Identification and characterization of pathogenicity-related factors in *Verticillium dahliae*

By

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ABSTRACT

Identification and characterization of pathogenicity-related factors in *Verticillium dahliae*

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Verticillium dahliae is the main causal agent of potato early dying (PED) disease in Canada and can lead to yield losses of up to 50%. The management of this disease is usually costly and inefficient. Therefore, it is important to understand the molecular interaction between the host and pathogen, in order to determine the key components leading to disease or resistance.

Dr. Daayf's lab previously identified several proteins and/or genes showing differentiatial activity between highly and weakly aggressive *V. dahliae* isolates. Isochorismatase Hydrolase, NADPH oxidases, exopolygalacturonase, Ras-GAP like protein, serine/threonine-protein kinase, Ubiquitin-conjugating enzyme variant MMS2, NADH-ubiquinone oxidoreductase, Thioredoxin, Pyruvate dehydrogenase E1 component subunit beta, myo-inositol 2-dehydrogenase, and HAD-superfamily hydrolases all showed higher activity in the highly aggressive isolate compared to the weakly aggressive one.

We hypothesized that Isochorismatase Hydrolase (VdICSH1) of *V. dahliae* may interfere with the SA synthesis pathway of potato during their interaction. We generated gene disruption mutants for *VdICSH1* in the highly aggressive isolate, and showed their reduced virulence compared with the wild type. Plant SA and JA accumulation showed different tendencies in the stems and roots at early infection stages, and similar tendencies in the leaves at later stages, when exposed to *V. dahliae* infection. It was also apparent that other isochorismatase family members in the *icsh1* mutants compensate for the absence of ICSH1.

Further, we investigated the function of ROS generated by NADPH oxidases in *V. dahliae*, by generating mutants for *NoxA*, *NoxB* and *NoxC* in the highly aggressive isolate. The *noxa* and *noxb* mutants lost their penetration ability and could not form penetration pegs after 72h on cellophane membrane, which led to a significant decrease in their virulence on potato.

Finally, we employed a new vector–free split-marker recombination method, derived from *Saccharomyces cerevisiae*, to quickly obtain knock-out mutants for the exopolygalacturonase (ExoPG) gene in the highly aggressive *V. dahliae* isolate. This gene showed more transcriptional activity in response to leaf and stem extracts, as well as during early infection stage.

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ABBREVIATIONS

PED: potato early dying

VdPDHB: Pyruvate dehydrogenase E1 component subunit beta

PAL: phenylalanine ammonia-lyase

Trx: thioredoxin

SAR: Systemic acquired resistance

SA: Salicylic acid

JA: Jasmonic acid

ROS: Reactive oxygen species

VdICSH1: *Verticillium dahliae* Isochorismatase Hydrolase 1

StICS: potato Isochorismate Synthase

AUDPC: total area under disease progress curve

DAI: days after inoculation

HPI: hours-post-inoculation

NoxA: NADPH oxidase

NoxB: NADPH oxidase

NoxC: NADPH oxidase

ExoPG: exopolygalacturonase

FOREWORD

This thesis has been written in paper style with the guidelines of Department of Plant Science, Faculty of Agriculture and Food Sciences, University of Manitoba. This thesis consists of five parts, which include: general introduction and literature review, as well as four manuscripts. Each Manuscripts comprises an abstract, introduction, materials and methods, results, and discussion. Thenceforth, following a general discussion and references. Chapter 3 (second manuscript) "*Verticillium dahliae's* isochorismatase hydrolase is a virulence factor that contributes to interference with potato's salicylate and jasmonate defense signaling" was published in Frontiers in Plant science in March, 2017 (doi: 10.3389/fpls.2017.00399).

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

The genus *Verticillium* is the main cause of vascular wilt in many plant species (Daayf, 2015). It is mainly composed of soil-borne fungal pathogens that can cause great losses in various crops (Daayf, 2015; Pegg and Brady, 2002; Subbarao et al., 1997). In 1816, *Verticillium* was first identified by Nees von Esenbeck (Isaac, 1967). It belongs to the Plectosphaerellaceae family (Zare et al., 2007) and has a close phylogenetic relation with *Colletotrichum,* which belongs to the Glomerellaceae family (Zhang et al., 2006). Both Plectosphaerellaceae and Glomerellaceae families are in the Hypocreomycetidae, which is a subclass of the fungal phylum Ascomycota (Lumbsch and Huhndorf, 2011; Zhang et al., 2006). One major characteristic of *Verticillium* is its erect, spiral branched conidiophores at the tip, there are flask-shaped spores (Isaac, 1967).

The major plant pathogenic fungi in this genera include *V. albo-atrum*, *V. nigrescens*, *V. longisporum* and *V. daliae* (Barbara and Clewes, 2003; Klosterman et al., 2009). The latest is the most widely detected *Verticillium* species on crop plants. In temperate and subtropical areas, it can cause *Verticillium* wilt on more than 200 host plants including crops, fruits, shrubs, and trees (Agrios, 2005; Bhat and Subbarao, 1999; Isaac, 1967; Pegg and Brady, 2002). *V. dahliae* isolates can be ranged into six vegetative compatibility groups (VCGs) (Bhat et al., 2003; Jiménez-Díaz et al., 2006).

According to data on FAOSTAT (http://faostat.fao.org/site/339/default.aspx), potato is the tenth largest crop produced in Canada, which ranged as the fourteenth largest potato producer in the world from 1996 to 2016. In 2016, the total global potato production was 377 million tonnes, with income receipt valued at 1.2 billion (http://www.agr.gc.ca/eng/industry-markets-and-trade/canadian-agri-food-sector-intelligence/horticulture/horticulture-sector-reports/potato-market-information-review-2015-2016/?id=1500402297688#a3.1). The average annual potato

yield over the past 20 years in Canada has been 4.6 million tonnes per year. China, India, and the Russia are the top three potato producers. The losses caused by *V. dahliae* on potato range from 10% to 15% decrease in annual yield, but still can be up to 50% under suitable conditions (Johnson and Dung, 2010; Johnson et al., 1986; Powelson and Rowe, 1993; Rowe and Powelson, 2002). *V. dahliae* is the primary component of the potato early dying complex, which may also comprise *V. albo-atrum* and root lesion nematodes (Pratylenchus spp.) (Rowe et al., 1987; Rowe and Powelson, 2002). Soft rot bacteria, *Colletotrichum coccodes* (Wallr.) Hughes, *Pratylenchus penetrans* (Cobb) Chitwood & Oteifa, and nutrient deficiencies are several other factors besides *V. dahliae* and *V. albo-atrum* to induce Early Dying syndrome (Davis and Huisman, 2004; Johnson and Dung, 2010; Mohan et al., 1992; Rowe et al., 1987). In potato, the infection by *V. dahliae* may happen during the early growing season, but the symptoms always occur in the rapid tuber-bulking period of the latter growing season (Davis and Huisman, 2004; Johnson and Dung, 2010; Mohan et al., 1992; Rowe et al., 1987). However, the above-ground symptoms do not always result into a decrease in production (Johnson and Dung, 2010; Nachmias and Krikun, 1984; Rowe et al., 1985). Although several strategies are used to control Verticillium wilt, i.e, crop rotation, green manures, soil fumigation, bio-fumigants and soil solarization, these are usually costly and efficiency-decreasing with repeated implementations (Johnson and Dung, 2010; Klosterman et al., 2009; Subbarao et al., 2007). Therefore, it is important to develop better strategies to control *Verticillium* wilt.

Previous studies in Dr. Fouad Daayf's lab tested several *V. dahliae* isolates over the years, with the intent to identify two *V. dahliae* isolates (Vd1396-9 and Vs06-14) consistently contrasting in their pathogenicity levels. Further proteomic studies using these two isolates identified several proteins uniquely detected in the highly, but not weakly aggressive isolate (El-Bebany et al., 2010). In addition, a transcriptomic study also identified several genes that were transcriptionally up-

regulated following elicitation with potato root extracts (El-Bebany et al., 2011). Thioredoxin and NADH-ubiquinone oxidoreductase, which are involved in maintaining cellular reactive oxygen species (ROS) level in the cell (Bazil et al., 2014; Huang et al., 2015; Kussmaul and Hirst, 2006), showed a significantly higher protein accumulation in the highly aggressive isolate (El-Bebany et al., 2010). These data indicated that ROS may be an important determinant for *V. dahliae* pathogenicity. Therefore, ROS-producing enzymes may be essential for its virulence.

In this project, I plan to investigate the activity of candidate genes, as indicated in previous findings of El-Bebany et al. (2010, 2011) in different aggressive isolates (Vd1396-9 and Vs06-07) *in vitro* and *in planta*. These two sets of treatments were established to mimic the activity of the pathogen in two important phases: (1.a) pre-infection when the pathogen is in the soil and senses the host via root or other exudates, and (1.b) the actual infection of the plant tissues. In order to have a better control of the experiments in this second phase, detached leaves with petioles were used, instead of whole plants. Then I will study the potential function of the most important genes that may play a role in pathogenicity. These important targets include Isochorismatase hydrolase (*ICSH1*), NADPH oxidase (Nox) family genes (*NoxA, NoxB*, and *NoxC*), and exopolygalacturonase (*ExoPG*).

1.2 Literature review

Given that *V. dahliae* has a wide range of hosts and the current disease management methods for verticillium wilt disease cannot fully protect plants, it is important to take a step back and try to better understand the molecular mechanisms underlying the *V. dahliae* infection of host plants. *V. dahliae* toxins, elicitors, cell wall-degrading enzymes and some known pathogenicity factors are key elements involved in the infection process. On the other side of the interaction,

plant defenses against *V. dahliae* can be manipulated for the sake of improving disease control. Plant hormones like salicylic acid (SA) and jasmonic acid (JA) are critical in the mediation of systemic acquired resistance against the pathogen infection. One of the mechanisms that are present in both plants and pathogens is ROS production. ROS as signal molecules are produced by plant pathogens to mediate the development of penetration structures on plant surface; plant can develop oxidative burst reactions by producing ROS to restrain the pathogen invasion. These common elements from both pathogen and plants are critical for the investigation of the interaction between pathogens and their hosts, and understanding how they mutually function may provide invaluable insights into the interaction of *V. dahliae* with its hosts.

1.2.1 The life cycle of *V. dahliae*

No sexual stage for reproduction of *V. dahliae* has been identified. Its asexual stage can be divided into three phases: parasitic, saprophytic, and dormant (Klimes and Dobinson, 2006). In the parasitic phase, *V. dahliae* firstly penetrates from the plant root surface, then colonizes the xylem while growth under a yeast-like morphology (Puhalla and Bell, 1981; Schnathorst, 1981). The fungus can go into a second phase—saprophytic, on dead plant tissues, where mycelia, conidiophores, and melanized microsclerotia are formed (Schnathorst, 1981; Wilhelm, 1955). At the end of the asexual stage, microsclerotia, the resting structures of this pathogen are produced for its long-term survival in the soil (Schnathorst, 1981). The infection process starts by the sprouting of microsclerotia stimulated by plant root exudates, followed by penetration on the elongation part of susceptible host root and colonization in the cortex (Chliyeh et al., 2014; Mol and Van Riessen, 1995). Invasive hypha penetrate the xylem vessels and produce spores that are then taken through the upper part of the plant by water (Chliyeh et al., 2014). The hypha of *V.*

dahliae grow in xylem vessels, and plant vascular cell walls produce coating materials such as cellulose, pectin and callose to block water absorption needed by the top part of the plants (Mol and Termorshuizen, 1995; Robb et al., 1979). As the infection progresses, plant stems and leaves begin to show wilt symptoms, including chlorosis and necrosis. Microsclerotia are lastly produced on both dead tissues and soil (Mol and Termorshuizen, 1995; Schnathorst, 1981), which can keep viability in the soil for up to 10 years (Klosterman et al., 2009).

1.2.2 *V. dahliae* virulence factors

1.2.2.1 *V. dahliae* toxins

In the past few decades, many researchers used fungal culture filtrates (FCFs) as a tool to investigate interactions between pathogens and their hosts. FCFs are expected to contain a series of toxic metabolites, i.e., a protein-lipopolysaccharide complex, that was isolated from cotton leaves infected by *V. albo-altrum* (Keen and Long, 1972). A phytotoxic protein-lipopolysaccharide complex (PLPC) was also found in *V. dahliae* culture filtrates. Treatment of cotton seedlings with purified PLPC caused wilting symptoms and induced plant defense reactions, including an increase in activity of pathogenesis-related proteins and phenylalanine ammonialyase (PAL) (Meyer et al., 1994). Comparison of metabolic profiles of a pathogenic strain and a non-pathogenic mutated strain of *V. dahliae* had revealed two PLPCs with totally different phytotoxic activities (Nachmias et al., 1982). These two PLPCs from the pathogenic strain but not from the mutated non-pathogenic strain induced pathogenicity in potato and many other host plants (Nachmias et al., 1982). The above studies all suggested that PlPs play important roles in *V. dahliae'*s pathogenicity.

A *V. dahliae* toxin (VD toxin) filtrate induced different levels of ultrastructural changes, and induced wilting symptoms, in callus cells of both susceptible and resistant cultivars (Zhen

and Li, 2004). In the susceptible cotton cultivar, VD toxin induced invaginated plasma membrane, extensive development of membrane-bound vesicles and cytoplasm agglutination. On the resistant cultivar, there was fewer disease symptoms in response to VD toxin, along with less invagination of plasma membranes, and a normal cytoplasm (Zhen and Li, 2004). However, application of both VD toxin and SA together on these two cultivars significantly reduced the deleterious effects of VD toxin and increased the level of b-1,3-glucanase in the host (Zhen and Li, 2004). The authors suggested then that the VD-toxin first target was the plasma membrane (Zhen and Li, 2004). In *Arabidopsis thaliana*, the high concentration of VD toxin disrupted microfilaments (MFs) around nuclei and cortical microtubules (MTs), while with low concentrations of VD toxin MF disruption was delayed (Yuan et al., 2006). Interestingly, the VD toxin could also induce the single ring-shaped nucleoli in the plant cell, after which MFs could not be recovered (Yuan et al., 2006). Therefore, the induction of ring-shaped nucleoli by the VD toxin suggested that the plant cells enter into the resting state and was followed with the resistance-related programmed cell death (Yuan et al., 2006).

1.2.3 *V. dahliae* Cell wall–degrading enzymes

Fungal strains from *Verticillium* genus can produce various extracellular hydrolytic enzymes, that are important for degrading polymers in the integument of their hosts (St Leger et al., 1997). *V. albo-atrum* and *V. dahliae* excrete high amounts of enzymes to degrade pectin, a major component of plant cell wall, and many proteases, which primarily degrade Bz-AA-AA-Arg-NA substrates (Bz, benzoyl; AA, various amino acids; NA, p-nitroanilide) of the plant cell wall components (Bidochka et al., 1999a; Bidochka et al., 1999b; St Leger et al., 1997). *Verticillium* plant pathogens produce much more cellulase and xylanase than *V. lecanii,* the insect

pathogen (Bidochka et al., 1999a; Bidochka et al., 1999b; St Leger et al., 1997). However, *V. albo-atrum*, *V. nigrescens* and *V. dahliae* do not secrete chitinase and produce less extracellular subtilisin-like protease than *V. lecanii* (Bidochka et al., 1999a; Bidochka et al., 1999b; St Leger et al., 1997). *V. lecanii,* alike to the mushroom pathogen *V. fungicola,* excretes a lot of chitinases and extracellular subtilisin-like protease against the chymotrypsin substrate (succinyl-Ala_2- Pro-Phe-NA) of the host integument (Bidochka et al., 1999a; Bidochka et al., 1999b; St Leger et al., 1997).

The primary plant cell wall is composed of three kinds of polysaccharides: pectic substances, cellulose, and hemicellulose, in addition to other substances (Novo et al., 2006; Schafer, 1994). *V. dahliae* can produce many cell wall–degrading enzymes (CWDE), but majority are pectinases and cellulases (Bidochka et al., 1999a; Bidochka et al., 1999b). The cellulase enzyme complex is composed of exo-β-1,4-glucanases, endo-β-1,4-glucanases, and β-1,4-glucosidases (De Lorenzo et al., 1997; Novo et al., 2006), which can degrade different kinds of polysaccharides from plant cell walls (Cooper and Wood, 1973). Pathogen-secreted pectinases and cellulases are involved in the induction of disease symptoms in the hosts (Pegg, 1981). During the infection in tomato, *V. albo-atrum*'s the endo-pectin lyase activity (endo-PL) increases greatly and seems to be the only CWDE showing such an increase (Cooper and Wood, 1980). Pectinase-deficient mutants of *V. albo-atrum* caused less severe or delayed symptoms on the host but the mutant did not influence the colonization of the host, indicating that pectinases are virulence factors (Calderone and Fonzi, 2001) but are not essential for pathogenicity (Durrands and Cooper, 1988; Podschun and Ullmann, 1998). Endoglucanase 1 is critical for the depolymerization of plant cellulose during the infection (De Lorenzo et al., 1997; Valášková and Baldrian, 2006). Maruthachalam et al. (2011) identified an endoglucanase 1 mutant of *V. dahliae* that lost virulence and was unable to form microsclerotia on solid PDA media or to colonize the host's xylem vessels. The protease identified

from *Uromyces viciaefabae* can degrade fibrous hydroxyproline-rich proteins and facilitate penetrating the plant cell wall (Rauscher et al., 1995). Proteases can degrade the plant proteins involved in host resistance (Pegg, 1981). In nutritionally-limited environments, proteases secreted in the vascular systems may play roles in survival and colonization of the hosts by hydrolysis of the cell wall proteins as a nutrient source (Dobinson et al., 1997; Pegg, 1981). Excessive catabolite can repress activities of CWDEs in *V. albo-atrum* (Cooper and Wood, 1975), and the disruption of a single gene of CWDEs in *V. dahliae* may not influence the pathogenicity due to the functional redundancy of multiple members of the same gene (Dobinson et al., 2004). A *V. dahliae* 30-kDa trypsin-like protein was identified and show to perform the serine protease activity, designated as VTP1 (Dobinson et al., 1997). However, disruption of the gene did not influence the growth or the pathogenicity of *V. dahliae* (Dobinson et al., 2004). The disruption of the β-1,6-glucanase homolog gene in *V. fungicola* reduced the virulence on mushroom *Agaricus bisporus* and the mutant's growth rate in media in presence of chitin decreased significantly, compared to the wild type, which indicated the important role of this gene in utilizing chitin as a carbon and nitrogen sources (Amey et al., 2003).

A sucrose nonfermenting 1 gene (VdSNF1) in a *V. dahliae* tomato race 1 strain is essential for the expression of pectate lyase genes in present of pectin, and can influence the utilization of pectin or galactose as a carbon source. *VdSNF1* is required for full virulence as its disruption significantly reduced the pathogenicity on tomato (Tzima et al., 2011).

1.2.4 Other pathogenicity factors

Microsclerotia originate from a single hyphal swelling which consequentially leads to the production of clusters of spherical pigmented cells (Hall and Ly, 1972; Heale and Isaac, 1965).

The development of microslerotia is important for the progress of *Verticillium* wilt.

In *V. dahliae*, *V. albo-atrum*, *V. longisporum*, *V. nigresecens*, and *V. tricorpus*, the homologs of class II hydrophobins, namely VDH1, are in a single copy, and in *V. dahliae* are important for the development of microsclerotia (Klimes and Dobinson, 2006). The deletion mutant of *VDH1* in *V. dahliae* cannot form microsclerotia, with no swollen, septate hyphae (Klimes and Dobinson, 2006). Interestingly, microsclerotia were detected in the mixture culture of a hyaline microsclerotia (albino) mutant *alm1* and *vdh1* mutant, suggesting that the exogenous VDH1 protein can restore the microsclerotia production in the *vdh1* mutant (Klimes and Dobinson, 2006). However, the loss of VDH1 did not affect the virulence of *V. dahliae* (Klimes and Dobinson, 2006). A *Verticillium* mitogen-activated protein (MAP) kinase, VMK1, identified in both lettuce isolate VdLs.17 and tomato isolate Dvd-T5 (Rauyaree et al., 2005). The disruption of the *VMK1* gene, significantly reduced microslerotia development in these two *V. dahliae* isolates (Rauyaree et al., 2005). The mutants from these two isolates exhibited reduces virulence on various kinds of host plants, when compared to wild type (Rauyaree et al., 2005). This indicates that VMK1 function is fundamental and conserved among different *V. dahliae* isolates (Rauyaree et al., 2005). However, *Verticillium* species infecting plants, a homolog of pmk1-like MAPK in *V. fungicola* was not required for the virulence on host mushroom *A. bisporus* and other phenotypes like sporulation and mycelial growth rate, indicating that this MAPK does not play important roles in fungal-fungal interaction (Collopy et al., 2010).

