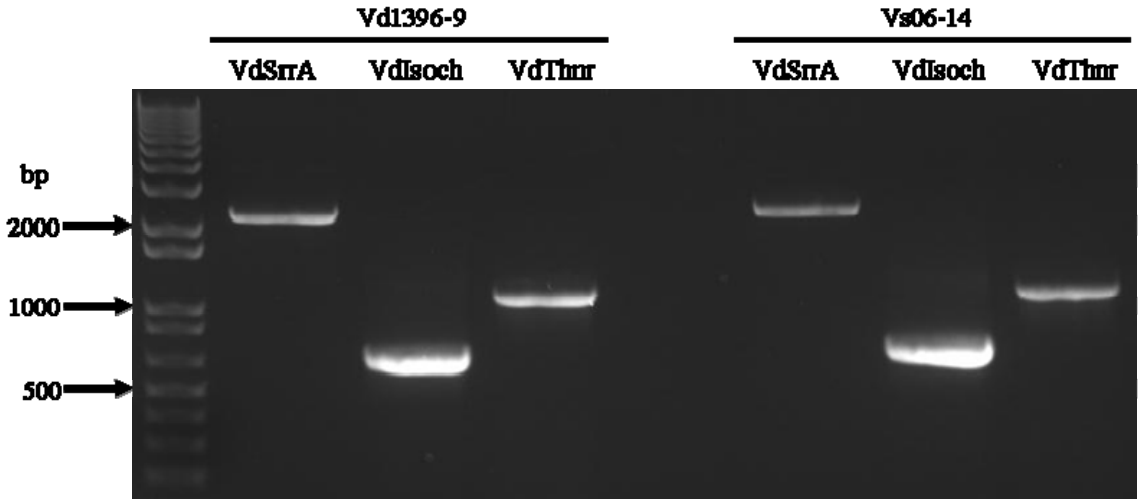


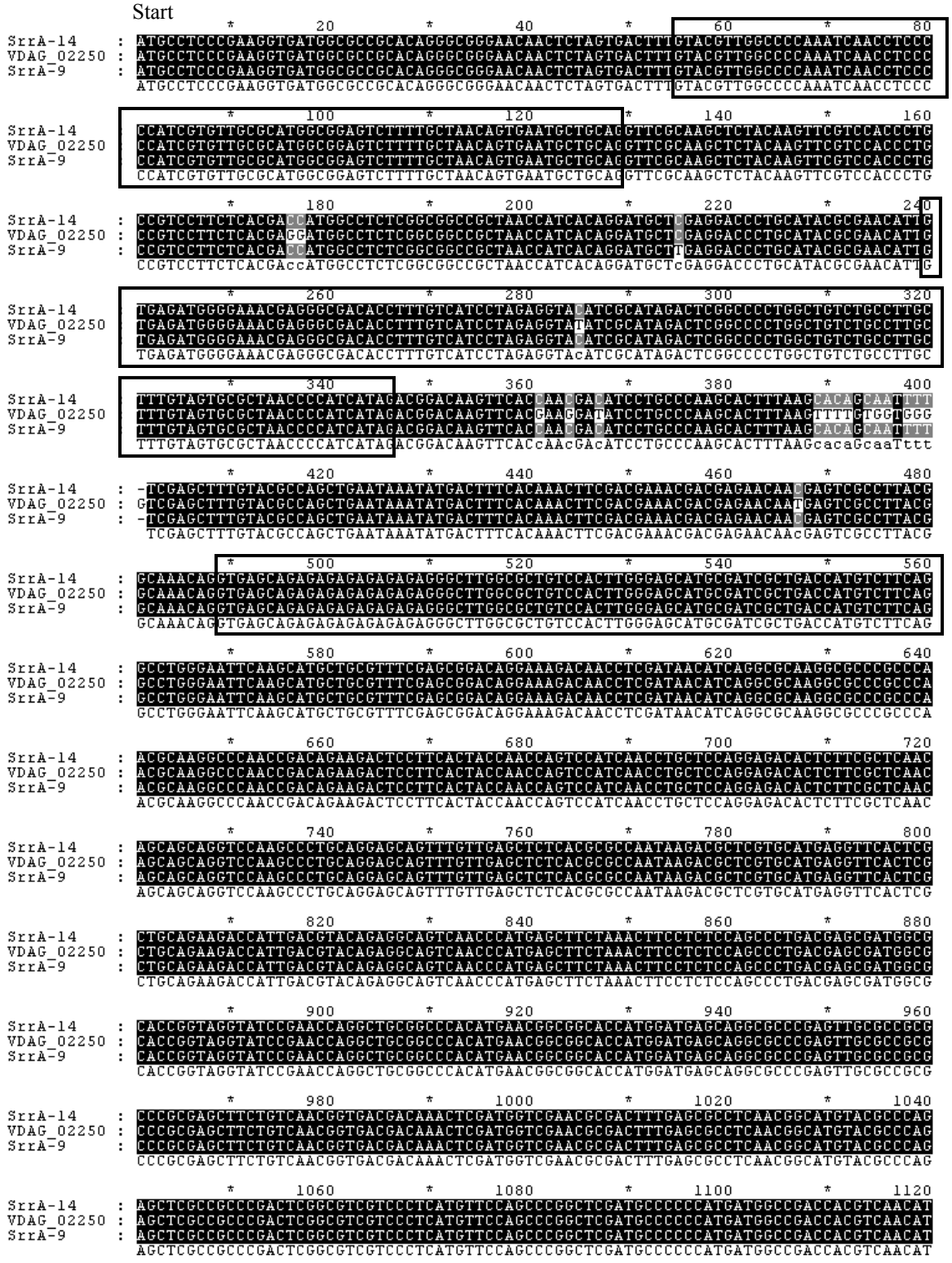
Figure 6.1. PCR amplification of stress response regulator A (*VdSrrA*), isochorismatase hydrolase (*VdIsoch*), and tetrahydroxynaphthalene reductase (*VdThnr*) genes from the genomic DNA of *V. dahliae* isolates Vd1396-9 (highly aggressive) and Vs06-14 (weakly aggressive).

aggressive). The sequences of the three genes from the two isolates were compared to those of the Broad institute *V. dahliae* isolate VdLs.17.

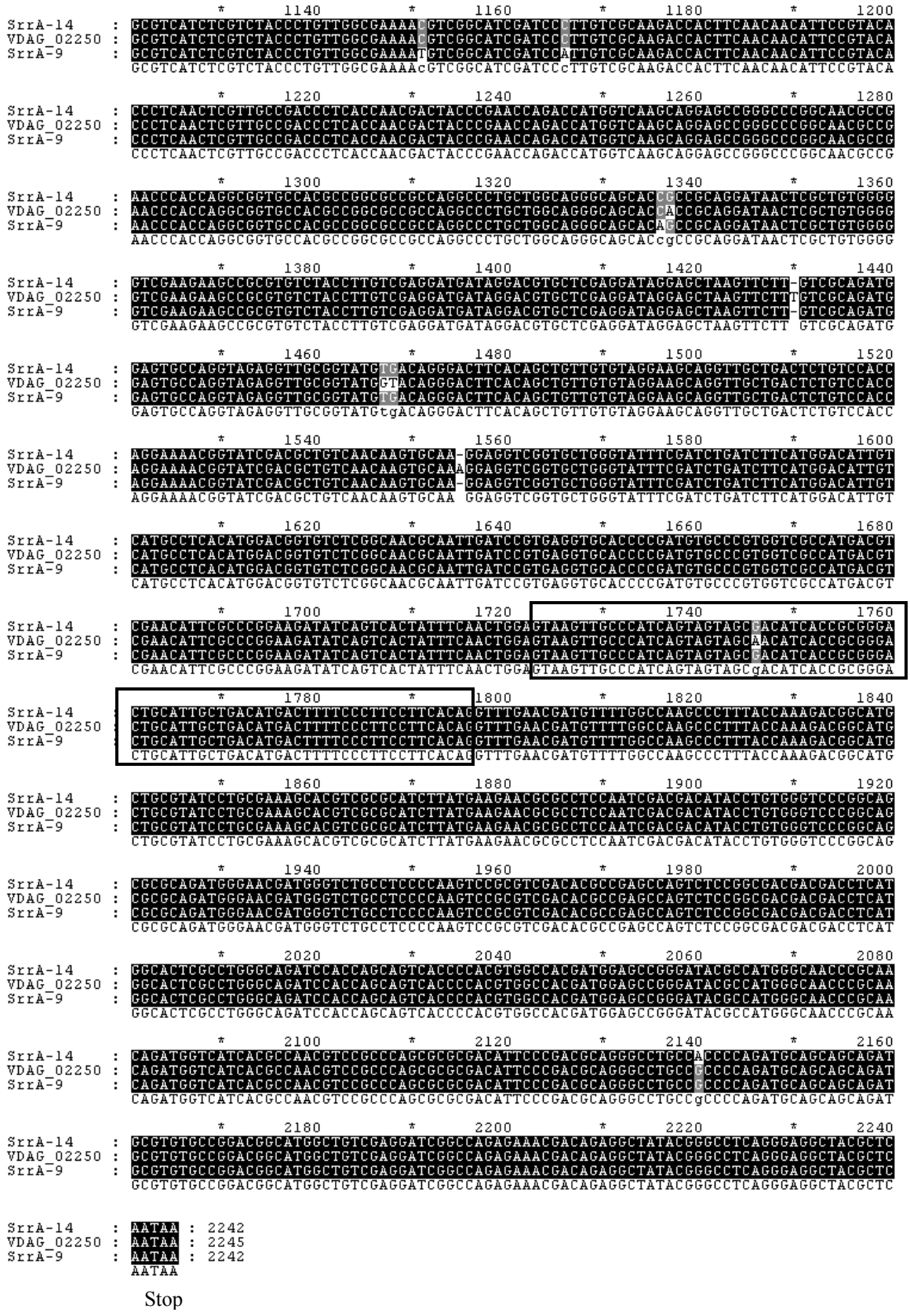
6.4.1 Stress response regulator A gene

The oxidative burst is a well known mechanism in plant disease resistance. Reactive oxygen species affect pathogens directly and activate plant defense mechanisms (e.g., production of phytoalexins and PR proteins) to resist pathogen infection (Bolwell 1999). Environmental stress tolerance, including oxidative stress, has been studied in several fungi (Gasch 2007). The stress response regulator A (*SrrA*) gene has been reported to play a role in the regulation of ROS stress response and found in several fungal species such as *Neurospora crassa*, *Magnaporthe grisea*, *Fusarium graminearum* and *Saccharomyces* spp. (Hagiwara et al. 2007). It has been shown that SrrA is important in oxidative stress tolerance *in A. nidulans* (Hagiwara et al. 2007). However, the role of this gene in *V. dahliae* has not been studied.

Three clones of the *SrrA* gene were sequenced for each isolate (Appendix 10.2). The full length sequence of *SrrA* gene from both Vd1396-9 and Vs06-14 isolates was 2242 nucleotides (nt), whereas in VdLs.17 isolate (the Broad Institute) the full length sequence is 2245 nt (Appendix 10.2). Multiple alignment of *SrrA* sequences from our isolates with VdLs.17 isolate revealed differences in 29 nt (Fig. 6.2). Twenty four nucleotide differences of the 29 were the same in both Vd1396-9 and Vs06-14 isolates as compared to VdLs.17 isolate. Whereas 4 and 1 differences were specific to isolates Vd1396-9 and Vs06-14, respectively, relative to VdLs.17.


Start
SrrA-14
VDAG_02250
SrrA-9

Continue,



Stop

Figure 6.2. Multiple alignment of the full length sequence of stress response regulator A gene from the highly aggressive *V. dahliae* Vd1396-9 (SrrA-9), the weakly aggressive isolate Vs06-14 (SrrA-14), and the Broad Institute genome database of isolate VdLs.17 (VDAG_02250). Introns sequences are labeled with boxes.

Multiple alignment of *SrrA* exons only showed 26 differences, 3 were detected in Vd1396-9, 1 was in Vs06-14 and 22 differences were shared in both isolates as compared to the exons sequences of VdLs.17 isolate (Appendix 10.2; Fig.10.1). The deduced amino acids sequence in both Vd1396-9 and Vs06-14 was 630 amino acids with many common changes in both of them as relative to VdLs.17. However, amino acid sequences in the N- and C-terminal sequences of the deduced proteins were identical in the three compared isolates (Appendix 10.2; Fig. 10.2). The observed changes in *SrrA* gene sequences might contribute to the differential expression of this gene in the highly and weakly aggressive *V. dahliae* isolates. However, the changes in the deduced amino acids sequences (Appendix 10.2; Fig. 10.2) are based on multiple alignments, whereas the regulation of gene expression *in vivo* is a complex network, especially during host-pathogen interactions (Kahmann and Basse 2001; Janky et al. 2009). Post-translational modifications could affect the differential activity of proteins. Therefore, the three deduced proteins from the sequences of the three tested isolates were predicted for phosphorylation sites using the available website (http://www.cbs.dtu.dk/services /NetPhos/) (Blom et al. 1999). The predicted serine, threonine and tyrosine phosphorylation sites were 40, 26 and 3 for isolate Vd1396-9; 39, 26 and 3 for isolate Vs06-14 and 21, 9 and 3 for isolate VdLs.17, respectively (Appendix 10.2; Fig. 10.3).

It has been reported that *SrrA* gene is a vital component in stress signal transduction and conidiospore production in *Aspergillus nidulans*. A disrupted *SrrA* gene mutant of *A. nidulans* resulted in H_2O_2 hypersensitivity and it was suggested that SrrA is acting as a transcriptional factor that activates a catalase gene in *A. nidulans* hyphae, which results in oxidative stress resistance (Hagiwara et al. 2007; Vargas-Pérez et al.

2007). Transcriptional regulation of oxidative stress is important for pathogenic fungi to overcome host defenses (Moye-Rowley 2003). However, the regulation of oxidative stress responses in plant pathogenic fungi is not much studied. Isolation and cloning of the *SrrA* gene from *V. dahliae* isolates of different aggressiveness is an initial step to investigating the role of this gene not only in the fungus itself but also during its interactions with host plants.

6.4.2 Isochorismatase hydrolase gene

Signaling in plant defense responses is an essential prerequisite for successful disease resistance/tolerance. Salicylic acid (SA) is a major signaling molecule that activates systemic acquired resistance in several plant-microbe interactions (Delaney et al. 1994). Isochorismatase hydrolase may act as a potential plant defense signaling suppressor through hydrolysis of isochorismate, which is involved in one of the SA biosynthesis pathways in plants.

The isochorismatase hydrolase gene was amplified and sequenced from the two *V. dahliae* isolates, Vd1396-9 (highly aggressive) and Vs06-14 (weakly aggressive), (Appendix 10.3). The full length sequence of the isochorismatase hydrolase gene from the two *V. dahliae* and isolate VdLs.17 (the Broad Institute isolate; Appendix 10.3) isolates were compared (Fig. 6.3). The sequence of isochorismatase hydrolase from the highly aggressive isolate, Vd1396-9, was identical to that of the Broad Institute isolate VdLs.17 (Appendix 10.3). Three nucleotide differences were detected only in the gene sequence from the weakly aggressive Vs06-14 isolate, at nucleotides 213, 396 and 492 (Fig. 6.3). The point insertion/deletion mutation in position 396 was found in an intron

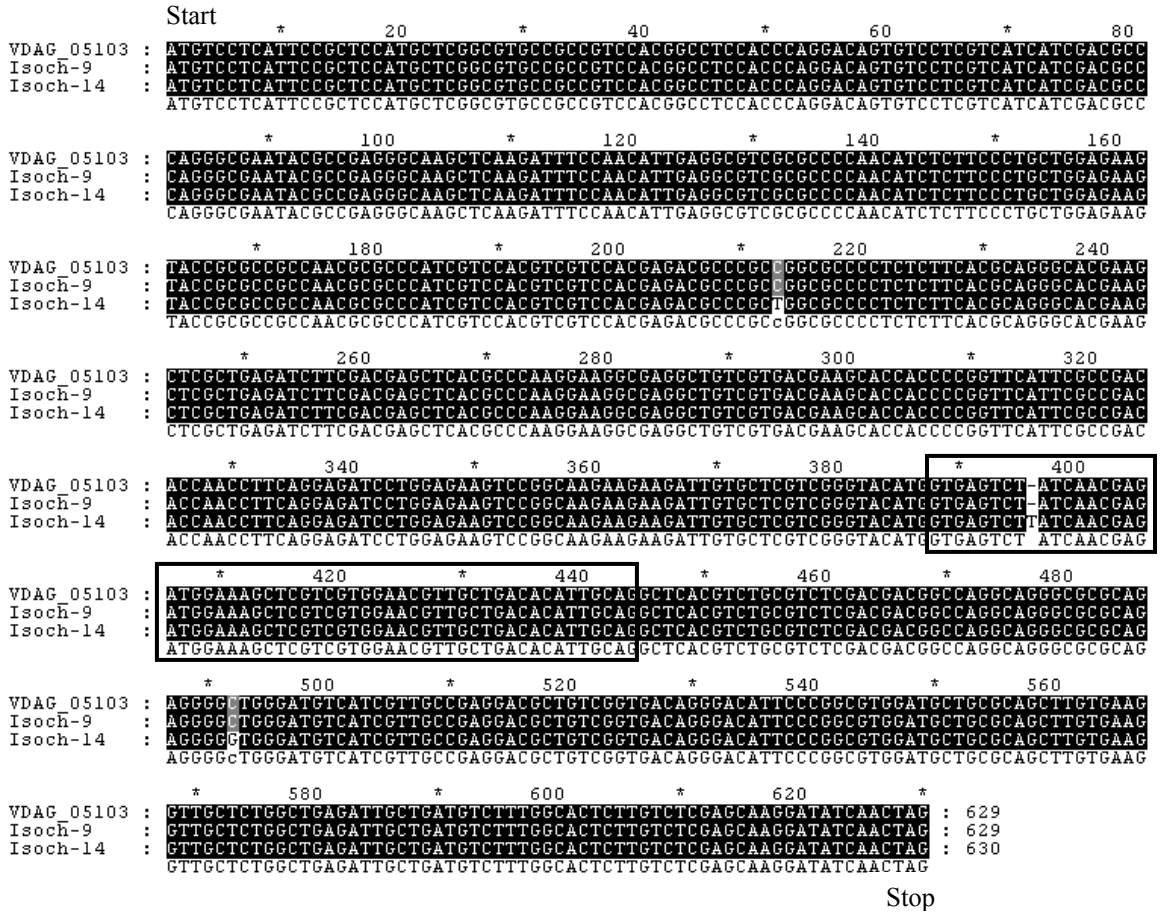


Figure 6.3. Multiple alignment of the full length sequence of isochorismatase hydrolase gene from the highly aggressive *V. dahliae* Vd1396-9 (Isoch-9), the weakly aggressive isolate Vs06-14 (Isoch-14), and the Broad Institute genome database of isolate VdLs.17 (VDAG_05103). Intron sequence is labeled with box.

sequence (The Broad Institute; Appendix 10.3), and therefore, it is removed when the exons sequences are aligned (Appendix 10.3; Fig. 10.4) and none of these three differences result in changes in the theoretical deduced amino acids (Appendix 10.3; Fig. 10.5). However, the differential expression of this gene in the two highly and weakly aggressive isolates could be regulated at several levels (transcriptional, translational and post-translational).

Mutations in introns could affect their splicing and, consequently, the regulation of gene expression (Swida et al. 1986; Fouser and Friesen 1986; Epstein et al. 1993) in addition to their known role in the evolution of organisms (Rogers 1990). The detected insertion/deletion point mutation may contribute to pathogen evolution from weakly to highly aggressive isolates, or *vice versa*, and the expression of isochorismatase hydrolase during *V. dahliae*-host interactions. There are many factors that could be involved in the interactions due to the living habitat of the pathogen in the vascular system of host plants. More studies are required to explain the role of isochorismatase hydrolase during *V. dahliae*-potato interactions.

6.4.3 Tetrahydroxynaphthalene reductase gene

The role of melanin pigment in pathogenesis of fungal plant pathogens is well known in several plant-fungal interactions. It facilitates the penetration step in foliar fungal diseases and is essential for production of survival structures of soilborne fungi including *V. dahliae* (Wheeler et al.1976). It was noticed that Vd1396-9 (the highly aggressive) isolate produced microsclerotia rapidly in both solid and liquid media as compared to Vs06-14 (the weakly aggressive) isolate (Section 3.0; Fig. 3.1).

Tetrahydroxynaphthalene reductase, an essential enzyme in melanin biosynthesis, was differentially expressed in isolates Vd1396-9 and Vs06-14 after 2 weeks of growth on PDA (Section 5.0; Fig. 5.2).

The full length sequence of the tetrahydroxynaphthalene reductase gene from *V. dahliae* isolates Vd1396-9 (highly aggressive), Vs06-14 (weakly aggressive), and VdLs.17 (The Broad Institute isolate) were aligned (Fig. 6.4). The sequences of three clones of each isolate are shown in Appendix 10.4. The length of the tetrahydroxynaphthalene reductase gene in both Vd1396-9 and Vs06-14 isolate is shorter than VdLs.17 by one nucleotide, 1055 nt (Fig. 6.4). In total, 15 differences were observed in the sequences in comparison with the reference isolate VdLs.17. 2 differences were common to both isolates, while 7 were specific to Vs06-14 and 6 to Vd1396-9. After removal of intron sequences according to the information available from the Broad Institute, 7 differences remained in exon sequences (Appendix 10.4; Fig. 10.6). One insertion/deletion is found in Vs06-14 (the weakly aggressive) in position 15, which caused a frame shift of the open reading frame that resulted in a different sequence of the deduced amino acid in this isolate while, the deduced amino acids in both VdLs.17 and Vd1396-9 highly aggressive isolates were identical (Appendix 10.4; Fig. 10.7). The insertion/deletion frame shift mutation seems to be the reason for the differential morphological appearance and expression of this gene that was observed in the proteomics analysis of Vd1396-9 and Vs06-14 isolates (Section 5.0; Fig. 5.2).

The differential expression of this gene in the two *V. dahliae* isolates might affect their levels of pathogenicity. It has been reported that melanin is a significant pathogenicity factor in several phytopathogenic fungi including *Rhizoctonia solani* (Kim

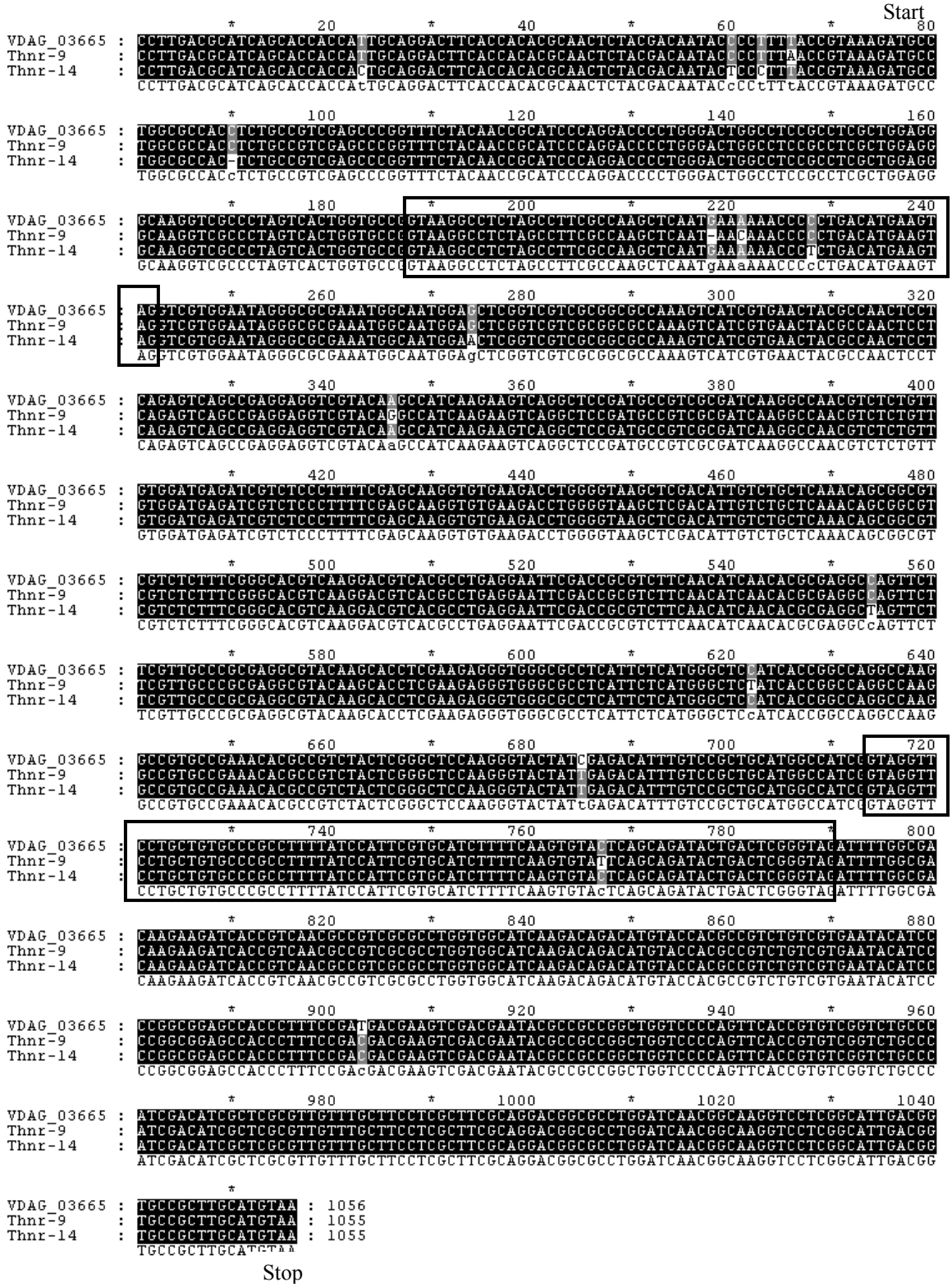


Figure 6.4. Multiple alignment of the full length sequence of tetrahydroxynaphthalene reductase gene from the highly aggressive *V. dahliae* Vd1396-9 (Thnr-9), the weakly aggressive isolate Vs06-14 (Thnr-14), and the Broad Institute genome database of isolate VdLs.17 (VDAG_03665). Introns sequences are labeled with boxes.

et al. 2001b), *Colletotrichum lagenarium* (Suzuki et al. 1982; Tsuji et al. 2003), and *Magnaporthe grisea* (Woloshuk et al. 1980). Using fungicides that act as melanin biosynthesis inhibitors has been suggested to control rice blast disease (Kurahashi 2001).

Isolation and cloning of stress response regulator A, isochorismatase hydrolase, and tetrahydroxynaphthalene reductase genes from the highly and weakly aggressive *V. dahliae* isolates lays the groundwork for further molecular and functional characterization of these genes in *V. dahliae*-host interactions. Knowing the strengths and weaknesses of the pathogen's behavior when attacking plants or neutralizing their defense responses will provide information for the development of more efficient management strategies for the control of Verticillium wilt disease.