Using *Agrobacterium tumefaciens*-mediated transformation (ATMT) Maruthachalam et al. (2011) got 20 transformants that failed to induce *V. dahliae* pathogenicity on lettuce, and another 31 transformants that showed reduced virulence. A T-DNA insert mutant of Endoglucanase 1 (EG-1) was shown to be essential for full virulence on its host (Maruthachalam et al., 2011). It did not

form microsclerotia on solid PDA media and could not colonize the host's xylem vessels (Maruthachalam et al., 2011). Another identified pathogenicity factor is hydroxymethylglutaryl-CoA (HMG-CoA) synthase gene, which is necessary for the necrosis and root discoloration induced on host (Maruthachalam et al., 2011). HMG-CoA synthase protein has been shown to participate in the sterol biosynthesis pathway in plant species (de Souza and Rodrigues, 2009) as well as in trichothecene mycotoxin production in *Fusarium* (Goswami and Kistler, 2004; Maruthachalam et al., 2011). Other identified pathogenicity factors include facilitator superfamily transporter (MFS) gene, which has roles in toxic compounds and fungicidal resistance (Kapoor et al., 2009; Prasad and Kapoor, 2005). Research on *Brassica juncea*, *Lycopersicon esculentum*, *Saccharomyces cerevisiae*, and *Stagonospora nodorum*, showed that the glyoxalase I gene is induced in response to osmotic stress (Espartero et al., 1995; Reddy and Sopory, 1999; Solomon and Oliver, 2004). Overexpression of this gene in *B. juncea* led to better tolerance to high salt stress (Reddy and Sopory, 1999), since glyoxalase I was able to transform the toxic metabolic by-product methylglyoxal (MG) into S-D–lactoylglutathione. A glyoxalase I gene was identified in *V. dahliae* by Klimes et al. (2006), however the gene disruption had no effect on pathogenicity (Klimes et al., 2006).

In *V. dahliae*, a catalytic subunit genes of cAMP-dependent protein kinase A (PKA), namely VdPKAC1, was necessary for the cAMP-dependent signaling transduction in *V. dahliae*. Disruption of VdPKAC1 caused reduction of mycelia growth and conidial production, as well as lower production of pathogen-derived ethylene and reduced virulence on tomato and eggplant. These results suggested the comprehensive role of VdPKAC1 in regulation of development, conidiation and virulence of *V. dahliae* (Tzima et al., 2011). A G protein ß subunit gene (VGB) in *V. dahliae* was identified and shown to be required for plant-derived ethylene production and full

pathogen virulence but negatively regulated the microsclerotia production and conidiation, compared with the wild type (Tzima et al., 2012). Interestingly, expression of *VdPKAC1* under the native promoter in the *VGB* gene mutant, recovered mutant with the wildtype phenotype of growth pattern, ethylene production, and pathogen virulence, as well as conidiation (Tzima et al., 2012).

1.2.5 Other *V. dahliae* genes in the pathogen-host interaction

Plant pathogens secret effector proteins as virulence factors to help develop symptoms in their host. However, these virulence factors could also be recognized by host as elicitors to induce plant defense, such as synthesis of phytoalexins (Hahn et al., 1981), ROS, and lipid peroxides (Anderson et al., 1991; Apostol et al., 1989; Dixon and Lamb, 1990; Lamb et al., 1989), plant lignin deposition (Kogel et al., 1988), phenolic compounds (Anderson et al., 1991), pathogenesis-related (PR) genes (Ryan, 1987; Showalter et al., 1985) and hydroxyproline-rich proteins (Showalter et al., 1985).

1.2.5.1 *V. dahliae* elicitors

An endopolygalacturonase isolated from *Rhizopius stolonifer*, stimulating the release of endogenous components in infected plant cells, was recognized as an elicitor promoting the synthesis of phytoalexins in castor bean (West, 1981). The *Phytophthora megasperma f.sp. glycinea* cell wall contained hepta-ß-glucan, which could induce synthesis of isoflavanoid phytoalexins in soybean cotyledons (Cheong and Hahn, 1991; Ebel, 1986; Sharp et al., 1984). *Phytophthora cryptogea* and *P. capsici* both secreted low molecular weight proteins that promoted capsidiol production in tobacco (Blein et al., 1991). Two *Avirulence* (Avr) gene -encoding proteins in *Cladosporium fulvum* can induce production of phytoalexins, PR proteins, hydroxyproline rich

proteins and deposition of cell wall-ralated components in tomato (De Wit, 1992). In *V. dahliae*, a 65 kDa heat-stable glycoprotein was isolated and could be de-glycosylated into a 53 kDa protein by N-glycosidase F (PNGase-F), and both could elicit production of phytoalexins in cotton cells (Davis et al., 1998). Chu et al. (1998) found another 26 kDa glycoprotein in *V. dahliae*, which can induce wilting symptoms and production of phytoalexins such as sesquiterpene aldehydes in cotton (Chu et al., 1998).

A necrosis- and ethylene-inducing protein (VdNEP) was isolated from *V. dahliae* Vd-8 originating from cotton (Wang et al., 2004). This 233 amino acid-contained protein composed of a signal peptide for eukaryotic protein secretion and was able to induce the necrosis symptom and the activation of SA-, ethylene- and JA-dependent defense pathways in *Nicotiana benthamiana* and *A. thaliana* leaves (Wang et al., 2004). Another group of proteins ranging from 24 kDa to 26 KDa, namely Nep1-like proteins (NLPs) with the similar function was found in several bacteria, oomycetes and fungi (Gijzen and Nürnberger, 2006; Pemberton and Salmond, 2004; Qutob et al., 2006), which exhibited cytotoxic activities on dicots but not on monocots (Gijzen and Nürnberger, 2006; Qutob et al., 2006). Klosterman et al. (2011) found eight NPLs in *V. dahliae* and seven NPLs in *V. albo-atrum* through a comparative genomic research. Zhou et al. (2012) cloned nine NPLs in the *V. dahliae* isolate V592 and were designated as NLP1 to NLP9, as NLP1 was the homolog of a VdNEP from *V. dahliae* isolate Vd-8. Only NLP1 and NLP2 induce necrosis lesions on *N. benthamiana* leaves via *Agrobacterium*-mediated transient expression (Zhou et al., 2012). In addition, purified NLP1 and NLP2 also promote the production of ROS and stimulate the activities of defense-related genes in *N. benthamiana*， cotton, and *A. thaliana* (Zhou et al., 2012). The gene deletion mutant of NLP1 and NLP2 did not have an impact on the virulence of *V. dahliae* towards cotton (Zhou et al., 2012). However, Santhanam et al. (2013) found NLP1 and NLP2 of the tomato-

pathogenic JR2 strain of *V. dahliae* were involved in the virulence development on tomato and able to induce necrosis on *N. benthamiana* leaves. PevD1 a 16.23 kDa putative protein secreted by *V. dahliae* with non-reported function, was able to induce the hypersensitive-like response, ROS burst, callose deposition and accumulation of phenolics and lignin on tobacco (Wang et al., 2012). Also, treatment of the tobacco leaves with purified PevD1 have increased resistance to the tobacco mosaic virus (TMV) (Wang et al., 2012).

The tomato immune receptor Ve1 controls resistance to the race 1 strain of *V. dahliae* and *V. albo-atrum* (Fradin et al., 2009; Kawchuk et al., 2001; Schaible et al., 1951; Vossen et al., 2010). Using the high-throughput population genome sequencing, de Jonge et al. (2012) found an *Ave1* (Avirulence on Ve1 tomato) was only present in *V. dahliae* race 1 but not race 2. Heterologous expression of the *Ave1* on Ve1 tomato induced the hypersensitive response on leaves, and co-expression of *Ave1* and *Ve1* in tobacco leaves also activated the hypersensitive response (de Jonge et al., 2012). Moreover, disruption of *Ave1* in the race 1 *V. dahliae* strain JR2 resulted in recover of pathogenicity on Ve1 tomato (de Jonge et al., 2012).

Santhanam et al. (2013) found a transcriptional regulator Sge1 in *V. dahliae* was required for conidia production and virulence on tomato. In *Fusarium oxysporum* f.sp. Lycopersic, the Sge1 homolog was essential for pathogenicity and expression of some effector proteins (Michielse et al., 2009). It remains to explore whether Sge1 in *V. dahliae* regulates the expression of effector proteins for induction of virulence.

1.2.5.2 Isochorismatase hydrolase and relative enzymes

Isochorismatase catalyzes isochorismate to 2,3-dihydroxybenzoate and pyruvate (Soanes et al., 2008). This reduces the production of SA in plants because isochorismate is a very important

precursor of SA synthesis (Wildermuth et al., 2001). In bacteria, a streptothricin hydrolase (SttH) belongs to the isochorismatase-like hydrolase (ILH) super family, has been identified and found to play an critical role in streptothricin (ST) resistance (Maruyama and Hamano, 2009). In *Pseudomonas aeruginosa*, PchB as best characterized isochorismate pyruvate lyase (IPL) rearranges chorismate into prephenate and catalyzes isochorismate conversion into pyruvate and salicylate (Künzler et al., 2005). In *P. aeruginosa*, an isochorismatase PhzD plays a role in production of trans-2,3-dihydro-3-hydroxyanthranilic acid from vinyl ether functional group of 2-amino-2-deoxyisochorismate for phenazine biosynthesis (Parsons et al., 2003). In addition, the PhzD can also hydrolyze vinyl ethers isochorismate, chorismate, and 4-amino-4-deoxychorismate. In *Escherichia coli*, EntB, an isochorismate lyase, participates in the enterobactin biosynthesis through catalysis of chorismate into 2,3-dihydroxybenzoate (Gehring et al., 1997). In *V. dahliae*, an isochorismatase hydrolase was only found in a highly aggressive isolate using proteomics comparison and not in a weakly aggressive one (El-Bebany et al., 2010). A protein containing a isochorismatase motif was identified in the secretomes of five different filamentous ascomycete phytopathogen species, but was absent in the non-pathogenic ones (Soanes et al., 2008).

1.2.6 Plant interaction with *V. dahliae*

After insect and pathogen attacks, plants produce a set of hormones, quickly inducing the activity of PR proteins (Ryan, 1987; Showalter et al., 1985), production of ROS, and a hypersensitive reaction (HR) at the infection site (Anderson et al., 1991; Apostol et al., 1989; Dixon and Lamb, 1990; Lamb et al., 1989). Plant hormones usually consist of the following groups: auxins or indoleacetic acid (IAA), gibberellins (GA), cytokinins (CK), abscisic acid (ABA), ethylene (ET), brassinosteroids, JA, SA, peptides and polyamines (Davies, 2010). Three types are

considered to mainly functioning in plant defense response to pathogen infection: JA, SA and ET. I.e., SA induces the activities of PR proteins and plant "oxidative bursts", as well as activate the systemic acquired resistance (SAR) and other defense response genes (Pasqualini et al., 2003; Wildermuth et al., 2001). In turn, ROS could also stimulate the production of SA (Holuigue et al., 2007).

1.2.6.1 Salicylic acid (SA)

Plants have a variety of receptors to recognize both elicitors secreted by microbes and phytohormones produced for plant systemic acquired resistance in response to pathogen infection. Treatment with SA enhances plant resistance against pathogens, while mutants with a deficiency in SA synthesis exhibit increased susceptibility to pathogens (Loake and Grant, 2007).

There are mainly two pathways to synthesize SA in plants, (i) the phenylpropanoid pathway which is facilitate by the enzyme phenylalanine ammonium lyase (PAL) (Coquoz et al., 1998; Pasqualini et al., 2003), and (ii) the pathway involving isochorismate synthase (ICS) to produce SA (Pasqualini et al., 2003; Wildermuth et al., 2001). In *A. thaliana*, the SA synthesized through isochorismate synthase 1 (ICS1) plays a essential role in plant defense responses to pathogens (Wildermuth et al., 2001). It is required for both PR1 gene expression and SAR defense response (Wildermuth et al., 2001). In tobacco and *A. thaliana*, SA synthesized through the PAL pathway participates in cell death in response to certain pathogen elicitors such as fungal glycoproteins, since ICS1 mutants can still exhibit cell death after infection by necrotizing pathogens (Dorey et al., 1997; Wildermuth et al., 2001). In *A. thaliana*, the SA synthesized via the PAL pathway, but not ICS pathway, mediates lesion development and local defense at the infection sites (Ferrari et al., 2003).

Under environmental, physiological or biotic stress, the amount of ROS increases dramatically (Holuigue et al., 2007). The NADPH-oxidases in the plasma membrane and the peroxidases in the apoplast are the main elements that contribute to oxidative burst in plants after pathogen attacks (Holuigue et al., 2007). SA co-ordinates with ROS to regulate the activities of defense response genes in plant (Sticher et al., 1997). During the HR, SA and ROS accumulate greatly around the infected plant cells (Pasqualini et al., 2003). However, systemic defense responses in uninfected tissues surround HR lesions and distal tissues show a relatively lower increase in the levels of SA and ROS (Alvarez et al., 1998; Durrant and Dong, 2004). Such an increase is too low to trigger programmed cell death (PCD) in these tissues but is able to activate defense genes (Sticher et al., 1997). H_2O_2 can trigger biosynthesis of SA via the PAL pathway (Holuigue et al., 2007). High levels of SA can also activate the production of ROS by different ways (Lemaire et al., 2004), and can contribute to inhibit antioxidant enzymes such as ascorbate peroxidase (APX) and catalase (CAT), resulting in large amounts of ROS production (Durner and Klessig, 1995). Thioredoxins (TRXs) may play a role in regulating the activities of these enzymes and thus, is important for redox signal transduction in programmed cell death response (Lemaire et al., 2004; Yamazaki et al., 2004).

1.2.6.2 Systemic acquired resistance (SAR)

Systemic acquired resistance (SAR) is a mechanism of inducing enduring defense responses against a broad spectrum of microorganisms, such as viruses, bacteria, fungi, and oomycetes (Durrant and Dong, 2004). SA plays an important role during SAR as a systemic signal due to the high level of SA in systemic leaves and phloem (Dempsey et al., 1999). Other components such as lipid-based molecules may function as mobile signals for SAR (Durrant and

Dong, 2004; Maldonado et al., 2002). In *A. thaliana*, the initiation of SAR requires an oxidative burst following the infection by the avirulent strain of *Pseudomonas syringae* (Alvarez et al., 1998). SA can bind to enzymes scavenging H_2O_2 and inhibit their activity to increase the ROS levels, thus inducing the expression of PR genes (Chen et al., 1993; Durner and Klessig, 1995). SAR signaling requires the interaction between the TGA transcription factors and the non expressor of pathogenesis-related genes 1 (NPR1) to regulate the expression of SAR signaling genes, such as PR genes (Fobert and Després, 2005). Oxidoreduction in critical Cys residues in NPR1 and TGA1 is an effective strategy of modulating protein activities for eliciting specific cellular responses (Després et al., 2003; Haddad, 2004; Mou et al., 2003; Shelton et al., 2005; Toledano et al., 2004). Reduction of C82 and C216 residues is crucial for NPR1 activation (Mou et al., 2003), while two conserved cysteines C260 and C266 of TGA1 determine its interaction with NPR1 under the treatment of SA (Després et al., 2003). The plant promoter sequence octopine synthase (ocs) element has proved to be closely related to defense/stress responses (Foley and Singh, 2004). In *A. thaliana*, decreasing the level of TGA4 by an intron-containing hairpin (ihp) can increase the activity of the ocs element (Foley and Singh, 2004). In contrast, the ihpTGA5 reduces its activity, thus TGA4 and/or TGA1 may be a negative regulator(s) of the ocs element activity, while TGA5 functioned as a positive regulator of it in *A. thaliana* roots (Foley and Singh, 2004). Thioredoxins (TRX) and glutaredoxins (GRX) may regulate redox changes of NPR1 and TGA1 or TGA4 with lack of data to clarify it (Fobert and Després, 2005).

1.2.6.3 Jasmonates (JAs)

Jasmonates (JAs) are fatty acid-derived cyclopentanone compounds and members of the oxylipin family that occur ubiquitously in a huge number of plants (Davies, 2010). JAs have

multiple functions in plants. Firstly, they are involved in diverse processes of plant growth and development, i.e., stomatal opening, seed germination, leaf senescence, root growth, fruit ripeness, tuber formation and tendril coiling (Bari and Jones, 2009). Secondly, JAs also play critical roles in response to abiotic and biotic stresses, as JA concentration rise significantly locally in response to tissue damage or pathogen infection (Bari and Jones, 2009). The concentration of JA is higher in younger tissues under unstressed condition, and increases rapidly and transiently under both abiotic and biotic stress (Wasternack and Hause, 2002).

The plant peptide hormone systemin and JA participate in systemic defense responses (Li et al., 2002). Application of systemin on leaves leads to JA accumulation through the activation of proteinase inhibitor (PI) and the octadecanoid pathway (Davies, 2010). Analysis of the tomato mutant *spr2* (defective in JA biosynthesis), *jai1* (defective in JA perception), and *spr1* (defective in systemin perception) with the grafting technique revealed that JA perception but not the production was required for recognizing the transmissible wound signals (Stratmann, 2003). JA accumulation is necessary for producing the systemic signal in wounded leaves but not in undamaged leaves (Stratmann, 2003). Overexpression of the JA-specific methyl transferase encoding gene (*JMT)* in *A. thaliana* results in higher accumulation of the MeJA, constitutively expressing the JA-responsive genes and reducing susceptibility to pathogens (Seo et al., 2001).

The accumulation of JA, in response to abiotic and biotic stress, leads to changes in the transcription levels of genes involved in plant defense and production of the secondary metabolites. In *A. thaliana*, an AP2-domain transcription factor, the ERF1, regulates defense response genes of the ethylene/JA-dependent signal pathway (Lorenzo et al., 2003). Both ethylene and jasmonate can rapidly activate the expression of *ERF1*, as block of either ethylene or jasmonate signaling pathways inhibited the ERF1 activation (Lorenzo et al., 2003). Overexpression of *ERF1* in the

JA-insensitive mutant *coi1* (coronative insensitive 1) or ET-insensitive mutant *ein2* (ethylene insensitive 2) was able to restore the phenotypes in both mutants, indicating both ethylene and jasmonate signaling pathways acted upstream of ERF1 (Lorenzo et al., 2003). Above all, ERF1 plays a critical role in the integration of the two signal pathways for regulating the expression of defense response genes that sit at the downstream of the two pathways (Lorenzo et al., 2003).

The *A. thaliana* CORONATINE-INSENSITIVE 1 (COI1) encodes an F-box protein, that is an important element of SCF^{COI1}, a E3-type ubiquitin ligase complex (Xie et al., 1998). Besides the F-box protein, this complex contains another 2 components: Skp1 and Cullin (Xie et al., 1998). COI1 is important for JA-mediated response due to its involvement in the receptor proteins degradation mediated by both SCF and 26S proteasome, and the mutant of *coi1* is insensitive to exogenous JA or coronatine (Xie et al., 1998). JA amino acid synthetase encoded by *JAR1*, is involved in conjugating the JA with amino acid isoleucine (Ile) to produce jasmonoyl-L-isoleucine (JA-Ile), the latter is a bioactive form of JA (Staswick and Tiryaki, 2004). *MYC2* encodes a bHLH transcription factor that can regulate the expression of some JA-responsive genes (Lorenzo et al., 2004). The jasmonate ZIM-domain (JAZ) protein can negatively regulate JA signal pathway (Chini et al., 2007). Thines et al. (2007) confirmed that JAZ protein is a substrate of the SCF^{COI1} complex. Davies (2010) describes the model of JA impact on expression of plant defense genes, in which the repressor proteins JAZ binds to MYC2 transcription factors to repress the expression of JA-responsive genes under low levels of JA and increasing JA signals the COI1 protein to recruit JAZ with SCF^{COI1} for degradation through the ubiquitin/26 proteasome pathway.

1.2.6.4 The cross-talk of JA- and SA-pathways

Both JA and Salicylic acid (SA) pathways play important roles in induction of defense

response against pathogens. These two pathways are usually antagonistic to each other, which helps plants to regulate their defense (Davies, 2010). Phytoalexin Deficient 4 (PAD4) and Enhanced Disease Susceptibility 1 (EDS1) are both required for SA synthesis and ROS accumulation for HR response at the infection sites (Falk et al., 1999; Jirage et al., 1999; Rustérucci et al., 2001). In *A. thaliana,* EDS1 and PAD4 have been demonstrated to be repressors of the JA/ET signaling pathway (Petersen et al., 2000). The mitogen activated protein (MAP) kinase MPK4, as a key element in mediating the antagonistic effect between JA- and SA-mediated signaling in plant, could suppress the SA pathway but simultaneously activate the JA/ET pathway (Davies, 2010; Petersen et al., 2000). In *A. thaliana,* SA levels was induced in the *mpk4* mutant to drive the constitutively activaction of SA responsive PR genes, resulting in enhanced resistance to biotrophic pathogens. JA responsive genes were transcriptionally down regulated in the *mpk4* mutant, which was more susceptible to necrotrophic pathogens (Brodersen et al., 2006; Petersen et al., 2000). Interestingly, MAPK4 negatively regulates EDS1 and PAD4, as double disruption of *EDS1* and *PAD4* in the *mpk4* mutant can suppress the phenotypes associated with activation of SA pathway and block of JA/ET pathway (Brodersen et al., 2006; Petersen et al., 2000).

However, in *A. thaliana* NPR1 has been proven to be critical for the SA-mediated suppression of JA pathway (Derksen et al., 2013b; Spoel et al., 2007). In *A. thaliana,* ectopic expression of a glutaredoxin-encoding gene *GRX480* mimiced the effect of SA treatment in wild type with induction of SA pathway and suppression of the JA-responsive gene *PDF1.2* (Ndamukong et al., 2007). Additional studies suggested GRX480 functioned in complex with TGA transcription factors to suppress the JA-signaling following the regulation of NPR1 in response to SA (Ndamukong et al., 2007). Another important regulator orchestrating the interaction between SA- and JA-signaling pathways is the WRKY transcription factor WRKY70 that functions at the

downstream of NPR1 and negatively regulates the expression of JA-responsive gene *PDF1.2* (Li et al., 2006; Li et al., 2004a). Other WRKY transcription factors function in the similar machinery with WRKY70 includes WRKY62 that is induced by induced by MeJA and SA to suppress the expression of JA-responsive genes *LOX2* and *VSP2* and WRKY53 that interacts with epithiospecifying senescence regulator (ESR/ESP), a JA-inducible protein, to negatively impact the JA-signaling pathway (Mao et al., 2007; Miao and Zentgraf, 2007).