7.0 PROFILING OF SECONDARY METABOLITES IN POTATO AFTER INOCULATION WITH HIGHLY *VS* WEAKLY AGGRESSIVE ISOLATES OF *VERTICILLIUM DAHLIAE*

7.1 Abstract

Verticillium wilt of potato is an important disease caused by the soilborne fungus *Verticillium dahilae* (Kleb.). Production of secondary metabolites is one of the defense mechanisms that are used by host plants to resist pathogens. Several genes/proteins that are potentially involved in overcoming plant defense mechanisms were identified in two isolates of the fungus (Sections 4.0; 5.0; 6.0). In the present chapter, potato cv. Kennebec was inoculated with either highly (Vd1396-9) or weakly aggressive (Vs06-14) *V. dahliae* isolates. Secondary metabolites were extracted from roots, stems, and leaves of the inoculated plants over time and putatively identified using high performance liquid chromatography (HPLC) equipped with a photodiode array detector and co-elution with commercial standards. The identified compounds include chlorogenic, caffeic and ferulic acids, *cis*-jasmone and rutin. Variations in the levels of these compounds have been detected in response to inoculation with *V. dahliae*. Some phenolics, such as caffeic and ferulic acids, accumulated in roots and stems after inoculation with the *V. dahliae* isolates, especially Vd1396-9. However, in the stems, the accumulation of rutin in response to inoculation was inhibited with the highly aggressive *V. dahliae* isolate as compared to the weakly aggressive one. Together with proteomics and transcriptomics analyses of the same *V. dahliae* isolates, profiling of secondary metabolites during

potato-*V. dahliae* interactions could help the understanding of this pathosystem and the future development of an efficient control method for Verticillium wilt disease.

7.2 Introduction

Potato Verticillium wilt is mainly caused by the soilborne deuteromycete *Verticillium dahliae* Kleb. The pathogen affects a broad range of dicotyledonous annual, perennial and woody plants worldwide (Rowe and Powelson 2002). Verticillium wilt symptoms include chlorosis, necrosis, vascular discoloration, stunting, and eventually wilting (Pegg and Brady, 2002). In potato, yield and tuber quality are affected. *Verticillium dahliae* survives in the soil for 10-15 years as microsclerotia (Wilhelm 1955). Microsclerotia are stimulated by potato root exudates, germinate, develop hyphae, penetrate and colonize the roots. The hyphae grow into the xylem vessels and reproduce asexually by conidiospores that are able to move upward in the vascular system (Rowe and Powelson 2002). At the end of the disease cycle, the fungus forms microsclerotia as survival structures. Current control methods for this disease depend on soil fumigation, soil solarization, crop rotation, the use of tolerant cultivars, where available (Johnson and Dung, 2010). Due to the low efficiency of these methods, the development of more successful Verticillium wilt management method is needed.

Host defenses against pathogens can be either constitutive and/or induced. The constitutive mechanisms include structural barriers such as waxes and cuticles and chemicals such as phytoanticipins (Osbourn 1996). The induced defense mechanisms consist of structural (e.g. cell wall fortification, cork layers and tyloses formation) and chemical (e.g. phytoalexins and pathogenesis related proteins) responses (Pegg and

Brady, 2002; Agrios 2005; van Loon et al. 2006; Hückelhoven 2007). Several molecules such as reactive oxygen species, salicylic, jasmonic, abscisic acids, and ethylene are involved in plant defense signaling, which then triggers defense mechanisms (Hammond-Kosack and Jones 1996) with a very complex cross-talk among these signaling molecules (Kunkel and Brooks 2002; Vlot et al. 2009). Salicylic acid and its glucoside were shown to increase in the xylem sap of *Brassica napus* inoculated with *V. longisporum* (Ratzinger et al. 2009). Ethylene involvement in signaling plant defenses in the *Arabidopsis thaliana*-*V. dahliae* pathosystem was investigated (Pantelides et al. 2010). Nitric oxide, another ROS, was reported in defense signaling in *A. thaliana* treated with a toxin from *V. dahliae* (Shi et al. 2009).

Phenolic compounds are well known in plant disease resistance, and are either constitutive or induced upon infection (Kosuge 1969; Nicholson 1992). Daayf et al. (1997) reported that terpenoid and phenolic compounds were increased as an early reaction in cotton seedlings inoculated with *V. dahliae.* In the same pathosystem, it was reported that terpenoid aldehyde and other derivatives accumulated in the xylem of infected cotton (Mace 1983; Mace et al. 1989). Moreover, lignin, lignin-like phenolics deposition and phenylalanine ammonia-lyase, cinnamyl alcohol dehydrogenase and peroxidase activity, hemigossypol, desoxyhemigossypol induction and δ-cadinene synthase were reported in cotton after *V. dahliae* infection (Smit and Dubery, 1997; Bianchini et al. 1999). It has been also reported that phenolics accumulated and phenylalanine ammonia-lyase (PAL) was activated in tomato cell cultures after inoculation with the wilt fungus *V. albo-atrum*. This accumulation was confirmed using PAL inhibitor (Bernards and Ellis, 1991). In the pepper-*V. dahliae* pathosystem, using an

amendment with organic compounds that contain SA was effective in diminishing the symptoms caused by *V. dahliae* infection in parallel to an early accumulation of phenolic compounds, which explained pepper resistance to *V. dahliae* (Goicoechea et al. 2004). It has been suggested that *V. dahliae* can utilize rutin, a defense-related phenolic compound, by cleaving its sugar moiety, use it as a carbon source, and metabolize the remaining flavonol (El Hadrami et al. 2009a,b). Therefore, *V. dahliae* could suppress the signalling of potato defense responses and/or detoxify phenolic compounds that are produced by the host plant, and overcome host plant defenses (El Hadrami et al. 2009a,b). However, potato defense mechanisms against *V. dahliae* are not fully understood. Several genes (e.g. stress response regulator A) and proteins (e.g. isochorismatase hydrolase) may be involved in overcoming and/or suppressing potato defense mechanisms (Sections 4.0; 5.0; 6.0). An investigation of the potato-*V. dahliae* interaction on a metabolomics level is needed to complement the transcriptomic and proteomics findings.

The main objective of this chapter was to screen secondary metabolites potentially involved in the potato-*V. dahliae* interaction, which could help to better understand this pathosystem and develop more sustainable Verticillium wilt management strategies.

7.3 Materials and Methods

7.3.1 Potato, *V. dahliae* and inoculation procedure

Potato tuber pieces of cv. Kennebec were planted in a mixture of sand and soil (1:1, v/v) supplemented with 50 gm of NPK fertilizer granules (16:20:16) for five weeks

in a greenhouse (20/16 ± 2°C day/night, 16 hours photoperiod, light intensity of 350 μmol m^{-2} s^{-1}). In parallel, two isolate of contrasting aggressiveness (*V. dahliae* isolates Vd1396-9 (highly aggressive) and Vs06-14 (weakly aggressive)) were maintained on PDA medium for two weeks. Conidiospores were harvested from the *V. dahliae* cultures in sterilized distilled water and a concentration of 10^6 conidiospores/ml was used for inoculation using a root dipping method (Alkher et al. 2009). Samples of roots, stems, and leaves from 3 replicates of un-wounded, un-inoculated control plants at time zero, just before inoculation, were harvested and kept at -80ºC. The plants inoculated with either *V. dahliae* isolate Vd1396-9 or Vs06-14, along with un-inoculated wounded control plants, were transplanted into 16 cm-diameter clay pots filled with an autoclaved mixture of soil, sand, peat and perlite (4:4:4:1, v/v/v/v), received 5 g/l NPK (20:20:20) fertilizer and were moved into the greenhouse under the same conditions mentioned earlier. All of the plants were distributed in a random complete block design in the greenhouse. Samples of roots, stems, and leaves were collected at 1, 3, 7, and 14 days after inoculation from three independent replicates for each time point. The same number of samples was collected from the un-inoculated wounded control replicates. The samples were put immediately in liquid nitrogen and kept at -80ºC until used for the extraction of secondary metabolites.

7.3.2 Phenolics extraction and high performance liquid chromatography (HPLC) analysis

Soluble phenolics were extracted from roots, stems and leaves of potato cv. Kennebec inoculated with *V. dahliae* isolates Vs06-14 or Vd1396-9 along with the un-

wounded, un-inoculated and wounded, un-inoculated controls. Phenolics extraction and HPLC analysis were carried out as described by Daayf et al. (2000). An amount of 0.5-0.75 g of fresh weight of each sample was ground in liquid nitrogen and used for phenolics extraction. An equal amount of 80% methanol (0.5-0.75 ml) was added onto the sample. Then, samples were homogenized using a vortex for 10 sec. every 15 min. for a total of 2 hrs. Samples were centrifuged at 9,600 *g* for 15 min., and the supernatant was used for injection into the HPLC. The HPLC analysis was performed using a Water 2695 separations module (Waters Corp., Milford, MA) equipped with a Waters 996 Photodiode Array detector, and a 250 x 4 mm RP-C18 (LiChroCART 4-4, Darmstadt, Germany) column with a particle size of 5 µm. The solvents were acidified water (1ml phosphoric acid/litre of nanopure water) (solvent A) and acetonitrile HPLC grade (solvent B) with an elution flow rate of 1 ml/min. The gradient used was as follows, time (min)/ %A / %B: 0/100/0, 5/95/5, 10/95/5, 14/90/10, 20/80/20, 23/80/20, 30/65/35, 35/ 65/35, 43/50/50, 48/25/75, 55/0/100 and 60/0/100. The injected volume was 50 µl for each sample.

Three biological replicates for each treatment/time point, for a total of 117 samples, were extracted and injected independently into the HPLC. Empower 2 Chromatography Data Software (Waters Corp., Milford, MA) was used to analyze the chromatograms. Retention time (RT) and maximum absorbance (nm) spectra, as compared to standards, were used for the identification of the detected phenolics.

Standard curves of chlorogenic, caffeic, ferulic acids, *cis*-jasmone and rutin (Sigma-Aldrich Canada Ltd, Ontario, Canada) were carried out at a concentration series of 1, 5, 10, 25, 50, 75 and 100 µg/ml. Three replicates for each concentration were run in the HPLC. The standards were dissolved in 80% methanol and run under the same

conditions as the extracted samples. Linear regressions were generated between peak area and concentration. The obtained equations were used to calculate the concentration of each phenolic compound in the analyzed samples. Standard curves are shown in Appendix 10.5 (Figs 10.8 to 10.12).

7.3.3 Statistical analysis

Content of the identified phenolic compounds were calculated based on the standard curves. Data were statistically analyzed using SAS 9.1 software (SAS Institute Inc., Cary, NC, USA). Differences among the means were compared using the least significant difference (LSD) test at $p<0.05$.

7.4 Results and Discussion

In the present study, three putatively identified phenolic compounds varied in potato roots in response to inoculation, chlorogenic, caffeic and ferulic acids and in the stems, three compounds, caffeic acid, rutin, and *cis*-jasmone. Of these compounds, only rutin presented variations in the leaves. Table 7.1 shows a list of the identified phenolics compounds with their retention time (min) and maximum absorbance wavelengths (nm). The number of the identified compounds in the roots and stems was higher than that in the leaves, which is not a surprise since *V. dahliae* is a soilborne pathogen that interacts first with potato roots, colonizes the root cortex, then moves and reproduces in the vascular system of the stem.

Phenolic compounds are mainly biosynthesized *via* the phenylpropanoid pathway, which primarily branches from the metabolism of the amino acid phenylalanine, followed

by several biochemical reactions to produce cinnamic acid, caffeic acid, flavones and other secondary metabolites (Hahlbrock and Scheel, 1989). The role of secondary metabolites (i.e. phenolic compounds) in plant-microbe interactions is an important topic to understand host resistance/susceptibility to pathogens (Hammerschmidt 1999; Dixon 2001). Phenolics were reported in plant defense against the vascular wilt pathogens *V. dahliae* and *Fusarium oxysporum* f. sp. *elaeidis* in several plants including cotton, olive and oil palm (Daayf et al. 1997; Báidez et al. 2007; Sékou et al. 2010).

7.4.1 Secondary metabolites identified in potato roots after inoculation with *V. dahliae* isolates

Chlorogenic acid was detected in potato roots of inoculated and control plants in concentrations ranging from 0.6 to 2.9 $\mu g.g^{-1}$ root fresh weight (Fig. 7.1). Chlorogenic acid accumulated in response to both wounding and *V. dahliae* inoculation, however, no significant differences were found. Chlorogenic acid was previously identified as part of a defense response in potato after infection with *Helminthosporium carbonium* (Kuć et al. 1956), *Phytophthora infestans* (Friend et al. 1973), *Streptomyces scabies* (Johnson and Schaal, 1952), and upon tuber physical injury (Johnson and Schaal, 1957).

Caffeic acid content of potato roots in wounded control, Vs06-14 and Vd1396-9 inoculated plants was variable, but did not differ from the unwounded control plant roots sampled at time zero (Fig. 7.2). Plants inoculated with Vs06-14 did not differ significantly in caffeic acid content. Wounded control plant roots and roots of plants inoculated with Vd1396-9 had significantly higher levels of caffeic acid at 3 d.a.i. than at

Table 7.1. List of secondary metabolites putatively identified in different parts of potato cv. Kennebec after inoculation with Vd1396-9 (highly aggressive) and Vs06-14 (weakly aggressive) *V. dahliae* isolates

Plant part	Putative identified compound	Retention time (min.)	Maximum absorbance (nm)
Root			
	Chlorogenic acid	19.7	217.9-241.4-326.6
	Caffeic acid	23.3	217.9-239.1-324.2
	Ferulic acid	28.0	217.9-235.5-323.0
Stem			
	Caffeic acid	22.1	217.9-239.1-324.2
	Rutin	25.3	205.0-255.6-355.2
	cis-Jasmone	50.6	236.7
Leaf			
	Rutin	25.3	205.0-255.6-355.2

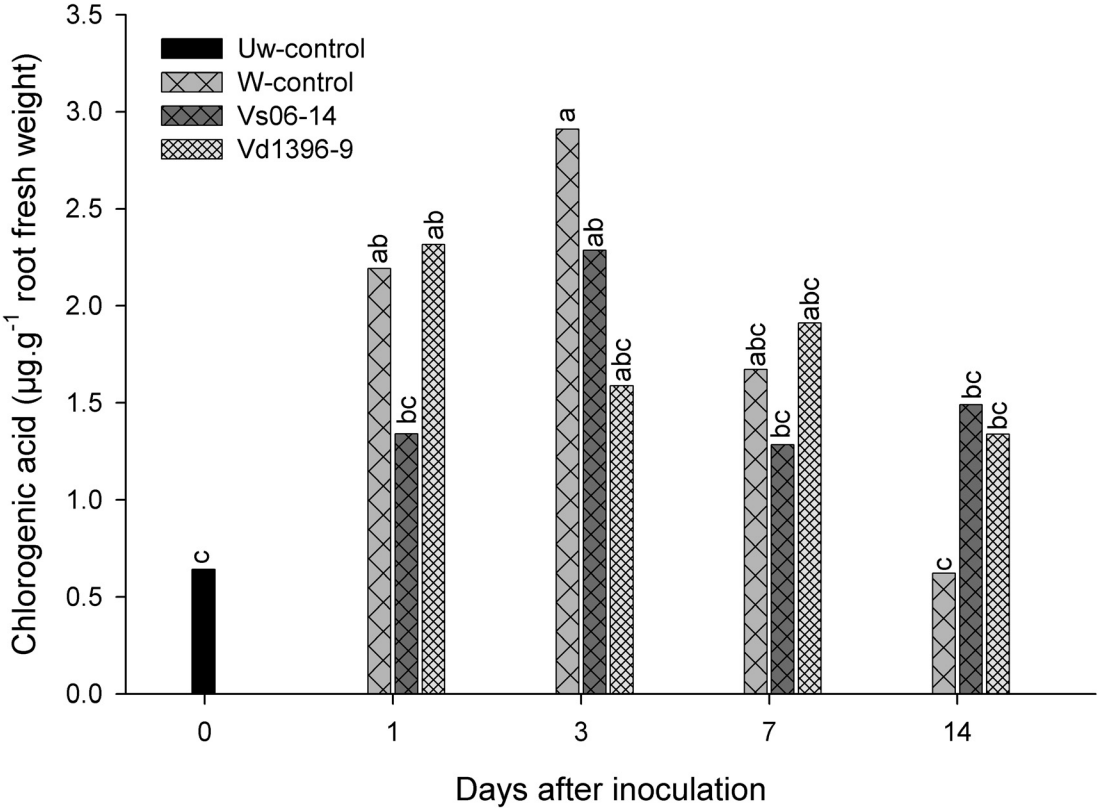


Figure 7.1. Chlorogenic acid content (µg.g^{-1} fresh weight) in roots of potato cv. Kennebec wounded, un-inoculated control plants (W-control), inoculated with the weakly (Vs06-14) or the highly (Vd1396-9) aggressive *V. dahliae* isolates at 1, 3, 7 and 14 days after inoculation. Uw-control represents chlorogenic acid in the un-wounded, un-inoculated control plants at the day of inoculation. Bars with the same letter(s) are not significantly different according to the least significant difference test at $p<0.05$.

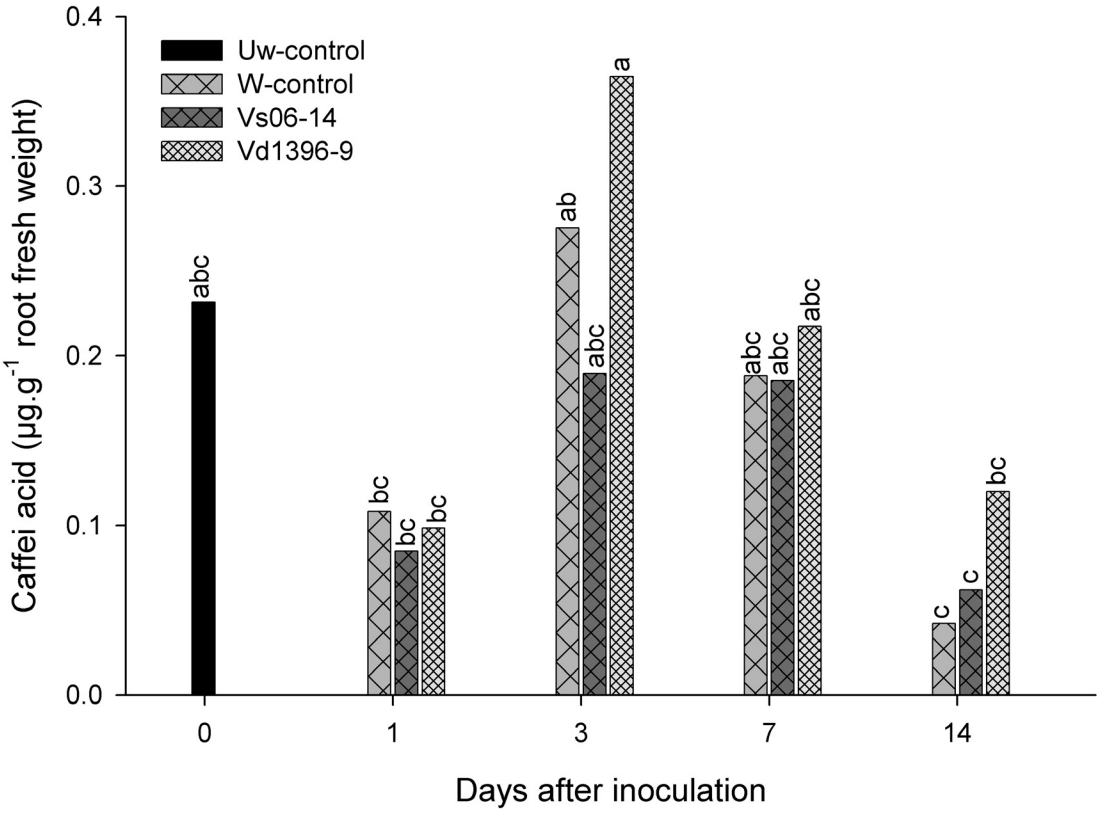


Figure 7.2. Caffeic acid content (µg.g^{-1} fresh weight) in roots of potato cv. Kennebec wounded, un-inoculated control plants (W-control), inoculated with the weakly (Vs06-14) or the highly (Vd1396-9) aggressive *V. dahliae* isolates at 1, 3, 7 and 14 days after inoculation. Uw-control represents caffeic acid in the un-wounded, un-inoculated control plants at the day of inoculation. Bars with the same letter(s) are not significantly different according to the least significant difference test at $p<0.05$.

14 d.a.i. It has been reported that caffeic acid accumulated in potato in response to infection by other fungi (Kuć et al. 1956).

Ferulic acid showed gradual accumulation in the roots over time after inoculation with the highly (Vd1396-9) or the weakly (Vs06-14) aggressive isolate, whereas there was an increase in ferulic acid in the wounded control at 1 d.a.i. and 3 d.a.i. followed by a significant decline at 14 d.a.i. (Fig. 7.3). The content of ferulic acid was 1.78, 1.15 and 0.11 $\mu g.g^{-1}$ root fresh weight of Vd1396-9-inoculated, Vs06-14-inoculated and wounded control plants, respectively, with significant differences at 14 d.a.i.. Ferulic acid has been reported as an inhibitor of mycelial growth, conidial germination and hydrolytic enzyme activity of the wilt fungus *Fusarium oxysporum* f. sp. *niveum* (Wu et al. 2010).

Chlorogenic, caffeic, and ferulic acids have been suggested to be factors in disease resistance in many plant-microbe interactions including tobacco-*V. dahliae* (Sheppard and Peterson, 1976), canola-*Leptosphaeria* spp. (El Hadrami and Daayf 2009), and saskatoon- *Entomosporium mespili* (Wolski et al. 2010). It seems that chlorogenic and ferulic acids could play a role in the roots of potato in the early three days after inoculation, to resist the establishment of *V. dahliae* infection.

7.4.2 Secondary metabolites identified in potato stems after inoculation with *V. dahliae* isolates

Caffeic acid, rutin, and *cis*-jasmone were identified in stems of potato after inoculation with the *V. dahliae* isolates. Caffeic acid was detected in both roots and stems, however, its content in stems was relatively higher, 2-fold than in the roots, especially in the case of plants inoculated with the highly aggressive isolate, Vd1396-9

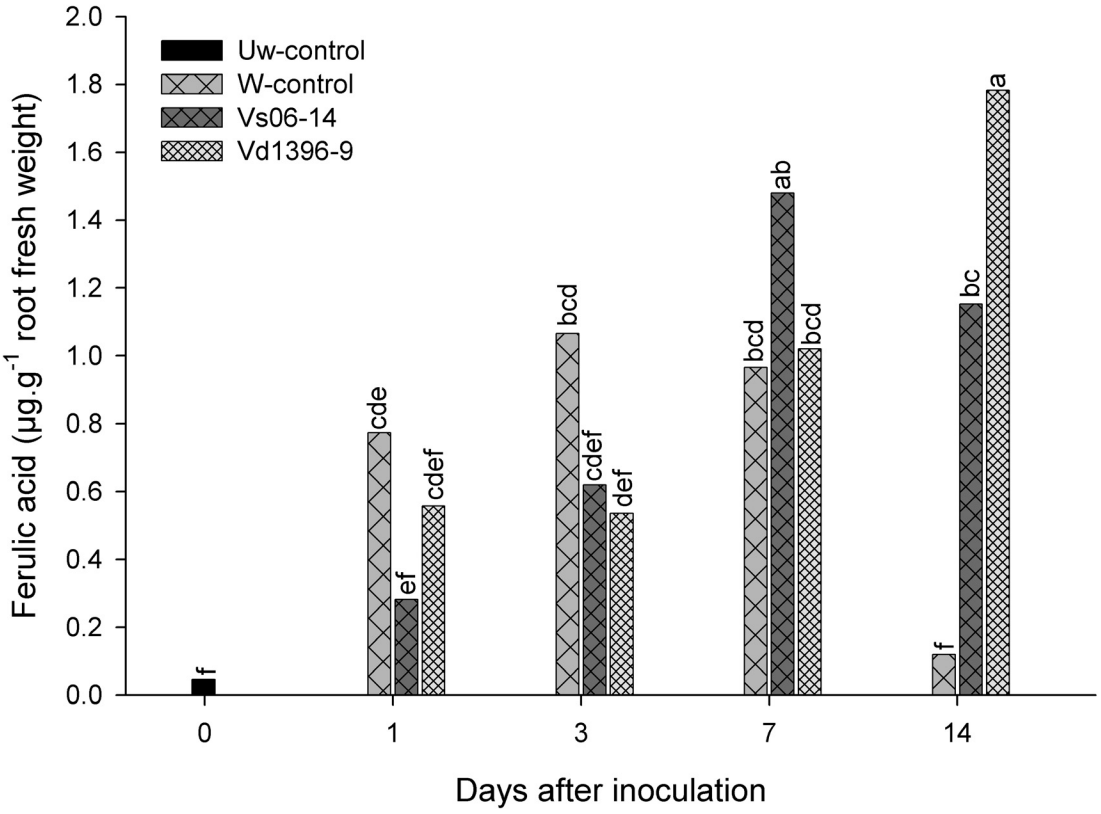


Figure 7.3. Ferulic acid content (µg.g^{-1} fresh weight) in roots of potato cv. Kennebec wounded, un-inoculated control plants (W-control), inoculated with the weakly (Vs06-14) or the highly (Vd1396-9) aggressive *V. dahliae* isolates at 1, 3, 7 and 14 days after inoculation. Uw-control represents ferulic acid in the un-wounded, un-inoculated control plants at the day of inoculation. Bars with the same letter(s) are not significantly different according to the least significant difference test at $p<0.05$.

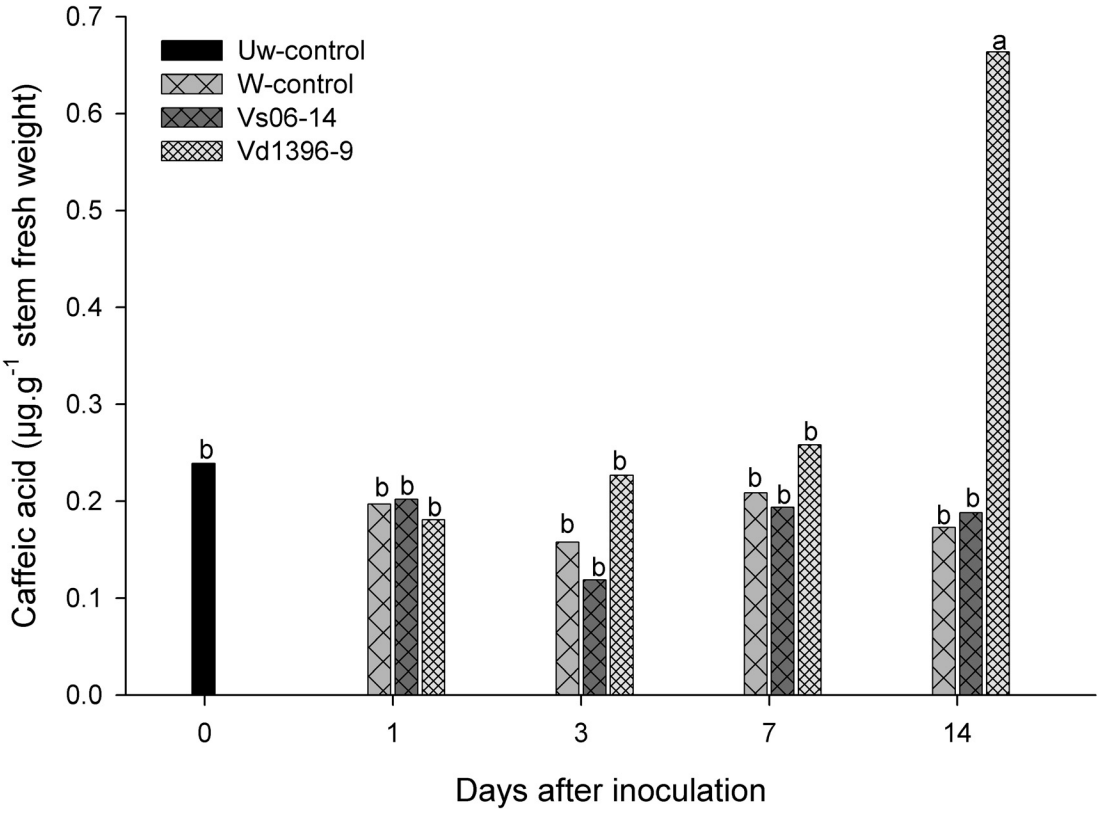


Figure 7.4. Caffeic acid content (µg.g^{-1} fresh weight) in stems of potato cv. Kennebec wounded, un-inoculated control plants (W-control), inoculated with the weakly (Vs06-14) or the highly (Vd1396-9) aggressive *V. dahliae* isolates at 1, 3, 7 and 14 days after inoculation. Uw-control represents caffeic acid in the un-wounded, un-inoculated control plants at the day of inoculation. Bars with the same letter(s) are not significantly different according to the least significant difference test at $p<0.05$.