1.2.6.5 Reactive oxygen species (ROS) and NADPH oxidase (NOX)

1.2.6.5.1 Reactive oxygen species (ROS)

Eukaryotic and prokaryotic organisms produce reactive oxygen species (ROS) during aerobic respiration (Aguirre et al., 2005). The varied roles of ROS in cell physiology have been described in many studies. Mammalians produce a great amount of ROS and induce "oxidative bursts" in phagocyte cells during the host defense against pathogen infections and inflammation (Lambeth, 2004; Morgenstern et al., 1997; Rothfork et al., 2004). Plants also produce ROS in response to pathogen infections (Simon‐Plas et al., 2002), which also plays key roles in plant growth and development (Foreman et al., 2003; Monshausen et al., 2007). ROS play important roles in fungal cellular differentiation, fruiting body formation, ascospore production, and germination (Lara‐Ortíz et al., 2003; Malagnac et al., 2004; Wang et al., 2014), as well as facilitates the defense against other fungal competitors (Haedens et al., 2005; Silar, 2005). There are enzymatic and non-enzymatic system responses for ROS generation (Heller and Tudzynski, 2011). Mitochondria is the main source of non-enzymatic ROS production (Heller and Tudzynski, 2011). The mitochondrial electron transport chain produces low but continuous superoxide (O^{2-}), which is then converts into hydrogen peroxide (H_2O_2) by mitochondrial superoxide dismutase

(SOD) (Aguirre et al., 2005). The most important enzymatic system for producing ROS are the NADPH oxidase (Nox) enzymes that produce ROS by transporting electrons from an intracellular donors like NADPH to extracellular acceptors like oxygen (Bedard et al., 2007; Tudzynski et al., 2012).

1.2.6.5.2 The scavenging system of ROS and tioredoxins (TRX)

The homeostasis of cellular ROS requires the balance of the scavenging system, which includes non-enzymatic systems such as glutathione (GSH) and thioredoxin (TRX) and enzymatic systems such as catalase(CAT), hydrogen peroxidase, glutathione peroxidase (GPx) and superoxide dismutases(SOD) (Aguirre et al., 2005; Heller and Tudzynski, 2011).

TRXs containing two redox active cysteines (WCGPC) in the conserved domain are divided into three types in higher plants, including Trx-f and Trx-m in chloroplasts, and Trx-h in the cytosol (Buchanan, 1991; Buchanan et al., 2002; Foley and Singh, 2004; Jacquot et al., 2002; Rivera-Madrid et al., 1995). TRXs as oxidoreductases can catalyze dithiol-disulfide changes in Cys residues of target proteins (Jacquot et al., 2002). Other research showed that TRXs were involved in regulation of ARX and CAT activities (Lemaire et al., 2004; Yamazaki et al., 2004), which were critical for ROS scavenging (Holuigue et al., 2007). According to previous studies in Dr. Daayf's lab, a TRX was identified to be uniquely present in a highly aggressive *V. dahliae* isolate through proteomic comparison analysis with a weakly aggressive isolate (El-Bebany et al., 2010).

1.2.6.5.3 NADPH oxidase (NOX)

Nox homologues are wildly present in eukaryotic organisms. The best studied Nox enzyme is the mammalian gp^{91phox}, also known as Nox2 (Aguirre and Lambeth, 2010). In total, there are

seven Nox homologous in human, belonging to three suberfamilies by structures (Aguirre and Lambeth, 2010). Nox1 to Nox4 contain the basic catalytic structure of a N-terminal transmembrane domain with two heme binding sites and a C-terminal dehydrogenase domain with NADPH and FAD binding sites (Aguirre and Lambeth, 2010). Nox5 additionally harbors a Ca^{2+}-binding EF-hand motif in front of the catalytic structure (Bánfi et al., 2004). Based on Nox5 structure, Duox1 and Duox2 additionally contain a heme-containing peroxidase domain at the extreme N-terminus (Ameziane-El-Hassani et al., 2005; Edens et al., 2001). All of Nox5, Duox1 and Duox2 are subjected to regulation by intracellular Ca^{2+} level (Ameziane-El-Hassani et al., 2005). In *A. thaliana*, there are 10 Nox enzymes known as respiratory burst oxidase homologs (Rboh), structurally similar to the mammalian Nox5 (Sagi and Fluhr, 2006). In fungi, there are three Nox homologues: NoxA, NoxB and NoxC (Takemoto et al., 2007). NoxA and NoxB are structurally similar to the mammalian gp^{91phox} (Lara‐Ortíz et al., 2003; Malagnac et al., 2004; Tanaka et al., 2006), with NoxB containing an additional 40 amino acids motif at the N-terminus (Malagnac et al., 2004; Tanaka et al., 2006), while NoxC is most closely homologous to the mammalian Nox5 (Lewit-Bentley and Réty, 2000).

In bacteria, ROS such as superoxide and hydrogen peroxide (H_2O_2) participate in the regulation of oxidative stress responsive genes through activating redox-responsive transcription regulators SoxR and OxyR (Pomposiello and Demple, 2001). No Nox homologues have been identified in bacteria so far. However, bacteria generates ROS by the respiratory chain (Bedard et al., 2007), and the bacterial non-proton pumping type II NADH dehydrogenase (NDH-2) plays an important role in respiratory metabolism (Heikal et al., 2014). In spite of wide presence of the NDH-2 homologue in bacteria (Heikal et al., 2014; Melo et al., 2004), archaea (Gomes et al., 2001), fungi (de Vries and Grivell, 1988) and plants (Moller and Lin, 1986), its function still remains

unclear. The bacterial NDH-2 facilitates the non-energy conserving transfer of electrons from cytoplasmic NAD(P)H to ubiquinone (Melo et al., 2004). It has two membrane-anchoring regions, separately harboring two FAD binding sites and two NAD(P)H binding sites (Heikal et al., 2014). However, the sequence similarity between bacterial NDH and Nox homologue is low (Bedard et al., 2007). NDH-2 also called rotenone-insensitive NADH:quinone oxidoreductases (Melo et al., 2004). Computational biology predicted that the NADH:ubiquinone oxidoreductase (complex I) is involved in ROS generation from flavin mononucleotide (FMN) (Bazil et al., 2014). Interestingly, El-Bebany et al. (2010) identified an NADH-ubiquinone oxidoreductase uniquely present in a highly aggressive isolate but not in a weakly aggressive one in *V. dahliae*.

In mammals, generation of ROS requires the formation of an enzyme complex, including the catalytic subunit gp^{91phox} and the adaptor protein $p22^{phox}$ providing a binding site for regulatory subunits like $p40^{phox}$, $p47^{phox}$, $p67^{phox}$, and the small GTPase Rac2 (Diebold and Bokoch, 2001; Lambeth, 2004). In fungi, the activation of gp^{91phox} homologues NoxA and NoxB also require the formation of the Nox complex. Whether the synchronous interaction among the catalytic and regulatory subunits that is necessary for the Nox complex activation in fungi still remains unknown (Tudzynski et al., 2012). In *Epichloë festucae*, a homologue of the $p67^{phox}$ NoxR interacts with small GTPase RacA to regulate the NoxA activity and ROS production in planta (Takemoto et al., 2006; Tanaka et al., 2008). In *Botrytis cinerea*, NoxR is involved in the activation of NoxA and NoxB and is required for the formation of sclerotia, penetration and colonization in plant tissues (Segmüller et al., 2008). In *Podospora anserina* and *Neurospora crassa*, NoxR regulates the activity of both Nox1 and Nox2 (Brun et al., 2009; Cano-Domínguez et al., 2008). A fungal homologue of $p40^{phox}$, namely BemA, and the guanyl-exchange factor Cdc24 were both identified in *Epichloë festucae*, and can interact with NoxR to regulate cellular localization of NoxR

(Takemoto et al., 2011).

It was suggested that Nox homologues are involved in multicellular development due to their presence in all multicellular organisms and then absence in unicellular lives (Takemoto et al., 2007). The Nox homologues always play critical functions in the virulence of pathogen. In *Aspergillius nidulans*, deletion of NoxA as the only mammalian gp^{91phox} homologue blocks the fruiting body development from cleitothecia initials to mature cleitothecia, but does not affect asexual development or hyphal growth (Lara‐Ortíz et al., 2003). Other studies indicated NoxA was negatively regulated by the MAP kinase (MAPK), as the expression of *NoxA* was enhanced in the *mapk* mutant and resulted in premature development and increase number of cleistothecia (Lara‐Ortíz et al., 2003). In *A. nidulans,* NoxR is required for both sexual and asexual development (Semighini and Harris, 2008). In *Claviceps purpurea*, Nox1 is essential for conidial germination and pathogenicity, as the gene deletion mutant caused dramatic reduction of virulence on host rye due to defected colonization in the ovarian tissues (Giesbert et al., 2008). In *Fusarium graminearum*, NoxA and NoxB are both necessary for full fungal virulence but only NoxA is involved in ROS production and perithecia development (Wang et al., 2014). The NoxR in *F. graminearum* interacts with NoxA and RacA (Zhang et al., 2016). The *noxr* mutants have defective sexual development with lack of perithecia production, and display reduced virulence on host plants (Zhang et al., 2016). In *Magnaporthe grisea*, ROS generation is required for development of the specialized infection cell called an appressorium (Egan et al., 2007). The NADPH oxidase encoding genes *NOX1* and *NOX2* were both required for pathogenicity on host rice and barley leaves (Egan et al., 2007). *NOX1* has been shown to regulate ROS production at the plasma membrane for facilitating cell wall biosynthesis and the appressorium penetration on the plant surface (Egan et al., 2007). In *Botrytis cinerea* both *NoxA* and *NoxB* are essential for full fungal

virulence on the host and formation of sclerotia (Segmüller et al., 2008). The *NoxR* regulatory subunit showed similar functions in penetration and colonization of plant tissues when compared to NoxA and NoxB (Segmüller et al., 2008).

1.3 Objectives

My general goal for the Ph.D projects was to characterize pathogenicity related-genes in *V. dahliae* and investigate their roles during the interaction between *V. dahliae* and potato. To achieve this goal, the project was divided into the following four main parts:

1) Expression analysis of 20 selected genes after elicitation with potato root, stem and leaf extracts, as well as during the *in planta* stage of infecting detached potato leaves. Among them, fifteen genes were studied in chapter 2. The studies on the remaining five genes were conducted in three following chapters, focusing on functional characterization and mechanism revelation.

2) Isochorismatase hydrolase (ICSH1) gene was identified to be significantly induced during the infection in objective 1). Homologues of the isochorismatase family catalyze isochorismate into other compounds (Soanes et al., 2008; Wildermuth et al., 2001), therefore it is hypothesized that VdICSH1 may disturb the potato SA synthesis pathway and change the ratio of host SA and JA dynamics to overcome potato defense against *V. dahliae*. The second objective was divided into four sub-objectives: (i) determine the role of potato ICS pathway during defense against *V. dahliae*; (ii) generating *VdICSH1* gene mutant for functional analysis; (iii) determining the activities of other isochorismatase family members in the *icsh1* mutant and the wild type; (iv) comparing changes in SA and JA accumulation between potato plants infected by *icsh1* mutants and wild type, as well as non-infected control plants.

3) Previous studies in Dr. Daayf's lab indicated that ROS may be important for *V. dahliae*

pathogenicity, and the expression of *NoxA* and *NoxB* in the highly aggressive isolate increased significantly during infection, compared with the weakly aggressive one. As a result, we hypothesized that members of the Nox gene family may be involved in facilitating the infection on potato and for the interaction with the host. The third objective was divided into four sub-objectives: (i) generating the gene mutants for *NoxA*, *NoxB,* and *NoxC,* respectively, and determining the phenotypes and pathogenicity of all gene mutants; (ii) determining the difference in resistant responses of the *noxa*, *noxb*, *noxc* mutants and the wild type strain to oxidative and osmotic stresses; (iii) comparing cell wall biosynthesis and integrity among *noxa*, *noxb*, *noxc* mutants and the wild type strain; (iv) comparing the penetration ability between the *noxa*, *noxb*, *noxc* mutants and the wild type strain.

4) The expression of exopolygalacturonase (ExoPG) gene increased significantly in the highly aggressive isolate under elicitation of potato leaf and stem extracts, as well as during the early infecting stage, according to results from objective 1). It was thus hypothesized that ExoPG may also have important functions in fungal virulence. Generation of the *ExoPG* gene mutant was critical for this objective. *Agrobacterium*-mediated T-DNA insertion was the most efficient method for creating gene disruption mutants in *V. dahliae* (Tian et al., 2015; Zhu et al., 2017). A vector-free split-marker recombination approach is being employed for quickly processing gene disruption in *Saccharomyces cerevisiae* (Catlett et al., 2003), but there is no record that this method has been applied to *V. dahliae*. The fourth objective was also divided into three sub-objectives: (i) generating the *ExoPG* gene deletion mutant in *V. dahliae* using the vector-free split-marker recombination method; (ii) determining the phenotypes and pathogenicity of the Δ*exopg* mutants; (iii) determining the total exopolygalacturonase activity in the Δ*exopg* mutants and the wild type strain.

CHAPTER 2

MONITORING GENE EXPRESSION OF POTENTIAL PATHOGENICITY-RELATED GENES IN *VERTICILLIUM DAHLIAE* IN RESPONSE TO INFECTION AND ELICITATION WITH POTATO EXTRACTS BY QUANTITATIVE REAL TIME PCR (RT-QPCR)

Abstract

Previous studies used comparative proteomics and subtractive hybridization / cDNA-AFLP methods to compare highly and weakly aggressive isolates of *Verticillium dahliae* when exposed to potato root extracts, and identified genes and peptides putatively involved in important cellular processes such as germination and pathogenicity. However, quantitation assessment of these genes' expression during potato-*V. dahliae* interaction has not been done. Here, we monitored the expression of 15 of those genes by quantitative real time PCR (RT-qPCR), after inoculation of petioles of detached potato leaves with two isolates of *V. dahliae* (one highly and one weakly aggressive), as well as in response to elicitation with potato (leaf, stem and root) extracts. Several genes had strong expression or upregulation in the highly aggressive isolate in response to treatments with plant extracts or during petiole infection. These included genes encoding for: Ras-GAP like protein, serine/threonine-protein kinase, Ubiquitin-conjugating enzyme variant MMS2, NADH-ubiquinone oxidoreductase, Thioredoxin, Pyruvate dehydrogenase E1 component subunit beta (VdPDHB), myo-inositol 2-dehydrogenase, and HAD-superfamily hydrolase. The stronger activities of these genes in the highly aggressive isolate may partially explain its higher aggressiveness. Further functional characterization of these genes related to pathogenicity and virulence is expected to improve our understanding of fungal pathogenesis and may help exploring new ways in managing Verticillium wilt.

2.1 Introduction

Verticillium wilt caused by *V. dahliae* or *V. albo-atrum*, is a major disease, that causes significant yield losses in potato production valued at millions of dollars. Also referred to as potato early dying (PED), this disease can cause 5 to 12 metric tons of tuber yield loss per hectare, and result in up to 90% disease severity (Krikun and Orion, 1979; Molina et al., 2014; Pegg and Brady, 2002; Rowe and Powelson, 2002). Previous research showed a correlation between the density of *V. albo-atrum* in the soil and severity of wilt symptoms under favorable conditions (Nnodu and Harrison, 1979). *V. dahliae* has a larger host range than *V. albo-atrum* (Pegg and Brady, 2002), affecting more than 200 dicotyledonous plant species including flowers, oilseed and fiber crops, fruits, vegetables and woody perennials (Pegg and Brady, 2002; Rowe and Powelson, 2002).

V. dahliae produces microsclerotia that can be released in the soil and remain viable for 10-15 years (Rowe and Powelson, 2002; Wilhelm, 1955). Microsclerotia germinate in response to stimulation by root exudates secreted in the rhizosphere (Olsson and Nordbring-Hertz, 1985) and reach the plant roots (Fradin and Thomma, 2006). *V. dahliae* can enter the susceptible plant from the root tip, then cross the endodermis and penetrate the young xylem elements, finally reaching the vascular cylinder (Bowers et al., 1996). *V. dahliae* sporulates in the vascular tissue and moves following the sap stream but may be trapped in pit cavities (Fradin and Thomma, 2006). The conidia germinate in the vessel end walls and penetrate the adjacent elements for future propagation in the host (Fradin and Thomma, 2006).

Plants have evolved a series of defense mechanisms against *V. dahliae* (Daayf, 2015). Many plants such as tomato, pea and cotton, can compromise the expansion of *V. dahliae* by increasing the production of lignin-like phenolic polymers or lignin depositions in cortical plant cell walls (Griffiths, 1971; Smit and Dubery, 1997). In tomato, *Ve*-mediated plant resistance to *V.dahliae*

involves the accumulation of lignin (Gayoso et al., 2010), which can decrease microsclerotia's viability even in crop residues in the soil (Debode et al., 2005). Some enzymes, including peroxidase, phenylalanine ammonia-lyase (PAL), and cinnamyl alcohol dehydrogenase, are involved in plant resistance to *V. dahliae* (Gayoso et al., 2010; Smit and Dubery, 1997). Cotton plant cells reinforce their walls by the accumulation of polysaccharides (callose and cellulose) and release phytoalexins (such as desoxyhemigossypol) in response to infection by *V. dahliae* (Daayf et al., 1997; Mace et al., 1989, 1990). When *V. dahliae* enters the xylem vessels, plant vascular cell walls may produce coating materials such as cellulose to prevent fungal horizontal spread. In addition, the neighboring parenchyma cells produce paramural deposition such as pectin, callose and cellulose (Robb et al., 1979).

Commonly used strategies to manage Verticillium wilt in crops include cultural practices such as crop rotations, and sometimes the use of fumigants. However, none of these methods provides full control of the disease. Crop rotation research showed that up to 5 years' separation cannot effectively suppress the disease (Davis et al., 1994) due to the long-lasting survival of microsclerotia in soil and the wide host range of *V. dahliae* (Pegg and Brady, 2002; Wilhelm, 1955). In addition, once *V. dahliae* enters the plant vascular tissues, no fungicides can control it (Fradin and Thomma, 2006).

Alternative control approaches relying on the understanding of the molecular mechanism of *V. dahliae* infection, including the identification of genes that are critical for pathogenicity, are needed more than ever to control this disease. Previous studies demonstrated differential expression of genes that are upregulated in a highly aggressive isolate in response to potato root extracts, and differential accumulation of a series of proteins in a highly aggressive *V. dahliae* isolate, as compared to a weakly aggressive isolate (El-Bebany et al., 2011; El-Bebany et al., 2010).

The presence and activity of these proteins and genes in the highly aggressive isolate may explain the differential virulence of *V. dahliae* isolates. One of these genes, an isochorismatase hydrolase gene was proved to be involved in *V. dahliae*'s interference with potato's SA defense pathway (Zhu et al., 2017). A series of 15 other genes of interest included: Thioredoxin, NADH-ubiquinone oxidoreductase, Pyruvate dehydrogenase E1 component subunit beta, Ubiquitin-conjugating enzyme variant MMS2, HAD-superfamily hydrolase, Serine 3-dehydrogenase, Wos2, Ras-GAP like protein, Xanthine dehydrogenase, myo-inositol 2-dehydrogenase, DNA lyase, serine/threonine-protein kinase, glucan endo-1,3-alpha-glucosidase, RAD51 and nuc-1 negative regulatory protein preg (El-Bebany et al., 2011; El-Bebany et al., 2010). So far, no studies have assessed the expression of these genes to determine their potential involvement in pathogenicity and virulence of *V. dahliae*. The objective of this study was to assess the expression of these 15 genes clustered in different groups according to their functions, (i) in response to elicitation with different potato extracts, and (ii) during infection of the petiole of detached potato leaves by two *V. dahliae* isolates with different levels of aggressiveness.

2.2 Materials and Methods

2.2.1 *V. dahliae* isolates

Two *V. dahliae* isolates, Vs06-07 and Vd1396-9, a weakly and highly aggressive isolate respectively (Alkher et al., 2009; Uppal et al., 2007), were selected for this study. Both isolates were cultured on potato dextrose agar (PDA) 24±0.5°C for 14 days.

2.2.2 Pathogenicity tests of *V. dahliae* isolates

Kennebec, a susceptible potato cultivar to *V. dahliae* (Alkher et al., 2009), was used for the

detached leaves inoculation and pathogenicity tests. Plants were grown in a mixture of sand, soil and peat moss with a ratio of 16:4:1, under a day/light temperature regimen of 22/18 °C and a photoperiod of 16/8 h.

Assessment of the pathogenicity of both *V. dahliae* isolates was performed on potato cv. Kennebec as described by Zhu et al. (2017). Briefly, three Kennebec plants (3-week-old) were trimmed at the root tips and placed in the conidia suspension (10^6 conidia/mL) of the weakly or highly aggressive *V. dahliae* isolates, then planted in 6-inch pots with a pasteurized mixture of sand, soil and peat moss (16:4:1). The total area under disease progress curve (AUDPC) of disease severity and of percentage of infection were evaluated as described by Zhu et al. (2017).