(Figs 7.2 & 7.4). The highest content of caffeic acid was found in the stems 14 days after inoculation with Vd1396-9 and was significantly higher than all other treatments tested.

The highest content of caffeic acid in roots (0.36 $\mu g.g^{-1}$ fresh weight) was detected three days after inoculation, whereas in stems (0.66 $\mu g.g^{-1}$ fresh weight), this occurred 14 days after inoculation (Figs. 7.2 and 7.4). The difference might be due to the time spent by *V. dahliae* to colonize the vascular system of the potato stem. In lettuce, colonization of cortical tissues and entering the vascular system of roots occurred in 2 weeks after inoculation while the vascular discoloration of taproot and crown area was observed in 8-10 weeks after inoculation (Vallad and Subbarao 2008). The increase of caffeic acid was noticed only in response to the highly aggressive isolate, which supports its aggressive role in the potato-*V. dahliae* interaction.

The flavonoid, rutin, was identified in potato stems after inoculation with *V. dahliae* in all treatments. However, it was not detected in the un-wounded-uninoculated control plants at inoculation time zero (Fig. 7.5). It seems that rutin accumulated in stems as a response to either wounding or infection of the plant roots. Rutin concentration was significantly higher at 14 days after inoculation, with the weakly aggressive, Vs06-14, isolate. The content of rutin at 14 d.a.i. was 2.91 and 0.31 $\mu g.g^{-1}$ stem fresh weight in *V. dahliae* isolates Vs06-14 and Vd1396-9 inoculated plants, respectively. It has been reported that many fungal plant pathogens can detoxify secondary metabolites produced by plants (Bouarab et al. 2002; Pedras and Ahiahonu, 2005) and it has been suggested that the highly aggressive *V. dahliae* isolate' could detoxify rutin and overcome potato defense responses (El Hadrami et al. 2009a,b).

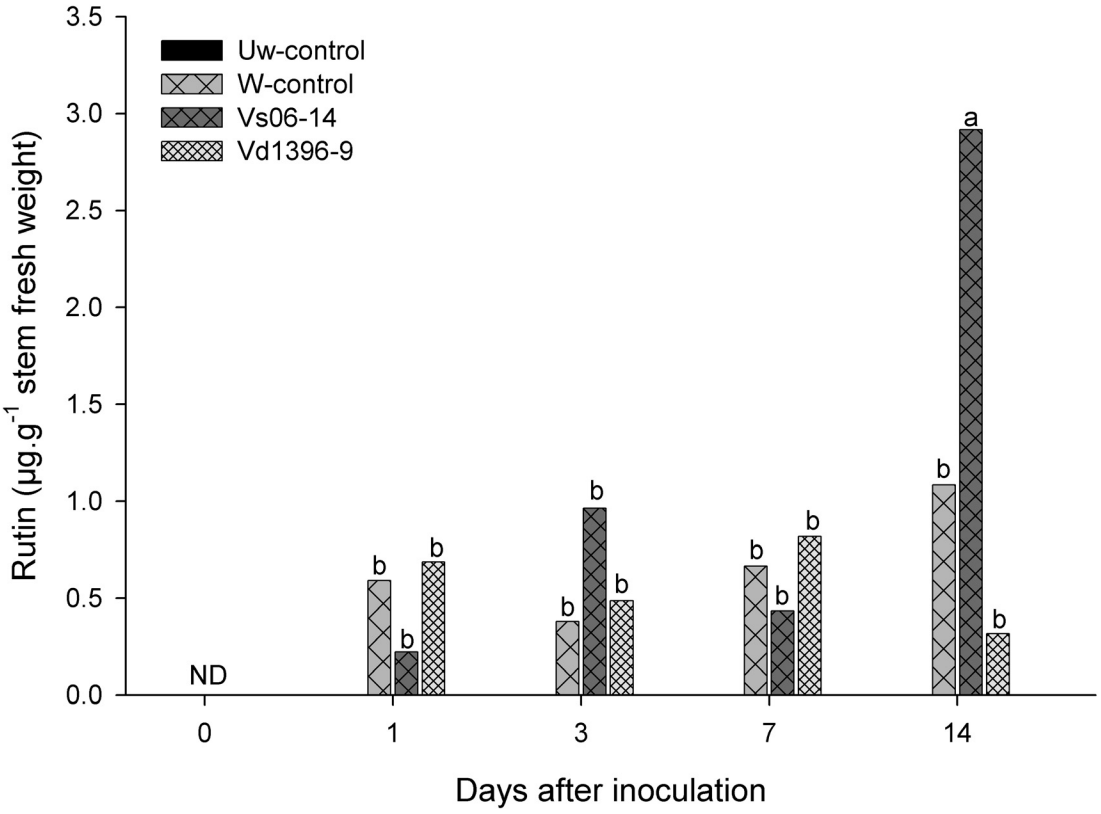


Figure 7.5. Rutin content (µg.g^{-1} fresh weight) in stems of potato cv. Kennebec wounded, un-inoculated control plants (W-control), inoculated with the weakly (Vs06-14) or the highly (Vd1396-9) aggressive *V. dahliae* isolates at 1, 3, 7 and 14 days after inoculation. Rutin was not detected (ND) in the un-wounded, un-inoculated control plants (Uw-control) at the day of inoculation. Bars with the same letter(s) are not significantly different according to the least significant difference test at $p<0.05$.

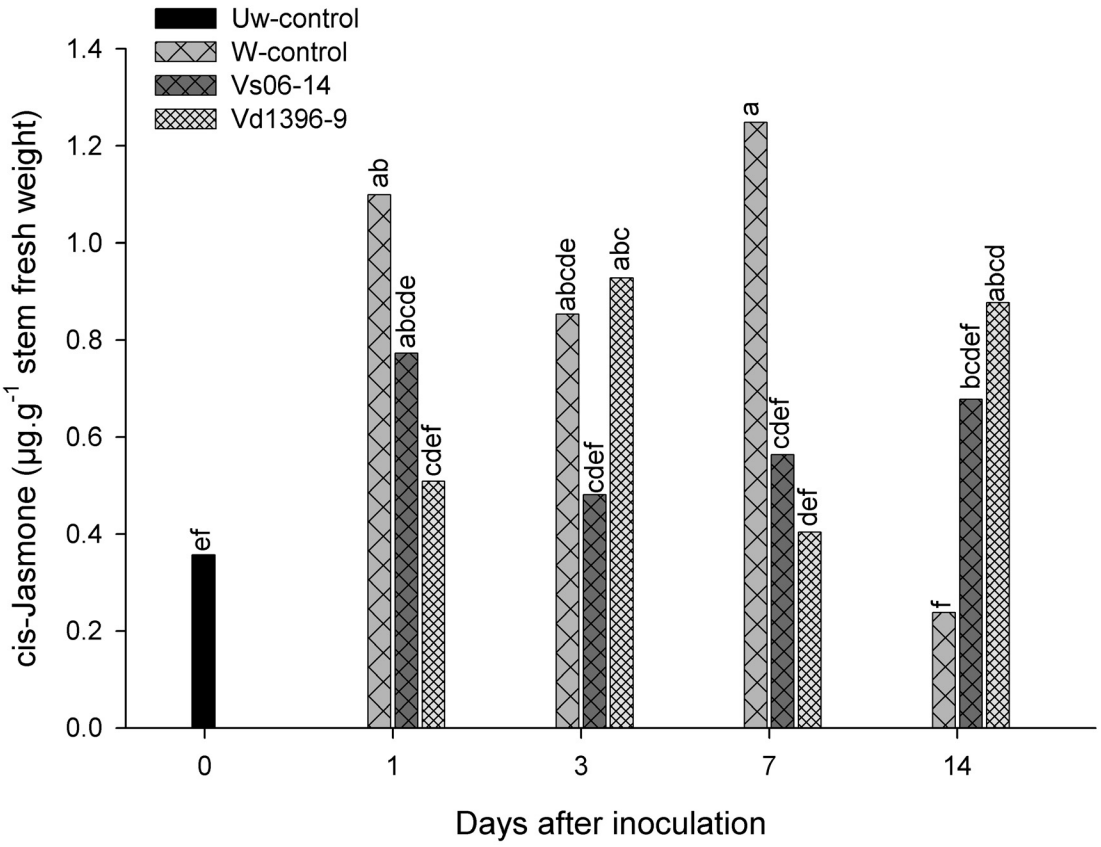


Figure 7.6. *cis*-Jasmone content (µg.g^{-1} fresh weight) in stems of potato cv. Kennebec wounded, un-inoculated control plants (W-control), inoculated with the weakly (Vs06-14) or the highly (Vd1396-9) aggressive *V. dahliae* isolates at 1, 3, 7 and 14 days after inoculation. Uw-control represents *cis*-jasmone in the un-wounded, un-inoculated control plants at the day of inoculation. Bars with the same letter(s) are not significantly different according to the least significant difference test at $p<0.05$.

Cis-jasmone is a volatile compound known in plant defense against insects (Birkett et al. 2000). The content of *cis*-jasmone ranged from 0.23 to 1.24 $\mu g.g^{-1}$ stem fresh weight. There was no significant difference in *cis*-jasmone content in stems of control and potato inoculated with *V. dahliae* isolates Vs06-14 and Vd1396-9 (Fig. 7.6).

7.4.3 Secondary metabolites identified in potato leaves after inoculation with *V. dahliae* isolates

Rutin was identified in the leaves of potato inoculated with *V. dahliae* isolates, un-wounded and wounded control plants. The concentration of rutin in leaves reached 74.7 $\mu g.g^{-1}$ fresh weight one day after inoculation with the highly aggressive isolate (Fig. 7.7), while the highest concentration of rutin was 2.91 $\mu g.g^{-1}$ fresh weight in stems of potato inoculated with the weakly aggressive isolate (Fig. 7.5). Rutin accumulated significantly in the leaves as a response to all treatments, as compared to the un-wounded un-inoculated plants. Starting after one day of inoculation, rutin content gradually decreased over time in both wounded control and Vd1396-9-inoculated plants. However, there was no decrease in rutin content of leaves of plants inoculated with Vs06-14, the weakly aggressive isolate.

7.5 Concluding Remarks

Phenolic compounds are important in plant defense mechanisms to a wide range of biotic diseases and abiotic disorders. Profiling of secondary metabolites during potato-*V. dahliae* interaction indicates that chlorogenic, caffeic and ferulic acids may play a role in the early stage of root infection to restrict the spreading of the fungus. Later, once the

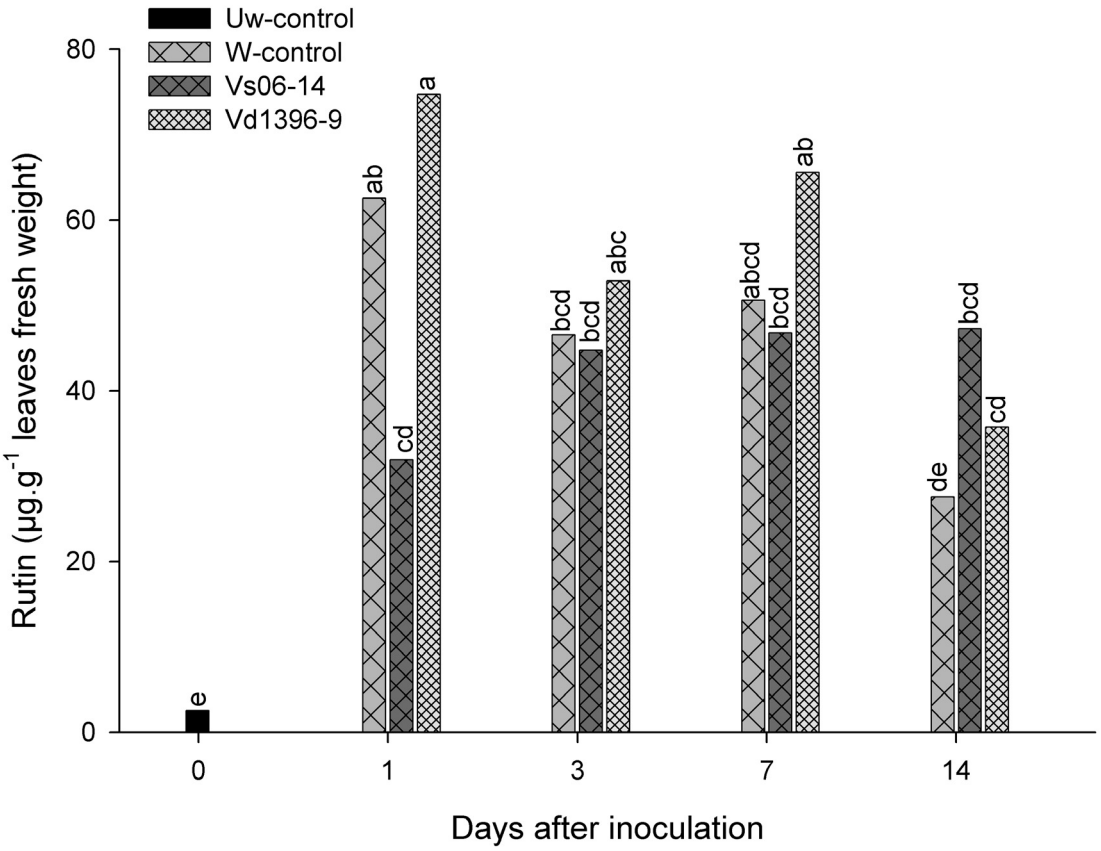


Figure 7.7. Rutin content (µg.g^{-1} fresh weight) in leaves of potato cv. Kennebec wounded, un-inoculated control plants (W-control), inoculated with the weakly (Vs06-14) or the highly (Vd1396-9) aggressive *V. dahliae* isolates at 1, 3, 7 and 14 days after inoculation. Uw-control represents rutin in the un-wounded, un-inoculated control plants at the day of inoculation. Bars with the same letter(s) are not significantly different according to the least significant difference test at $p<0.05$.

fungus reaches the foliar parts, caffeic acid, *cis*-jasmone and rutin accumulated in response to the inoculation. The variations in the pattern of phenolics accumulation in plant parts against the highly *vs.* weakly aggressive *V. dahliae* isolate suggest that in the early stage of infection, both isolates induced phenolics in the host plant. Then, either the host plant could not recognize the highly aggressive isolate, thereby resulting in a decrease in the accumulation of phenolics, or, the highly aggressive isolate was able to manipulate these compounds. From another point of view, the highly aggressive isolate might have used other pathogenicity factors to suppress the signaling of the plant defense responses, perhaps, by neutralizing the reactive oxygen species and salicylic acid signaling molecules and, subsequently inhibiting the production of phenolic compounds.

8.0 GENERAL DISCUSSION AND CONCLUSIONS

Verticillium wilt is an important disease that affects a very broad range of crops worldwide, including potato. The pathogen, *V. dahliae*, produces microsclerotia as resting structures at the end of the disease cycle, which can tolerate harsh environmental conditions and remain viable in the soil for more than 10 years as a source of infection. Verticillium wilt diminishes the yield and quality of host plants. Management of Verticillium wilt is very difficult due to the persistent nature of the microsclerotia in soils. The current control recommendations rely on applying non-ecofriendly soil fumigants, introducing non-host plants into the cultural rotation, soil solarization or using partially resistant cultivars where available. However, control of Verticillium wilt has not been completely achieved. Many of the previous studies in this pathosystem focused on plant defense reactions to *V. dahliae*, whereas the pathogenesis of *V. dahliae* has not been fully understood. The successful management of this disease should rely on understanding both host and pathogen, and their interactions.

The current study investigated the molecular basis of *V. dahliae* pathogenesis on potato. In the first chapter (section 3.0), a differential pathosystem of *V. dahliae*-potato was established. Assessment of *V. dahliae* isolates (Vd1396-9, Vs04-28, Vs06-13 and Vs06-14) on two potato cultivars (Kennebec and Ranger Russet, susceptible and moderately resistant cultivars, respectively) was carried out by evaluating disease severity, vascular discoloration and growth measurements of the inoculated plants over time. Isolate Vd1396-9 caused chlorosis, necrosis and vascular discoloration symptoms greater than Vs06-14 on both cultivars, which indicated the high level of aggressiveness

of this isolate. This might be, in part, due to the high rate of conidiospore production and microsclerotia that were observed with this isolate. The results indicated and confirmed that *V. dahliae* isolates Vd1396-9 and Vs06-14 were highly and weakly aggressive, respectively, on potato.

These isolates were chosen for the transcriptomics and proteomics analyses in order to investigate their pathogenicity factors. In the second chapter (section 4.0), potato root extracts were used to induce pathogenicity-related genes in *V. dahliae*. In this pathosystem, the potato root exudates stimulate the germination of microsclerotia, which produce penetrating hyphae and start the infection. The expressed genes in this early stage of *V. dahliae*-potato interactions had not been previously investigated. In the present study, 573 differentially expressed transcripts were detected in the *V. dahliae* isolates after elicitation by potato root extracts. Among them, 185 transcripts of interest were recovered, re-amplified, sequenced and putatively identified in one and/or the other isolate after elicitation by root extracts derived from either the susceptible (Kennebec) or the moderately resistant (Ranger Russet) potato cultivars. The number of the identified, differentially expressed transcripts in Vd1396-9 was relatively higher than in Vs06-14, and in response to the root extracts from Kennebec relative to Ranger Russet cultivar root extracts. This indicates that isolate Vd1396-9 may have more factors to infect the host and perhaps neutralize its defense reactions. Numerous differentially expressed transcripts exhibited homology with genes involved in pathogenesis-related functions such as cell-wall degradation, oxidative burst tolerance and signaling pathogenesis in *V. dahliae* and/or other fungi.

In the third chapter (section 5.0), the first proteomic map of *V. dahliae* was reported with comparative analysis of the differentially expressed proteins in the highly (Vd1396-9) and weakly (Vs06-14) aggressive isolates. The strength of conducting the proteomics analysis comes from the direct relation of proteins to biological functions. Twenty five differentially expressed proteins were putatively identified, 18 from isolate Vd1396-9 and 7 from isolate Vs06-14. The identification of these proteins revealed their potential roles in *V. dahliae* pathogenesis such as melanin production, stress tolerance and suppression of plant defense signaling. The majority of the pathogenicity-related proteins were identified in the highly aggressive isolate, which supports their roles in pathogenesis.

Based on the results from the transcriptomics and proteomics analyses and among many of the pathogenicity-related candidate genes/proteins, three were chosen for further characterization in order to investigate their differential expression in the two isolates. The fourth chapter (section 6.0) describes isolation, cloning and sequence analysis of stress response regulator A, isochorismatase hydrolase, and tetrahydroxynaphthalene reductase from Vd1396-9 and Vs06-14 isolates. These genes were selected to represent oxidative stress tolerance, suppression plant signaling and microsclerotium formation in *V. dahliae*. It seems that these functions contribute to the aggressiveness of Vd1396-9 isolate. The comparative sequence analysis showed various differences including insertion/deletion and substitution mutations. The detected differences of sequences of the three genes in Vd1396-9 and Vs06-14, especially those in the coding regions, may elucidate their differential expression and, consequently, the level of aggressiveness of the two isolates.

In the fifth chapter (section 7.0), phenolic compounds were profiled in potato cultivar Kennebec after inoculation with either the highly (Vd1396-9) or the weakly aggressive (Vs06-14) *V. dahliae* isolate. Accumulation of chlorogenic, caffeic, and ferulic acids, and *cis*-jasmone and rutin was observed in roots, stems and leaves of inoculated plants over time as compared to control plants. These compounds were induced in the early (1-3) days after inoculation, followed by a decrease in their levels, especially in the plants inoculated with the highly aggressive isolate. It might be due to overcoming or suppressing of plant defenses by the highly aggressive isolate, which is in agreement with the earlier findings on the transcriptome and proteome levels.

It seems that pathogenesis of *V. dahliae* is complex and the co-evolution of the pathogen with the plant has resulted in the development of counter-defense tactics by the pathogen to overcome and suppress the host resistance. The present study is a contribution to the knowledge about the vascular wilt pathogen, *V. dahliae*, and its interaction with potato at the transcriptomic, proteomic and metabolic levels. Figure 8.1 collectively describes potato-*V. dahliae* interactions based on results derived from the three levels as four phases; host plant root exudation, stimulation and activation of pathogenicity-related factors in the fungus to attack to the host plant, activation of plant defense mechanisms and pathogen counter-defenses. Integrating information from the three levels helped to identify potential factors which could be targeted for disease control by neutralizing their action in *V. dahliae*-host interactions. The findings of the current study will improve our knowledge about Verticillium wilt and may help to develop novel management strategies.

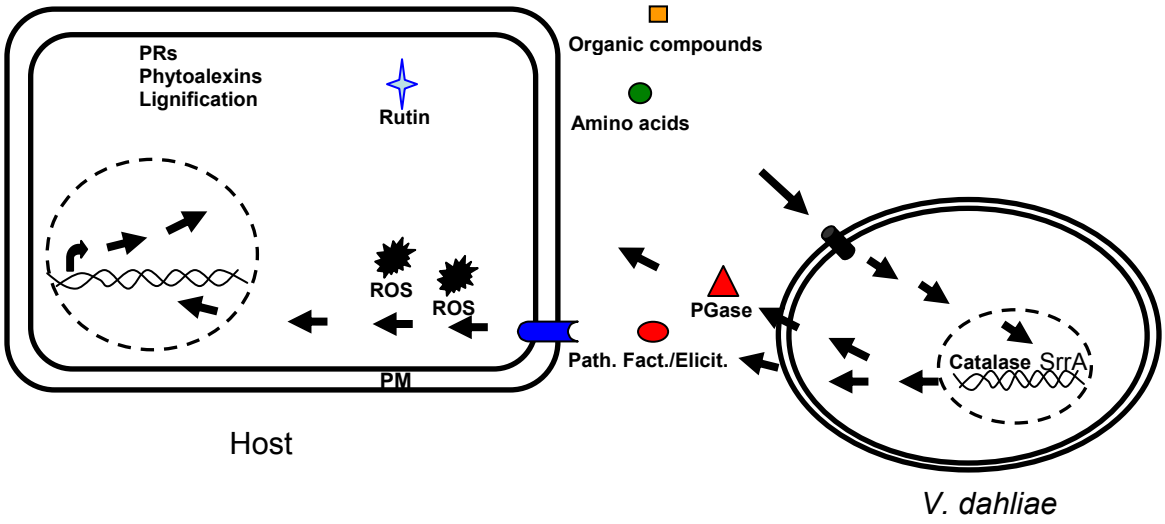


Figure 8.1. A model describing host-*V. dahliae* interactions based on integration of results from transcriptomic, proteomic, and metabolic analyses. Interactions could be divided into four phases: 1) The host plant releases root exudates, compounds such as organic and amino acids, which are recognized by the pathogen; 2) These root exudates stimulate the pathogen germination and activate expression of signaling and pathogenicity-related factors (e.g., serine/threonine-protein kinase, cell-wall degrading enzymes) or elicitors; 3) Some of these factors could be recognized by the host and activate plant defense mechanisms including production of reactive oxygen species and accumulation of phenolics (e.g., rutin, ferulic acid); 4) The pathogen can develop counter-defense strategies to overcome plant defenses such as expression of stress response regulator A that activates a catalase enzyme which detoxifies reactive oxygen species, and protects the pathogen from the oxidative burst.

8.1 Recommendations for future research

Pathogenesis in soilborne fungi is very complex. The present study initiated research for further investigation into the *V. dahli*ae-host interaction. In-depth functional and molecular characterization of selected genes is needed. Generating knocked-out and over-expressed mutants in the highly and weakly aggressive isolate, respectively, for each gene could be the next step. These genes could be compared in many different isolates and correlated those results with the isolates' aggressiveness. The expression and protein product localization of these genes *in planta* could be studied using green fluorescence protein and confocal microscopy. Investigating global gene expression using microarray in parallel to proteomics techniques of plants inoculated with the same two model isolates could provide us with a detailed view of the *V. dahliae*-host interactions.

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10.0 APPENDICES

10.1. Nucleotide sequences of the differentially expressed fragments in *V. dahliae* isolates:

>Vd1396-9-1-1 (344 bp)
GATCCGTGACTACATGCCTGCTGGATGCACAGTTCAGGATGAGTTTTGACTGGACTGGGTTACGTCACGAAAGACAATGGCGCTGTGAT
GGCGCGGCATTGAGGGTGGATTGTGTGCAAATACTCTCTCTCAACATGTAAACAGGCATCTTGGAGGATAACAGTCCGATACCCTTGAC
CGTCTACTGCCGTTCTTGGGTCATACCTTGGTCTGAGAGAGACGCTGTGGCTACTAAGGAAGTTGCATGAAACCACAGACTTGGACAAT
TGCTCCCGTGATCAATGAAGGCATGATATGAGCCTACGACCTGAGCTAGATCCATTTACTCACGACTCATCACACAA
>Vd1396-9-1-2 (265 bp)
GATAAGGGCTCATGGAGAGCAGGTATCATCTCTAGCGTGTTTCAGATTCTCGCCACTGCCTTCGTTGTTGGCTATCCCCTTGGTCCTGC
ACGACTTGCCCAGAACTGCATACCGAACTGTAACTGACAAAGGGTGAATGAATCGCTCTTGCCCTGTTCAGACCTTGCTTTTCCTCGTC
CTTAACGCCTTTGCAATTTTTTTTTATTGAATAGGGAACTTTCCCCCCTGCGCCTCTCAAATTTTAAGTTGTTGCCGCCCCCACAAA
>Vd1396-9-1-3 (237bp)
GATAGCGCGCCTAGCCGCCATGGATGTCATGTAGGCAGACCGGTATGTATATCAGTTCTTCGGAGACATTGTCGAGCTGCTTATATGGA
GCACACAACACTTGGCCTAGTGCACCATCATTCTCTGCAGCTGTGAAGAGCTGTAGGTAAAATTGAATGTGATCATCAATCACCCGCGA
TGACCCAGATTGCGCACCCTGtATGTAGCGAGTGATGAATTTGGCTACAGCACATCACA
>Vd1396-9-1-4 (170 bp)
GATTACGAGCGCACAGTTCCTGATTGCGGACGAATTCATGTGGAGCAGCACCAATACAGTGATCTGCTGTGAGAAATGTGCAGGAGGTG
TAGAGAATCCGAACGCTCTAGCCGTTTGTGTCTTATGCGTCTTCGATGCTAGGAGCTTGTCTCCCCCTATGCCGTGCTTTA
>Vd1396-9-1-5 (105 bp)
GATATCTTTGACAGGTAGGACTCTAGGCCCTCTGTCACGCTCTCGATTTCAGAGGGGGGATTTTGATATACAGCACGTGTGAAATGTAC
CATCCATTCCAGTCCC
>Vd1396-9-1-6 (64 bp)
GTCATTTTATCTGATCGGAGGTGACAATCTGGGTCGCTGAGATTTGAATTGGCACGCAGCTCAA
>Vd1396-9-2-13 (101 bp)
ACGTAATATCCTATTATGGATCGTCCGCGCAGGCTTGAACCCTGCTGCTGTCGTCACAATTTTTGTGAATTGGTACGCAGTCAACGAAT
TGGTACGCCCTT
>Vd1396-9-2-14 (67 bp)
GACTGCATGTACTATTGTCGGACGGTCCCCCAAAGACTTGCTCCCCATATCTGCTGAACTGATTCCT
>Vd1396-9-5-19 (70 bp)
GGTTTTGATCTTCTGGTGGTGCTCCCTCTCGTGTGAATGTGGCTTTCGAAAAAATTGGCCCCCCAATCAA
>Vd1396-9-7-20 (46 bp)
GCTCCCTCCgATAGACGCGCGAACGCGATATGTAGTGTGAATTGCA
>Vd1396-9-9-21 (292 bp)
CATACTGCACATCGGAAGGCTCGCAACACACGCAGCATGGTGAGGTGAGGCGTGACGACTTGTTGACGGAACACTGCTATACCGTGCGA
GAGGCTGACTGCATTCAACGGCATCCTTCTGATACCGTGTTCAAAGGAAAAAGCTGATACGGATCGATACGGATCGCAAGGTTGGTTTT
CTGCCCGCTCCATGTGCCGTGCTCCTTGCTACGTCCGTTCCTTTGTTGGCGCTCGTTTCTCAGAAACACGAGGCCGTTTGATAGCCTTG
AAAACTAGAGCCGGAAGGGGGACGT
>Vd1396-9-10-32 (380 bp)
GGCTCGGCTGAGCGTCTCCGCTCAAGAGATTTGTCTGGAAGGGGTGCTTGAAAGCCGCTGCTTGCCCTTAAGGGACGTCTCCGGAAAAC
TTGACATGTGCGTCAATCCAAGTTCTGGTAAACCAAAAGACTCGGAGAGCCCACCATGATCTTGTCTTCCAGACGAACGGGTATGTCGT
TGAGTGAAGGCCAGGTCCAGTGGCTTGGCGATGACGAAGAGCTCGTCTTTGGGCAGGTGAAGCAGTCCGCCGTTGTTGGGCTGTCTGAG
TCCCTCTAGGAATCATAGCGAAATGTCGTCGGTATCAAGCTCAGATGAGGGTATCCTGGTCGCTTTGCGACGATGACCTTTGGTACTCC
GCGCTAGGTAGGGCTGCTTTTCTC
>Vd1396-9-10-33 (362 bp)
GCTTATGGTAGACACCGCCCTCCGCGGGCATGTAGACGACAGGAACATGACGTTAGTGACATCTGCGCTTGTGAAAACAGACCCTTGGT
GACTTGCGGTGACATGACTTCTGGGAACATGTTGCTAGAACAATCCTACCGCACCTTCTATTTCACACCGATGAACATCTGATGCCACC
GGCAGGGATAAACCTAATGACGGTCATCCCGACGAGCATGCATCGTGCTGCTTTTGCCTCCCCCAGACTACTGATGTCCACTCTTGTGG
CGCATTGGCAGAACACCCAATGATTATCAGCGTACCTACGCCCCGTACACATGTCGCCGCACACTCTCTGCTCACTTTCCTCTGCCTTG
CGTGGT
>Vd1396-9-10-34 (371 bp)
GAGCTCGGGCGGCTGCTATCTGTGGGGAGGGGAGCTTGTACACCTTGGAATGGCCTTTAGGGACGTGGTCACTTAGGTGGCATTGGCCA
TACTGCACCACCAGGAAATGGCGAGTAGAAAAAGCACTGCGTACAACAACTCGTCTCTCACACCACAGGGCTATGAGCGCTCATCAAGG
CATGCACCGAGTGACTTGACTACGATCGAGGATTCCTCATGCTGCTTGACAAGTGACCTGACGATCTTGACCTCTCTTAGTCCCTTTGT
GAACAATAGCATAACTTTGCAGGCGTCACTAACACCCCTGACTATCCGGGTCAATGTGTCAAGAGGGAATTGGTACGCAGTCATTGGGT
AGTTCTGCCTTTCTC
>Vd1396-9-10-35 (311 bp)
GGAGACTTGAGATTCTCCCAGTCATCGCCTCGCCATCTTTTCCCGATGAGGTCCTGCTCTTTTTCCTGCTTTGCCTCCGGCTGGAACCC
AGTTGTCAGGGTTCATTGAGGCGAGGAGAGCGTTTCGCAGCAACTTGTCTTTCTGTTTGGTTGTTCTCTCTCTTTTTTTTTATTAGCTG
GCCGGCGTGCAAGGGGTTCTCAGGAACAGGTCTTGAAAAGCACCAGGCCAGGGGGTGCAGGGCGGTTGGGATGGGAGGGACGGTTGGAT
GGTTGCTGGTCGGCATGCGACCAAGTGCGTCCTACGAAGCCTTT
>Vd1396-9-10-37 (163 bp)
TTGGAGACACGCGGCATGGTATGCAACTCGCCATAGGGACCAGATCTTACACGGTGCTGCCCATCGGGATGTGGGACCAGACCAATCAG
CGAAACCGGATGCCAGGCATGTGGTCGCATCAGGCCGCACACATTCTGCTCCACCACACCACATTACGATTCGC
>Vd1396-9-10-38 (427 bp)
CCGTGCCATCAGCATGGATAGCAGGCAGTGTTCGTACGcCCGACACACCGGACGCACCGCCTTAGTACCTGAAGGCACGCGTCCCAATG
GCCCATGTAATGGGCAGGtAGCTGGCAGAGTAGGTGTGAGGTAAGCGGCAATGACAGGTATCTTCACCTGGCCGAACAGAGCTGCCCAT

GTGAGGTGTTCGTATCTCCCTTACTCTCTCGAAGTCGGTCGCACTCGTTGCTTATGCGTCATCGTGAATGAAACGGCTAGAGCGAAAGC
TCGACAGGTAATCCTTCTGCGATCTAGCTGTGCGCAGGCCACACTGATGCTCCTCACACATTCATCGTCTCGACTCAAGCATCCAACAG
TCGCACGTCAAGTCTTGGCCGCTGTACAAGTGAATGCTGACCACTGTGAAGTTGAGCTACAGCACGATCAA

>Vd1396-9-10-39 (400 bp)

ACTAGCGGAcCTCCTGTCGCCATCTaTCAGCaCGGGaGGGATTGCGTGTGTGgAGCATCGTCTCTGTAGCGAACGTCATCCACTTGGTG
AGGATGACGTGACTTCGCCCACTACTCgGtAGTCTCTCAGACTTAGAGAACTcTCTGGAAGGATATGGCATAGACGAGCGCATCTTGGC
TTGCGCATATCGAAGTAGCTCCAGAGTGAAGCCAGACTGgTGTGTTAATCAATCGACCGTAGTCGTGTCCTCCACGTCCTCCAGCGTAC
TGTTGCGCAGAAACGCAGTATTACATAGACGCGATCGAGGGCGTCTCATGCGAAAGTACGACTGTCTACATAGTTCGCAACCCGAAAGC
AGCTTACATCCTCGATATCGAACGAGCGTCTCAATCCGTAGCGG

>Vd1396-9-10-40 (256 bp)

GAAACGATGTCTCATCATTCCATCGTTTCACATTCATGTCTAGCGAGGTATTCACCTAACCTCAATGTGGTCTAAACTACGATGTGCAG
CATGCTGACTTCATGGTGCCGATGATCGTCACATATTTCAACACTGGATCACTATCTTTCATTGTGAAATTTGATAATACTGTTTTGCT
TCTGTTGTGCTTTCCTGGCATGAGGTGCTCTGGCGAGGCTGCCTCCTAAGCATGTTTCCAAGCATCACGCATGTCACT

>Vd1396-9-10-41(265 bp)

GCATTCACCTCTGGGAGGACACGAGCCAAGCTTGATGCTTTATACCATCTCATAAGCCGCCGAATGCCATGGCGTGTTGTCATCTACCT
CAGTGTACGATGTCGCGAGGACTTCCCATAGTGCCGAGAACCGGGAATGGCGTCATCGGGCGCATACCTGGTTGGCTGCTCTAGCCTAT
GGCCCAACGCAATGATGGCTCATCTTGCATCAACGGCCAATTGCCGCTTTTTGAGCAGGGATCTAGTCGGAATTGGCACGCAGTCAA

>Vd1396-9-10-42 (262 bp)

CTCGACAATGGGATGGCACGAGCCAAGGtTGACGCTTCCTACCCATCTCATAAGGCGCCCCTTTCGATGGCATTTGAGAACTACGTCGG
TGCACGATGTCCCGTGGACTTCCCAGAGTGCCTAGAACCGGGAATGGAGTCATCGGGCGCATACCTGGTTGGCTGATCTAGCCTATGGC
CCAACGCATTGATGGATCATCCTGCATCAACGGAGAACTGCCGCTTTTTGAGCATGGAACTAGTAGGAATTGGCACGCAATCAA

>Vd1396-9-10-43 (213 bp)

GCTAGACGATAAAGCTATCTCCGTGGAGTGCAGGTCATTATGTTGAATTTGCACAAGGAGTCTCTAGTGAGAGACATCTAGACGGGAAC
GCCGCTGGAGAGTTCTATGAATTACGCGAATCCCGCCCCCACAGCCTTTATTTCCCGCATCCTAGTGCAGGCGACGGCGCCCTCTGCTG
ATGCGCGTCAAAAGCTCCCGATCTGACCTAATCTG

>Vd1396-9-10-44 (240 bp)

GATGAGGTCGACTCCGGATGTATCCTCTCGCACTAGCTGTGTCTGAACAGGGGTGGGCTGCACGGCCTTAGGAGATCGACAGACATAGG
GCGCTCGGCTAGCCGAACGGATGTCTCACCCTCGCCATCGCCGAGACTCCGTCCCGTAGCGTTGTCGTCGGCGATGATCCTCCACGCGC
ATCCCCAGTCTTTCCAGTCCGGATGGTCGCACGTCACCGGGGAAGGAATTCGCACACAGTCA

>Vd1396-9-10-45 (187 bp)

GAACAGCCCAGGCCGTGTCGGAGGTCCGAGGACGGCAGAAGCTCAGGGTAGATGAAGCCACCTCGCTTTCGCTGCCAGGCAGTGCGCCG
TCGCTTGACTTCCATCTGGTCTCGTCTGTCAGCGGGACGTGTCAAGCAAACATCTGTCAGGGGCGACGGGCGCGAAAACTGAGTTGGGA
GAATGGCGG

>Vd1396-9-10-46 (175 bp)

CTGATGCTATCTGCGGATGAGTTGATGTCAGAGTATAAGATGGCATGATGATGGCGATGGATCCCGTTTGGATATCGCGGTTGAGACCT
GAGGTGGGACGGGGAATGACGACGAGGAATGGCTTGGGAAACTTGAGAAGCGCCGTGCTTGATCAGGAGGGAGAGAAGATGAGGCG

>Vd1396-9-10-47 (196 bp)

GTATGAGTGCCATCTGCGACCTTgACGTCAGGAGTTAAGATGGCATGAATGACGGCAATGGATCCCCGTTTGGATATCCCGATTGATAC
CTGCGTTGAGACAGGTAATGACCACGAGGAAGGGCTTGGGAAACTTGACACACCCCGTGCTTAATCACGACGCCGATAAGATGAGGCGG
GTTTTGCCACCCACACAA

>Vd1396-9-10-48 (241 bp)

TCTGTTCCGCCATCGATGTACAAAAGCCCACGGTGTAGCGGAGATGGAAGCATATCGGAGTCTTTGCTCTTTGCGCCTGATTTATGCAG
CGCGCTTAGCTTGATGTGAGGCTCTGAGGCTAACACTGTCCGGACCTGATTAAGCACGTTCTAGCGTCATACTCGGAGAATCgTGCGGA
ACGGAGATCGCTCGGAACTGCCTGTATGCACTCGATGAGGAGATTGCGTCACGCCACGTCACA

>Vd1396-9-10-49 (183 bp)

GCTGAGAGGGGCGGCCTGCAGTGGCTCCGGGCGCTGAATCGAAATCGGCACTACGTTGACCCGCTGTCTGCATTTCGGCTTGTCTAATG
CATAGAAGGCGTCAGGCCATGAACACTACAGCCAAATGGATACAATCCATTCGCGGCAAATGCCTCATTCTGCGGGGATGTGGCACACA
CACAA

>Vd1396-9-10-50 (137 bp)

GAGCAGTTGTGCtTGTAGCATGTGGTGTCGTCTCGGTGACGGGTCGGAGACCAGGTAATGTTCATTGCTCCCATGTTGGGACGTTGATA
CAAACTAGGCCTGCAGTGTGAGAGAACAATGCCTAACTGGCGATGGTT

>Vd1396-9-10-51 (118 bp)

GTCTCGCATTCTTGTACAATTTTTATGGGTTGGAAACTCTCGCAAGGAGACTGTGACGGATTGTTCTGCCCAACCACGGGGAATCGATC
TCATTGTTTCTGAAGTGTATTGATTATTA

>Vd1396-9-10-52 (145 bp)

CTGCGAGTTTAGACTGAGACACTAGGATATGGAGCATCGGTCTGGCTACATGTCTCCGATTGTATAGTGAGGTATCGGTAGAGAGAACG
CGCTTAGCGCATGGTCTCCTGCCTTTGCTCAGGAAGTAGGTGCGCACATCACAACA

>Vd1396-9-10-53 (107 bp)

GACTCGtCGTCTGGTCACAGGAAGTAGAGGCGCCTTTCGCAGGGCCTTGCCTCAAACTCCAATTCTTGACCCTCCTTTCGATTGTGATG
CTGTCTCCTATTTCTCTC

>Vd1396-9-10-54 (124 bp)

CTCTAGCATGGGCTGGACATTATAGCGTAGGAGCTTTTCGCGGCTATTGCCATCGGGCATTGGATGGATCCTAGTCGCGATTGAGCTAT
CCTACCAGCTTAC

>Vd1396-9-10-55 (97 bp)

ATGGATTGACGGTAGTATCCACCATCAATGTTCTTAGCGAGATTCTTGCTAATGCTCTGGAGTATCTGTCAGTTGACGCACACTTGTCG
AAGTCTTG

>Vd1396-9-10-56 (105 bp)

GCTACAGGCATCTGACGAGGTATCCCAACCATCAGATGTTCTTAGCGAGATTCTTGCTAATGCTCTGGAGTATCTGTCAGTTGACGCAC
ACTTGTCGAAGTCTTG

>Vd1396-9-10-57 (138 bp)

GCGTCCGAGTATTGACCATATGgATCCCAACGATCAGAGCTTCGTTTGCAACATTCCTCGCCAAAGCGGTCGTGAGCATCCTGGCAGGA
TAACTGCACATCTTCTGGAACGTCTGTGAAAATTTGACACACATCACAA

>Vd1396-9-10-58 (117 bp)

GTACGTTAgGACGTAGTGACCTTCCTTTATATTCTTCTATTATCTTCGAGAAGATTAGAATATTATCGCAGGTATACTATTACGAAGAC
GGTCGTAGGAATTGCGTACCGCAGTCAA

>Vd1396-9-10-59 (149 bp)

GCATTTGTGCcTCAGTACGtGGATACTACCTGGAGgAACCAGGATCCACGtGGGTGCCAGTATGCTCAAGTACGCACCTGGCCCGAGCC
CGGTCACGCTCTACCGTACATCATAGTCCCAGGTGGAAATTTGGTTACGCCAGTTCACAA

>Vd1396-9-10-60(134 bp)

TCAGACGGGGATACTACCGGAGGAACCAGGACGCACGCGCGGGCCAGTAGCGCAAGTAGCGACgTGGCACAGGCACGgTCAGGCCTTAC
CGTACGTATTGGTTGAGGGGGAGATTGTGGCTCCCGCACACACAA

>Vd1396-9-10-61(97 bp)

GCGTGTCGAGAAATTGCCTGGGTTAGATTTTCCATTGGGTATCCCGCTGCTAAAATGCCTCCGTGCTCCTTGGCGAATTGGGTGCCCCG
CTCAAAAA

>Vd1396-9-10-62 (69 bp)

GGCGCGTCAGAACTTCGtTGGGtAGATTTTCCATTGGTTCCCAgTCCTAAAATGCTCCTGGCTCCTTTG

>Vd1396-9-10-63 (66 bp)

GCGTCTCAGAACTTCGTTGGGTGATTTTCCATTGGTTCCCAGTCCTAAAATGCTCCATGCTCCTTG

>Vd1396-9-10-64 (104 bp)

AGCGCGGCGCCTAGGGGCAAGCATAAGCAAGCGTTAACTGATGGACACCAAGTCGCTTGTTGGCGATCACGCTGTCTTGGGAATAGGGT
ACCGCAGCTCAACCA

>Vd1396-9-10-65 (60 bp)

GACTTCACAGGGCAGCATAGCAAGCGTACTGTGGCACAAGCCTTTTGCCGTCACGTGCGT

>Vd1396-9-10-66 (61 bp)

GGTCTTGTGGGTGACGGGGAGATGAGGGCGTTTTTAGCGGTTGGGAATTGGACCACATATA

>Vd1396-9-10-68 (66 bp)

GATTGACATATGGGCGTCCCGACGCCGCGTGGGAACCTTGGGTGTGGGGAATGTGGCACACACACA

>Vd1396-9-11-71 (222 bp)

GTGGGGACAAGCCGAGTCACGCCCCCTCGCCTGCATGTTGTTGGCGAAGCACAGCCTGGCCCACGTCCGGCTTCACAGCATCAGATGAG
GGCTGGGGCAGTCGCAGCGCAAAAGCTGGCCAACTGGAATTTCACTTTGCGTT
TGTCCCGCCGGGTCATGTTTCACGAAGGTTGGGTCCGCGTCTGCTCCGTCAGTCGGGTGGAACGGCAAGCGCACAAAGAG

>Vd1396-9-11-74 (215 bp)

CAAGGAGCACGCTCTGAGACCAGCCAAGTCACTACAGGATAAAGCTTCCGGAAGATACTCCCCGCTGATGTCTCTGTCATGTTTCTGCC
CACCCTGAGCCTCATTATCCTCGGCTCTTACCGTGCATACGTTTCCCTCGGCACTCTTACGCAACATTCTGACCAATGTTTGTCACGCA
AGGGTCATCGTCGCGGGCACCCAGGACGCGTTCACAG

>Vd1396-9-11-75 (209 bp)

ATTTTTCTCTCTGTCTCTCTCTCTGCTTTGCGCCTGAATCTAAGGCatgCCGGGCGATCAGGGCACCACGCTTGCCGACGAACCCTGTT
GCGAATCCGGAACATAATTGTTCTCCATTATGCGATGCGCGGTTGGGGCAAATaGCCTTGTTTGTCGGGGGAAACCAGTGGTTGGTAGG
CAAGGCCTGAGaCGGGAACGGGGAATTGGTA

>Vd1396-9-11-79 (141 bp)

CATCGTTTACGGCTAGGACTACTGGGGTATCTAATCCCATTCGCTCCCCTAGCTTTCGTCcCtgaGTGTCAGTTACGGCCTAGCAGAGC
GCTTTCGCCACCGGTGTTCTTCCCAATCTCTACGCATTTCACCGCTACACTG

>Vd1396-9-11-82 (86 bp)

GTCTCTTTTTGAGAGAGCCATAATCTAGCAATCCAAGTAGCGAAGGTTGTCACGGCATGACTTGTAGTCATAATCATGCTGTCGCT

>Vd1396-9-11-85 (69 bp)

GTCGCAGCCATGTGTTTACATCTGGTCTCGTGTCATCATGTTCTTGGGGTGGGCGCATGGCATCGCGTT

>Vd1396-9-11-88 (51 bp)

TTCGAGCTATTTGTGCTACCTTACCTAGTATCCCTCTAGGTGGAATTGGTA

>Vd1396-9-12-93 (338 bp)

GCTAGTATCTGGAGTGGACCATACACAACGACGGCATGGTGACCACGCGCCGACTCACTGATTAGCCACGACTTAGGTGTACATCTTCT
ACGTGGACGGGAATTCACGTGATCATCCTTATAGATTCGTGGGGCTCGGACGATAACCCTTCCCTTTGACGCAGAACTTCAGGTGTCTA
GTAGAAACACCAAGGCGGTGCGCCTATAGGACGACACTCCATATGTGAAGGGGATGTCGGTGCGCCCTGAACATTGCGAAGAACTACAA
TGGAACGTATAAGGAAGCTGCATTTCGCAAGACGTAGGAGGATACCTACACCCATGAGCTCAAGGAGTGGT

>Vd1396-9-12-94 (269 bp)

GATGGTTCCTACTTCTCGACGGAATTGACTTCCACGTGGGCAGCCGTGCGTCAGGACAGTCAAGCCGGACCCATATTCGCCTTCTCGTC
TGCCGCGACTCTAAGGCTTGATAGATGTCTGCATCCGAACAGGGGCTACTCCACGATGCTCTCCTGTCTCGTCAACCGTACCTCCGCCC
CCGACTCTCGCCACAGACAAGGGAACTGGCGATAGCATCCAAAACCACGAACGACCCAGGATCCAGGACACAGGCTCGATGCTCACGGT
TG

>Vd1396-9-12-95 (270 bp)

GCAGGTACCGCGCCAGCCGATGCTCACTGCCGCCGCCGGGCCTTGCCCATCAAGCAGGACCTAGCTGTACCAGAACCCAAGGTGTCTCT
GGGACATCACCACCGTTGACGACTGGCCTCCACCAGAACATATGGACCAAAAGACAGCGTAAACTCCGGACACTGCCACAACACATTAC
GTCAATAGAAAGAAGGAGCAAGCTTGAACATATCATGTCTTCATCATGGTCTCCTACAACCCCCGACCCATAGTGTTTACGTACGTACA
CGT

>Vd1396-9-12-96 (244 bp)

GCGAATCGCGACCGGCTGCTCCGAGCTcCGCGTGAGTCATAGCCATCGACAGAAGCTGGCTCGGCAGCTCGACTGAACGAAGGAAGGTC
GCGATTCGATTTTGTCGGGCTCATTGATGGCAAATCATATGTCGACTTGGTCGGGCTCTTCGAGGGAAGATCGTAGCTAGATTTGGTAG
GGCTCATTGACGGAAGGTCGTACGTCCGTTTTGCTGGTTCGGACCGCAGCCGGGTAGATTCGAGGT

>Vd1396-9-12-98 (268 bp)

CCTGTATACACGGAGtCGGCATGCTTCCGAGCTCTCCCGTGTAGATCATAGGCTATCGCACAGCAAGCTGGACTCGGCAGACTCGTACT
GAATGAAGGGAGAGGTCGCAGATCTCAGATTTCGTCGGAAATCATTGATGGCAAATCCTATGGGGACTTGCCTCGGGCTCTTCGAAGGA