2.2.3 Gene selection and primers' design

Fifteen genes were selected based on proteomic and subtractive hybridization/cDNA-AFLP analyses of weakly and highly aggressive isolates (El-Bebany et al., 2011; El-Bebany et al., 2010). These genes include: the Ras-GAP like protein (VDAG_01012), Pyruvate dehydrogenase E1 component subunit beta (VdPDHB) (VDAG_01642), DNA-(apurinic or apyrimidinic site) lyase (DNA AP lyase) (VDAG_02445), glucan endo-1,3-alpha-glucosidase agn1 (VDAG_04101), Thioredoxin (VdTRX) (VDAG_04529), serine/threonine-protein kinase (VDAG_04632), Ubiquitin-conjugating enzyme variantMMS2 (VDAG_05365), nuc-1 negative regulatory protein preg (VdPREG) (VDAG_06766), Xanthine dehydrogenase (VDAG_07735), myo-inositol 2-dehydrogenase (VDAG_08205), HAD-superfamily hydrolase (VDAG_08490), DNA repair protein RAD51 (VDAG_08796), Wos2 (VDAG_08865), NADH-ubiquinone oxidoreductase (VDAG_09026), and Serine 3-dehydrogenase (VDAG_09532). Primer pairs for the target genes were designed based on the available *Verticillium* sequences from Verticillium comparative

genomic project of the Broad Institute (https://www.broadinstitute.org/scientific-community/science/projects/fungal-genome-initiative/verticillium-comparative-genomics-pro). The Histone H3 gene (VDAG_10035) was treated as a housekeeping gene (Zhu et al., 2017) (**Table 2.1**).

Table 2.1 Primers used in RT-qPCR for amplifying the target genes

Primer's name	Primer sequence	Tm (°C)	Accession number	Amplification length (bp)
Ras-GAP like protein-F	ACGCTGTCCAACCTTCAC	53	VDAG_01012	273
Ras-GAP like protein-R	GTTGATCTTGTCCCAGTCG	52.7	VDAG_01012	
Pyruvate dehydrogenase E1 component subunit beta-F	CTTCGGCGACAAGAGGGT	58.1	VDAG_01642	283
Pyruvate dehydrogenase E1 component subunit beta-R	GGGAATGCTGCCATACCAC	58.2	VDAG_01642	
DNA-(apurinic or apyrimidinic site) lyase-F	CCGGCTGGGACGTTTA	55.1	VDAG_02445	152
DNA-(apurinic or apyrimidinic site) lyase-R	GCGGAATCTGGTGGTTG	54.2	VDAG_02445	
glucan endo-1,3-alpha-glucosidase agn1-F	GCCTTCGGAAACCTCAAT	54.6	VDAG_04101	307
glucan endo-1,3-alpha-glucosidase agn1-R	CTCCCATGAACTCATACGC	53.2	VDAG_04101	
Thioredoxin (VdTRX)-F	GCTGCTCCTGTTTATGCCTTTCCA	66.8	VDAG_04529	145
Thioredoxin (VdTRX)-R	GAGGTTATGCGGCTTGTTCGT	61.8	VDAG_04529	
serine/threonine-protein kinase-F	GGTGGGTGCGGTCAAATA	56.9	VDAG_04632	272
serine/threonine-protein kinase-R	AGGCATCCGTAGCACGAC	56.2	VDAG_04632	
Ubiquitin-conjugating enzyme variantMMS2-F	CATCCTCGGTCCTCCTCA	55.5	VDAG_05365	235
Ubiquitin-conjugating enzyme variantMMS2-R	CGCCATGTACCTCCTGATC	55.6	VDAG_05365	
VdPREG-F	GGGAATCTGACTAGGTTTCATT	54.7	VDAG_06766	227
VdPREG-R	GAGTCGGACAGACCTTTGG	54.7	VDAG_06766	
Xanthine dehydrogenase-F	GGCTGCTGCATGGATAAG	54.4	VDAG_07735	227
Xanthine dehydrogenase-R	CCGACAAATACCGACACG	54.9	VDAG_07735	

myo-inositol 2-dehydrogenase-F	AGTCTGGCATCGACAATAAC	52.3	VDAG_08205	192
myo-inositol 2-dehydrogenase-R	GCAGTCTCAACACGCAAA	52.6	VDAG_08205	
HAD-superfamily hydrolase-F	AGCCCGACCCTGCCATCTA	62.6	VDAG_08490	225
HAD-superfamily hydrolase-R	GGAACTCTTGCCAGTCCTTCA	59.2	VDAG_08490	
DNA repair protein RAD51-F	ATGGTGAGGGCGAGATGG	58.3	VDAG_08796	153
DNA repair protein RAD51-R	GGGTGTAAGCGACGGATT	55.4	VDAG_08796	
VdWos2-F	GTCTGCTACCAAGGCAACTCC	58.7	VDAG_08865	227
VdWos2-R	TCTCCTCCGTGTCAATCTCG	58.1	VDAG_08865	
NADH-ubiquinone oxidoreductase-F	ATCGGGGCGGGTCTCATT	62.1	VDAG_09026	116
NADH-ubiquinone oxidoreductase-R	CCTTCGGCAGGCTTCTCC	60.3	VDAG_09026	
Serine 3-dehydrogenase-F	ACTTGGGCATTAAGGTGGTC	56.5	VDAG_09532	348
Serine 3-dehydrogenase-R	CATCGCAGTCAGTTGTCGTAG	56.6	VDAG_09532	
His3-F(Zhu et al., 2017)	ATGGCTCGCACTAAGCAA	54.8	VDAG_10035	238
His3-R(Zhu et al., 2017)	TGAAGTCCTGGGCAATCT	52.7	VDAG_10035	

Note: The accession numbers of the genes are available in the Verticillium comparative genomic project of the Broad Institute (https://www.broadinstitute.org/scientific-community/science/projects/fungal-genome-initiative/verticillium-comparative-genomics-pro).

2.2.4 Elicitation and inoculation methods

The elicitation of both *V. dahliae* isolates with potato extracts was done following the protocol described by Zhu et al. (2017) and the preparation of potato extracts was done following the protocol described by El-Bebany et al. (2011). Briefly, one milliliter of 10^8 conidia of both isolates were added to 100 ml Czapek-Dox Broth (CDB) liquid media (Difco Laboratories, Sparks, MD, USA) and incubated at 24±0.5°C for 7 days. One milliliter of prepared potato leaf, stem, and

root extracts was added into CDB medium and mycelium samples were harvested after 7 days of incubation in the same conditions. Three biological replicates were prepared for each treatment.

For the detached leaves infection, the protocol was also described by Zhu et al. (2017). *V. dahliae* isolates Vd1396-9 and Vs06-07 were grown in PDA at 24±0.5°C for 21 days, conidia were collected and diluted into a concentration of 3×10^7 conidia/mL. Clones of potato cv. Kennebec plants were grow in LA4 soil mix (SunGro Horticulture, Agawam, MA 01001, USA) for 4 weeks, then one leaf was cut, placed into 1ml of the conidia suspension and maintained under a photoperiod of 16/8 h at 24°C. Sterilized water, was used as a control for inoculation. Four to six individual detached leaves, each from separate individual plants were pooled as one sample. This was repeated three times for each treatment at each time-point (1, 3, 5, 8 days after inoculation (DAI)). Samples representing the three biological replications were immediately frozen separately in liquid nitrogen and stored at -80°C until used for RNA extraction.

2.2.5 RNA extraction and RT- qPCR

Total RNA was extracted from mycelium, and potato detached leaves using the Omega Fungal RNA kit (Omega Moi-Tek, Inc., GA, USA), following the manufacturer's protocol. The first strand cDNA was synthesized following the manufacturer's protocol of the Superscript first strand synthesis kit (Life Technologies Carlsbad, CA, USA).

Quantitative Real-Time PCR was performed for each target gene as well as for the reference gene using SsoFast EvaGreen Super mix (Bio-Rad Lab, PA, USA) following the manufacturer recommendations. The $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001) has been used to evaluate expression of the above mentioned genes.

2.2.6 Statistical Analysis

Pathogenicity data and gene expression under different treatments were analyzed with PROC MIXED in SAS Statistical Analysis Software (SAS Institute, Cary, NC, USA; release 9.1 for Windows). The normality of all data (three biological replicates, in infection experiments each representing 4-6 leaves samples from 4-6 separate plants) was determined with PROC UNIVARIATE, and analysis showed that data from different treatments were all qualified for normal distribution with Shapiro–Wilk test (>0.9) and p value (>0.05), and qualified for homogeneity based on residuals comparison to studentized residuals critical values (Lund, 1975). Log_{10} transformation was applied to some sets of gene expression data statistical analysis. The macro PDMIX800.sas (Saxton, 1998), $\alpha = 0.05$ was applied to mean values separated by least squared means, and into grouped letters results. Mean values (n = 3) with the same letters indicate non-significant difference from each other ($P<0.05$) in different sets of experiments.

2.3 Results

2.3.1 Isolate aggressiveness and disease assessment

The highly aggressive isolate (Vd1396-9) induced significantly more disease measured as total AUDPC of “percentage of infection” and “disease severity” than the weakly aggressive isolate (Vs06-07) or water control (**Figures 2.1A &B**). The plant growth rate of potato inoculated with Vd1396-9 was significantly lower than that of the other two treatments (**Figure 2.1C**). Vd1396-9 inoculated potato plants exhibited more symptoms at 5 weeks after inoculation (WAI), than plants inoculated with Vs06-07 or water (**Figure 2.1D**).

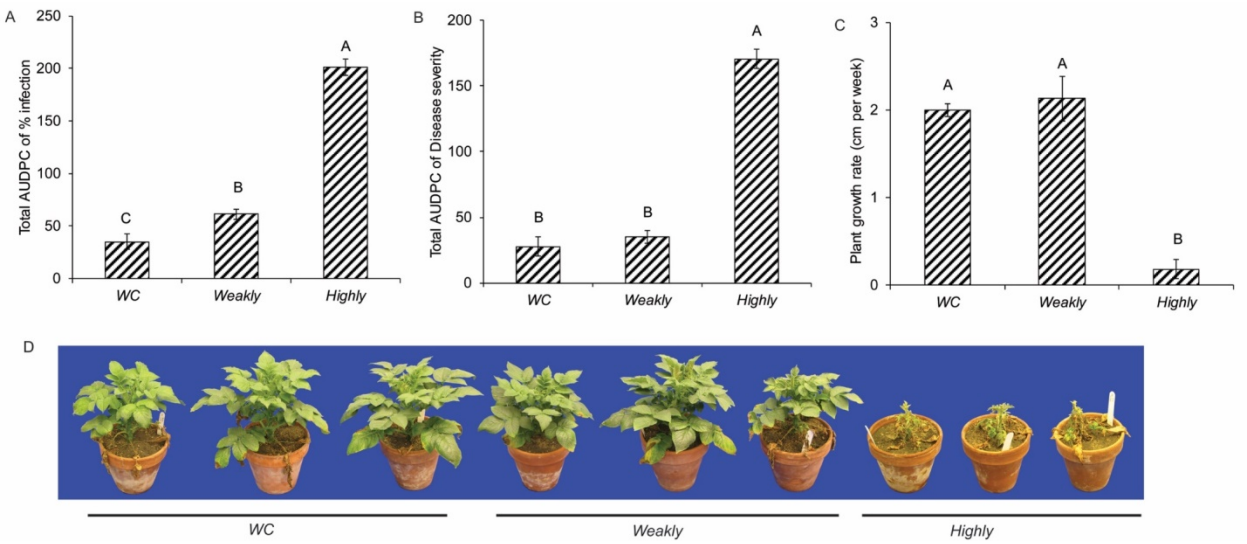


Figure 2.1 Pathogenicity analysis of the highly (Vd1396-9) and weakly aggressive (Vs06-07) *V. dahliae* isolates on the susceptible potato cultivar Kennebec.
(**A**) Total AUDPC of percentage of infection; (**B**) Total AUDPC of disease severity; (**C**) Growth rate of potato plants per week (cm); (**D**) Potato plants infected with WC, Weakly and Highly aggressive isolates at 5 weeks after inoculation.
WC: Water control treatment; Weakly: weakly aggressive *V. dahliae* isolate Vs06-07; Highly: highly aggressive *V. dahliae* isolate Vd1396-9. Error bars refer to standard error. The macro PDMIX800.sas (Saxton, 1998), $\alpha = 0.05$ was applied to mean values separated by least squared means, and into grouped letters results. Values (n = 3) with the same letters are not significantly different ($P<0.05$).

2.3.2 Differential fungal genes expression in response to elicitation with potato extracts and inoculation

El-Bebany et al. (2010) conducted a proteomic study to analyze the differential expression of fungal proteins in two *V. dahliae* isolates varying in their aggressiveness level. The differentially expressed proteins Thioredoxin (VdTRX) (VDAG_04529), NADH-ubiquinone oxidoreductase (VDAG_09026), Pyruvate dehydrogenase E1 component subunit beta (VdPDHB) (VDAG_01642), Ubiquitin-conjugating enzyme variant MMS2 (VDAG_05365), HAD-superfamily hydrolase (VDAG_08490), Serine 3-dehydrogenase (VDAG_09532) and Wos2 (VDAG_08865), were visible only in the highly aggressive isolate's profile (El-Bebany et al.,

2010). A combined subtractive hybridization / cDNA-AFLP method was used to identify genes with differential expression in response to root extracts from both moderately resistant and susceptible potato cultivars between the same highly and weakly aggressive isolates (El-Bebany et al., 2011). Genes encoding Ras-GAP like protein (VDAG_01012), Xanthine dehydrogenase (VDAG_07735), myo-inositol 2-dehydrogenase (VDAG_08205), and DNA-(apurinic or apyrimidinic site) lyase (DNA AP lyase) (VDAG_02445), were also upregulated in the highly aggressive isolate in response to root extracts from both susceptible and moderately resistant potato cultivars (El-Bebany et al., 2011). Genes such as serine/threonine-protein kinase (VDAG_04632), glucan endo-1,3-alpha-glucosidase agn1 (VDAG_04101), DNA repair protein RAD51 (VDAG_08796) and nuc-1 negative regulatory protein preg (PREG) (VDAG_06766) were upregulated in both highly and weakly aggressive isolates in response to potato root extracts (El-Bebany et al., 2011). However, all these experiments only compared the relative level of gene expression within highly or weakly aggressive isolates under root extracts with water control treatment, but without assessing the quantitative changes between highly and weakly aggressive isolates.

Most of the selected investigated genes in the current study, showed higher expression in the highly aggressive isolate during infection of the detached leaves. However, the same genes exhibited different expression trends depending on elicitation of potato root, stem, or leaf extracts. In this study, gene expression with fold change $\geqslant 2$ were defined as up-regulated, and fold change $\leqslant 0.5$ were defined as down-regulated.

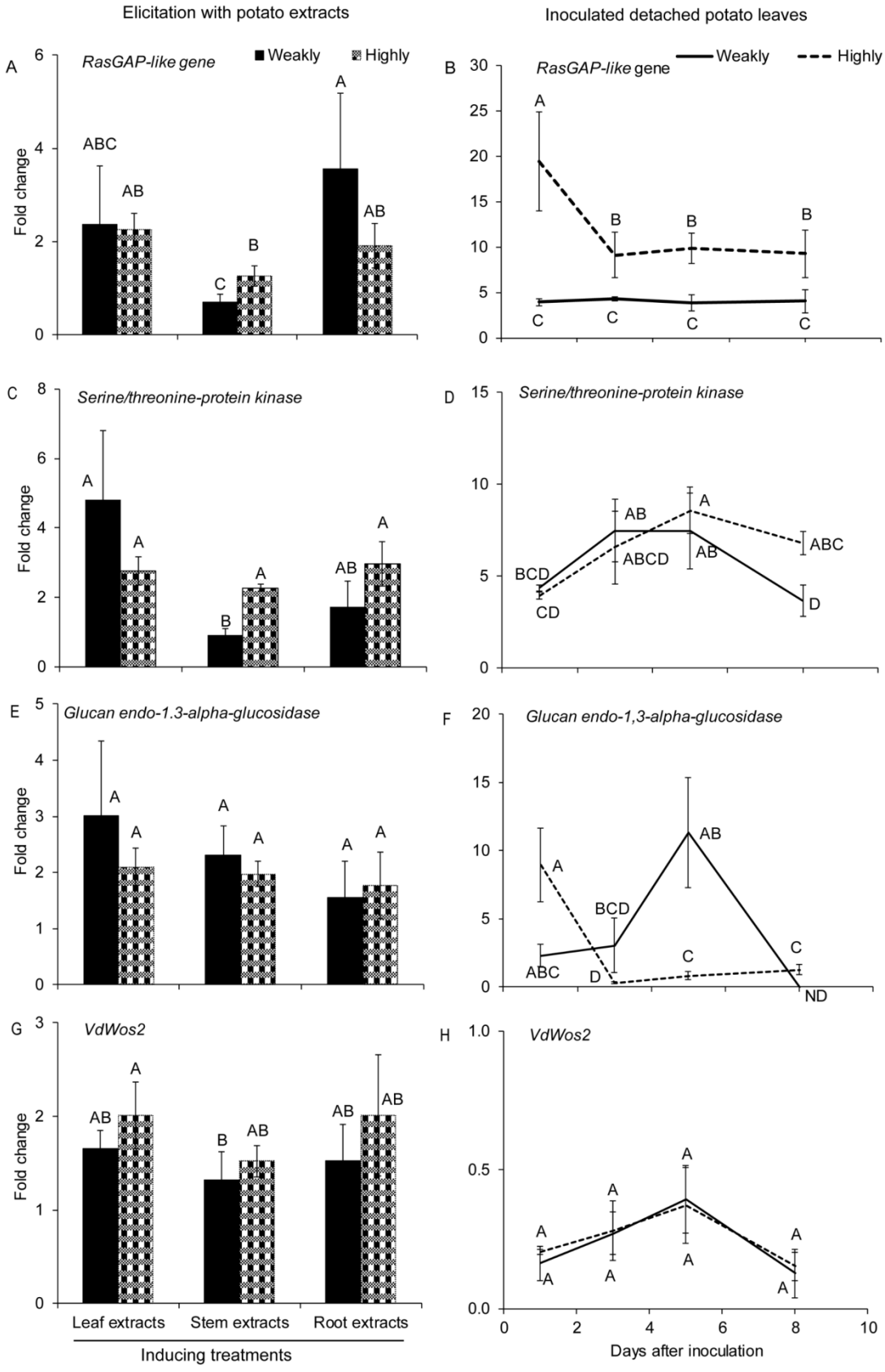
2.3.2.1 Genes involved in cell differentiation and proliferation

Four genes involved in cell differentiation and proliferation were assessed: Ras protein,

serine/threonine-protein kinase, Wos2, and glucan endo-1,3-alpha-glucosidase. Most were upregulated (fold change ≥ 2) in both highly and weakly aggressive isolates of *V. dahliae* in response to elicitation with different potato extracts (**Figures 2.2A, C, E&G**). This increased activity suggests an involvement of these genes in the pre-infection stage for both the highly and weakly aggressive isolates of *V. dahliae* (**Figures 2.2A, C, E&G**). The expression of both Ras-GAP and serine/threonine protein kinase was more noticeable in the highly than the weakly aggressive isolate in response to stem extracts (**Figures 2.2A&C**).

During infection of detached potato leaves, both tested isolates induced increases in transcripts of Ras-GAP, serine/threonine protein kinase, and endo-1,3-alpha-glucosidase (**Figures 2.2B, D&F**), indicating that these three genes may play a role in the infection process. The accumulation of Ras-GAP transcripts was much higher in the highly aggressive isolate than the weakly aggressive one (**Figure 2.2B**). This was evident throughout the entire monitored period but especially so, at the early stages of infection (**Figure 2.2B**). Serine/threonine protein kinase gene reached a maximum expression in the highly aggressive isolate at 8 DAI during infection, which was also significantly higher than weak one (**Figure 2.2D**). The expression of endo-1,3-alpha-glucosidase in the weakly aggressive isolate peaked at 5DAI and was higher than the highly aggressive one during infection of detached leaves. In the same experiment, the expression of *Wos2* was downregulated in both isolates (fold change ≤ 0.5) (**Figures 2.2F&H**).

The increased genes' activity during exposure to potato extracts, or during infection process, show that Ras-GAP, Serine/threonine protein kinases, endo-1,3-alpha-glucosidase, and Wos2 may be involved in different cellular processes leading to infection by *V. dahliae*.


Elicitation with potato extracts
Inoculated detached potato leaves
A
RasGAP-like gene
Weakly
Highly
Fold change
ABC
AB
C
B
A
AB
B
RasGAP-like gene
Weakly
Highly
A
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Serine/threonine-protein kinase
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Serine/threonine-protein kinase
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ABC
BCD
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CD
E
Glucan endo-1.3-alpha-glucosidase
A
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A
F
Glucan endo-1,3-alpha-glucosidase
AB
A
BCD
C
C
ABC
D
ND
G
VdWos2
AB
A
B
AB
AB
AB
Leaf extracts
Stem extracts
Root extracts
Inducing treatments
H
VdWos2
A
A
A
A
A
A
A
Days after inoculation

Figure 2.2 Expression of genes involved in cell differential and proliferation under different treatments.
Expression of (**A**) Ras-GAP like protein, (**C**) Serine/threonine protein kinases, (**E**) glucan endo-1,3-alpha-glucosidase and (**G**) VdWos2 in the highly (Vd1396-9) and the weakly (Vs06-07) aggressive *V. dahliae* isolates in response to elicitation with different potato extracts: leaf, stem, root extracts.
Expression of (**B**) Ras-GAP like protein, (**D**) Serine/threonine protein kinases, (**F**) glucan endo-1,3-alpha-glucosidase and (**H**) VdWos2 in the highly (Vd1396-9) and the weakly (Vs06-07) aggressive *V. dahliae* isolates in response to infection of detached potato leaves at 1,3,5, and 8 DAI.
WC: Water control treatment; Weakly: weakly aggressive *V. dahliae* isolate Vs06-07; Highly: highly aggressive *V. dahliae* isolate Vd1396-9; ND: Non-detectable. Error bars refer to standard error. The macro PDMIX800.sas (Saxton, 1998), $\alpha = 0.05$ was applied to mean values separated by least squared means, and into grouped letters results. Values ($n = 3$) with the same letters are not significantly different ($P<0.05$).