AGATCCTATCTACATTTGGCAGGGCTCATTGACGGAACGTCCAACGTCCGTTTACCAGGTTCACACCGCAACCGGGACAGATCACCAGG
T
>Vd1396-9-12-99 (232 bp)
CCGACTGGATCTGACAGTTGCGGACATGACATACTCCTCCCGTTCGTGCCATATTGCGAAGGAGACATGCTGGAAAGGTTACCGGTGTC
GGCCTAGGGAGCACTGATGGCCACTCAGAGCTCAGACTTAGTTGATCCAAGGGAAGTCCTATTTACAGTTATTGATGGTACACGCTCTT
CTGTTCGCACGTTATTTGGAGTATCTCTATCAGATAATATTGGCACACACACAA
>Vd1396-9-12-100 (226 bp)
GCTCTCGCCAGCCTACAGCTGCCTGCCTTCTCCAGCTTCAACGTTCGGGACGCACGAGCAACATGTCCCGCAAGGTGCTCGGCCGGTCA
ACTTGAGATGGGCATACGCTTGCCTGTCTCCCATATGATTGGGAGATACACACACTCTGGGACCATGAGGGTTGATTTGTGGGTTGGTA
ATCGGCGAGGCTGTCCTGGTCGAGATGGGGGAATTGGTCCGCAGTCAA
>Vd1396-9-12-101 (195 bp)
GGGACGACTGAGGAGACTTGACGCTGGCGCCGCGATTCAAGTCTCGGAAACAAGTTCGAGGCCGTCATCGACAACATGACTGACTATGT
CGACACGTACATCCGCGACGTTTTTGACAAGGTGCAAGAACAAGGCGTCAGCAGTAGCGCTTTTGACCTGCCTCCGCTTGACGTGGATC
TGAGCCTTGACATTCAG
>Vd1396-9-12-102 (178 bp)
TCTCTGTTGACACCTCACACCAAGAACTGGTGCAGCCTGATACGATTGAGAAGCTCAACCAGATCGCCGAGGCTCTTCAAGCGAGGGAC
TACGACGGGGCGCAGGCCCTGCAGGTGGAAGTTCAGCGTGAGAAGACGACGGAGTGCGGCAACTGGATGGTACGTTTGTCTCCAAGTTT
>Vd1396-9-12-103 (237 bp)
GATTAATCTAGACGCTGTTATAAGTTCAACAAGTCTTCAGGAACTGGAAATCTACTCACCGGAAACAAGAAGGCACACTCGCTGAATTG
TACCTGATAGCTGAAGGACTAGCACTCGCTTGGTCAGTGGGtACACCGCATATCACGTTATAGCGTTTAGACGAGCTCCTCTGGGGGAC
CTTGcGTGACACCGTGTTAAGTAAGCCCGTCGGTAATAATTTGCGACAGCACATCATCA
>Vd1396-9-12-104 (174 bp)
CGTTAATGAAGGCAGCTCACTTATACTCTGTGTGGCGCCGGAACACCTTGTGTCTCGTGTATGCGATCTAGAGACTTTCTTTAGCGAAG
GCGTTTTATCATGCGGCGTGTCAGAGGGCAAGACGAAGGGGTTCCGTCTCTCGGAGCCTGATTTTTGTCGTGTTCTGGCGGTTTT
>Vd1396-9-12-105 (184 bp)
CATAATTGAGACACCGGACGGTTAGGCTCCTAGCCAGCCGTCATAATCATTGCTGCTGGAGATACTCAAGCCGAATAGAAGAAAACAAC
ACTCGAAAAAAAAAACCTTCGATTGTAGGGAAAATCAAATGCGGTCTAAGACTCCTGAACCAGACACTTGGCATGGAATTGGTGCCCCGT
CAACAC
>Vd1396-9-12-106 (156 bp)
ATCGCTTGCCCGCTGATAAATTGACTTTCACAAACTTCGACGAAACGACGAGAACAACGAGTCGCCTTACGGCAAACAGGTGAGCAGAG
AGAGAGAGAGAGGGCTTGGCGCTGTCCACTTGGGAGCATGCGATCGCTGACCATGTCTTCAGGCCTG
>Vd1396-9-12-107 (149 bp)
GCTTGCTTGTTCGGGGCGGTCCCCCGCCAATCTATGTACAGGTCTAAAACGACCATGCGCTACGCGATCCGGCGTCTGTGGCATGGCAT
GACATCCCTACTCTTTTTGTTCCTACACCGCCAAAGAATCGGACAAGCAACGTCCCTTTC
>Vd1396-9-12-109 (207 bp)
TCATTCGGCTTCACAGGATTGCTTCCAGAACAGTGGGTCATCCTCTCTGGAGCTACCCAGGTGCACTGATAGGGATCTATGCAACTTTC
TATCTGATGACAGGATATGTATATCCAGCCTCACACGTAGGTCCTTCTCGATACACATTCACCCAGTAGACGTGCCACTACATCGGTTG
CGAGATATGTGtGATCACGACATCTACAA
>Vd1396-9-12-110 (113 bp)
GCGCGCTCGATCTGGTGGGAAGCTCGAAAGGCTTGGGGGGGACAACGCTGATGGTGGGAGCCTTGAGTTTGCAGGTAAGAATGACTGGC
CACAGACCACTTGGACCTGCTCTT
>Vd1396-9-12-111 (167 bp)
GACTTCGCTTCGCAtTCTGTGCAGGGAACAAGCTCGAAAAGGTCTTGAGAGGGGGTACAACGACATAACTGGATGGGAAGCCGTCGCAG
TTTTGCAGGCTAAGTAATGACTGTGCCACAGCACCACATTGGACGCTGCCCGTGTGGATATTGGCTACAGCACATCAA
>Vd1396-9-12-112 (111 bp)
GGAATATGGATGTGGCCCGAGGCTCCCTGGTAGTGCCATCGTCTGAAGGGAGAGAATTGAACGGAATGCCGTATGCCACGTTGCCACCC
GCATTCATGGCATCGCTGGAAT
>Vd1396-9-12-113 (113 bp)
GCGGATCTACCGTTCCCTGGGGCTCGGGGCAGAACTACGCTGGGGACGGCCTGCCTCCTCTGATACTGGATGGCGCCACTGATGCCTGC
AAAGATTCATTGATTCGCGGAATT
>Vd1396-9-12-114 (110 bp)
TGCACTGGATGGcGAGGTAGGCACGATGAAATCCGTCGGGGCTCACCGATTAGTGGAGATCGCACGCTTCAACGTTCCCCGGAGTTCTA
AGCAGTCCACCAGCACTTACG
>Vd1396-9-12-115 (84 bp)
GACTTCCTGCAGATAGTTGCTGTCGGACTCTCGTGTCCAGGTGTTCTGAGAAGAGTCCGATCGATTGTCCTCTTTCCTGGGCTG
>Vd1396-9-12-116 (90 bp)
GGAATGCCTAGGGATTGATTCCCATGCCGATCTCCCCCGTGACAACATCCATGTACCTCAACGGGATGGATCACGTGCATCATATACTG
G
>Vd1396-9-12-117 (89 bp)
AATGCCTTCGGGATTGATTCCTCATGCCGATCTCAACCCGAGCAACATCCAATACCACAACGGGGCGGATCACGGGCATCATCGACTGG
>**Vd1396-9-12-118 (87 bp)**
TCTTCGGTATTGATTCCTCATGACCGCATCTCCCCCGGAACGACATCCTTATCCTCTGCGGGTCGAGATTGCGGGCCTCATAGACTG
>Vd1396-9-12-119 (64 bp)
GAGCAGCGATGAGTTTTGAGGTCTGGGGGTGAGGTCCGATAGACGCTCCTTTGGGTCGTTGCAG
>Vd1396-9-12-120 (53 bp)
GGGATTGCTCGTCGGGCTACGATCCCGGATGAGTCATGGGAATGGTCGCACCA
>Vd1396-9-12-122 (50 bp)
GCATATTGTCCCGCCCCGCTTCGGCGAATCTTCCCACGTGTTTCTGGTCT
>Vd1396-9-13-124 (280 bp)
CGAGATCAGCAGTATATCCTCACGCATCTCGTGGACATGCTGCCTAGGAGTGACGTTACACAGTTACGGTATGATGACCAGGAAGCGAG
TTTTATGACGGCTTTGGACACATTTACATGAAGTGCTTTCGATGGACGTCAGATGACTAAACGTGAGGCGCCCCTGCTGTGTATATAGA

CGCTCCGCCTCCCATCTACGAACTGAACCACCGTCTTATGGGGTGCCAATGCTGCTCTGGTGGTGCAAATCACAGACAAAGGACACTGA
AATTCCCATGTCT

>Vd1396-9-13-125 (247 bp)

CAGCTTGGGCGATCTGGAGAACACCGGTCATTCTCCAATCTTCAATATCTGCATCAAGACCAAACCAGACGAGAACCTGACCGAAGCCC
ACGCCATGCGATTTATCGACCAGCCATACCTCGCATCCCCGTGGCGAAGGTTTACTTAAGAATTCACCTATAAAGGGGACTCATACATC
GTCATGCGCCATATCAAAGGACAAATGGCAGGGCATGGTTGGCTAAGCCGTACAGAAGAATCGAAAATG

>Vd1396-9-13-126 (243 bp)

AGTCTAGGGGCTAGGAGTAAGACTCTCAGTAGAACAAAAGGGTAAAAGTCCCCTTGATTTTGATTTTCAGTGTGAATACAAACCATGAA
AGTGTGGCCTATCGATCCTTTAGTCCCTCGGAGTTTGAGGCTAGAGGTGCCAGAAAAGTTACCACAGGGATAGACTGGCTTGTGGCGGC
CAAGCGTTCATAGCGACGTCACTTTTTGATCCTTCGATGTCGGCTCTTCCTATCATTCCGAAATG

>Vd1396-9-13-128 (192 bp)

CGAGCACTCCATGGACTGAACATACCATCCACTCTTGCCTAGGTTGTCGCACAAGTAGTCCTCTGGCAGCTCTGTGGCCTGGTTCGGGA
CATGATCGGTCCCCCACATCGAGCCGACGGCGACAGGCGTGTTGGTCAACTCACGGGGGATGATGTCAGGGTTGCCGTTGCTGCAAATT
GGCACGCACACACA

>Vd1396-9-13-129 (279 bp)

CTTGCGCGAACTGGACGAACcTAGgaATGTCAGCGTGTCGTTTGACTGACACGCAAGAGTGGAATACAGGCAgATATTGCACGTGACAC
AACACACATGTACTCGAATCTCACATATGCACAGCGAGAGTtTGAGTGCAGACGCAATGGAAGAAGTACCGTACGTATCGTGCCCTGCA
GCTGCCGTTAGCTTCGCCCTCTCGAGTAGTCGAAGTCATTCTACTCTAGCGTAGCCACAGTTGCGTTACGTCGGAAATTTGGGCTAACG
GCCCGATCCAAA

>Vd1396-9-13-130 (211 bp)

GCGACGCGACGTCTCCTGCAGAAGCCTTATCATTGCGCGTAGGACGACTATCTGTGAGCCGCTTATTGCGGTTTGTTGCAAGCCGTGAG
CTCATTCGCTACGAGCAGCGAGAACTTTGCTCAATCTCTCAATCTGTCTTAGACATCTGGGATGCCTTAGCAACCATTCATACAAGTTT
GGACCTTACGACATAGTTGGCTACCCACATCAA

>Vd1396-9-13-132 (107 bp)

CGCATCATACAAATGCCACTCCGAGgTATCGACAATCCGGCCTCTTTTTCTTCGAACCCCCGCATTCAGTTCGACGACCACACCATAAG
TCCCTCGCCGCCTCCTCC

>Vd1396-9-13-133 (145 bp)

AAGACAATAGTTACACTTTACCGTTTCATTAATGCCGCATCATCTTTGCAGTGCGTTGGcTATCGcGAATGTCATAGCACGATCCATAC
ACCCAGGCAGTCTTTTCATCGGAAGCTGTTCCCGAAATTTGGTACCGCCAGTCAAA

>Vd1396-9-13-134 (113 bp)

GGCGTCATTTCGCTGATGAGAGACTGCTTCTGTATGTGTAAGTGACAGTACTACCGTATCGGGATGACGGTGCTCCGTCCGCGCCGGTG
CATCGTTAATTGGTACACACTCAA

>Vd1396-9-13-137 (81 bp)

CTGCGaCAGACGTGTTGGTGGGCCTCCTTTCGGCGCAGGAATTCTCGCTCTCGGGACCGAAATTGGGTTCCGCCGGTCAAT

>Vd1396-9-14-140 (265 bp)

GATGGATGAATACGTGACTTCTTCCTGATGAGTGAACAAGCAATCACTCTCAGAGATGCCCTGAGTCTGTCGTAGATCTTGAGCCATAC
GGGTTTCACCTCTATCCCTTTTTCAAACTGATCCTCGTTGTGCTGGCGTGAGCAATGATATTGCCTCCGATAGGTTTCTTGGGATCAGG
GCTGAACATCTGACTCGGGCCGCCGTCCACCTGCGCCACAACTTGGTTCTTGCTGACCACCGCTATGCCAAATTGGTCCCCAGCCAT

>Vd1396-9-14-141 (172 bp)

GGCTAAGGTTGTAGTGAACGATGCACGCGTGAAGACAAACCCCCGTCTCTTCTGAGTTCAACACGTCCTGCTGTTATGTACTTCAAAAA
AGGAAGCTTGATACAAATTCTCAAAACACACCAAAGCAAGCATAGGTGTTGCCCCACAGGTTGGTGCACGCGCTGCCGGCGCC

>Vd1396-9-14-142 (202 bp)

GCCGTTCACCCACATATGGACACTGGACAATCCGTGAGTGGATGCCGTCTTAGTCCTCTTGCCGTACTCCTGTCCTGGATTTCACGCGC
ATGCCGCCTGTGAATCACATTGGTTCTGGAATCACCATCTAGCCTTTGAGTCACGAGACATCTGGCGAAGGCACATACACTTATCGGTG
GAGGGCCAATTGGTACCCAATCAA

>Vd1396-9-14-144 (60 bp)

ATTGAGCCCCGCTCTACTGTCATGGACTCTACGTTGATCGCCTCTGCGCCCTCTACCCCG

>Vd1396-9-15-150 (69 bp)

TTCATCATCAtACcCTTCTGAACGGATCATGATACTGTTGACTTGCCGCAATTGGCTACCCACATCACA

>Vd1396-9-15-151 (75 bp)

AGGCATCAGCCATCACTAcTGATGGCAACGTAATCaTCTCGtTCTGATGGGcTCGAATTGGGTACGCAGCTCCAA

>Vd1396-9-16-152 (315 bp)

GGTAAGCTTTTGCTGGAGTAGTGCTAGCCCTTGGTTGGGAGAATCATCACAGCTGTCAAATCTTGCGCGGCGTTCCCTGTTCAGTCCAG
GACCACGTCATTCCAACGCCTTGATCCGGTATCGATATGACGAGCTTCATGTACCTGTCTCTCATATGGTCAAGGCCTTCTCAAGAGCA
CGCAAGTGTGGCGCGCCGGGTTGAAGACATTGTCGACACACGGGTTCAGCGTACTTGAACGGGGTGAGGAGGATACGTGATTGAAATGC
GTATCGCAGACTTGATAGGTGGGCACTGATTAGCTCGTTCGCTCGTCC

>Vd1396-9-16-153 (266 bp)

GAGTGCGTAGACACAGAACGGAGAAACCAGACTGGCTGGTGGACGGAATCAGGGTATCTTGGAACCGCACCGCAGCCTATGCGAGAGCA
TGACTAACTTTCACTTTCGCGCATACCGTCGAAAAGAGGCGGAGGCTGCTCCACGCACCCTTCTTGTAGCGGCCGGTGTCTACAATGCC
AAGGATGGTAGGCACAGCTCATCTGCTCATTGACGCACCGGGATGGGTCCACATGTCCAATTGGTACAATTGCAACGCAACCAAGTCA

>Vd1396-9-16-154 (179 bp)

TTACGTCGCAGACTCTCCTTGGCGGCTGTGTCGTGGGGTTCGAGTGTAAAGTGGAACCGCGCAAGGACACCACAGCTCGGCTGCCGGCC
GGCTGCTCCGTGGCTTGATATGCAGGGGGGGACGGCAGCGATTTGACTAAATGGACGTTCCGCGACTCCTGCGAGTTGCTCCGCCATCA
C

>Vd1396-9-16-155 (97 bp)

AGCCTGCGAGCTGGTATTCCTCTTTCCTCTTCTCCTTCAGTGATTCCATGAACTGACGGAAGGTAGCTTGCTCGTAGTGATCGTACAAG
TCCCTGGG

>Vd1396-9-16-156 (51 bp)

GCGCGTATCGACAATCGGCGGTTCGGGTCGCCTTCGCATGATGCCGATCTC

>Vd1396-9-2-158 (195 bp)

AACCGCCGAAACTCATCGCAATCATCGAGCGCCGGGGCATGGCCATCGGGGGCGCCGAAGCACCACCGACAGGAGATATTTAGGCACCG
CGTGTCACGTTCACTGGGAACGCACCGCCGGCAGGCAACTCATGCATAGCACACGCGAGGACCGCTAACGGCATCCACCTTACCCCTCT
GAATTGCAACGCAGTCA

>Vd1396-9-2-159 (155 bp)

CGAAGCACAATGGACAATCCGCAGCCAAGCCTCTAAGTCTCTTGCGACTCTGCCTGCATTCACGGCATCCGCCTGTAAGCAGTTGGTTC
TGATTCAGAATCGGCCTTTGAGTCACGAGAGATATGGGGAAGGTTCAGAGACTTGACGGGGGTGGG

>Vd1396-9-2-160 (127 bp)

GACGCCGCGAACGTGTACGCAGCACGGTTGATGTGCGCATTGGTCTCTACGATCCCCCATTGCGTTGCGGTTAAGGTAACCGACACATG
CGCCACGGTTGCAAGACAGATATCGCATGGATCCGTGT

>Vd1396-9-2-161 (186 bp)

CCCGCATGGTTACGCCGCGGATTGGTACGCAGATCACtGCAATTGATGAGTCGGCGCAATGAGAGTGGGTACATATTGTAGTGACGGTG
TATAAGTGCTGTGCAGGAATGAACTTCTATGATGGGTGTTATTGGCTCGCAGATCACAAGAGATATGGGGAAGGTTCAGAGACTTGACG
GGGGTGGG

>Vd1396-9-2-162 (60 bp)

CGTACTGGCTTTCTGTCGACAAACGATCAATCAAATTCTTCCGTGAATGGACGCAGTCAA

>Vd1396-9-3-163 (339 bp)

CACCAATCGTTCCGTTCAGCGCGAATTGATTGCTCCAGCTTCGTAACGTAATGACAAAAGGACTGTGTCCCCTAATGGCACGTGCCCGA
AAGTGGTCGGCATAAAATGAACGATCGGCTCGACAATCTCCTCTGTACCGGCACAACATCACGTTTGACACCCGCGGCCACACACTCAC
CTCGGACAACGTCACACATTGCGTAAAACTACAAATCGGGGACAATGATTTTTCTCCCTGGATCCACATGTTGCACGCCCCATCACTGG
GTCGGTCTGCCTTGTCCGCCCATGCTGTACCTCTGCTCTCACCGTCCCAAGTTGATTCGCAACTCAAGTCAA

>Vd1396-9-3-164 (234 bp)

AGCTCGCAAGAGCAGATCTCTCTGGTCGAATCGTTTCGGAACATGCCGGCGGGCAGCTTGACAGTTTGGTTGTATTTCGACTCCATGTC
AATCTCCGTGACCGTTATGTATCGAACGTTGCCGAAACCACAGAACTCATGTCTATTAGAATATCCTAGCTGGCGTGGAAATATTCACT
TTCGTTTCCCAAAATCAAGTATTGCGGTACTGGTGTGATCGGTACCGCTCCCGCTG

>Vd1396-9-3-165 (258 bp)

CAAGCGtATGCAACAAGTGCGaTCAAGTCCGTCTGAGCCAAGTTGCATCGTTATGCACATCGTGTGAATCACCTCATGTCTGTAGAAGT
ATCAGCGgAGTTCTCTCCACCGGGGGTGACCGACCTTGCGGTATTGTACAATTCACTATGTCCCGACCaTTTGTGACCGTCGCTCACaT
GATTACTGGCCAGAGATGATTGCCCATGAGCTCATGACGTGCGATGTCGCGTGCATTGTAGATTGcGCTACATCAACTCA

>Vd1396-9-3-167 (120 bp)

TCATCCTCCCATTGATGAGGACGCTTGTAAGTAGAATCTTATGTAGATTGCACACGTCCAGAGCAATGAATAAATATTGCCCCTTGATC
AACGCCCCGGCATGAATTGGCACGCACTCAA

>Vd1396-9-3-168 (64 bp)

CAATAAGTATACGCACCGAAACGCGATATCTAGCGGTGAATTGGcTAAATTGGCACCCAAACAA

>Vd1396-9-3-169 (64 bp)

GGGCCGACGACGgATAGGACCGCTATACATGTACGTCGACACTCTGAATTTGGACCCACTCAAA

>Vd1396-9-4-170 (316 bp)

AGGAGGGGTTGAGGGGGCAGGACAGGTTCTCTCTCTCTGCTACTGTTGGACACGGACACCCACCCAAAAGAGGCCACATTTTTGGAAAG
GGGACCTACCCGCCTGGGGTCGCTTGCAGTGGAGTGCGTGCGGCGAGCTGTCACCACCGACCAGGTGTGAACCGGCGCGCCGATGTTCT
TCCGACACCTGCTTGGTTCCAAAGGCAAATTCCAGGCCTCTTGTCCGTGCCTCTAGGCTCCACCCGACCGCGCCTGCATCTCTCCAGGT
TTCCCCACCAGCCGCCCGCTATGGGATGTGATCTGGCCCCCTCACACGT

>Vd1396-9-4-171 (262 bp)

GGAATCGGATGACACCAGTCGGCCAGGGTGATACCTTCCGAACAGGATACAGGCAGTCACAGGGATAATATGAAGCTACGAATCATGAC
ACACATTCGTGGCTAGGCAAACATGGCCGCCGGTACCTGGATAGCCTATCGTGTGAGCCAGAGTATTTGTCACTTCTACCAGGACATGC
CTCCACCCATGTGTGGCAGCCCTATGAGGGATGTTGAGTGAAAAAGGGTTCGCCAAAGTAGTCTGCTGAATTGGCACACACACA

>Vd1396-9-4-172 (169 bp)

CTCCGCTGATCTTACCGAGGGATGGACGTGTTCACCCCGGGTAGATTGAGCTTACGCGGTCTGGAATCTGGCGCAATGCCTTGGAAGCG
GGACGTTTGAGACGACTGGACCGATGCTTCCGCGATTTGTCAGTATACATGGGACTACGCGCACGGGATTTGACCTGATC

>Vd1396-9-4-173 (101 bp)

GACCAACGGGCCCTGGGGCTCGGGGTTGAACGACGCTCGGACAGGCATGCCTCCCAGGATACTGGAAGGCGCCATGTGCGTTCAAAGAT
TCGATGATTCAC

>Vd1396-9-4-174 (109 bp)

GTATCCACATTTGATTAAGCGTCTGCCTGGTCTTGGCGTCAGATCGTTGAATTGGTACGCAGTCAGGAaGGCGCCATGTGCGTTCAAAG
ATTCGATGATTCACTGAATT

>Vd1396-9-4-175 (50 bp)

CGACTGACCGGTGTTGAGATATCCCCTGGCGGCAgAGTTTGATGTGCTGA

>Vd1396-9-5-176 (235 bp)

AGGGCTCTGGAGACAGAATCTCCAGTGAAAAAAGGGTAAAAGTCCCCTTGATTTTGATTTTCAGTGTGAATACAAACCATGAAAGTGTG
GCCTATCGATCCTTTAGTCCCTCGGAGTTTGAGGCTAGAGGTGCCAGAAAAGTTACCACAGGGATAACTGGCTTGTGGCGGCCAAGCGT
TCATAGCGACGTCGCTTTTTGATCCTTCGATGTCGGCTCTTCCTATCATACCGAAGC

>Vd1396-9-5-178 (254 bp)

TGTGGAGTTAGAGAGGAGTATGCGGTATCGATTAGAATGTAGAGACACGGGAGTCCTTCAATGAAAGGGGTGGCTAAATTCGTGTACTG
ATCCTGGCTCATTAGTGGATGCGTGTGTGTGACCAATGGACCACTAACTAGTGTGTTACTCTACCGAATGGTGCTGATTTGAGTAAGAA
TTGGCCCACAATCACCGTCGCTTTTTGATCCTTCGATGTCGGCTCTTCCTATCATACCGAAGCAGAATTGGTACGC

>Vd1396-9-5-179 (160 bp)

GATAATGAAATTGGTGCACCTGGTGTTCCGTTTATGACTCTTTGTGATCCGTTGTGGTCGGATATGTGGATATTTTCCATGCACAACTC
GGATTGGTAGACAGGTAGTGCAAACTAGCGAGTGGTTTTTCATAGGCATTTTTTGACCTGAAAAAGCCTCC

>Vd1396-9-5-180 (163 bp)

ACGGAGACGGCACGCTGGCGGTGGCAGCACGAAAAGCCAGTCAAGCTTGCTCAACCGCGCGAGACCGCCCGACTGTTGACTGTTGCGGT
TCATCGTGACCCGACGATTTGTCGAGTCAACAAAGTGTGTCCCAAGTCGTCTGCACTCTCTTCAGACCTAAGCA

>Vd1396-9-5-181 (101 bp)

AGGGATGGGAAGGGGTGAGGCCGATGGATGCGACATGCCTTTAGGAGCGAGAACGACCTCAGGTTCAACGGCACGTACTGGGCGCTCCA
GATATTGTGGCA
>Vd1396-9-5-182 (88 bp)
AGAGATGCCTgTCAGTAACAATTGCCGATTTTGAAAGAGGTCACCACCACATTTGCGAACTCCAGAAATTGTGTACGCAGCTCACAAA
>Vd1396-9-5-183 (62 bp)
CGCGCCGCGGCGGACATGCGCTTGCCGCCGGTGCTGGTGTTTCCAGATTGGGCCCCCACCCA
>Vd1396-9-6-184 (334 bp)
GCCAGCTGTAGACGGCAGAGACTATCTGGCTGGGGCGGTCTCTCTGAGGCAGATGACATTCGTCTGATGAGGGGCGCTCATGTCTCAGA
TATGTTCTACTCTATCAAAGGGGTGTAAATTCCACTTGATTCAGATTGACGGGGAGGACACCTCCTTTGAAAGTGCTGCCTCTCATCTT
GGTAGTCCCACATACTGAGACGCTGAACGTGGAACACACATCATCACTGATATTCTGGGTTAGGGGGTCACCTACGTCTATTGTATCGA
CTCTTAGGGAGGAGACCAGTATCTCCTCTTCCTATGACACCAAAGAAATAGTGCCACACCCACAACA
>Vd1396-9-6-185 (276 bp)
TCTGTGGTTAGGAGAATGAATTCTCTGATGTACGTGGGTTCAGATTCCCCCCTGAAGTGCGATTTTCACGAGCGAAGAGCATTCCATCG
TATAGCGAGGACATATCGAACCTATGTAGCCAAGGGGATTTATGGAGGTTATATCGGGCTGATTAGTTACCACATGGAATACATGTGGC
TTGTGCGTGGCCAACAGGTGCTTTGCGTATCGGCGCTGTTTGTTCCGTCGATGTTGGCTCTTGTTATCATTCCAAAAATTAATTGGCAC
TCACACAAA
>Vd1396-9-6-186 (191 bp)
GGGTCTTCGCTAATCGCCCCAGCTCCTCGTGGCCTGTGACTGCATTAAGGTGCATAGGAATCCATTGATGGACCCCGGTGCGATTCTGG
TTTCGTGGGACTGACGCCACCTCACATAGGGTATTCCTGGCCTCTCATGTCATGTTGTGCTCCCGCATCCTTGGTACTCTCGTGAATTG
GGCCCCCATCAAC
>Vd1396-9-6-187 (154 bp)
CGATGGGCTCATTCTGAACTGCTTCGTTGAAGCTCATCTTTTGTTTTCGGCCCGTTCATCTTTTGAGATCTTTTTAGGGAGCCATGATG
TCTGACTTTTGTCCATGGGAACAGTCAGGGCTGCTGCCTAATGTCATAGTCTGAAAGAAGACTTG
>Vd1396-9-6-188 (158 bp)
GGAATcAGCATGGCCAGTCGCCAGAGCTATGTGGATGTTTGCCAAGTCATCGCTTCATCACTACGGGATGCGCTAGACGGCGGTTGGCT
CCCGCACGTAGATCGCTTCACCTGCCCCTGCGGTTCATGATGCCAAGAATTTGGGTACCGCACATCAAA
>Vd1396-9-6-189 (88 bp)
GGGGGGGTCACGGTGCAGCGGGCGGAGCGTCCGGGGTACAGCTTGTAGATGCCATCGTCTCGGAGAAGTTGGGTTCCGCCGCACACAA
>Vd1396-9-6-190 (50 bp)
CTCCTCTCGTAGACGCGCGCACGCGATAGATGTGTGAATTGCAGAATTGG
>Vd1396-9-7-192 (254 bp)
GGTCTTGGCGATCAGACGCAACGAAACCCCACGTGTAATGTGAGGTGCACAATTGCGTTGCTTTTCATTTTTCGGGTGTGAATACAATC
CATGTAAAGTGATGCGCCTATCTATCCGTTTAGTCCCACGGTATTTTGAGGCCTAGAGGTGCCCGAAAAGTAACCACAGGGTATAACTG
GCTTGTGGCGGCCAACCTTACATAGTAACTGCTCTTTTCGATCCTTGCATGTCGGTTCTTCCTATCATACCGAACC
>Vd1396-9-7-194 (121 bp)
CTGATGTGAGACGCGCGAACGCGATATGTAGTGTGAATTGCAGAATTGGTACGCAGtCATCCTCTGGCGGGTGACACTCCCCTACTTCT
CTCCGACCCCCAGAAATGTGGCCCCCACACAA
>Vd1396-9-7-195 (50 bp)
CAGCCTgCCgAGAAGACGCAGCGAACGCGATATGTAGTGTGAATTGCAGA
>Vd1396-9-8-197 (314 bp)
GTCTACGGGCTAGGAATACAGAATCTCCTTAGAACATAACGGTAATAGTCCCCTTGATATTGATTTTCAGCGTGAATACGCACCATGAA
AGTGTGGCCTATCGATCCTTTAGTCCCTCAGAGTTTGATGCATTAGGTGCCACATAAGTTACCACGCGGAGAACTGGGTTGGGCCGGCC
AAGCGTTCATAGCGACGTCACTTTTTGATCCTTCGATGTCAGCTCTTCCTATCATACCGAATCAGAATTGGTTCGCACGTCATCAAGAC
gTCGCTTTTTGATCCTTCGATGTCGGCTCTTCCTATCATACCGAAGC
>Vd1396-9-8-198 (189 bp)
GCTCAGGATGCGCGATGGTACGTTCGCCTTTGAGATCGGGAACAAGCTGTGCGACAGGGTCGCCTTGGAACTAGGTGAAAGCCCAGTAA
ATAGATCCTTTGTGAGCATGGTGTGCGCAACGCTTCTCTTTGATATAAAGCTACGATTCCACTTTGGATCGTTTTGTTCCAAAAGGGTA
CCCCATCCAAA
>Vd1396-9-8-199 (116 bp)
GGTTTGATATGAGCCTGTCGTCGCTGGCTATAAGTGGTAGCTCCGCTTCGGTGACAAGGTCGCGGGGGACTCGGGGTTGCCCTCCTGTG
TGCTGCAGAATTGGTGCGCAGTCACAA
>Vd1396-9-8-200 (56 bp)
GGGGGGGTTGGCTGTGGGGTGCGCTGGGAGGGGTGTGGAGAATGGTCGCGTCAAAT
>Vs06-14-1-201 (88 bp)
TCGTTAGATCGACTACATGGTTGCTACTGATGTCCAAGTTGCCTATGTGTTGTCTGATAGACTGTAATAGGCGCGCTGGCCTGCCGGT
>Vs06-14-1-202 (106 bp)
ATAAAATAATTCAAATACAGGGTTTTGATGAAGTGTGGATGTTAGATGTTACGTTGTAATTTTTGATGGGGATTTTCATACTTGGTGAA
TGGGCCACCACAACAAA
>Vs06-14-1-203 (104 bp)
ATTGCATAATTTATCTTCAGCATTGCATGCTATTGACCTTATTTTACGTGTAGTTATGGACCTATTTTTATCTGGATCTCTGTGTGAAT
TTGGACCCCCACAAA
>Vs06-14-1-204 (50 bp)
TAATTAATAATTGAACTACTATATTTTGAACCAGGAGAGAAATTTGAATT
>Vs06-14-2-205 (86 bp)
ACAGGTAGGTCCCACGGTCAGCTGATCTCTCGCTCAAGCCTGGTTCTCGACGGTATTTCTGCGCTTCTAAAGTTGGGCATCACTGT
>Vs06-14-3-206 (205 bp)
CATGGTGACTGAGCTCGTTCTaTTGAGGGCGCGGTGATGTCCAGTAGTGGCGCCGCAAGCACCACCGCAAGGAGATATTCGAGTCACCG
GGTGTCACGATTACGCTGGGAACGCACCGCGCGGTAGGGAACTCACCGCATATCACCTCGAGGACCGACTAACCGGCATCCACGCTTAC
CCCGTGTGAATTGGtCACGCACAACAA
>Vs06-14-3-207 (100 bp)

GGGCGGGCAGCGAGGAACATCTCTGCAGATAACGGCACTACTTGGTCCTGGTGCGGTCGATGTGGACTCTTTGATCGCCACCTAACCTG
AGGGTTTGCCT

>Vs06-14-3-208 (141 bp)

ACGAACGTATATgATgTGCTTCGTTCAGAACCGGGGAACAGCAGTGGGGCGTCCTGCAATAGGCTACGTCATTTCATTCGGTGTGGGAT
GGAATATTTACGTTCTCGTGTTGCCTGGAAATTGTGTACCACACATCAACAA

>Vs06-14-3-209 (105 bp)

ACGATAGTTAGTTTCTTGAATTACTGTAACTGAGATGGTACAATTGGTACGTCTTTATATTTCATGAGATATCTACTTCTTGTGAATTG
GAACGCACCCAACTCA

>Vs06-14-4-210 (136 bp)

GAGAGATCCGCACGGGCCGCGTGGAGCTCTGGGATTGAAACGAGTCTTCGTGTACAGGTCATGCCATCCCAGGCATACTTTAAGGCGCC
ATGCTGCGTCAAAGATTCGAAGATGCGCCGCACTCAAACGCAGTCAA

>Vs06-14-4-211 (148 bp)

GCGAGTGTcACGTCGGAGCAGTGTCGGCTCGATTTATGCAACCTGCTTAATTCACTGGTCACGTACGCATCCCATGGCATGAGTGTTGC
AGGAGCCAGCGGCACTGACTGTTCTCTAGTATGTAAATTGTGGTACCGCAGGTCACAAG

>Vs06-14-4-212 (127 bp)

TTCTAGGTGTCACTCGGCCTCTATCGCTATGCTTGTGAGCGTGTTGCTTGAGGATGTTGCAGATAGGCCAGTTCTGAATGCCCTACTGC
CTCGTGGTGCGGTGGGAATTGTGGGCCCGACCACACAA

>Vs06-14-4-213 (92 bp)

GGCCGAACATTGTCTTGTGGTGTGAGCGGTCTCGAGAGCTACATCGGTATGAGTGTGTGTGGTGTAATTTGTGGTCCACCACAGTCACA
ATT

>Vs06-14-5-214 (116 bp)

GTTGTCGTCTTGGGGTTGGTGCTGGTCGCTGCTAGTGGGGGGGTGATTGTTGGATTGAGGTGTGTAATTTGGTTCTTTGGTGTGTATTG
GTGGCGTAAAAATTGGCCCCCATCAAA

>Vs06-14-5-215 (117 bp)

TGTTTTGATGGGATGTTTGCTGGTTTGAGAAGGATCTGTCGGGTTTTGTATTTGTTCGTGATGGTGTTGTGTTGTTGTCCCTTGATTGT
TTGGAGAGAATTGGGTCCGCCATTCACA

>Vs06-14-5-216 (109 bp)

GGCTGTGGATGGGATTTGTGGCGTTTTGTCGGTGGCATGAAATTGGGTTGAATATTCGCCGGTGATTTCGGGGACCGAGTATACTTTTG
ATAGAATTGGTCCGCAGTAA

>Vs06-14-5-217 (73 bp)

TGTGTCTCGCACGaTgTGATGCGCTGTATGAGTGCGGTCCTTGTGTGTATCCAGAAATTGGTGCCCACGTCCA

>Vs06-14-6-218 (93 bp)

GGCATTTGGTTGTGCTGCTTGATATTCTTATACATTGTATATTTACAATTAGTTGAAGGAAAACCTGCAAGTAGAAATCCTTGAATGAG
TGTA

>Vs06-14-6-220 (106 bp)

TATATCTGTGTGCTGCTGATTATCTTATCATTGTATATTTACGATTAGTTGAAGGAAAACCTGCAAGTAGAAATCCTTGAATGAGTGTA
GGGTTGCCACCCAATCA

>Vs06-14-7-221 (103 bp)

TGATATACGCTCGATCTTGATATGCATTGTGATTGCACAATTGGTGCGCGATCACCTGCGGTAGAATTCCTTGAATGAGTGTAGAAAAG
GTGCGCCCCCACCA

>Vs06-14-7-222 (126 bp)

CACACACTGGGACACTGCTCGAATCTTCATATGCATCCGTGTATTtGACAAATTATGTTGACGCGAAACACCTGCAAGGTGTAAATCCT
TTGGAATGAGTTGTAGAAATTGGCTACCCACATCCAC

>Vs06-14-7-223 (100 bp)

TCTCTGAGAGACGCCCCCACACATGTATACCGCTCTTTGCGGCATTGTCCGCGGTCATATTCCAGGGACTTTTTGGGTGGCAAAAATGT
GGCCGCATCCA

>Vs06-14-8-224 (129 bp)

GATCGAGgTTCGAGCATAATATGGGATGATTTCAGTAGATAGAGATGCGATCCTTCATGTACAACTACTCTTTTCTAGGGAAGAATACG
TAGTAAGACAGGAAGCTGGAAATTGGGGATCACAGTTCAA

>Vs06-14-8-225 (85 bp)

AGATCGAGTTCGAGAATATATGGATAATTTAGTAGATAGAATGGATCCTCATTACTATACTTACCAAATAAGAATAGTAGAAGAC

>Vs06-14-9-228 (156 bp)

TACTATTGCGCAAGGATACATGATCATCGGATCACGACATCTGGACCTAGATGCAGAAGCCTGTCAACCATCGTGAGTCATTGTTGATT
CTGCACTGCGCACATCTGGTCGCAATGTCTTCTCGCGTGAAGGTAAGTTGCGCCACGATCACATCAA

>Vs06-14-9-229 (93 bp)

CTGCATTGCCAATAGAGTGTTTCAAATCTGCTCTGTCTAAGGGAACGTTCAACTCTGTGAGTTGAATGCACACAACACAAGGGAAGTTA
TCTG

>Vs06-14-9-230 (101 bp)

TTATGGCAATTGGTTGTTCAGCATGGACTGATATGTTAACATAGATCTGTGTCTTGCGTTTGTGGAGAGTTCTTCTGGGTGGGAATTGG
GCCCCACCCAAA

>Vs06-14-10-231 (78 bp)

GGCATTGCGTTCCGTATTTCTTCTTCATCTTCGAGAAATTAGAATATTATCGAGGTATATTATTACGAAGACGTCTAG

>Vs06-14-13-232 (160 bp)

GCGCGCGCCGTTGGCACGTAACCCGTGCcTGTCAATGCGCCTCATTGTTTTATCATCGCATGGACTTTGGTGTGGTCGTACCATACACT
TCTGTACCAGAGTCCTCGCTTTGTATGAGAAAACCGTTTTCGCACATATGTGTGGCTACACGCACACACAA

>Vs06-14-14-233 (102 bp)

GCTTTCTGTGCGCTCGGCACGgTCGATAAGCACCCGCGGTGCCTTCGTAGGTCTTATACTAAGGCTGCCTAGAGGTCGGACCTCTCTGG
CTAGTCCTTTTGT

>Vs06-14-14-234 (85 bp)

GTATTTTTTGTGTCCATGTAGGTTGTTTTTGTGGGGACTTGCTGTCTCTTTAGAGGTACGTGTTTGCTCCGAATTGGTCCAACAA

>Vs06-14-14-235 (109 bp)

ATGTGGACTTTCTTGGCGGACGCTCTCACGTTCGGCTGATATGGGTGGGCCTGTTCGATTCATGGTGTTTTGCCGTCTGTTCTTCTTGC
GAATTGGGACCCCCATCAAA
>Vs06-14-14-236 (153 bp)
TTGATCTTtCCTGgATGCGTCTGCCGTGTTATAGCGACGTTGTCGTATAGTCGGGGGCGGCGACGCTGTATGCGTTTGTCCGGCTGTGT
TATGGCCGCGCATATACTTCCTTCTTCTGCCGTATGTGGAGCTTCGGCTACACACAAATCAACA
>Vs06-14-15-237 (177 bp)
GCAGATAGCGTCCGCACGACAAAAGAGAGGATTTGTTGCCCTGGCGACTTTGCCGTTCGTAGCCTTTACTATGGCATCGCTGATGGACT
TGCGAAAGAGGGCGGCATTCGTTTGCCAGTCCATGTTCACTGCGCACGCCGAAGCTCCGAAGTTCTTGGTTGGATTCGGACCAGTTGC
>Vs06-14-15-238 (170 bp)
CAACTCCACGTACTATTACTAGAGAAGCGTATGATGTGATGTGCCGTTTTGACCTTCCTAAACATTTTTCTCGCATGGGTTACTTAGTT
CATGTGCAGGGTCGAATCATGTGGCAGGTCCTTTCTGCTTCGGTTCTAAAGACTACCGGATCGCTTTGCGGTTTTGCTGCC
>Vs06-14-15-240 (137 bp)
GTAGCGTAGCCACGGTATGACGCTCTAGGCGCGTTCGAAGGACGGGCTCGTTCACGACGCGATACTCTGTCGACACCCCTTTAGTTCGA
TCCCTGACATCGCCTTTGCCGCCGAATGTGTGGCGCCCCACACACACA
>Vs06-14-15-241 (130 bp)
GGACATAGTCACTGCATACTCTCTGTTCGCGGTGAGGGATGTGACTTTTGCACGTGGAATATCTGATGACATCCGGTATAATTGAATCC
TGACATTGCCTTTGCCCGAAATTGGTGTGCGCACATCACAC
>Vs06-14-15-242 (80 bp)
GACGATTACGCATCCTGGCGCCCGTTGAGACCGGCTTTGAGTGATTGCCGCGCATCATTAAAGAACGTGCAAATGCTTTC
>Vs06-14-15-243 (104 bp)
TCGTGGACGTCTCACCGACGCATTCAcGACCCGGAGGGCACGTTGACATTCGTCGCATCGcGGCATCGTCGTCGCGAAATTGTGGTACG
CGCAGGATCACAATT
>Vs06-14-15-244 (60 bp)
CAGTCCGCGTGAGAGGCCGAGACGGATTCGTCTCGGCTCGCTAATTGGGCCGCACCCATT
>Vs06-14-16-246 (164 bp)
CCGTCAGTCcTGgCCGcGgTCTTCAtCGGATCCGTGCCAGAGTGcACCATGGCCTCACGCGCGTCTTGCGACCgTATCGTTCTGACGAA
CATCGATTTTGTGCCAGTGCAGGCGTGGCTGGCGGCACCCCTGTGTCACGAGAGTTGCGCTACCGCcAGCATCAA
>Vs06-14-16-247 (81 bp)
GTTGAGGGTAAGTGTGTTTCGACGTGTAACTAGATGGCTTGAGTTGTTGGGGTCAACATCCTACACGAATGGTCGCAGTCA
>Vs06-14-16-248 (116 bp)
GGCTTCTGCACCTTGGAGTGTTGGAATCCTTGCGCAAGGGAGGTAGATGGCAGTCTCCTGGGTAGGGTAGTTTAGTTAACAACATGCCT
TGTCGCAATTGTGTTCCCCGCTTAACA
>Vs06-14-16-249 (59 bp)
GGAATCCTCTTTCGTGACTAGAAGAGTAACGGGAAACGGCCTGGTGGTGGAGGAATCGG
>Vs06-14-16-251 (102 bp)
GTGTAGCCGTaTGTCATGAACGGAGTGCGGTGCGGAGACGTTGACTTGGTCATTGCATCTCGCGTCGTCTCCGCGAAATTGGGGTACGC
CaCGAGTCACAAT
>Vs06-14-16-252 (83 bp)
GTCTGTAGATGCATACCGATGGTCTAAACGCGTGAGATGCAAGTGTGcCATGTATCCGCGATGGTGTGTCCCGCAGACAAATT

10.2. Stress response regulator A gene-full sequence

The Broad Institute *V. dahliae* isolate VdLs.17
http://www.broadinstitute.org/annotation/genome/verticillium_dahliae/MultiHome.html)
VDAG_02250.1 stress response regulator SrrA (Transcript:VDAG_02250T0)
http://www.broadinstitute.org/annotation/genome/verticillium_dahliae/GeneDetails.html?sp=S7000001884957719
http://www.broadinstitute.org/annotation/genome/verticillium_dahliae/TranscriptDetails.html?sp=S7000001884957720
Coding sequence (including *introns*):2245 nt
ATGCCTCCCGAAGGTGATGGCGCCGCACAGGGCGGGAACAACTCTAGTGACTTTGTACGTTGGCCCCAAATCAACCTCCCCCATCGTGT
TGCGCATGGCGGAGTCTTTTGCTAACAGTGAATGCTGCAGGTTCGCAAGCTCTACAAGTTCGTCCACCCTGCCGTCCTTCTCACGAGGA
TGGCCTCTCGGCGGCCGCTAACCATCACAGGATGCTCGAGGACCCTGCATACGCGAACATTGTGAGATGGGGAAACGAGGGCGACACCT
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TCACGAAGGATATCCTGCCCAAGCACTTTAAGTTTTGTGGTGGGGTCGAGCTTTGTACGCCAGCTGAATAAATATGACTTTCACAAACT
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CGCAAGGCGCCCGCCCAACGCAAGGCCCAACCGACAGAAGACTCCTTCACTACCAACCAGTCCATCAACCTGCTCCAGGAGACACTCTT
CGCTCAACAGCAGCAGGTCCAAGCCCTGCAGGAGCAGTTTGTTGAGCTCTCACGCGCCAATAAGACGCTCGTGCATGAGGTTCACTCGC
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TATCCGAACCAGGCTGCGGCCCACATGAACGGCGGCACCATGGATGAGCAGGCGCCCGAGTTGCGCCGCGCCCGCGAGCTTCTGTCAAC
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TGCCTCACATGGACGGTGTCTCGGCAACGCAATTGATCCGTGAGGTGCACCCCGATGTGCCCGTGGTCGCCATGACGTCGAACATTCGC
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TTCCCTTCCTTCACAGGTTTGAACGATGTTTTGGCCAAGCCCTTTACCAAAGACGGCATGCTGCGTATCCTGCGAAAGCACGTCGCGCA
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GCCGCCCCAGATGCAGCAGCAGATGCGTGTGCCGGACGGCATGGCTGTCGAGGATCGGCCAGAGAAACGACAGAGGCTATACGGGCCTC
AGGGAGGCTACGCTCAATAA

The full length sequence of SrrA isolated from the highly aggressive *V. dahliae* isolate Vd1396-9

Vd1396-9-SrrA-clone 1

ATGCCTCCCGAAGGTGATGGCGCCGCACAGGGCGGGAACAACTCTAGTGACTTTGTACGTTGGCCCCAAATCAACCTCCCCCATCGTGT
TGCGCATGGCGGAGTCTTTTGCTAACAGTGAATGCTGCAGGTTCGCAAGCTCTACAAGTTCGTCCACCCTGCCGTCCTTCTCACGACCA
TGGCCTCTCGGCGGCCGCTAACCATCACAGGATGCTTGAGGACCCTGCATACGCGAACATTGTGAGATGGGGAAACGAGGGCGACACCT
TTGTCATCCTAGAGGTACATCGCATAGACTCGGCCCCTGGCTGTCTGCCTTGCTTTGTAGTGCGCTAACCCCATCATAGACGGACAAGT
TCACCAACGACATCCTGCCCAAGCACTTTAAGCACAGCAATTTTTCGAGCTTTGTACGCCAGCTGAATAAATATGACTTTCACAAACTT
CGACGAAACGACGAGAACAACGAGTCGCCTTACGGCAAACAGGTGAGCAGAGAGAGAGAGAGAGAGGGCTTGGCGCTGTCCACTTGGGAGC
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GCAAGGCGCCCGCCCAACGCAAGGCCCAACCGACAGAAGACTCCTTCACTACCAACCAGTCCATCAACCTGCTCCAGGAGACACTCTTC
GCTCAACAGCAGCAGGTCCAAGCCCTGCAGGAGCAGTTTGTTGAGCTCTCACGCGCCAATAAGACGCTCGTGCATGAGGTTCACTCGCT
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CATGTTCCAGCCCGGCTCGATGCCCCCCATGATGGCCGACCACGTCAACATGCGTCATCTCGTCTACCCTGTTGGCGAAAATGTCGGCA
TCGATCCATTGTCGCAAGACCACTTCAACAACATTCCGTACACCCTCAACTCGTTGCCGACCCTCACCAACGACTACCCGAACCAGACC
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GCCCCAGATGCAGCAGCAGATGCGTGTGCCGGACGGCATGGCTGTCGAGGATCGGCCAGAGAAACGACAGAGGCTATACGGGCCTCAGG
GAGGCTACGCTCAATAA

Vd1396-9-SrrA-clone 2

ATGCCTCCCGAAGGTGATGGCGCCGCACAGGGCGGGAACAACTCTAGTGACTTTGTACGTTGGCCCCAAATCAACCTCCCCCATCGTGT
TGCGCATGGCGGAGTCTTTTGCTAACAGTGAATGCTGCAGGTTCGCAAGCTCTACAAGTTCGTCCACCCTGCCGTCCTTCTCACGACCA
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GCAAGGCGCCCGCCCAACGCAAGGCCCAACCGACAGAAGACTCCTTCACTACCAACCAGTCCATCAACCTGCTCCAGGAGACACTCTTC
GCTCAACAGCAGCAGGTCCAAGCCCTGCAGGAGCAGTTTGTTGAGCTCTCACGCGCCAATAAGACGCTCGTGCATGAGGTTCACTCGCT
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TCGATCCATTGTCGCAAGACCACTTCAACAACATTCCGTACACCCTCAACTCGTTGCCGACCCTCACCAACGACTACCCGAACCAGACC
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CCTTCCTTCACAGGTTTGAACGATGTTTTGGCCAAGCCCTTTACCAAAGACGGCATGCTGCGTATCCTGCGAAAGCACGTCGCGCATCT
TATGAAGAACGCGCCTCCAATCGACGACATACCTGTGGGTCCCGGCAGCGCGCAGATGGGAACGATGGGTCTGCCTCCCCAAGTCCGCG
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GAGCCGGGATACGCCATGGGCAACCCGCAACAGATGGTCATCACGCCAACGTCCGCCCAGCGCGCGACATTCCCGACGCAGGGCCTGCC
GCCCCAGATGCAGCAGCAGATGCGTGTGCCGGACGGCATGGCTGTCGAGGATCGGCCAGAGAAACGACAGAGGCTATACGGGCCTCAGG
GAGGCTACGCTCAATAA

Vd1396-9-SrrA-clone 3

ATGCCTCCCGAAGGTGATGGCGCCGCACAGGGCGGGAACAACTCTAGTGACTTTGTACGTTGGCCCCAAATCAACCTCCCCCATCGTGT
TGCGCATGGCGGAGTCTTTTGCTAACAGTGAATGCTGCAGGTTCGCAAGCTCTACAAGTTCGTCCACCCTGCCGTCCTTCTCACGACCA
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GCTCAACAGCAGCAGGTCCAAGCCCTGCAGGAGCAGTTTGTTGAGCTCTCACGCGCCAATAAGACGCTCGTGCATGAGGTTCACTCGCT
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TCTTGTCGCAGATGGAGTGCCAGGTAGAGGTTGCGGTATGTGACAGGGACTTCACAGCTGTTGTGTAGGAAGCAGGTTGCTGACTCTGT
CCACCAGGAAAACGGTATCGACGCTGTCAACAAGTGCAAGGAGGTCGGTGCTGGGTATTTCGATCTGATCTTCATGGACATTGTCATGC
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CCTTCCTTCACAGGTTTGAACGATGTTTTGGCCAAGCCCTTTACCAAAGACGGCATGCTGCGTATCCTGCGAAAGCACGTCGCGCATCT
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TCGACACGCCGAGCCAGTCTCCGGCGACGACGACCTCATGGCACTCGCCTGGGCAGATCCACCAGCAGTCACCCCACGTGGCCACGATG
GAGCCGGGATACGCCATGGGCAACCCGCAACAGATGGTCATCACGCCAACGTCCGCCCAGCGCGCGACATTCCCGACGCAGGGCCTGCC
GCCCCAGATGCAGCAGCAGATGCGTGTGCCGGACGGCATGGCTGTCGAGGATCGGCCAGAGAAACGACAGAGGCTATACGGGCCTCAGG
GAGGCTACGCTCAATAA

The full length sequence of SrrA isolated from the weakly aggressive *V. dahliae* isolate Vs06-14

Vs06-14-SrrA-clone 1

ATGCCTCCCGAAGGTGATGGCGCCGCACAGGGCGGGAACAACTCTAGTGACTTTGTACGTTGGCCCCAAATCAACCTCCCCCATCGTGT
TGCGCATGGCGGAGTCTTTTGCTAACAGTGAATGCTGCAGGTTCGCAAGCTCTACAAGTTCGTCCACCCTGCCGTCCTTCTCACGACCA
TGGCCTCTCGGCGGCCGCTAACCATCACAGGATGCTCGAGGACCCTGCATACGCGAACATTGTGAGATGGGGAAACGAGGGCGACACCT
TTGTCATCCTAGAGGTACATCGCATAGACTCGGCCCCTGGCTGTCTGCCTTGCTTTGTAGTGCGCTAACCCCATCATAGACGGACAAGT
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TATGAAGAACGCGCCTCCAATCGACGACATACCTGTGGGTCCCGGCAGCGCGCAGATGGGAACGATGGGTCTGCCTCCCCAAGTCCGCG
TCGACACGCCGAGCCAGTCTCCGGCGACGACGACCTCATGGCACTCGCCTGGGCAGATCCACCAGCAGTCACCCCACGTGGCCACGATG
GAGCCGGGATACGCCATGGGCAACCCGCAACAGATGGTCATCACGCCAACGTCCGCCCAGCGCGCGACATTCCCGACGCAGGGCCTGCC
ACCCCAGATGCAGCAGCAGATGCGTGTGCCGGACGGCATGGCTGTCGAGGATCGGCCAGAGAAACGACAGAGGCTATACGGGCCTCAGG
GAGGCTACGCTCAATAA

Vs06-14-SrrA-clone 2

ATGCCTCCCGAAGGTGATGGCGCCGCACAGGGCGGGAACAACTCTAGTGACTTTGTACGTTGGCCCCAAATCAACCTCCCCCATCGTGT
TGCGCATGGCGGAGTCTTTTGCTAACAGTGAATGCTGCAGGTTCGCAAGCTCTACAAGTTCGTCCACCCTGCCGTCCTTCTCACGACCA
TGGCCTCTCGGCGGCCGCTAACCATCACAGGATGCTCGAGGACCCTGCATACGCGAACATTGTGAGATGGGGAAACGAGGGCGACACCT
TTGTCATCCTAGAGGTACATCGCATAGACTCGGCCCCTGGCTGTCTGCCTTGCTTTGTAGTGCGCTAACCCCATCATAGACGGACAAGT
TCACCAACGACATCCTGCCCAAGCACTTTAAGCACAGCAATTTTTCGAGCTTTGTACGCCAGCTGAATAAATATGACTTTCACAAACTT
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GCTCAACAGCAGCAGGTCCAAGCCCTGCAGGAGCAGTTTGTTGAGCTCTCACGCGCCAATAAGACGCTCGTGCATGAGGTTCACTCGCT
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CATGTTCCAGCCCGGCTCGATGCCCCCCATGATGGCCGACCACGTCAACATGCGTCATCTCGTCTACCCTGTTGGCGAAAACGTCGGCA
TCGATCCCTTGTCGCAAGACCACTTCAACAACATTCCGTACACCCTCAACTCGTTGCCGACCCTCACCAACGACTACCCGAACCAGACC
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TCTTGTCGCAGATGGAGTGCCAGGTAGAGGTTGCGGTATGTGACAGGGACTTCACAGCTGTTGTGTAGGAAGCAGGTTGCTGACTCTGT