2.3.2.2 Genes involved in DNA repair

We assessed expression of three genes involved in DNA damage repair of post-replicative processes. DNA-(apurinic or apyrimidinic site) lyase, ubiquitin-conjugating enzyme variant MMS2, and DNA repair protein RAD51 have been well investigated in yeast and mammals (Aburatani et al., 1997; Davies et al., 2001; Hofmann and Pickart, 1999; Nakamura and Swenberg, 1999; Ulrich and Jentsch, 2000).

Generally, the expression of these three genes were up regulated in both isolates in response to potato extracts (Figure 3 A, C&E). The expression of DNA-(apurinic or apyrimidinic site) lyase and RAD51 were higher in the weakly aggressive isolate in response to potato root extract (**Figures 2.3 A&E**) but RAD51 also showed the same significant response to the leaf extract (**Figure 2.3E**). We also noted increased expression of RAD51 in the pre-infection stage in the weakly aggressive isolate (**Figure 2.3E**). During infection of detached leaves, only the DNA-(apurinic or apyrimidinic site) lyase showed increases (fold change ≥ 2) in both *V. dahliae* isolates. The expression of this gene also increased with root extract elicitation, suggesting its involvement

in both pre-infection and infection stages. The expression of MMS2 in the weakly aggressive isolate during infection was suppressed (fold change ≤ 0.5) and significantly lower than the highly aggressive one (**Figure 2.3D**). The expression of RAD51 was opposite to MMS2, in that its expression in the highly aggressive one was suppressed and lower than in the weakly one at 1 and 8 DAI during infection (**Figure 2.3F**).

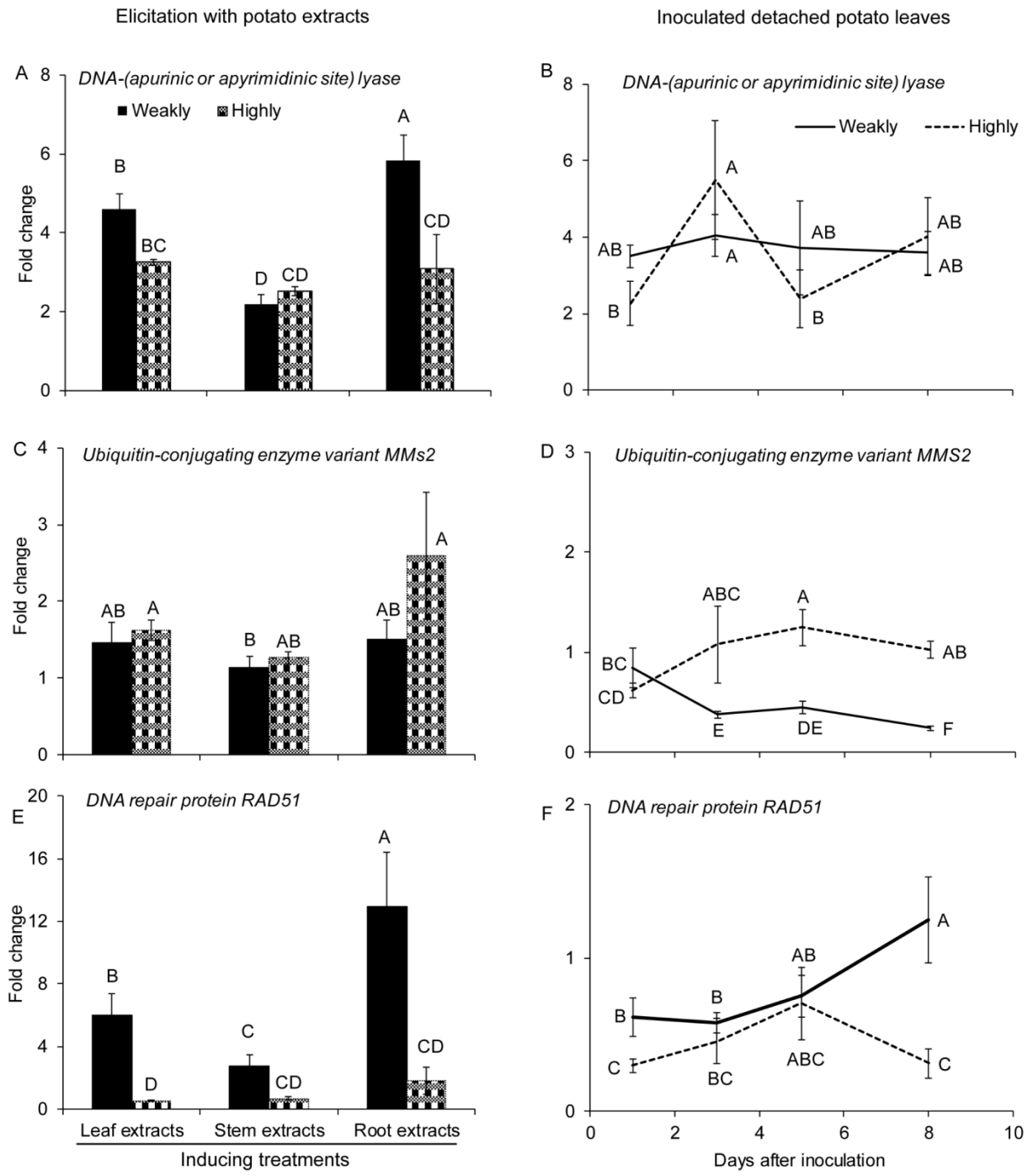

Figure 2.3 Expression of genes involved in DNA repair under different treatments.
Expression of (**A**) DNA-(apurinic or apyrimidinic site) lyase, (**C**) Ubiquitin-conjugating enzyme variant MMS2 and (**E**) DNA repair protein RAD51
in the highly (Vd1396-9) and the weakly (Vs06-07) aggressive *V. dahliae* isolates in response to elicitation with different potato extracts: leaf, stem, root extracts.
Expression of (**B**) DNA-(apurinic or apyrimidinic site) lyase, (**D**) Ubiquitin-conjugating enzyme variant MMS2 and (**F**) DNA repair protein RAD51 in the highly (Vd1396-9) and the weakly

(Vs06-07) aggressive *V. dahliae* isolates in response to infection of detached potato leaves at 1,3,5, and 8 DAI.
WC: Water control treatment; Weakly: weakly aggressive *V. dahliae* isolate Vs06-07; Highly: highly aggressive *V. dahliae* isolate Vd1396-9. Error bars refer to standard error. The macro PDMIX800.sas (Saxton, 1998), $\alpha = 0.05$ was applied to mean values separated by least squared means, and into grouped letters results. Values ($n = 3$) with the same letters are not significantly different ($P<0.05$).

2.3.2.3 Genes related to ROS production and cleavage

Two genes related to ROS balance, thioredoxin (Trx) and NADPH ubiquinone oxidoreductase were assessed by qRT-PCR. Both isolates increased their expression of Trx in response to potato root extract elicitation and during detached leaf infection (**Figures 2.4A&B**). However, during detached leaves infection, in the highly aggressive isolate, Trx reached a dramatic high expression level at 5 DAI and then fell back down at 8 DAI (**Figure 2.4B**). Trx expression was still higher than the weakly aggressive one overall (**Figure 2.4B**). The expression of NADH-ubiquinone oxidoreductase increased in the highly aggressive isolate in response to all forms of potato extracts, though only the stem extract was statistically significant (**Figure 2.4C**). During detached leaf infection, its expression in the highly aggressive isolate was only slightly increased at 8 DAI, while its expression in the weakly aggressive one was suppressed and significantly lower than in highly aggressive one from 5 DAI (**Figure 2.4D**).

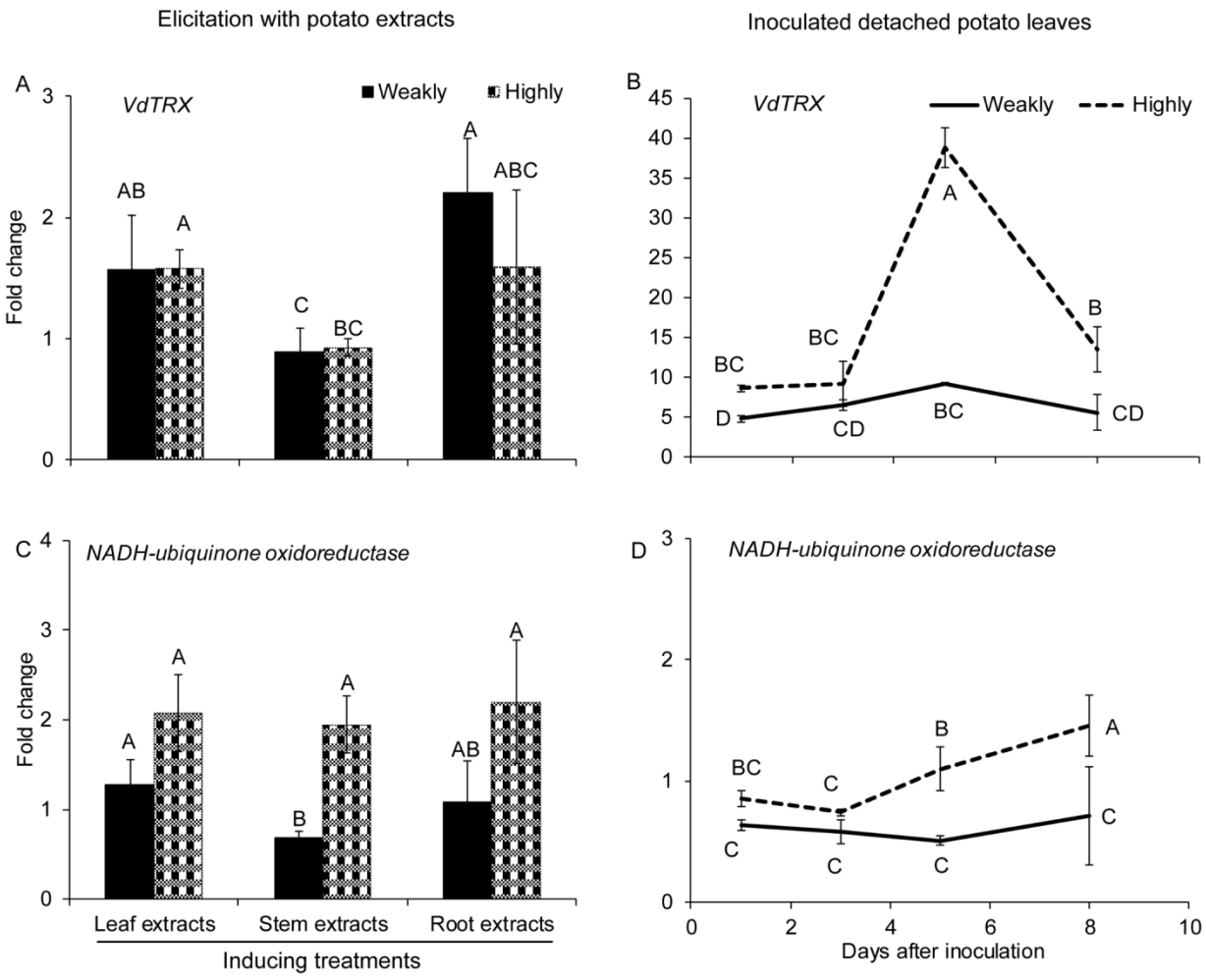

Figure 2.4 Expression of genes involved in ROS regulation under different treatments.
Expression of (**A**) Thioredoxin (Trx) and (**C**) NADH-ubiquinone oxidoreductase in the highly (Vd1396-9) and the weakly (Vs06-07) aggressive *V. dahliae* isolates in response to elicitation with different potato extracts: leaf, stem, root extracts.
Expression of (**B**) Trx and (**D**) NADH-ubiquinone oxidoreductase in the highly (Vd1396-9) and the weakly (Vs06-07) aggressive *V. dahliae* isolates in response to infection of detached potato leaves at 1,3,5, and 8 DAI.
WC: Water control treatment; Weakly: weakly aggressive *V. dahliae* isolate Vs06-07; Highly: highly aggressive *V. dahliae* isolate Vd1396-9. Error bars refer to standard error. The macro PDMIX800.sas (Saxton, 1998), $\alpha = 0.05$ was applied to mean values separated by least squared means, and into grouped letters results. Values (n = 3) with the same letters are not significantly different ($P<0.05$).

2.3.2.4 Genes related to cellular metabolism

Cellular metabolism includes many chemical reactions involved in maintaining normal life for an organism. The expression of Serine 3-dehydrogenase, pyruvate dehydrogenase E1 component subunit beta, xanthine dehydrogenase, myo-inositol dehydrogenase and nuc-1 negative regulatory protein *VdPREG*, all involved in cell metabolism were assessed. These genes are in turn involved in catabolism of serine, conversion of pyruvate to acetyl-CoA, oxidative metabolism of purines and pyrimidines, inositol and inositol phosphate metabolism and the regulation of the activity of transcription factor NUC-1 (Bittner et al., 2001; Dallo et al., 2002; Hille and Nishino, 1995; Jiang et al., 2001; Kang and Metzenberg, 1993; Kato et al., 2008; Peleg et al., 1996; Seifert et al., 2007; Tchigvintsev et al., 2012; Yamazawa et al., 2011).

In general, the expression of Serine 3-dehydrogenase, pyruvate dehydrogenase E1 component subunit beta and xanthine dehydrogenase increased in both *V. dahliae* isolates in response to potato extracts (**Figures 2.5A, C&E**). During infection, the expression of Serine 3-dehydrogenase and xanthine dehydrogenase was significantly up-regulated in the highly aggressive *V. dahliae* isolate over time. However, the expression in the weakly aggressive isolate was repressed or not changed (**Figures 2.5B&F**). In addition, the highly aggressive isolate exhibited more transcripts of serine 3-dehydrogenase in response to potato root extracts and pyruvate dehydrogenase E1 component subunit beta and xanthine dehydrogenase in response to stem extracts (**Figures 2.5A, C, & E**). This was also true for the highly aggressive isolate's expression during infection of detached leaves (**Figures 2.5B, D, &F**). The Serine 3-dehydrogenase, pyruvate dehydrogenase E1 component subunit beta, and xanthine dehydrogenase, all exhibited higher activities in the highly than the weakly aggressive *V. dahliae* isolate (**Figures 2.5 A, B, C, D, E & F**).

The expression of myo-inositol dehydrogenase in the highly aggressive isolate was up-regulated in response to potato extracts and during infection of detached leaves, while we were unable to detect the expression in the weakly aggressive isolate in all the treatments (**Figure 2.5 G & H**). The expressions of nuc-1 negative regulatory protein *VdPREG* in weakly aggressive *V. dahliae* isolates were significantly higher than in the highly aggressive one under all the treatments, as well as during infection at 8 DAI (**Figure 2.5I & J**).

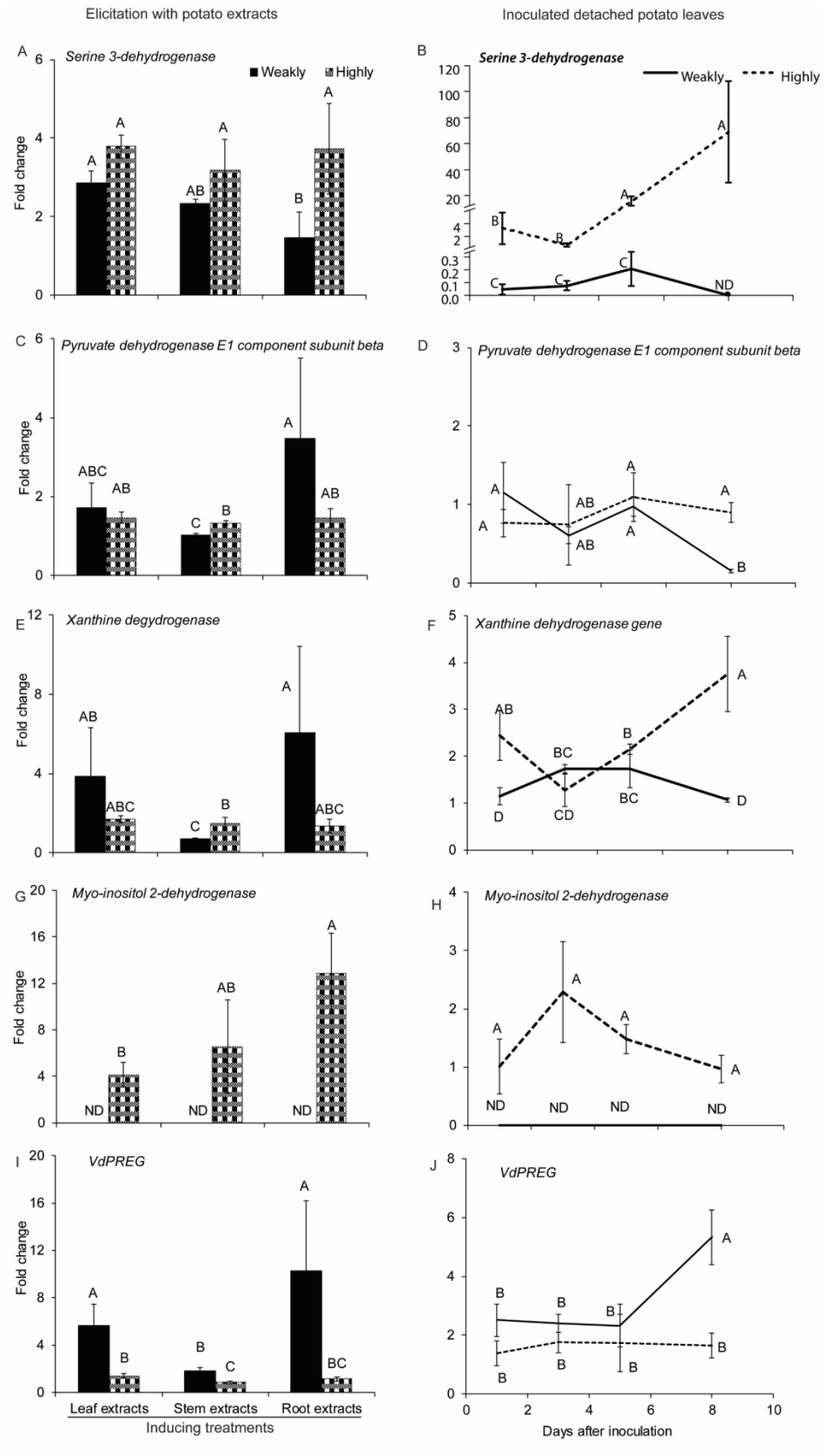
Elicitation with potato extracts
Inoculated detached potato leaves
A
Serine 3-dehydrogenase
Weakly
Highly
Fold change
B
Serine 3-dehydrogenase
Weakly
Highly
C
Pyruvate dehydrogenase E1 component subunit beta
D
Pyruvate dehydrogenase E1 component subunit beta
E
Xanthine degydrogenase
F
Xanthine dehydrogenase gene
G
Myo-inositol 2-dehydrogenase
H
Myo-inositol 2-dehydrogenase
I
VdPREG
J
VdPREG
Leaf extracts
Stem extracts
Root extracts
Inducing treatments
Days after inoculation

Figure 2.5 Expression of genes related to cellular metabolism under different treatments.
Expression of (**A**) Serine 3-dehydrogenase, (**C**) Pyruvate dehydrogenase E1 component subunit beta, (**E**) Xanthine dehydrogenase, (**G**) Myo-inositol dehydrogenase and (**I**)*VdPREG* in the highly (Vd1396-9) and the weakly (Vs06-07) aggressive *V. dahliae* isolates in response to elicitation with different potato extracts: leaf, stem, root extracts.
Expression of (**B**) Serine 3-dehydrogenase, (**D**) Pyruvate dehydrogenase E1 component subunit beta, (**F**) Xanthine dehydrogenase, (**H**) Myo-inositol dehydrogenase and (**J**) *VdPREG* in the highly (Vd1396-9) and the weakly (Vs06-07) aggressive *V. dahliae* isolates in response to infection of detached potato leaves at 1,3,5, and 8 DAI.
WC: Water control treatment; Weakly: weakly aggressive *V. dahliae* isolate Vs06-07; Highly: highly aggressive *V. dahliae* isolate Vd1396-9; ND: Non-detectable. Error bars refer to standard error. The macro PDMIX800.sas (Saxton, 1998), $\alpha = 0.05$ was applied to mean values separated by least squared means, and into grouped letters results. Values ($n = 3$) with the same letters are not significantly different ($P<0.05$).

2.3.2.5 Genes related to detoxification

HAD superfamily hydrolase constitute the largest branch of phosphatases superfamily (Burroughs et al., 2006; Koonin and Tatusov, 1994). In *Escherichia coli* and *S. cerevisiae*, several homologues of HAD-like hydrolase were identified (Kuznetsova et al., 2015; Kuznetsova et al., 2006). These homologues were demonstrated to have a role in detoxification of 2-deoxyglucose, or phosphorylated glycerol phosphates and carbohydrates (Kuznetsova et al., 2015; Kuznetsova et al., 2006). The expression of this gene in *V. dahliae* was not significantly different among the two isolates in response to potato extracts (**Figure 2.6A**). However, during the detached leaves infection, HAD superfamily hydrolase transcripts accumulated more in the highly aggressive isolate (**Figure 2.6B**). Following the infection, there was an increasing trend in the highly aggressive isolate and a mirrored decrease in the weakly aggressive one (**Figure 2.6B**).

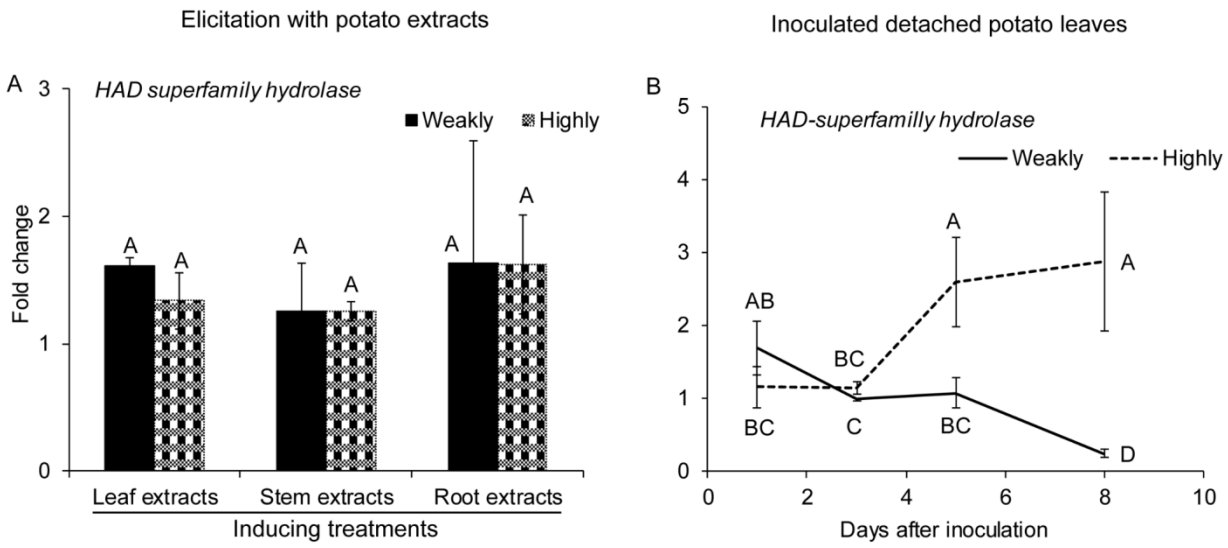

Figure 2.6 Expression of genes related to detoxification under different treatments.
(**A**) Expression of HAD superfamily hydrolase in the highly (Vd1396-9) and the weakly (Vs06-07) aggressive *V. dahliae* isolates in response to elicitation with different potato extracts: leaf, stem, root extracts.
(**B**) Expression of HAD superfamily hydrolase in the highly (Vd1396-9) and the weakly (Vs06-07) aggressive *V. dahliae* isolates in response to infection of detached potato leaves at 1,3,5, and 8 DAI.
WC: Water control treatment; Weakly: weakly aggressive *V. dahliae* isolate Vs06-07; Highly: highly aggressive *V. dahliae* isolate Vd1396-9. Error bars refer to standard error. The macro PDMIX800.sas (Saxton, 1998), $\alpha = 0.05$ was applied to mean values separated by least squared means, and into grouped letters results. Values (n = 3) with the same letters are not significantly different ($P<0.05$).

2.4 Discussion

V. dahliae causes one of the most devastating potato diseases, causing great losses in crop production worldwide (Pegg and Brady, 2002). Comprehending the molecular mechanisms of infection, colonization and tissue damage are important tools towards sustainable management of this disease in the field (Pegg and Brady, 2002; Rowe and Powelson, 2002). This study provides more details about the quantitative expression of 15 genes involved in different cellular processes in two *V. dahliae* isolates possessing differential levels of aggressiveness (Vd1396-9 and Vs06-

07). In order to provide as complete a picture as possible, we assessed the expression of those 15 *V. dahliae* genes in two different settings that would relate to two different phases of *V. dahliae*'s interaction with potato. Firstly, our assessment of gene expression in *V. dahliae* in response to plant exudates, represents the pre-infection phase. Secondly, our assessment of gene expression in detached leaves represents the host infection process.

2.4.1 Genes involved in cell differentiation and proliferation

Ras-GAP-like protein, serine/threonine protein kinase, Wos2 and glucan endo-1,3-alpha-glucosidase are involved in regulating cell differentiation and proliferation. The increased expression of Ras-GAP and Wos2 in response to potato extracts suggests that they are important for spore germination and pre-infection by *V. dahliae*. This result is in line with previous findings in *Candida albicans* (behaves as a pathogenic yeast in human under specific conditions), which showed transient increases in Rho1-GAP (Rho1 belongs to Ras family) and WOS2 during a morphogenesis switch from a less virulent yeast form to a virulent filamentous form (Nantel et al., 2002). Moreover, Ras-GAP's involvement corresponds with findings that Ras-GAP is important for cell differentiation, such as yeast in bud formation of *Saccharomyces cerevisiae* (Cvrckova and Nasmyth, 1993), and hyphal growth, conidiation and mitosis of *Aspergillus nidulans* (Harispe et al., 2008).

The similar expression trends of Ras-GAP and serine/threonine protein kinase during the detached leaves' infection mirror those from the stem extract treatment, which suggests that Ras-GAP and serine/threonine protein kinase may work in a similar model, or even in the same signal pathway, in *V. dahliae*. It has been shown in numerous studies that Ras-GAP-related Ras is essential for virulence or infection-related structure formation in *F. graminearum*, *M. Oryzae* and

A. fumigatus (Bluhm et al., 2007; Ding et al., 2009; Park et al., 2006), and its function involves serine/threonine protein kinases such as MAP kinase and sometimes PKA (Fortwendel et al., 2008; Norton and Fortwendel, 2014). This is in agreement with our findings that Ras-GAP and serine/threonine protein kinase express more in response to elicitation with stem extracts and infection of detached leaves by the highly aggressive isolate than the weakly aggressive one. All this indicates that the small GTPase signaling pathway in *V. dahliae*, including *Ras-GAP* and serine/threonine protein kinase, may have an important role in the infection and morphogenesis processes related to pathogenicity, and may also play important roles in differential aggressiveness between isolates.

In *M. Oryzae*, disruption of transcription factor *Tup1* induced a loss of the pathogenicity and an increase in glucan endo-1,3-alpha-glucosidase (MoAgn1) expression (Chen et al., 2015). This indicates that a high activity of MoAgn1 controlled by the pathogenicity-related transcription factor Tup1 may be associated with low pathogenicity. In our results, the expression of endo-1,3-alpha-glucosidase in *V. dahliae*, also exhibited a transient increase in the less virulent isolate. We hypothesized that the more aggressive *V. dahliae* isolate may have down-regulated the activity of endo-1,3-alpha-glucosidase to accommodate the stress condition present during infection of the host.

2.4.2 Genes involved in DNA repair

In response to pathogen attacks, plants produces a great number of ROS in order to induce DNA damage and restrict the expansion of the pathogen (Sharma et al., 2012). We suggest that these genes involved in the DNA repair pathway exhibit various functions based on different *V. dahliae* stages. In *V. dahliae*, these DNA repair proteins may help recover from DNA damage. In

S. cerevisiae, homologues of RAD6 and RAD18 are involved in both the error-free post-replication DNA repair pathway and the REV3-mediated mutagenesis pathway (Bailly et al., 1994; Bailly et al., 1997; Broomfield et al., 1998; Jentsch et al., 1987; Morrison et al., 1989; Prakash et al., 1993; Reynolds et al., 1985). MMS2 is only involved in the error-free post-replication DNA repair mode (Broomfield et al., 1998). All three genes (DNA-(apurinic or apyrimidinic site) lyase, ubiquitin-conjugating enzyme variant MMS2, and DNA repair protein RAD51) showed an increased expression in response to potato extracts. This is in line with a RAD14 transient induction during transition from a less virulent to a more virulent state in *C. albicans* (Nantel et al., 2002). In *Helicobacter pylori*, the disruption of a homologue of DNA-(apurinic or apyrimidinic site) lyase increased sensitivity to oxidative stress and reduced the colonization rate as compared to the wild type (Bailly and Verly, 1989; O'Rourke et al., 2003). This indicates that DNA-(apurinic or apyrimidinic site) lyase is involved in aiding bacteria's survival under stress conditions and plays a role in its ability to colonize the host (O'Rourke et al., 2003). This is similar to our findings and indicates that this homologue in both isolates may help *V. dahliae* survive during stress conditions, including DNA damage during infection.

Ubiquitin-conjugating enzyme variant MMS2 transcripts may contribute more in the highly aggressive isolate during the infection process. This is also similar to findings with the human pathogen *Paracoccidioides brasiliensis*, where MMS2 was significantly elevated while infecting oral keratinocytes cell (da Silva et al., 2011), indicating its function during infection.

2.4.3 Genes related to ROS production and cleavage

In fungi, ROS are critical for controlling infection morphogenesis, sexual development, ascospore germination and pathogenicity (Lara‐Ortíz et al., 2003; Malagnac et al., 2004; Wang

et al., 2014). Non-enzymatic ROS are produced in the mitochondria electron transport chain (Bedard et al., 2007; Heller and Tudzynski, 2011). NADH-ubiquinone oxidoreductase (complex I) is the predominant gene responsible for the production of ROS in mitochondria in mammalian and bacterial cells (Bazil et al., 2014; Brand et al., 2004; Kussmaul and Hirst, 2006; Turrens, 2003). Homeostasis of cellular ROS is maintained by scavenging systems which includes the thioredoxin (Trx) system, that consists mainly of Trx, Trx reductase (TrxR), and peroxidase (Prx) (Huang et al., 2015). Our findings based on TrxR and NADH-ubiquinone oxidoreductase in *V. dahliae* show that the ROS balance in *V. dahliae* responds differently in these two different aggressive isolates and when exposed to different parts of the potato plant.

The dramatic increase of *TRX* in the highly aggressive *V. dahliae* isolate, compared to the weakly aggressive one, suggests that the Trx function in reducing ROS and maintaining ROS balance in *V. dahliae*, may be important for infection-related morphology, differentiation and therefore critical during infection. These are in accordance with previous studies demonstrating that, the expression of Trx or TrxR in *Staphylococcus aureus, S. cerevisiae*, *S. pomb* and *C. albicans,* and *C. neoformans,* increased in response to various stress conditions like oxidative or nitrosative stress (Enjalbert et al., 2003; Fradin et al., 2003; Godon et al., 1998; Hong et al., 2006; Missall and Lodge, 2005; Salmon et al., 2004; Uziel et al., 2004). In *C. neoformans*, TrxR is also required for viability (Missall and Lodge, 2005). All of these findings indicate that the thioredoxin (Trx) ROS scavenging system may help the highly aggressive *V. dahliae* to respond to stress conditions or help during colonization at the time of infection. Our findings also indicate that ROS produced by the NADH-ubiquinone oxidoreductase-mediated non-enzymatic system is also involved in regulating spore germination during the pre-infection process. These results suggest that ROS regulation may be important for pathogenicity in *V. dahliae* and related to the differential

aggressiveness of the highly aggressive isolate

2.4.4 Genes related to cellular metabolism

The genes involved in cellular metabolism are essential for maintaining nutrition support and normal growth in various organisms (Berman and Magasanik, 1966; Bittner et al., 2001; Dallo et al., 2002; Engerson et al., 1987; Enroth et al., 2000; Galbraith et al., 1998; Hille and Nishino, 1995; Jiang et al., 2001; Kang, 1993; Kang and Metzenberg, 1990, 1993; Kato et al., 2008; Lessard and Perham, 1994; Peleg et al., 1996; Seifert et al., 2007; Tchigvintsev et al., 2012; Yamazawa et al., 2011). The expression of Serine 3-dehydrogenase, pyruvate dehydrogenase E1 component subunit beta, xanthine dehydrogenase andmyo-inositol dehydrogenase were upregulated in both isolates with potato extracts elicitation and during infection. *VdPREG* is a negative regulatory for phosphorus acquisition, indicates that phosphorus acquisition pathway may be more activated in highly than in weakly aggressive *V. dahliae* isolate.

Several other studies show that these components of cellular metabolisms are also important for pathogenicity or virulence. For instance, D-serine metabolism is essential for virulence and regulation of several virulence factors in *S. saprophyticus* (Korte-Berwanger et al., 2013). This supports our results in *V. dahliae* that Serine 3-dehydrogenase shows more activity in the highly aggressive isolate and suggests a role in pre-infection and infection. The importance of pyruvate dehydrogenase E1 component subunit beta in the highly aggressive *V. dahliae* isolate during the pre-infection and infection stages is suggested by its higher expression in response to stem extracts and infection (Figures 2.5C & D). This is correlated with the important role of Lipoamide dehydrogenase (Lpd), a member of the pyruvate dehydrogenase complex family, in the pathogenesis of *Mycobacterium tuberculosis* (Venugopal et al., 2011). Plants produce antifungal

compounds such as flavonoids to inhibit the activity of the pathogen's xanthine dehydrogenase in order to increase the resistance to fungi and bacteria (González et al., 1995; Hayashi et al., 1988; Khobragade et al., 2008; Kong et al., 2000; Nagao et al., 1999). Even though there is no direct evidence showing that xanthine dehydrogenase is essential for pathogenicity in fungi, our result showed higher activity of xanthine dehydrogenase in the highly aggressive isolate than the weakly aggressive one (**Figure 2.5E & F**). This may indicate that xanthine dehydrogenase in a higher virulent *V. dahliae* like Vd1396-9, may overcome these inhibitors from plants and play a role in infection. Our finding on myo-inositol dehydrogenase (**Figure 2.5 G & H**) also suggests that the highly aggressive isolate utilizes this protein during the pre-infection and infection process. This gene may play a function in helping *V. dahliae* in rhizopine utilization and viability for better colonization in the plant root. This was also demonstrated in *S. meliloti* and *S. fredii*, myo-inositol dehydrogenase homologue is important for inositol and inositol phosphate metabolism and rhizopine utilization (Berman and Magasanik, 1966; Galbraith et al., 1998; Jiang et al., 2001). In *N. crassa*, the negative regulatory factor PREG can control the activity of transcription factor NUC-1, while NUC-1 can up-regulate the activity of the phosphorus acquisition enzymes encoding genes during phosphorus starvation conditions (Kang, 1993; Kang and Metzenberg, 1990, 1993; Peleg et al., 1996). The lower activity of PREG in the higher virulent *V. dahliae* isolate, may indicate a higher activity of the phosphorus acquisition pathway. Phosphate acquisition is important for virulence in *Ustilago maydis* and *C. neoformans* and it was demonstrated that a connection between phosphate acquisition and the PKA pathway controls the fungal virulence in both pathogens (Boyce et al., 2006; Kretschmer et al., 2014). Our findings in PREG are consistent with their research as well as our hypothesis that a higher virulent *V. dahliae* may down-regulate *PREG* in order to achieve a higher activity in NUC1 and the phosphate acquisition pathway, as

well as the up-regulation of serine/threonine protein kinases (such as PKA). This may be important for virulence in the highly aggressive *V. dahliae*.

2.4.5 Genes related to detoxification

HAD-like hydrolase protein can be identified in different organisms, and studies reported their important roles in virulence in *C. neoformans* and in detoxification in *E. coli* and in *S. cerevisiae* (Jung et al., 2018; Kuznetsova et al., 2015; Kuznetsova et al., 2006). Plants produce various secondary metabolites, proteases and phytoalexins to ward off pathogens (Lattanzio et al., 2006; Loschke et al., 1983; Ryan, 1990; Wink, 1988; Wittstock and Gershenzon, 2002). Plants also release some endogenous peptides to induce plant defense (Kim et al., 2014). HAD superfamily hydrolase might help *V. dahliae* avoid plant toxins by detoxification and therefore play a role in virulence. This research is in line with our findings that HAD superfamily hydrolase may have a role to play in infection and differential virulence in isolates.

2.4.6 Conclusion

In conclusion, the majority of the genes we tested increased in both weakly and highly aggressive isolates in response to treatment with the various potato extracts. The genes involved in cellular metabolism were more stimulated in the highly aggressive isolate than the weakly aggressive one in response to potato extracts. This indicates that the metabolism of pyruvate, purines and pyrimidines, inositol and inositol phosphate, and phosphorus acquisition may be activated more in the highly aggressive isolate than in the weakly aggressive one in response to the various potato extracts (**Figure 2.7**). The genes involved in ROS production (NADH-ubiquinone oxidoreductase) and small GTPase signaling pathway (including Ras-GAP and

serine/threonine protein kinase) were also elevated in the highly aggressive isolate more than the weakly aggressive one when exposed to potato stem extracts, suggesting a higher activity in these pathways (**Figure 2.7**). During infection of potato detached leaves, there were more up-regulated genes in the highly aggressive isolate than the weak aggressive one. These genes include key elements in the small GTPase signaling pathway, which include Ras-GAP and serine/threonine protein kinase, DNA damage repair, ROS production and cleavage, cellular metabolism and detoxification. These results suggest an important role for all of these pathways during infection (Figure 2.7).

We tested these genes based on hints from the studies of El-Bebany et al. (2010, 2011), and confirmed their differential expression in highly and weakly aggressive isolates. Our results showed that the higher activities of the genes involved in cell differentiation and proliferation, DNA repair, ROS regulation, cellular metabolism and detoxification, may explain some of the differential aggressiveness of the highly aggressive isolates. Future projects should focus on functionally characterizing the genes' roles in the virulence of *V. dahliae* on its host plants. In particular, genes such as those encoding Ras-GAP like protein, serine/threonine protein kinases and Trx deserve more attention to investigate. Meanwhile, RNA-seq profiling and metabolite profiling on infected potatoes by highly and weakly aggressive isolates will reveal more of the host's defense and pathogen counter-defense mechanisms.

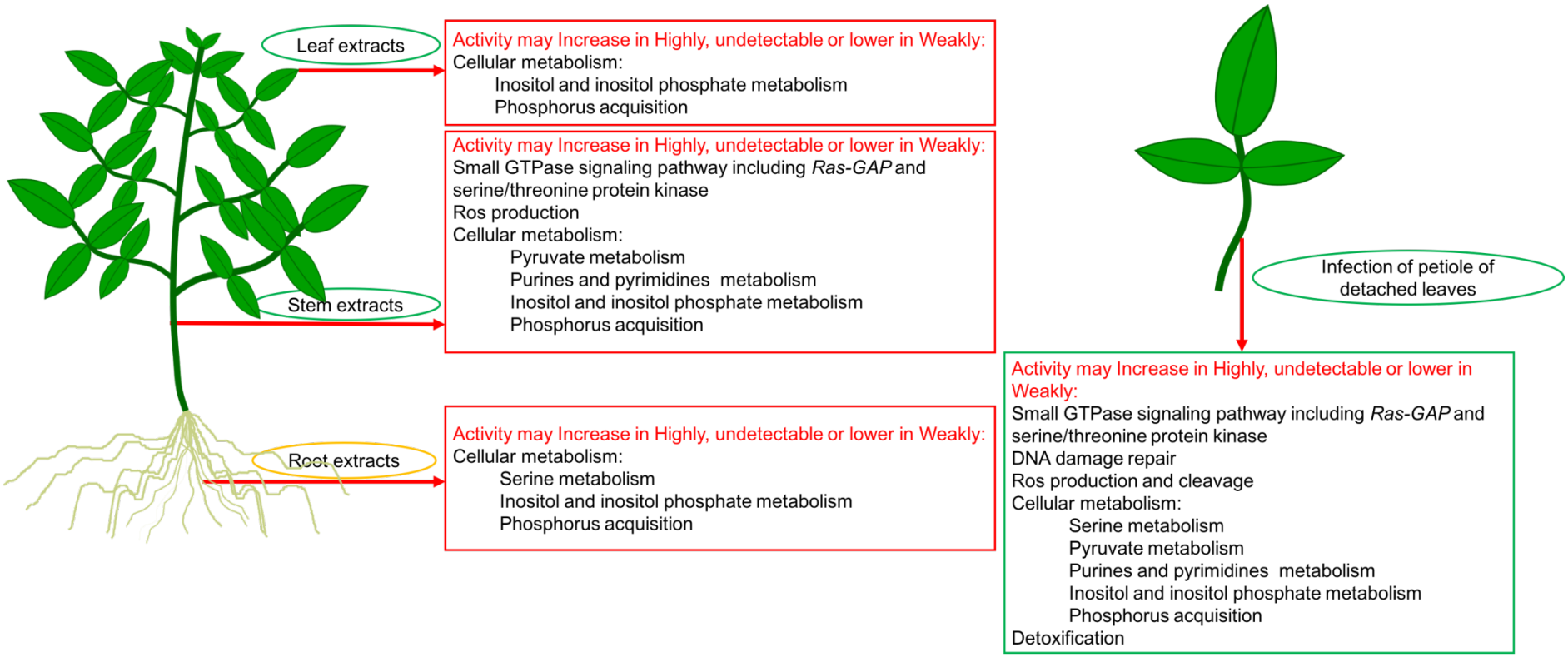

Figure 2.7 Potential involvement of pathways in response to potato extracts from various tissues and infection.