CCACCAGGAAAACGGTATCGACGCTGTCAACAAGTGCAAGGAGGTCGGTGCTGGGTATTTCGATCTGATCTTCATGGACATTGTCATGC
CTCACATGGACGGTGTCTCGGCAACGCAATTGATCCGTGAGGTGCACCCCGATGTGCCCGTGGTCGCCATGACGTCGAACATTCGCCCG
GAAGATATCAGTCACTATTTCAACTGGAGTAAGTTGCCCATCAGTAGTAGCGACATCACCGCGGGACTGCATTGCTGACATGACTTTTC
CCTTCCTTCACAGGTTTGAACGATGTTTTGGCCAAGCCCTTTACCAAAGACGGCATGCTGCGTATCCTGCGAAAGCACGTCGCGCATCT
TATGAAGAACGCGCCTCCAATCGACGACATACCTGTGGGTCCCGGCAGCGCGCAGATGGGAACGATGGGTCTGCCTCCCCAAGTCCGCG
TCGACACGCCGAGCCAGTCTCCGGCGACGACGACCTCATGGCACTCGCCTGGGCAGATCCACCAGCAGTCACCCCACGTGGCCACGATG
GAGCCGGGATACGCCATGGGCAACCCGCAACAGATGGTCATCACGCCAACGTCCGCCCAGCGCGCGACATTCCCGACGCAGGGCCTGCC
ACCCCAGATGCAGCAGCAGATGCGTGTGCCGGACGGCATGGCTGTCGAGGATCGGCCAGAGAAACGACAGAGGCTATACGGGCCTCAGG
GAGGCTACGCTCAATAA

Vs06-14-SrrA-clone 3

ATGCCTCCCGAAGGTGATGGCGCCGCACAGGGCGGGAACAACTCTAGTGACTTTGTACGTTGGCCCCAAATCAACCTCCCCCATCGTGT
TGCGCATGGCGGAGTCTTTTGCTAACAGTGAATGCTGCAGGTTCGCAAGCTCTACAAGTTCGTCCACCCTGCCGTCCTTCTCACGACCA
TGGCCTCTCGGCGGCCGCTAACCATCACAGGATGCTCGAGGACCCTGCATACGCGAACATTGTGAGATGGGGAAACGAGGGCGACACCT
TTGTCATCCTAGAGGTACATCGCATAGACTCGGCCCCTGGCTGTCTGCCTTGCTTTGTAGTGCGCTAACCCCATCATAGACGGACAAGT
TCACCAACGACATCCTGCCCAAGCACTTTAAGCACAGCAATTTTTCGAGCTTTGTACGCCAGCTGAATAAATATGACTTTCACAAACTT
CGACGAAACGACGAGAACAACGAGTCGCCTTACGGCAAACAGGTGAGCAGAGAGAGAGAGAGAGGGCTTGGCGCTGTCCACTTGGGAGC
ATGCGATCGCTGACCATGTCTTCAGGCCTGGGAATTCAAGCATGCTGCGTTTCGAGCGGACAGGAAAGACAACCTCGATAACATCAGGC
GCAAGGCGCCCGCCCAACGCAAGGCCCAACCGACAGAAGACTCCTTCACTACCAACCAGTCCATCAACCTGCTCCAGGAGACACTCTTC
GCTCAACAGCAGCAGGTCCAAGCCCTGCAGGAGCAGTTTGTTGAGCTCTCACGCGCCAATAAGACGCTCGTGCATGAGGTTCACTCGCT
GCAGAAGACCATTGACGTACAGAGGCAGTCAACCCATGAGCTTCTAAACTTCCTCTCCAGCCCTGACGAGCGATGGCGCACCGGTAGGT
ATCCGAACCAGGCTGCGGCCCACATGAACGGCGGCACCATGGATGAGCAGGCGCCCGAGTTGCGCCGCGCCCGCGAGCTTCTGTCAACG
GTGACGACAAACTCGATGGTCGAACGCGACTTTGAGCGCCTCAACGGCATGTACGCCCAGAGCTCGCCGCCCGACTCGGCGTCGTCCCT
CATGTTCCAGCCCGGCTCGATGCCCCCCATGATGGCCGACCACGTCAACATGCGTCATCTCGTCTACCCTGTTGGCGAAAACGTCGGCA
TCGATCCCTTGTCGCAAGACCACTTCAACAACATTCCGTACACCCTCAACTCGTTGCCGACCCTCACCAACGACTACCCGAACCAGACC
ATGGTCAAGCAGGAGCCGGGCCCGGCAACGCCGAACCCACCAGGCGGTGCCACGCCGGCGCCGCCAGGCCCTGCTGGCAGGGCAGCACC
GCCGCAGGATAACTCGCTGTGGGGGTCGAAGAAGCCGCGTGTCTACCTTGTCGAGGATGATAGGACGTGCTCGAGGATAGGAGCTAAGT
TCTTGTCGCAGATGGAGTGCCAGGTAGAGGTTGCGGTATGTGACAGGGACTTCACAGCTGTTGTGTAGGAAGCAGGTTGCTGACTCTGT
CCACCAGGAAAACGGTATCGACGCTGTCAACAAGTGCAAGGAGGTCGGTGCTGGGTATTTCGATCTGATCTTCATGGACATTGTCATGC
CTCACATGGACGGTGTCTCGGCAACGCAATTGATCCGTGAGGTGCACCCCGATGTGCCCGTGGTCGCCATGACGTCGAACATTCGCCCG
GAAGATATCAGTCACTATTTCAACTGGAGTAAGTTGCCCATCAGTAGTAGCGACATCACCGCGGGACTGCATTGCTGACATGACTTTTC
CCTTCCTTCACAGGTTTGAACGATGTTTTGGCCAAGCCCTTTACCAAAGACGGCATGCTGCGTATCCTGCGAAAGCACGTCGCGCATCT
TATGAAGAACGCGCCTCCAATCGACGACATACCTGTGGGTCCCGGCAGCGCGCAGATGGGAACGATGGGTCTGCCTCCCCAAGTCCGCG
TCGACACGCCGAGCCAGTCTCCGGCGACGACGACCTCATGGCACTCGCCTGGGCAGATCCACCAGCAGTCACCCCACGTGGCCACGATG
GAGCCGGGATACGCCATGGGCAACCCGCAACAGATGGTCATCACGCCAACGTCCGCCCAGCGCGCGACATTCCCGACGCAGGGCCTGCC
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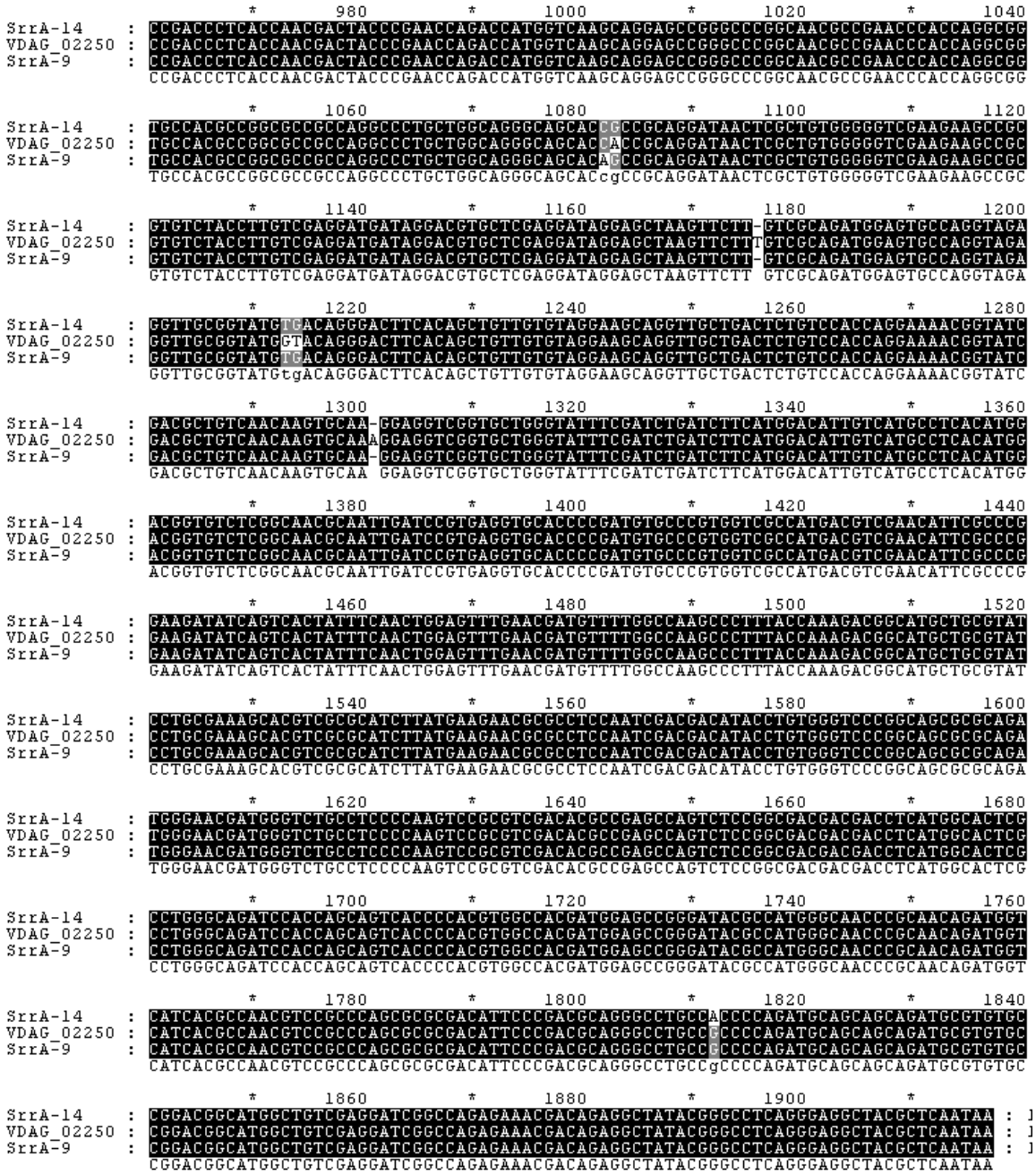

Figure 10.1. Multiple alignment of the sequences of exons only of stress response regulator A gene from the highly aggressive Vd1396-9 (SrrA-9), the weakly aggressive Vs06-14 (SrrA-14) and the Broad Institute VdLs.17 genome database (VDAG_02250) *V. dahliae* isolates.

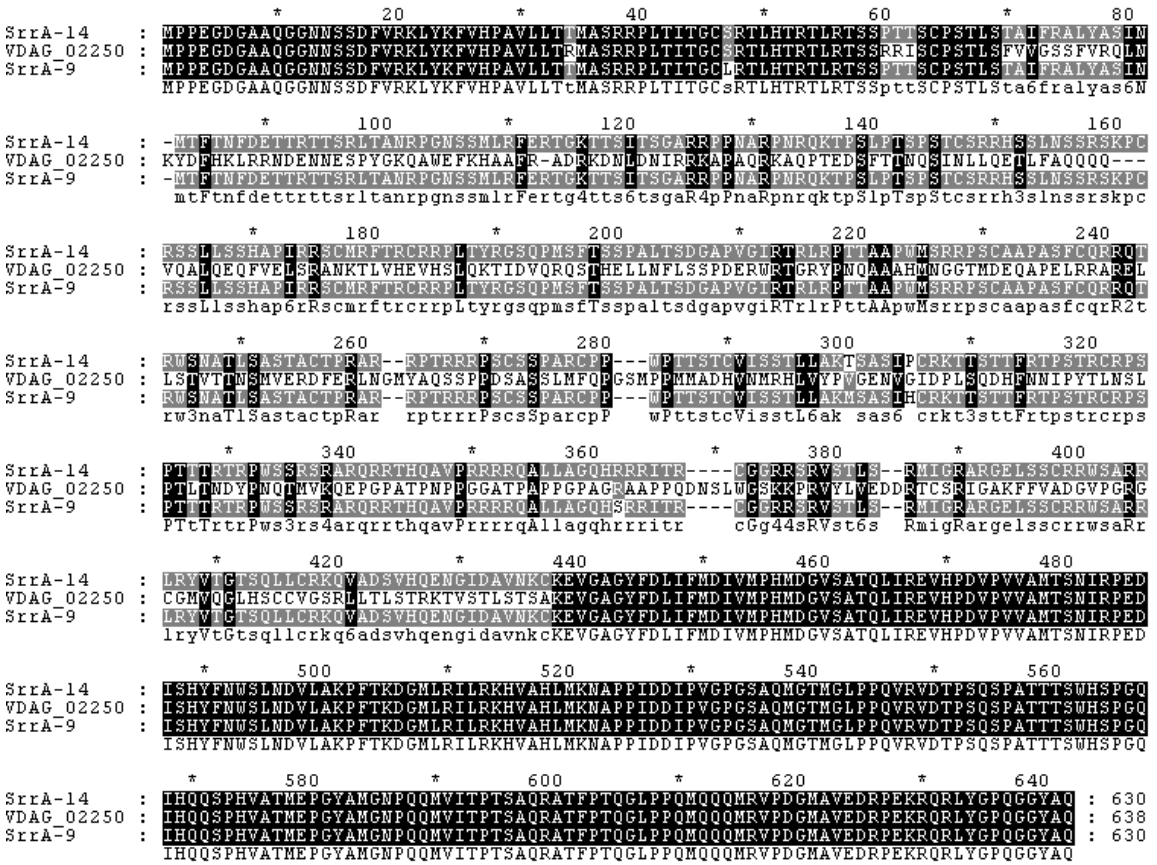

Figure 10.2. Multiple alignment of the deduced amino acids of stress response regulator A gene from the highly aggressive Vd1396-9 (SrrA-9), the weakly aggressive Vs06-14 (SrrA-14) and the Broad Institute VdLs.17 genome database (VDAG_02250) *V. dahliae* isolates.

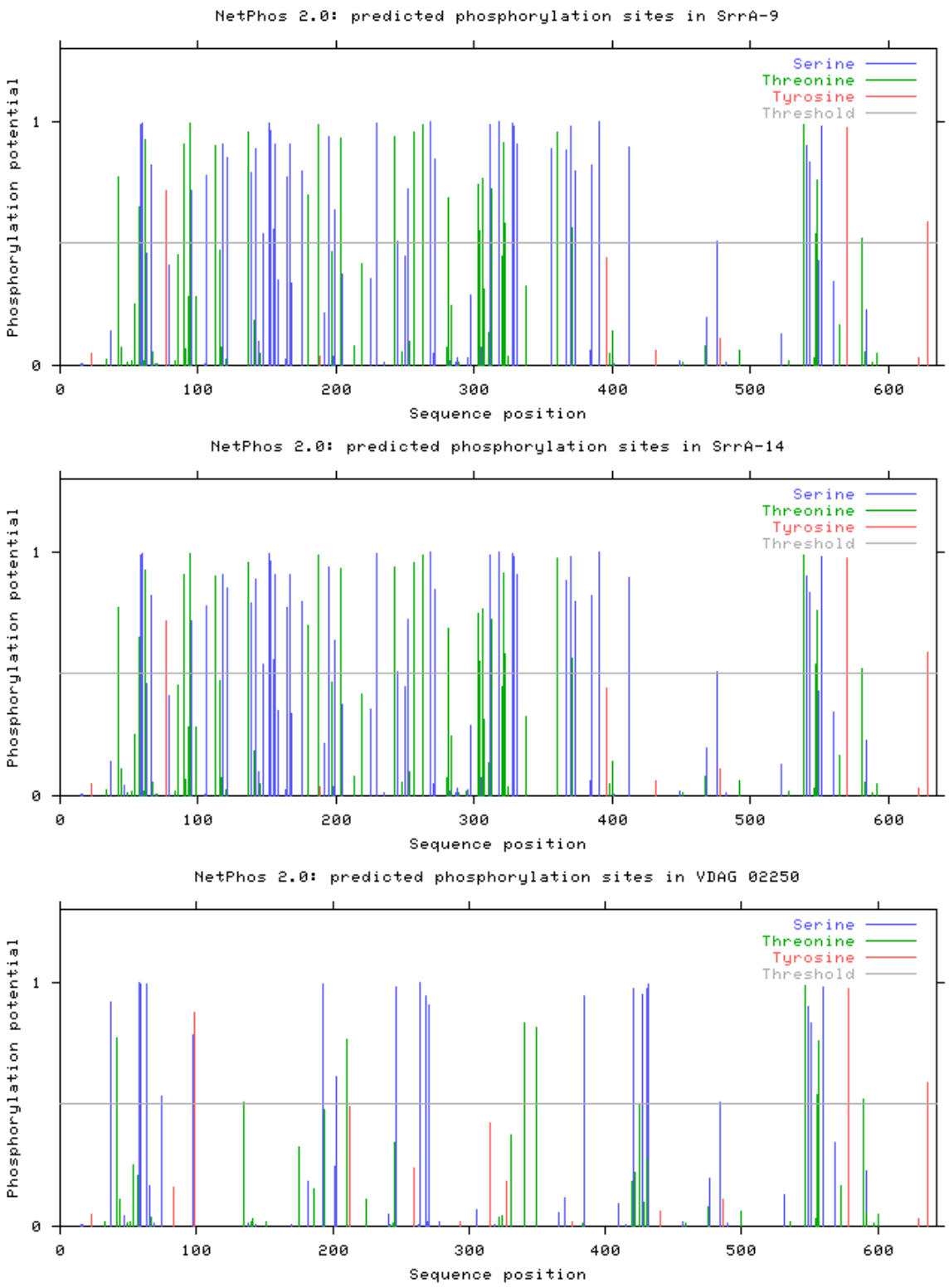

Figure 10.3. Prediction of phosphorylation sites in the deduced SrrA protein of *V. dahliae* isolates Vd1396-9 (SrrA-9), the highly aggressive, Vs06-14 (SrrA-14), the weakly aggressive, and the Broad Institute isolate VdLs.17 (VDAG_02250)

10.3. Isochorismatase hydrolase gene-full sequence

The Broad Institute *V. dahliae* isolate VdLs.17
http://www.broadinstitute.org/annotation/genome/verticillium_dahliae/MultiHome.html)
VDAG_05103.1 isochorismatase hydrolase (Transcript:VDAG_05103T0)
http://www.broadinstitute.org/annotation/genome/verticillium_dahliae/GeneDetails.html?sp=S7000001884959978
http://www.broadinstitute.org/annotation/genome/verticillium_dahliae/TranscriptDetails.html?sp=S7000001884959979

Coding sequence (including *introns*):629 nt

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ATACGCCGAGGGCAAGCTCAAGATTTCCAACATTGAGGCGTCGCGCCCCAACATCTCTTCCCTGCTGGAGAAGTACCGCGCCGCCAACG
CGCCCATCGTCCACGTCGTCCACGAGACGCCCGCCGGCGCCCCTCTCTTCACGCAGGGCACGAAGCTCGCTGAGATCTTCGACGAGCTC
ACGCCCAAGGAAGGCGAGGCTGTCGTGACGAAGCACCACCCCGGTTCATTCGCCGACACCAACCTTCAGGAGATCCTGGAGAAGTCCGG
CAAGAAGAAGATTGTGCTCGTCGGGTACATGGTGAGTCTATCAACGAGATGGAAAGCTCGTCGTGGAACGTTGCTGACACATTGCAGGC
TCACGTCTGCGTCTCGACGACGGCCAGGCAGGGCGCGCAGAGGGGCTGGGATGTCATCGTTGCCGAGGACGCTGTCGGTGACAGGGACA
TTCCCGGCGTGGATGCTGCGCAGCTTGTGAAGGTTGCTCTGGCTGAGATTGCTGATGTCTTTGGCACTCTTGTCTCGAGCAAGGATATC
AACTAG

The full length sequence of isochorismatase hydrolase isolated from the highly aggressive *V. dahliae* isolate Vd1396-9

Vd1396-9-isochorismatase hydrolase-clone 1

ATGTCCTCATTCCGCTCCATGCTCGGCGTGCCGCCGTCCACGGCCTCCACCCAGGACAGTGTCCTCGTCATCATCGACGCCCAGGGCGA
ATACGCCGAGGGCAAGCTCAAGATTTCCAACATTGAGGCGTCGCGCCCCAACATCTCTTCCCTGCTGGAGAAGTACCGCGCCGCCAACG
CGCCCATCGTCCACGTCGTCCACGAGACGCCCGCCGGCGCCCCTCTCTTCACGCAGGGCACGAAGCTCGCTGAGATCTTCGACGAGCTC
ACGCCCAAGGAAGGCGAGGCTGTCGTGACGAAGCACCACCCCGGTTCATTCGCCGACACCAACCTTCAGGAGATCCTGGAGAAGTCCGG
CAAGAAGAAGATTGTGCTCGTCGGGTACATGGTGAGTCTATCAACGAGATGGAAAGCTCGTCGTGGAACGTTGCTGACACATTGCAGGC
TCACGTCTGCGTCTCGACGACGGCCAGGCAGGGCGCGCAGAGGGGCTGGGATGTCATCGTTGCCGAGGACGCTGTCGGTGACAGGGACA
TTCCCGGCGTGGATGCTGCGCAGCTTGTGAAGGTTGCTCTGGCTGAGATTGCTGATGTCTTTGGCACTCTTGTCTCGAGCAAGGATATC
AACTAG

Vd1396-9-isochorismatase hydrolase-clone 2

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ATACGCCGAGGGCAAGCTCAAGATTTCCAACATTGAGGCGTCGCGCCCCAACATCTCTTCCCTGCTGGAGAAGTACCGCGCCGCCAACG
CGCCCATCGTCCACGTCGTCCACGAGACGCCCGCCGGCGCCCCTCTCTTCACGCAGGGCACGAAGCTCGCTGAGATCTTCGACGAGCTC
ACGCCCAAGGAAGGCGAGGCTGTCGTGACGAAGCACCACCCCGGTTCATTCGCCGACACCAACCTTCAGGAGATCCTGGAGAAGTCCGG
CAAGAAGAAGATTGTGCTCGTCGGGTACATGGTGAGTCTATCAACGAGATGGAAAGCTCGTCGTGGAACGTTGCTGACACATTGCAGGC
TCACGTCTGCGTCTCGACGACGGCCAGGCAGGGCGCGCAGAGGGGCTGGGATGTCATCGTTGCCGAGGACGCTGTCGGTGACAGGGACA
TTCCCGGCGTGGATGCTGCGCAGCTTGTGAAGGTTGCTCTGGCTGAGATTGCTGATGTCTTTGGCACTCTTGTCTCGAGCAAGGATATC
AACTAG

Vd1396-9-isochorismatase hydrolase-clone 3

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ATACGCCGAGGGCAAGCTCAAGATTTCCAACATTGAGGCGTCGCGCCCCAACATCTCTTCCCTGCTGGAGAAGTACCGCGCCGCCAACG
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ACGCCCAAGGAAGGCGAGGCTGTCGTGACGAAGCACCACCCCGGTTCATTCGCCGACACCAACCTTCAGGAGATCCTGGAGAAGTCCGG
CAAGAAGAAGATTGTGCTCGTCGGGTACATGGTGAGTCTATCAACGAGATGGAAAGCTCGTCGTGGAACGTTGCTGACACATTGCAGGC
TCACGTCTGCGTCTCGACGACGGCCAGGCAGGGCGCGCAGAGGGGCTGGGATGTCATCGTTGCCGAGGACGCTGTCGGTGACAGGGACA
TTCCCGGCGTGGATGCTGCGCAGCTTGTGAAGGTTGCTCTGGCTGAGATTGCTGATGTCTTTGGCACTCTTGTCTCGAGCAAGGATATC
AACTAG

The full length sequence of isochorismatase hydrolase isolated from the weakly aggressive *V. dahliae* isolate Vs06-14

Vs06-14-isochorismatase hydrolase-clone 1

ATGTCCTCATTCCGCTCCATGCTCGGCGTGCCGCCGTCCACGGCCTCCACCCAGGACAGTGTCCTCGTCATCATCGACGCCCAGGGCGA
ATACGCCGAGGGCAAGCTCAAGATTTCCAACATTGAGGCGTCGCGCCCCAACATCTCTTCCCTGCTGGAGAAGTACCGCGCCGCCAACG
CGCCCATCGTCCACGTCGTCCACGAGACGCCCGCTGGCGCCCCTCTCTTCACGCAGGGCACGAAGCTCGCTGAGATCTTCGACGAGCTC
ACGCCCAAGGAAGGCGAGGCTGTCGTGACGAAGCACCACCCCGGTTCATTCGCCGACACCAACCTTCAGGAGATCCTGGAGAAGTCCGG
CAAGAAGAAGATTGTGCTCGTCGGGTACATGGTGAGTCTTATCAACGAGATGGAAAGCTCGTCGTGGAACGTTGCTGACACATTGCAGG
CTCACGTCTGCGTCTCGACGACGGCCAGGCAGGGCGCGCAGAGGGGGTGGGATGTCATCGTTGCCGAGGACGCTGTCGGTGACAGGGAC
ATTCCCGGCGTGGATGCTGCGCAGCTTGTGAAGGTTGCTCTGGCTGAGATTGCTGATGTCTTTGGCACTCTTGTCTCGAGCAAGGATAT
CAACTAG

Vs06-14-isochorismatase hydrolase-clone 2

ATGTCCTCATTCCGCTCCATGCTCGGCGTGCCGCCGTCCACGGCCTCCACCCAGGACAGTGTCCTCGTCATCATCGACGCCCAGGGCGA
ATACGCCGAGGGCAAGCTCAAGATTTCCAACATTGAGGCGTCGCGCCCCAACATCTCTTCCCTGCTGGAGAAGTACCGCGCCGCCAACG
CGCCCATCGTCCACGTCGTCCACGAGACGCCCGCTGGCGCCCCTCTCTTCACGCAGGGCACGAAGCTCGCTGAGATCTTCGACGAGCTC

ACGCCCAAGGAAGGCGAGGCTGTCGTGACGAAGCACCACCCCGGTTCATTCGCCGACACCAACCTTCAGGAGATCCTGGAGAAGTCCGG
CAAGAAGAAGATTGTGCTCGTCGGGTACATGGTGAGTCTTATCAACGAGATGGAAAGCTCGTCGTGGAACGTTGCTGACACATTGCAGG
CTCACGTCTGCGTCTCGACGACGGCCAGGCAGGGCGCGCAGAGGGGGTGGGATGTCATCGTTGCCGAGGACGCTGTCGGTGACAGGGAC
ATTCCCGGCGTGGATGCTGCGCAGCTTGTGAAGGTTGCTCTGGCTGAGATTGCTGATGTCTTTGGCACTCTTGTCTCGAGCAAGGATAT
CAACTAG

Vs06-14-isochorismatase hydrolase-clone 3

ATGTCCTCATTCCGCTCCATGCTCGGCGTGCCGCCGTCCACGGCCTCCACCCAGGACAGTGTCCTCGTCATCATCGACGCCCAGGGCGA
ATACGCCGAGGGCAAGCTCAAGATTTCCAACATTGAGGCGTCGCGCCCCAACATCTCTTCCCTGCTGGAGAAGTACCGCGCCGCCAACG
CGCCCATCGTCCACGTCGTCCACGAGACGCCCGCTGGCGCCCCTCTCTTCACGCAGGGCACGAAGCTCGCTGAGATCTTCGACGAGCTC
ACGCCCAAGGAAGGCGAGGCTGTCGTGACGAAGCACCACCCCGGTTCATTCGCCGACACCAACCTTCAGGAGATCCTGGAGAAGTCCGG
CAAGAAGAAGATTGTGCTCGTCGGGTACATGGTGAGTCTTATCAACGAGATGGAAAGCTCGTCGTGGAACGTTGCTGACACATTGCAGG
CTCACGTCTGCGTCTCGACGACGGCCAGGCAGGGCGCGCAGAGGGGGTGGGATGTCATCGTTGCCGAGGACGCTGTCGGTGACAGGGAC
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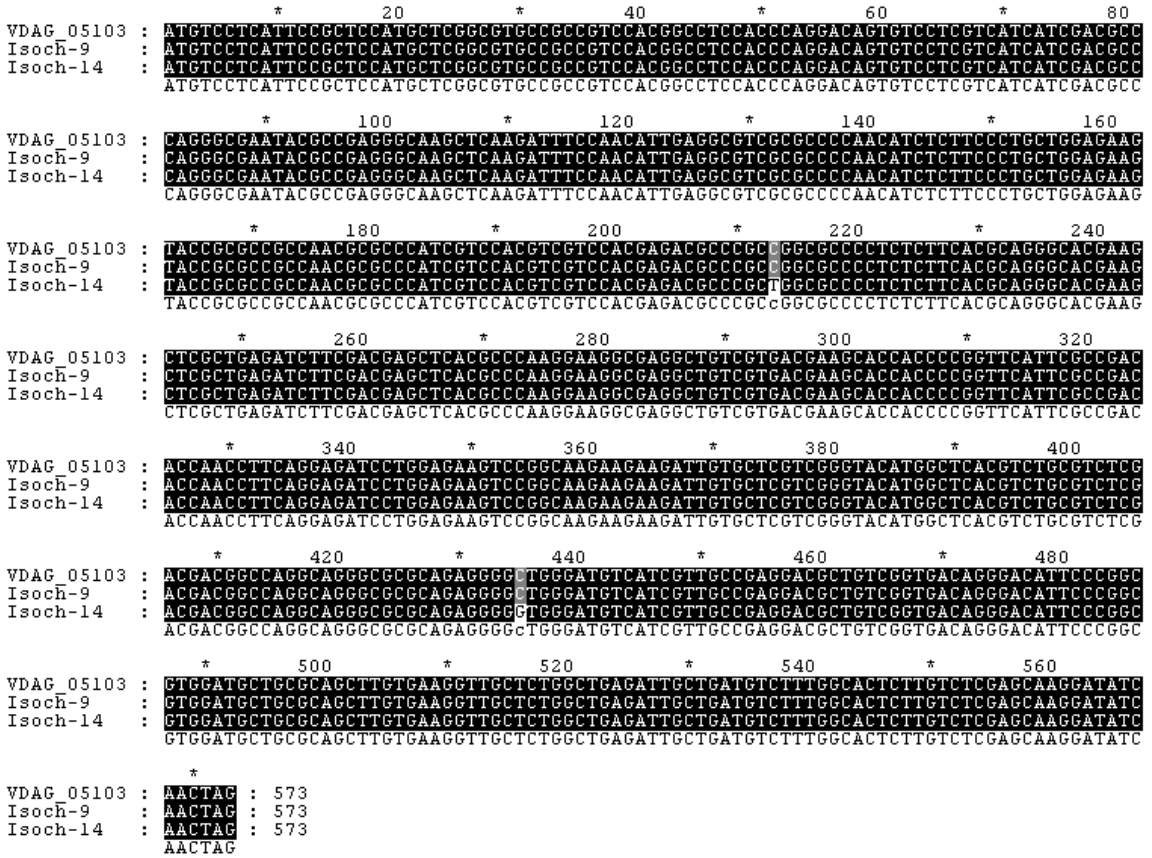

Figure 10.4. Multiple alignment of the sequences of exons only of isochorismatase hydrolase gene from the highly aggressive Vd1396-9 (Isoch-9), the weakly aggressive Vs06-14 (Isoch-14) and the Broad Institute VdLs.17 genome database (VDAG_05103) *V. dahliae* isolates.

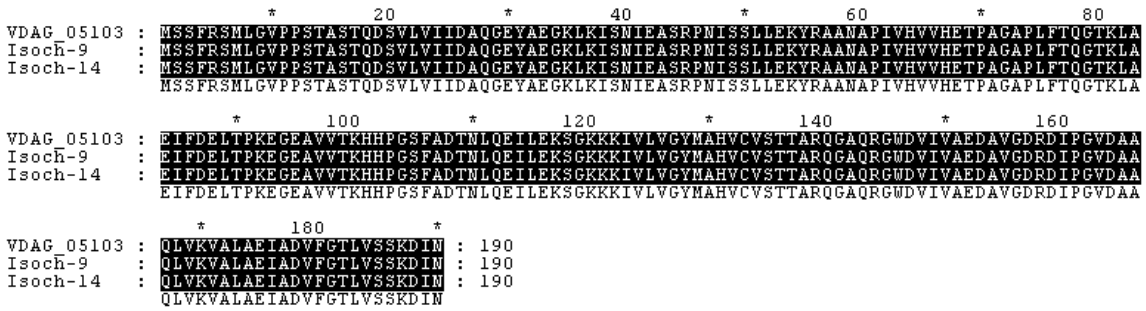

Figure 10.5. Multiple alignment of the deduced amino acids of isochorismatase hydrolase gene from the highly aggressive Vd1396-9 (Isoch-9), the weakly aggressive Vs06-14 (Isoch-14) and the Broad Institute VdLs.17 genome database (VDAG_05103) *V. dahliae* isolates.

10.4. Tetrahydroxynaphthalene reductase gene-full sequence

The Broad Institute *V. dahliae* isolate VdLs.17
http://www.broadinstitute.org/annotation/genome/verticillium_dahliae/MultiHome.html)
VDAG_03665.1 tetrahydroxynaphthalene reductase (Transcript:VDAG_03665T0)
http://www.broadinstitute.org/annotation/genome/verticillium_dahliae/GeneDetails.html?sp=S7000001884990859
http://www.broadinstitute.org/annotation/genome/verticillium_dahliae/TranscriptDetails.html?sp=S7000001884990860

Coding sequence (including *introns*): 1056 nt

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CTGGTGCCGGTAAGGCCTCTAGCCTTCGCCAAGCTCAATGAAAAAACCCCCTGACATGAAGTAGGTCGTGGAATAGGGCGCGAAATGGC
AATGGAGCTCGGTCGTCGCGGCGCCAAAGTCATCGTGAACTACGCCAACTCCTCAGAGTCAGCCGAGGAGGTCGTACAAGCCATCAAGA
AGTCAGGCTCCGATGCCGTCGCGATCAAGGCCAACGTCTCTGTTGTGGATGAGATCGTCTCCCTTTTCGAGCAAGGTGTGAAGACCTGG
GGTAAGCTCGACATTGTCTGCTCAAACAGCGGCGTCGTCTCTTTCGGGCACGTCAAGGACGTCACGCCTGAGGAATTCGACCGCGTCTT
CAACATCAACACGCGAGGCCAGTTCTTCGTTGCCCGCGAGGCGTACAAGCACCTCGAAGAGGGTGGGCGCCTCATTCTCATGGGCTCCA
TCACCGGCCAGGCCAAGGCCGTGCCGAAACACGCCGTCTACTCGGGCTCCAAGGGTACTATCGAGACATTTGTCCGCTGCATGGCCATC
GGTAGGTTCCTGCTGTGCCCGCCTTTTATCCATTCGTGCATCTTTTCAAGTGTACTCAGCAGATACTGACTCGGGTAGATTTTGGCGAC
AAGAAGATCACCGTCAACGCCGTCGCGCCTGGTGGCATCAAGACAGACATGTACCACGCCGTCTGTCGTGAATACATCCCCGGCGGAGC
CACCCTTTCCGATGACGAAGTCGACGAATACGCCGCCGGCTGGTCCCCAGTTCACCGTGTCGGTCTGCCCATCGACATCGCTCGCGTTG
TTTGCTTCCTCGCTTCGCAGGACGGCGCCTGGATCAACGGCAAGGTCCTCGGCATTGACGGTGCCGCTTGCATGTAA

The full length sequence of tetrahydroxynaphthalene reductase isolated from the highly aggressive *V. dahliae* isolate Vd1396-9

Vd1396-9- tetrahydroxynaphthalene reductase -clone 1
CCTTGACGCATCAGCACCACCATTGCAGGACTTCACCACACGCAACTCTACGACAATACCCCTTTAACCGTAAAGATGCCTGGCGCCAC
CTCTGCCGTCGAGCCCGGTTTCTACAACCGCATCCCAGGACCCCTGGGACTGGCCTCCGCCTCGCTGGAGGGCAAGGTCGCCCTAGTCA
CTGGTGCCGGTAAGGCCTCTAGCCTTCGCCAAGCTCAATAACAAACCCCCTGACATGAAGTAGGTCGTGGAATAGGGCGCGAAATGGCA
ATGGAGCTCGGTCGTCGCGGCGCCAAAGTCATCGTGAACTACGCCAACTCCTCAGAGTCAGCCGAGGAGGTCGTACAGGCCATCAAGAA
GTCAGGCTCCGATGCCGTCGCGATCAAGGCCAACGTCTCTGTTGTGGATGAGATCGTCTCCCTTTTCGAGCAAGGTGTGAAGACCTGGG
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GTAGGTTCCTGCTGTGCCCGCCTTTTATCCATTCGTGCATCTTTTCAAGTGTATTCAGCAGATACTGACTCGGGTAGATTTTGGCGACA
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Vd1396-9- tetrahydroxynaphthalene reductase -clone 2
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AACATCAACACGCGAGGCCAGTTCTTCGTTGCCCGCGAGGCGTACAAGCACCTCGAAGAGGGTGGGCGCCTCATTCTCATGGGCTCTAT
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AGAAGATCACCGTCAACGCCGTCGCGCCTGGTGGCATCAAGACAGACATGTACCACGCCGTCTGTCGTGAATACATCCCCGGCGGAGCC
ACCCTTTCCGACGACGAAGTCGACGAATACGCCGCCGGCTGGTCCCCAGTTCACCGTGTCGGTCTGCCCATCGACATCGCTCGCGTTGT
TTGCTTCCTCGCTTCGCAGGACGGCGCCTGGATCAACGGCAAGGTCCTCGGCATTGACGGTGCCGCTTGCATGTAA

Vd1396-9- tetrahydroxynaphthalene reductase -clone 3

CCTTGACGCATCAGCACCACCATTGCAGGACTTCACCACACGCAACTCTACGACAATACCCCTTTAACCGTAAAGATGCCTGGCGCCAC
CTCTGCCGTCGAGCCCGGTTTCTACAACCGCATCCCAGGACCCCTGGGACTGGCCTCCGCCTCGCTGGAGGGCAAGGTCGCCCTAGTCA
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GTCAGGCTCCGATGCCGTCGCGATCAAGGCCAACGTCTCTGTTGTGGATGAGATCGTCTCCCTTTTCGAGCAAGGTGTGAAGACCTGGG
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AACATCAACACGCGAGGCCAGTTCTTCGTTGCCCGCGAGGCGTACAAGCACCTCGAAGAGGGTGGGCGCCTCATTCTCATGGGCTCTAT
CACCGGCCAGGCCAAGGCCGTGCCGAAACACGCCGTCTACTCGGGCTCCAAGGGTACTATTGAGACATTTGTCCGCTGCATGGCCATCG
GTAGGTTCCTGCTGTGCCCGCCTTTTATCCATTCGTGCATCTTTTCAAGTGTATTCAGCAGATACTGACTCGGGTAGATTTTGGCGACA
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The full length sequence of tetrahydroxynaphthalene reductase isolated from the weakly aggressive *V. dahliae* isolate Vs06-14

Vs06-14- tetrahydroxynaphthalene reductase -clone 1

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TCTGCCGTCGAGCCCGGTTTCTACAACCGCATCCCAGGACCCCTGGGACTGGCCTCCGCCTCGCTGGAGGGCAAGGTCGCCCTAGTCAC
TGGTGCCGGTAAGGCCTCTAGCCTTCGCCAAGCTCAATGAAAAAACCCTCTGACATGAAGTAGGTCGTGGAATAGGGCGCGAAATGGCA
ATGGAACTCGGTCGTCGCGGCGCCAAAGTCATCGTGAACTACGCCAACTCCTCAGAGTCAGCCGAGGAGGTCGTACAAGCCATCAAGAA
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TTGCTTCCTCGCTTCGCAGGACGGCGCCTGGATCAACGGCAAGGTCCTCGGCATTGACGGTGCCGCTTGCATGTAA

Vs06-14- tetrahydroxynaphthalene reductase -clone 2

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Vs06-14- tetrahydroxynaphthalene reductase -clone 3

CCTTGACGCATCAGCACCACCACTGCAGGACTTCACCACACGCAACTCTACGACAATACTCCCTTTACCGTAAAGATGCCTGGCGCCAC
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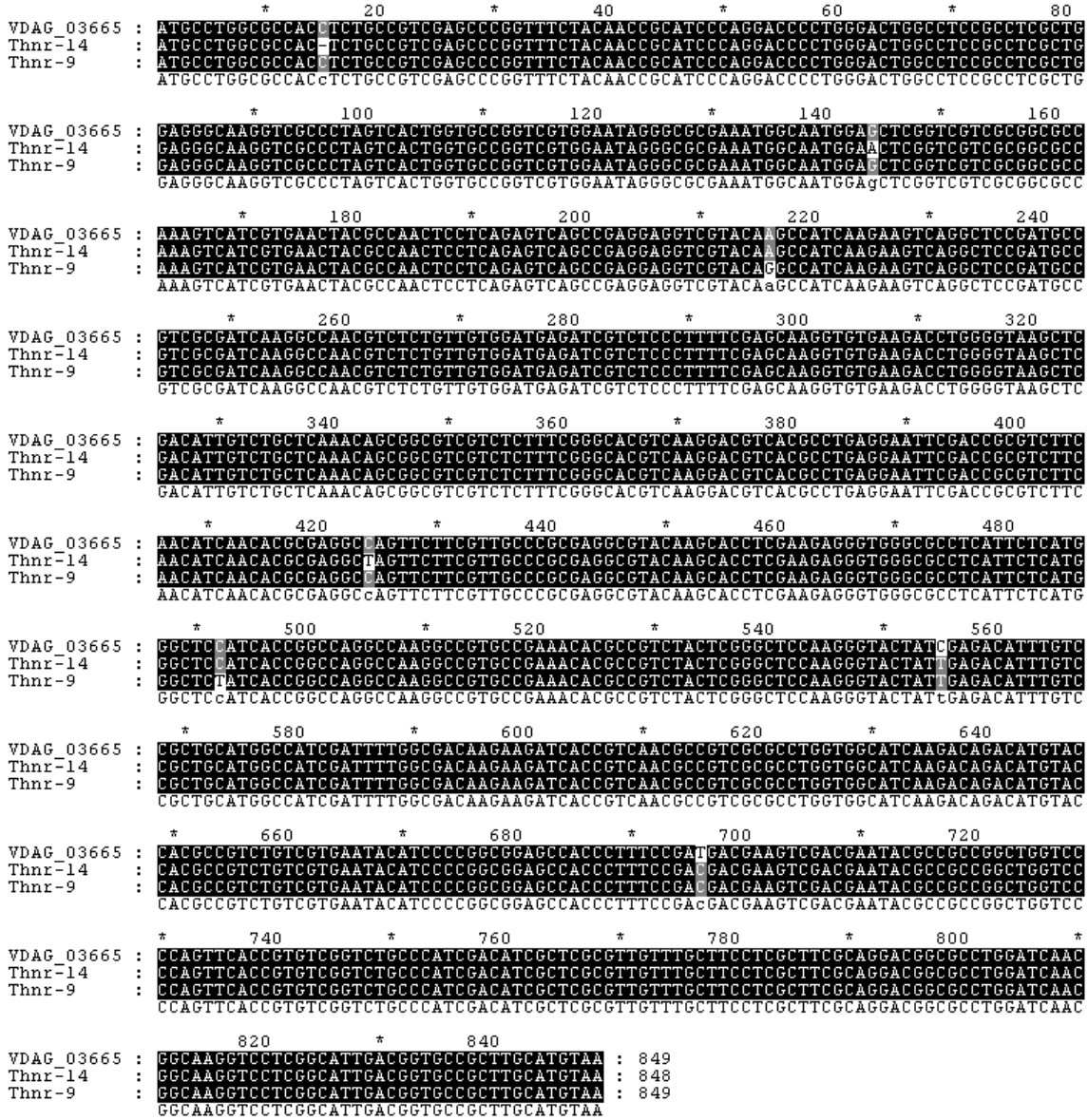

Figure 10.6. Multiple alignment of the sequences of exons only of tetrahydroxynaphthalene reductase gene from the highly aggressive Vd1396-9 (Thnr-9), the weakly aggressive Vs06-14 (Thnr-14) and the Broad Institute VdLs.17 genome database (VDAG_03665) *V. dahliae* isolates.

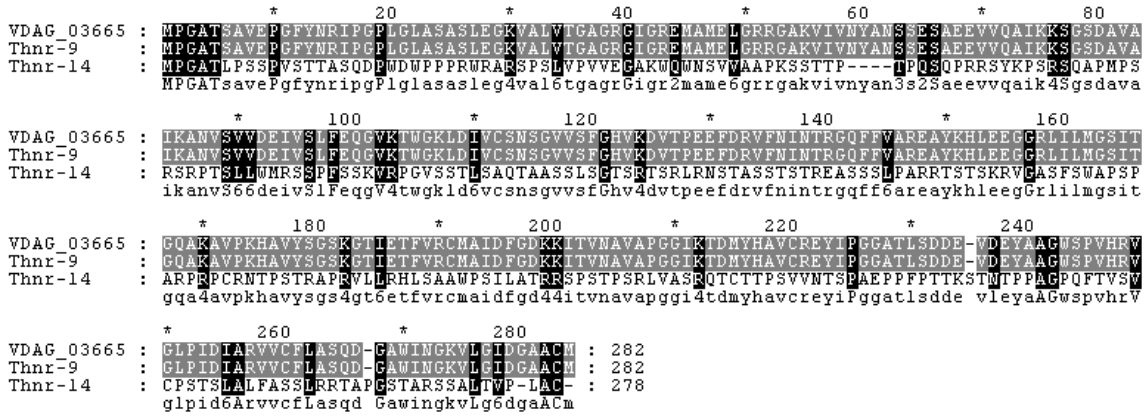

Figure 10.7. Multiple alignment of the deduced amino acids of tetrahydroxynaphthalene reductase gene from the highly aggressive Vd1396-9 (Thnr-9), the weakly aggressive Vs06-14 (Thnr-14) and the Broad Institute VdLs.17 genome database (VDAG_03665) *V. dahliae* isolates.

10.5 Standard curves of phenolic compounds

10.5.1 Standard curve of chlorogenic acid

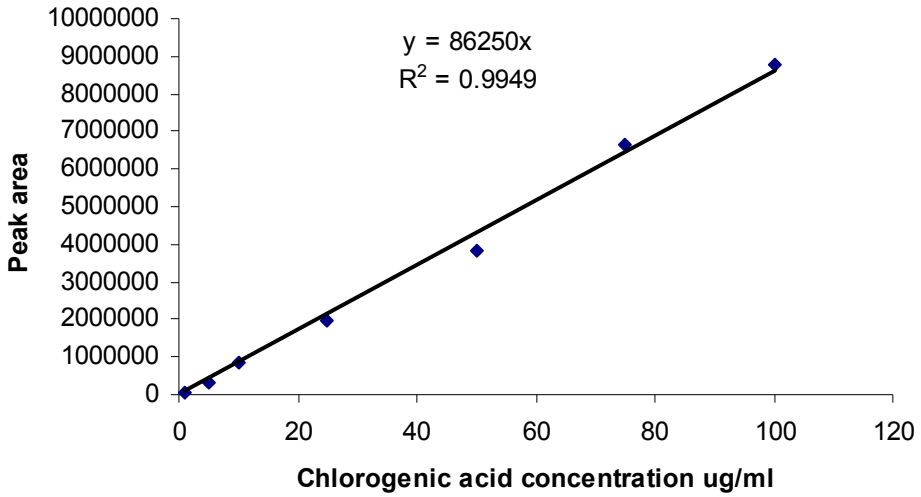

Figure 10.8. Standard curve of chlorogenic acid. Three replicates were used for each concentration. The equation of the standard curve was used to calculate chlorogenic acid content in the plant samples.

10.5.2 Standard curve of caffeic acid

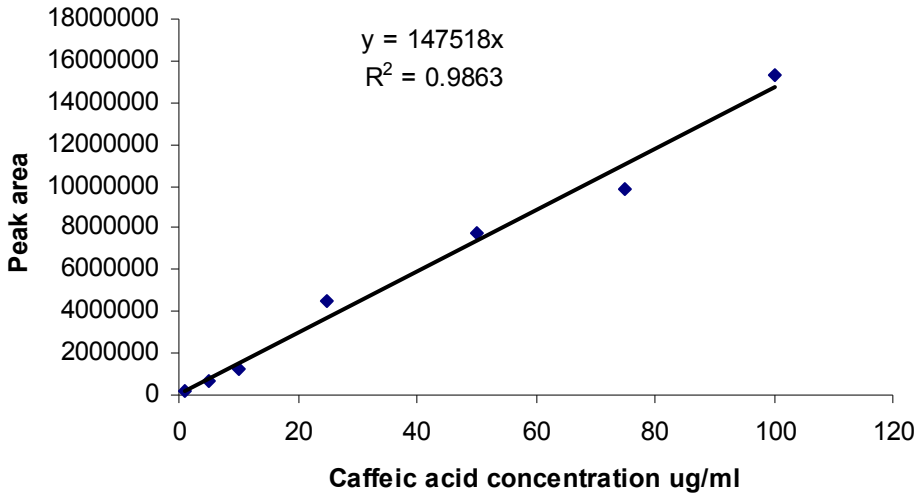

Figure 10.9. Standard curve of caffeic acid. Three replicates were used for each concentration. The equation of the standard curve was used to calculate caffeic acid content in the plant samples.

10.5.3 Standard curve of ferulic acid

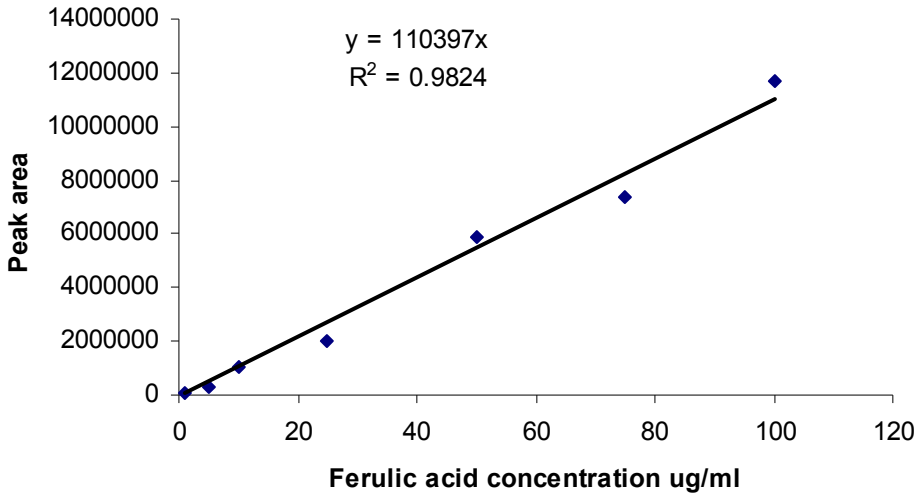

Figure 10.10. Standard curve of ferulic acid. Three replicates were used for each concentration. The equation of the standard curve was used to calculate ferulic acid content in the plant samples.

10.5.4 Standard curve of *cis*-jasmone

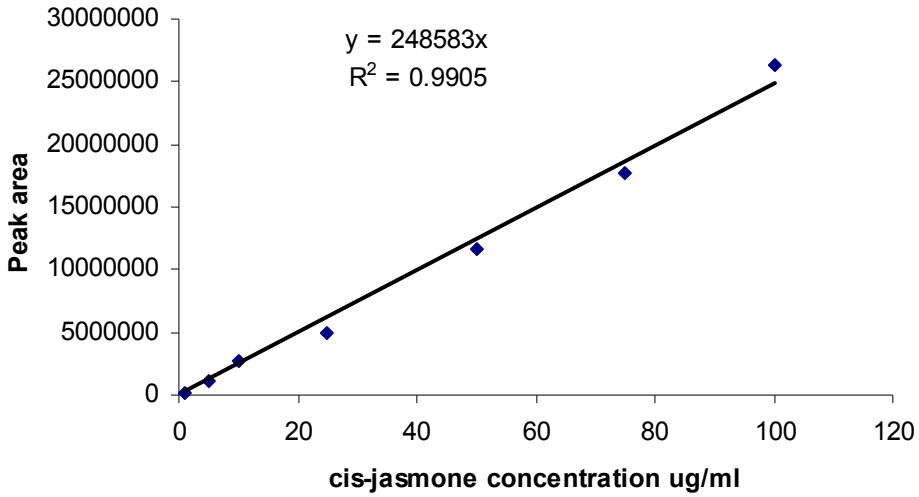

Figure 10.11. Standard curve of *cis*-jasmone. Three replicates were used for each concentration. The equation of the standard curve was used to calculate *cis*-jasmone content in the plant samples.

10.5.5 Standard curve of rutin

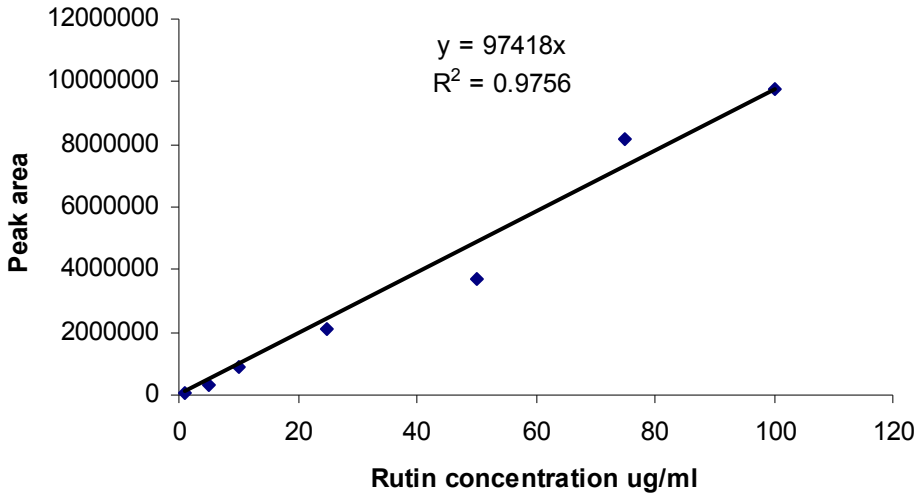

Figure 10.12. Standard curve of rutin. Three replicates were used for each concentration. The equation of the standard curve was used to calculate rutin content in the plant samples.