CHAPTER 3

VERTICILLIUM DAHLIAE'S ISOCHORISMATASE HYDROLASE IS A VIRULENCE FACTOR THAT CONTRIBUTES TO INTERFERENCE WITH POTATO'S SALICYLATE AND JASMONATE DEFENSE SIGNALING

Author contributions

Xiaohan Zhu planned and run most of the experiments in this manuscript and contributed to the writing of the results. **Atta soliman** provided technical assistance for the molecular work and writing of the manuscript. **Md. R. Islam** helped with the fungal transformation work. **Lorne Adam** provided technical assistance for inoculations and statistical analysis. **Fouad Daayf** contributed the initial idea, supervised the work, and contributed to the writing of the manuscript.

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Verticillium dahliae's Isochorismatase Hydrolase Is a Virulence Factor That Contributes to Interference With Potato's Salicylate and Jasmonate Defense Signaling

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This study aimed to dissect the function of the Isochorismatase Hydrolase (*ICSH1*) gene in *Verticillium dahliae*'s pathogenesis on potato. *VdICSH1* was up-regulated in *V. dahliae* after induction with extracts from potato tissues. Its expression increased more in response to root extracts than to leaf and stem extracts. However, such expression in response to root extracts was not significantly different in the highly and weakly aggressive isolates tested. During infection of detached potato leaves, *VdICSH1* expression increased significantly in the highly aggressive isolate compared to the weakly aggressive one. We generated *icsh1* mutants from a highly aggressive isolate of *V. dahliae* and compared their pathogenicity with that of the original wild type strain. The analysis showed that this gene is required for full virulence of *V. dahliae* on potatoes. When we previously found differential accumulation of ICSH1 protein in favor of the highly aggressive isolate, as opposed to the weakly aggressive one, we had hypothesized that ICSH would interfere with the host's defense SA-based signaling. Here, we measured the accumulation of both salicylic acid (SA) and jasmonic acid (JA) in potato plants inoculated with an *icsh1* mutant in comparison with the wild type strain. The higher accumulation of bound SA in the leaves in response to the *icsh1* mutant compared to the wild type confirms the hypothesis that ICSH1 interferes with SA. However, the different trends in SA and JA accumulation in potato in the roots and in the stems at the early infection stages compared to the leaves at later stages indicate that they are both associated to potato defenses against *V. dahliae*. The expression of members of the isochorismatase family in the *icsh1* mutants compensate that of ICSH1 transcripts, but this compensation disappears in presence of the potato leaf extracts. This study indicates ICSH1's involvement in *V. dahliae*'s pathogenicity and provides more insight into its alteration of the SA/JA defense signaling's networking.

Keywords: pathogenesis, *ICSH1*, salicylic acid, jasmonic acid, isochorismatase, virulence factor

Abstract

This study aimed to dissect the function of the *Isochorismatase Hydrolase* (*ICSH1*) gene in *Verticillium dahliae's* pathogenesis on potato. *VdICSH1* was up-regulated in *V. dahliae* after induction with extracts from potato tissues. Its expression increased more in response to root extracts than to leaf and stem extracts. However, such expression in response to root extracts was not significantly different in the highly and weakly aggressive isolates tested. During infection of detached potato leaves, *VdICSH1* expression increased significantly in the highly aggressive isolate compared to the weakly aggressive one. We generated *icsh1* mutants from a highly aggressive isolate of *V. dahliae* and compared their pathogenicity with that of the original wild type strain. The analysis showed that this gene is required for full virulence of *V. dahliae* on potatoes. When we previously found differential accumulation of ICSH1 protein in favor of the highly aggressive isolate, as opposed to the weakly aggressive one, we had hypothesized that ICSH would interfere with the host's defense SA-based signaling. Here, we measured the accumulation of both salicylic acid (SA) and jasmonic acid (JA) in potato plants inoculated with an *icsh1* mutant in comparison with the wild type strain. The higher accumulation of bound SA in the leaves in response to the icsh1 mutant compared to the wild type confirms the hypothesis that ICSH1 interferes with SA. However, the different trends in SA and JA accumulation in potato in the roots and in the stems at the early infection stages compared to the leaves at later stages indicate that they are both associated to potato defenses against *V. dahliae*. The expression of members of the isochorismatase family in the *icsh1* mutants compensate that of *ICSH1* transcripts, but this compensation disappears in presence of the potato leaf extracts. This study indicates ICSH1's involvement in *V. dahliae*'s pathogenicity and provides more insight into its alteration of the SA/JA defense signaling's networking.

3.1 Introduction

Verticillium dahliae Kleb., a soil-borne hemi-biotrophic pathogen, causes wilt in more than 200 dicotyledonous plant species and is considered the primary causal agent of the potato early dying (PED) syndrome (Johnson and Dung, 2010; Rowe and Powelson, 2002). The symptoms of Verticillium wilt include chlorosis and necrosis, starting in the lower leaves, as well as vascular discoloration, stunting, and wilting of the infected plant (Pegg and Brady, 2002). Ultimately, *V. dahliae* produces resting structures (microsclerotia) in the necrotic areas of infected tissues, which can sustain germination viability for long period of time up to 10 years or more in soil (Klosterman et al., 2009). When exposed to root exudate, the microsclerotia germinate and the mycelia penetrate the root epidermic cells, and then produce infectious hyphae to colonize the roots (Klosterman et al., 2009). The survival ability of microsclerotia and the wide host range of *V. dahliae* make management strategies for this wilt costly and inefficient, i.e., traditional cultural practices such as crop rotation do not provide useful solutions. Fungicides are ineffective since the infection and colonization occur in the plant's roots and vascular system before they move upward to the aerial plant parts (Daayf, 2015). Breeding of resistant lines would be paramount but has not been successful. In tomato, resistance to *V. dahliae*, mediated by the *Ve1* gene, has been reported, but is limited to race 1 (Fradin et al., 2009; Kawchuk et al., 2001). In potato (*Solanum tuberosum*), no similar genes have been described so far. However, other studies reported potato defense genes induced in response to this infection (Derksen et al., 2013a).

Plant defense against *V. dahliae* can also be induced using biological means such as non-pathogenic fungi (Veloso and Díaz, 2012), bacteria (Uppal et al., 2008), or plant extracts (El Hadrami et al., 2011). Exploring other new and more efficient Verticillium wilt management strategies require better understanding of the host-*V. dahliae* interaction, i.e., mechanisms of

virulence, defense, and how pathogen effector signaling interferes with the plant's ability to overcome diseases.

Several genes have been studied in relation to their role in *V. dahliae*'s pathogenicity on different plant species. However, little is known about their mechanisms of interference with host defenses (El Hadrami et al., 2011; El Hadrami et al., 2015). Several pathogens have been reported to interfere with their host's defense signaling, i.e., salicylic acid (SA), and jasmonic acid (JA), in order to successfully invade their tissues (El Oirdi et al., 2011). Potato plants have a high level of endogenous SA, particularly in the leaves (Navarre and Mayo, 2004; Yu et al., 1997). This phytohormone is essential for systemic acquired resistance (SAR) in many species, which makes it a prominent inducible defense hormone, especially against biotrophic pathogens (Koornneef and Pieterse, 2008; Yu et al., 1997). In plants, the phenylpropanoid and isochorismate pathways are two known pathways for SA synthesis (Coquoz et al., 1998; Pasqualini et al., 2003; Wildermuth et al., 2001). SA from the first pathway is apparently required for cell death in the infection site (Pasqualini et al., 2003), while the one from the second pathway seems to be required for SAR responses (Wildermuth et al., 2001). The JA pathway is known to generally interfere with the SA-defense pathway(Derksen et al., 2013b), and was reported to induce systemic tolerance to necrotrophic pathogens (Glazebrook, 1999; Kunkel and Brooks, 2002). The antagonistic effect between SA and JA has been reported in different studies, as part of more efficient defense processes (Davies, 2010; Pieterse et al., 2009). Exogenous SA application improved cotton's resistance of *V. dahliae* and its toxins (Li et al., 2003; Mo et al., 2016; Zhen and Li, 2004), and of *A. thaliana* to *V. dahliae* toxins (Jiang et al., 2005). Exogenous methyl jasmonate (MeJA), an analog of JA, increased resistance to *V. longisporum* in both wild type and R-gene-signaling-deficient mutant (*ndr1-1*) lines of *A. thaliana* (Johansson et al., 2006). Another study reported that

jasmonate-deficient tomato mutants (*def1*) were more susceptible to *V. dahliae* compared to the wild type control (Thaler et al., 2004). In *A. thaliana* mutants, such as ET-signaling-deficient mutants (*ein2-1*, *ein4-1*, *ein6-1*), and JA-signaling-deficient mutants (*esa1-1*, and *pad1-1*), but not SA-signaling-deficient mutants (*npr1-1*, *eds1-1*, *pad4-1*, *sid2- 1*) had enhanced susceptibility to *V. longisporum* (Johansson et al., 2006). Genes involved in the SA-signaling pathway such as *PAL1*, *PAL2*, and *PR-1* had a higher expression in the roots of the moderately resistant than the susceptible potato cultivar in response to *V. dahliae*. On the other hand, *PR-2* was up regulated in the leaves of the susceptible potato cultivar (Derksen et al., 2013a). Moreover JA pathway-related genes (*PR-3*, *PR-9*, *WIN2* and *POTLX3*) were up-regulated in a susceptible potato cultivar in response to *V. dahliae* (Derksen, 2011; Derksen et al., 2013a), which strongly suggested a partial involvement of SA in potato defense against *V. dahliae* (Derksen et al., 2013a).

The isochorismatase hydrolase (*ICSH1*) (VDAG_05103) of *V. dahliae* belongs to the isochorismatase family. In different organisms, this enzyme catalyzes the conversion of isochorismate into other components, such as 2,3-dihydroxybenzoate and pyruvate (Soanes et al., 2008; Wildermuth et al., 2001). As isochorismate is a very important precursor for SA biosynthesis in plants (Pasqualini et al., 2003), we had hypothesized the potential involvement of isochorismatase in *V. dahliae*'s virulence (El-Bebany et al., 2010), after it was only detected in the proteome of the highly aggressive *V. dahliae* isolate, but not in the weakly aggressive one (El-Bebany et al., 2010). Interestingly, proteins containing an isochorismatase motif have been found in the secretome of five kinds of phytopathogens, but were absent in the secretome of non-pathogenic filamentous ascomycetes (Soanes et al., 2008). Different members of the isochorismatase family were also identified in bacteria, leading the conversion of different substrates into different endpoint compounds (Gehring et al., 1997; Künzler et al., 2005;

Maruyama and Hamano, 2009; Parsons et al., 2003), i.e., a bacterial streptothricin hydrolase (SttH) has been identified as a member of the isochorismatase-like hydrolase (ILH) super family, with a primary role in streptothricin (ST)- resistance. The authors had suggested that SttH may have a function in molybdopterin modification (Maruyama and Hamano, 2009). An EntB in *Escherichia coli* has also been described as a isochorismate lyase involved in the enterobactin biosynthetic pathway through utilization of chorismate for the production of 2,3-dihydroxybenzoate (2,3-DHB) (Gehring et al., 1997). In *Pseudomonas aeruginosa*, PhzD was identified as an isochorismatase using 2-amino-2-deoxyisochorismate as a substrate, participating in the phenazine biosynthesis (Parsons et al., 2003). PhzD can also hydrolyze chorismate, vinyl ethers isochorismate, and 4-amino-4-deoxychorismate (Parsons et al., 2003). PchB, which possesses isochorismate pyruvate lyase (IPL) and chorismate mutase (CM) activities in *P. aeruginosa*, catalyzes the conversion of isochorismate into salicylate and pyruvate, and the rearrangement of chorismate into prephenate (Künzler et al., 2005).

Based on the proteomic analysis that showed isochorismatase hydrolase accumulation in the highly aggressive *V. dahliae* isolate Vd1396-9 but not in the weakly aggressive isolate Vs06-14 (El-Bebany et al., 2010), the differentially expressed genes in *V. dahliae*-potatoes interaction, and that both JA and SA signaling pathways seemed to be involved in potato defenses against *V. dahliae* (Derksen, 2011; Derksen et al., 2013a; El Hadrami et al., 2015; El-Bebany et al., 2011), we hypothesized that SA and isochorismatase hydrolase (ICSH1) are important components that partially mediate the interaction of *V. dahliae* with potato and other hosts. Indeed, further knocking out of the isochorismatase hydrolase gene in a *V. dahliae* isolate from cotton resulted in attenuating the aggressiveness of the pathogen on this plant species while it accumulated higher levels of SA (Liu et al., 2014). Our hypothesis was that the high level of isochorismatase hydrolase activity in

highly aggressive isolates may lead to hijacking the SA pathway in potato plants by hydrolyzing the isochorismate and may represent one of the reasons for higher aggressiveness in certain isolates. Given that previous studies reported that both SA and JA signaling pathways were activated in potato's response to *V. dahliae* (Derksen, 2011; Derksen et al., 2013a), we speculated that Isochorismatase hydrolase may have differential activities in different isolates, and may play different roles in response to different hosts.

In the present study, our objectives were to: (i) compare the expression of *VdICSH1* in isolates with different pathogenicity levels on potato under different treatments/conditions; (ii) perform a functional analysis of this gene by determining its effect on SA and JA accumulation in potato tissues, and thereby its role in *V. dahliae*'s pathogenicity on potato and selected alternative hosts.

3.2 Materials and methods

3.2.1 *V. dahliae* isolates

Verticillium dahliae isolates Vd1396-9 and Vs06-07, from Dr. Daayf 's lab collection, were grown on potato dextrose agar (PDA) media at 24°C for 2 weeks. *V. dahliae* Vd1396-9 and Vs06-07 have been characterized as highly and weakly aggressive isolates, respectively (Alkher et al., 2009; Uppal et al., 2007). The conidial spores were harvested by flooding culture plates with sterilized water, then the concentration was adjusted for each experiment.

3.2.2 *Agrobacterium*-mediated transformation of *V. dahliae*

The open reading frame (ORF) of *ICSH1* was amplified from genomic DNA of isolate Vd1396-9 with specific primers flanked with restriction sites of *Eco*RI and *Bam*HI (Table 3.1).

The DNA fragment was cloned into the binary vector pDHT (Mullins et al., 2001) and mutagenized using the EZ::TN transposon system (Epicentre Technologies, Madison, WI, USA). *Agrobacterium*-mediated transformation of *V. dahliae* was conducted according to the method described by Dobinson et al. (2004). The transformants were selected in PDA media containing hygromycin B (50 μg/mL). PCR was performed to confirm gene replacement using ICSH1-Up-F upstream the target sequence and ICSH1-EcoRI-F (**Table 3.1**). The positive candidate transformants were selected and the expression of *ICSH1* was confirmed by RT-PCR.

Table 3.1. Primer sequences used and accession numbers

Primer's name	Primer sequence	Tm	Accession number
ICSH1-EcoRI-F	GGAATTCATGTCCTCATTCCGCTCCAT	70.17	VDAG_05103
ICSH1-BamHI-R	CGGGATCC CTAGTTGATATCCTTGCT	66.01	VDAG_05103
qRTICSH1-F	ATGTCCTCATTCCGCTCCAT	60.27	VDAG_05103
qRTICSH1-R	CTAGTTGATATCCTTGCT	42.93	VDAG_05103
His3-F	ATGGCTCGCACTAAGCAA	54.8	VDAG_10035
His3-R	TGAAGTCCTGGGCAATCT	52.7	VDAG_10035
ICSH1-Up-F	TTTGGCTGCGAAAGACG	57.99	VDAG_05103
qrtVDAG03530F	AGGCTTTCAAACATCCAAC	52.2	VDAG_03530
qrtVDAG03530R	TTCAATAGCGAGTATGTCAGTT	52.4	VDAG_03530
qrtVDAG06170F	TTTCAACCGGCCCTCATT	58.4	VDAG_06170
qrtVDAG06170R	TGGGTGCCAGTCCTTGGT	58.7	VDAG_06171
qrtVDAG06346F	AACCTCTGCCCCAGCGTC	60.4	VDAG_06346
qrtVDAG06346R	GGATGACCTCGGCCTTGTC	59.8	VDAG_06347
potato-EF-F	GATGGTCAGACCCGTGAACAT	57	AJ536671.1
potato-EF-R	GGGGATTTTGTCAGGGTTGT	55.4	AJ536671.1

3.2.3 Genomic DNA extraction and southern blot analysis

V. dahliae was grown in CDB liquid media for 10 days, then mycelia were collected and ground in liquid nitrogen. The DNA extraction method followed the protocol of Al-Samarrai and Schmid (2000). Genomic DNA of *V. dahliae* (~20 µg) was digested by restriction enzyme EcoRV overnight at 37°C, and the digested DNA was separated in a 0.8% agarose gel for 6 h at 70 V. The DNA was transferred to a nylon membrane following the protocol of Maruthachalam et al. (2011). Hybridization and detection were conducted with Amersham AlkPhos Direct Labeling and Detection Systems (GE Healthcare Life Sciences, Mississauga, ON, Canada). The signal was visualized on X-ray film (Kodak, Rochester, NY, USA).

3.2.4 Growth rate and conidiation of mutants

V. dahliae mutants (*icsh1-2-3-1* and *icsh1-2-12-1*) and wild type were grown on PDA for 2 weeks, thereafter colony diameters were measured. Each isolate was replicated on 4 petriplates. Five 1.2 cm diameter mycelial plugs were randomly chosen from 4 week-old *V. dahliae* PDA plates and placed into 40 mL sterilized water, vortexed well then conidia concentration was determined using a hemocytometer.

3.2.5 Plant material

Potato cultivars Ranger Russet, a moderately resistant, and Kennebec, susceptible to Verticillium wilt, as well as the susceptible sunflower Hybrid IS8048 and the susceptible tomato variety Bonny Best were used in this study (Alkher et al., 2009; Madhosingh, 1996). Plants were grown in a soil mix containing sand, soil, and peat moss with 1:1:1 ratio in a growth room with a 16/8 h photoperiod and a 22/18°C temperature regimen.

3.2.6 *V. dahliae* gene expression in response to elicitation with different potato tissue extracts

Conidial suspensions of the *V. dahliae* highly aggressive isolate Vd1396-9, the weakly aggressive isolate Vs06-07 and the *icsh1-2-12-1* mutant were adjusted to a concentration of 10^8 conidia/mL, and 1 mL of each was placed into 100 mL Czapek-Dox Broth (CDB) liquid media (Difco Laboratories, Sparks, MD, USA), then incubated for 1 week at 23.5 ±1°C. Potato tissue extracts were prepared according to the protocol of El-Bebany et al. (2011). Briefly, 5 g of root, stem, or leaf tissues from 3-weekold healthy Kennebec potato plants were ground into powder using liquid nitrogen with mortars and pestles. Twenty-five mL of sterilized distilled water was added to the powder, then placed on a shaker at 120 rpm (C2 Platform Shaker, Edison, NJ, USA) for 4 h. The mixtures were centrifuged at 2000×*g* for 5 min, and the supernatant filtered through

0.45 µm syringe filters (Thermo Fisher Scientific,Wilmington, DE, USA). One milliliter of root, stem and leaf extracts were separately added to 1-weekold cultures in CDB media. The experiment was carried out in three replications for each isolate supplemented with different potato extracts. One milliliter of sterilized distilled water was added to the control. The mycelia were harvested a week after treatment, and immediately ground into a powder using liquid nitrogen with pre-chilled mortar and pestle. The mycelial powder was used for RNA extraction.

3.2.7 Gene expression analysis in infected potato detached leaves

Healthy leaves were cut from 4-week-old Kennebec potato plants, and then petioles placed into 1 mL conidial suspensions of Vs06-07 or Vd1396-9, with a final concentration of $3x10^7$ conidia/mL to ensure quick infection. Sterilized water was used for control plants. A half milliliter of sterilized water was added to the detached leaves every second day. The detached leaves were kept at 24±2°C, with a 16/8 h photoperiod day/night. Four to six leaves all from different potato plants were bulked as one replication, with three replications for each isolate for each time point (1 DAI, 3 DAI, 5 DAI, and 8 DAI). Gene expression was analyzed in all treatments. The leaf tissues were immediately frozen in with liquid nitrogen, then ground finely to a fine powder and used for total RNA extraction.

3.2.8 RNA extraction and cDNA synthesis

Total RNA was extracted from potato tissues (100 mg) following the manufacturer's protocol of the Omega Fungal RNA kit (Omega Bio-Tek, Inc., Norcross, GA, USA). The quantity and quality of the total RNA were analyzed using a NanoDrop 2000 (Thermo Fisher Scientific,Wilmington, DE, USA). The first DNA strand was synthesized from approximately one

microgram of the total RNA using Superscript first strand synthesis kit (Life Technologies, Carlsbad, CA, USA).

3.2.9 Quantitative Real-Time RT-PCR

Quantitative Real-Time RT-PCR was performed according to the protocol of SsoFast EvaGreen Super mix (Bio-Rad Lab, Philadelphia, PA, USA) using a CFX96 Thermal Cycler (Bio-Rad, Hercules, CA, USA). The expression level of the *ICSH1* transcripts was assessed using specific primer sets (Table 3.1). The Histone H3 gene (VDAG_10035) was used as a reference gene. The data was analyzed using the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001).

3.2.10 Pathogenicity analysis of *Vdicsh1* mutants

The potato, sunflower and tomato were sown in small plastic trays containing LA4 soil mix (SunGro Horticulture, Agawam, MA 01001, USA) in a growth room for 3 weeks, then roots were washed, trimmed 1–2 cm from the tip and placed in a conidial suspension. The conidia of *V. dahliae icsh1* mutants and wild type were harvested with sterile water and diluted to a concentration of 10^6 conidia/mL. Sterile water was used as a control treatment. The inoculated plants were transferred to pots containing a pasteurized mixture of sand and soil (2:1). The plants were grown in the same growth room under temperature and photoperiod of 24/18 ± 2°C and 16/8 h day/night, respectively. The total AUDPC of percentage of infection, disease severity, and plant height were analyzed. The vascular discoloration of the stem cross-sections were rated in the last week according to Alkher et al. (2009). Biological replications for potato cultivars Kennebec and Ranger Russet, sunflower Hybrid IS8048, and tomato variety Bonny Best, were 12, 6, 4, and 6, respectively for each treatment. The experiment for potato cultivar Kennebec, sunflower Hybrid IS8048 and tomato

variety Bonny Best were repeated with 3, 2, and 2 times, respectively with similar results.

3.2.11 Analysis of SA and JA Accumulation in potato tissues

Three-week-old plants of the potato cultivar Kennebec were inoculated using the root dipping method described above in one of the three conidial suspensions, including *icsh1* mutant (*icsh1-2-12-1*), wild type strain Vd1396-9, or the sterilized water control. The leaves, stems, and roots were sampled at 4, 9, 14, 21, and 35 DAI with three replications. The same tissues were split and extracted for SA and JA analysis.

The extraction and HPLC-PDA-Fluorescence analysis for SA was performed according to El Hadrami et al. (2015). Briefly, 500 mg of potato tissues were ground into powder with liquid nitrogen, then suspended with 490 µl of 80% methanol and 10 µl of 12.5µg/ml *O*-anisic acid (Sigma-Aldrich Canada Co.) as an internal standard (Meuwly and Metraux, 1993). The mixture was shaken overnight at 4°C and centrifuged at 5,300 rpm for 5 min., the supernatant was evaporated under N_2, then re-extracted by adding equal volumes of ethyl acetate twice. The acetate phases were combined into a new tube and dried under N_2. Finally, extracts were re-suspended in 500 µl pure methanol for free SA analysis. The aqueous phases from the previous step were used for bound SA extraction by hydrolyzing in an equal volume of 8N HCl at 100°C for 2 h followed by re-extraction in ethyl acetate. The samples were re-suspended in 500 µl pure methanol and run for SA on HPLC-PDA- Fluorescence (El Hadrami et al., 2015). Bound SA was determined by the amount of free SA released by hydrolysis.

The extraction and UPLC-MSMS analysis for JA was performed according to Henriquez et al. (2016). Briefly, 500 mg of potato tissues were ground into powder with liquid nitrogen, then suspended with 5 ml of extraction solvent (HCl:2-propanol: H2O in a ratio of 2:1:0.002) and 50µl

of 1µg/ml (±)-9,10-dihydrojasmonic acid DHJA (OlChemIm Ltd. Czech Republic) as an internal standard. They were shaken for 30 min at 4°C and mixed with 7 ml of dichloromethane, shaken again for an additional 30 min followed by centrifugation at 5300 rpm for 5 min. The lower phase was transferred into a new tube, dried under N_2 on ice, then re-suspended in 500 µl 50% methanol.

3.2.12 Statistical Analysis

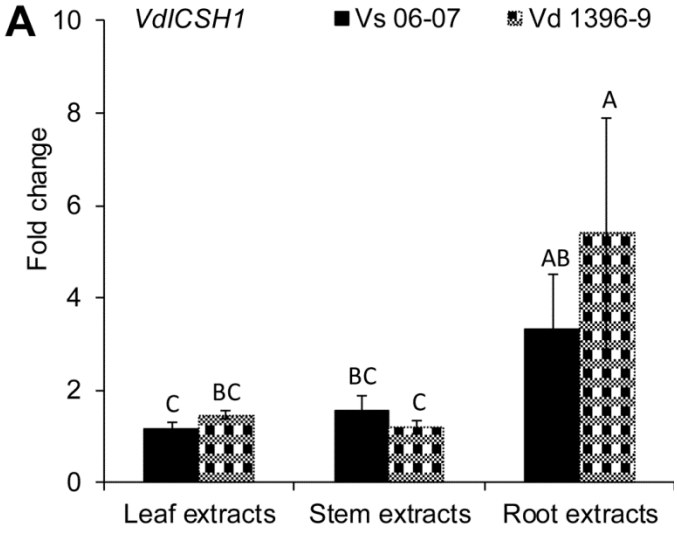
Statistical analyses were performed using PROC MIXED with Statistical Analysis Software (SAS) (SAS Institute, Cary, NC, USA; release 9.1 forWindows). Data were checked for normality with PROC UNIVARIATE and outliers were removed based on residuals comparison to critical values for studentized residuals (Lund, 1975) and the Shapiro–Wilk test for normality. Gene expression data were normalized by $\log^{10}$ transformation. Mean values were separated using least squared means and letters assigned by the macro PDMIX800.sas (Saxton, 1998) with $\alpha = 0.05$.

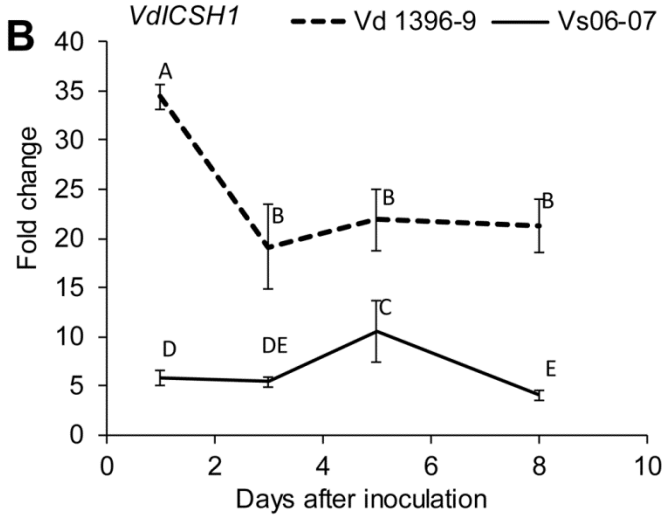
3.3. Results

3.3.1 Expression of *icsh1* in *V. dahliae* isolates under induction with plant extracts

According to previous research, Vd1396-9 is a highly aggressive *V. dahliae* isolate on potato, while Vs06-07 is a weakly aggressive one (Alkher et al., 2009; Uppal et al., 2007). We tested the expression of *ICSH1* in isolates with high and low levels of aggressiveness and under the elicitation of potato tissue extracts. The highly and the weekly aggressive isolates of *V. dahliae* were exposed to potato extracts from different plant parts. The expression of the *ICSH1* increased in response to all extract types, but was significantly higher, in both isolates, in response to the root extract than to the leaf and stem extracts. There were no significant differences between

responses of the highly and weekly aggressive isolates (**Figure 3.1A**).


A
VdICSH1
Vs 06-07
Vd 1396-9
Fold change
10
8
6
4
2
0
C
BC
BC
C
AB
A
Leaf extracts
Stem extracts
Root extracts


B
VdICSH1
Vd 1396-9
Vs06-07
Fold change
40
35
30
25
20
15
10
5
0
A
B
B
B
C
D
DE
E
0
2
4
6
8
10
Days after inoculation

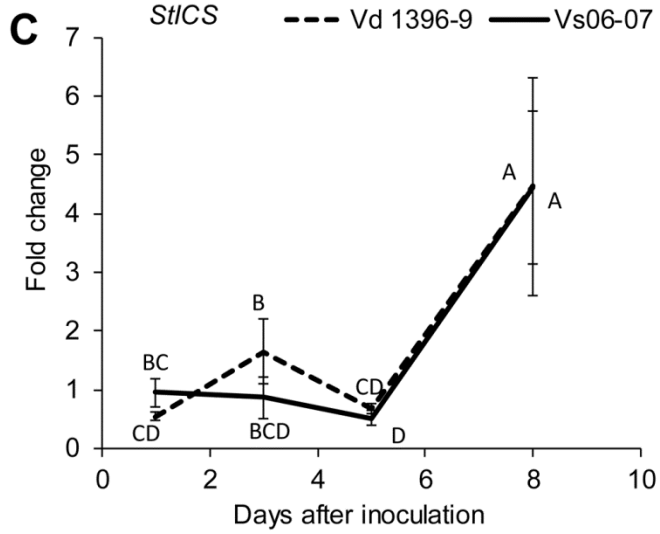

C
StICS
Vd 1396-9
Vs06-07
Fold change
7
6
5
4
3
2
1
0
BC
B
CD
A
A
CD
BCD
D
0
2
4
6
8
10
Days after inoculation

Figure 3.1 Expression of *V. dahliae ICSH1* and potato Isochorismate Synthase (StICS) under different treatments.

(A) Expression of *V. dahliae ICSH1* under elicitation with different potato tissue extracts. *V. dahliae* highly aggressive isolate Vd1396-9 and the weakly aggressive isolate Vs06-07 were induced in liquid media by different potato tissue extracts. Sterilized distilled water was used as a control treatment. QRT-PCR data were normalized using *V. dahliae* Histone H3. The expression data obtained for both isolates under different treatments were analyzed using the $2^{-\Delta\Delta C_T}$ method relative to its corresponding isolate cultured in CDB medium with water. The Bars represented by mean values (n = 3, with two technical replications) sharing the same letter are not significantly different from each other ($P < 0.05$).

(B) Expression of *V. dahliae ICSH1* during infection of detached potato leaves. Kennebec potato detached leaves (4-week-old) were placed in conidial suspensions of *V. dahliae* highly aggressive isolate Vd1396-9 and the weakly aggressive isolate Vs06-07; sterilized distilled water was used as a control treatment. Detached leaves from 12 and 18 different individual plants were combined to three biological replicates for each treatment at each time point. QRT-PCR data was normalized using *V. dahliae* Histone H3. The expression data obtained for both isolates under different treatments were analyzed using the $2^{-\Delta\Delta C_T}$ method relative to its corresponding isolate cultured in CDB medium. Point values are represented by mean values (n = 3, with two technical replications) sharing the same letter are not significantly different from each other ($P < 0.05$). This experiment was repeated twice.

(C) Expression analysis of potato Isochorismate Synthase (*StICS*) in response to inoculation with wild type *V. dahliae* isolate. Kennebec potato detached leaves (4-week-old) were placed in conidial suspensions of *V. dahliae* highly aggressive isolate Vd1396-9 and the weakly aggressive isolate Vs06-07; sterilized distilled water was used as a control treatment. Detached leaves from 12 and 18 different individual plants were combined to three biological replicates for each treatment at each time point. QRT-PCR data was normalized using potato elongation factor. The expression data obtained from infected detached leaves under different treatments were analyzed using the $2^{-\Delta\Delta C_T}$ method relative to that in uninfected detached leaves. Point values are represented by mean values (n = 3, with two technical replications) sharing the same letter are not significantly different from each other ($P < 0.05$).

3.3.2 Expression of *icsh1* in *V. dahliae* isolates after inoculation of potato detached leaves

The expression level of *ICSH1* was firstly analyzed in the highly and weakly aggressive isolates during the infection of susceptible potato plants (cv. Kennebec), but due to the low biomass of the weakly aggressive isolateVs06-07, it was not detected by QRT-PCR in the plant tissues (Data not show). Therefore, the inoculation was tested with conidial suspensions on detached potato leaves to analyze the expression of *ICSH1* at different time points after inoculation. The gene expression was analyzed for all the treatments, but there was no expression of *ICSH1* on

detached leaves without *V. dahliae* infection (**Figure 3.2**). The expression level of *ICSH1* transcripts in inoculated potato leaves followed the same trend with the two *V. dahliae* isolates tested. The expression started by increasing to a high level after 1 day and decreased thereafter. However, a significantly higher expression level was observed with the highly aggressive isolate Vd1396-9, as compared to the weekly aggressive one Vs06-07 (**Figure 3.1B**).

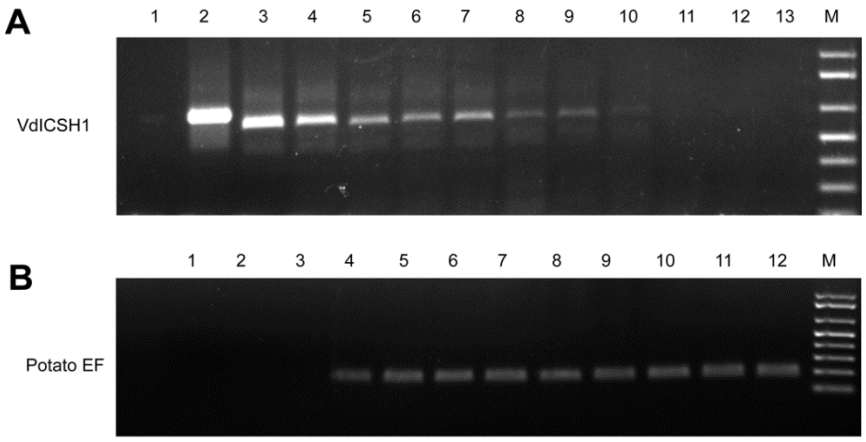


Figure 3.2 Amplification of *V. dahliae ICSH1* in all potato detached leaves' treatments at 1 DAI.
Kennebec potato detached leaves from 4-week-old plants were placed in conidial suspensions of the *V. dahliae* highly aggressive isolate Vd1396-9, the weakly aggressive isolate Vs06-07, or sterilized distilled water as a control treatment.
(A) Amplification of *VdICSH1* in all potato detached leaves' treatments at 1 DAI; lane 1: negative control; lane 2: Vd1396-9 genomic DNA; lane 3: cDNA of Vd1396-9; lane 4: cDNA of Vs06-07; lane 5–7: cDNA of potato detached leaves inoculated with Vd1396-9 at 1 DAI; lane 8–10: cDNA of potato detached leaves inoculated with Vs06-07 at 1 DAI; lane 11–13: cDNA of water control potato detached leaves at 1 DAI; M: DNA marker (1Kb Plus DNA Ladder, Invitrogen, USA).
(B) Amplification of potato elongation factor (EF) in all potato detached leaves' treatments at 1 DAI; lane 1: negative control; lane 2: cDNA of Vd1396-9; lane 3: cDNA of Vs06-07; lane 4–6: cDNA of potato detached leaves inoculated with Vd1396-9 at 1 DAI; lane 7–9: cDNA of potato detached leaves inoculated with Vs06-07 at 1 DAI; lane 10–12: cDNA of water control potato detached leaves at 1 DAI; M: DNA marker (1Kb Plus DNA Ladder, Invitrogen, USA).

3.3.3 Expression of ICS in potato in response to *V. dahliae*

PAL and ICS are two plant pathways that are both involved in SA synthesis (Pasqualini et al., 2003; Wildermuth et al., 2001). In a previous study, *PAL1* and *PAL2* were shown to be up-

regulated in potato in response to *V. dahliae* infection, indicating that PAL-mediated SA signaling pathway is involved in potato plant defense against *V. dahliae* (Derksen et al., 2013a). The expression of a key enzyme of the ICS pathway, Isochorismate synthase (StICS), was assessed during inoculation using the two isolates with contrasting levels of aggressiveness. The expression of isochorismate synthase (*StICS*) in potato under *V. dahliae* infection increased significantly 5 DAI, but with no significant difference between the tested isolates (**Figure 3.1C**).

3.3.4 ICSH1 Mutant of the highly aggressive *V. dahliae* isolate Vd1396-9

To determine the function of *ICSH1* in *V. dahliae*, Agrobacterium-mediated transformation was carried out to introduce the transposon insertion into *V. dahliae* Vd1396-9 and disrupt the *ICSH1* as described by Dobinson et al. (2004). A DNA fragment containing the chloramphenicol resistance gene and the hygromycin phosphotransferase gene inserted at the 449th bp of *ICSH1* ORF, was generated in the binary vector pDHt. Five positive *icsh1* mutants were identified by PCR (**Figure 3.3A**). The integration of the transposon cassette was confirmed by southern blot (**Figure 3.3B**). On the RNA level, the depletion of *icsh1* transcripts was confirmed by semi-quantitative PCR. The wild type and the ectopic control (*ICSH1-ECT-2-11-3*) (Random insertion in *V. dahliae* genome but without replacing the original *ICSH1* ORF) showed normal levels of *ICSH1* transcripts (**Figure 3.3C**). Out of the five identified mutants, two (*icsh1-2-3-1* and *icsh1-2-12-1*) were selected randomly for the next steps of phenotype analysis.

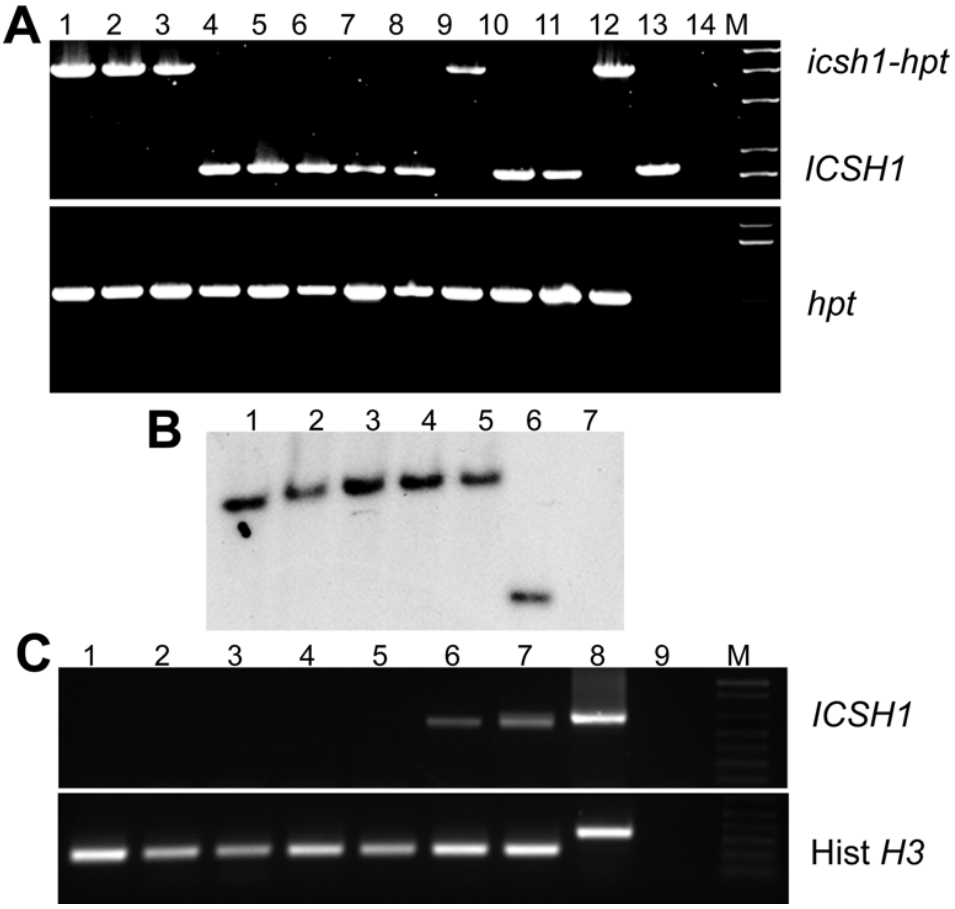


Figure 3.3 Identification of *V. dahliae icsh1* mutants.
(A) PCR analysis of transformants of the *icsh1* mutants. Lane 1–12 represent transformants; lane 13, wild type strain Vd1396-9; lane 14, negative control. *hpt*: Hygromycin phosphotransferase gene; *icsh1-hpt*: Isochorismatase hydrolase gene ORF disrupted by insertion of a transposon cassette containing the chloramphenicol resistance gene and the hygromycin phosphotransferase gene; M: DNA marker (1Kb Plus DNA Ladder, Invitrogen, USA)
(B) Southern blot analysis of the *icsh1* candidate transformants using *hpt* probe. Lane 1–5, positive transformants; lane 6, ectopic control (random insertion without replacing the original *ICSH1* ORF); lane 7, wild type strain Vd1396-9.
(C) Screening of the transformants of *icsh1* mutant by RT-PCR. Lane 1–5, positive transformants; lane 6, ectopic control; lane 7, wild type strain Vd1396-9; lane 8, DNA of wild type strain Vd1396-9; lane 9, negative control; *Hist H3*: *V. dahliae* Histone H3; M: DNA marker (1Kb Plus DNA Ladder, Invitrogen, USA)

3.3.5 Characterization of the *icsh1* mutants

The phenotype, growth rate, conidiation, microsclerotia formation of *icsh1* mutants were tested on PDA plates, while the pathogenicity was tested on susceptible and moderately resistant

potato cultivars, on sunflower and on tomato plants. The morphology, conidiation and microsclerotia formation were not affected in the tested *V. dahliae icsh1-2-3-1* and *icsh1-2- 12-1* mutants (**Figure 3.4**). There was no significant difference among the *icsh1* mutants and the wild type neither in growth rate nor in conidial formation. On the other hand, the total area under disease progress curve (AUDPC) of infection and disease severity showed significant reduction with *V. dahliae* mutants compared to the wild type in both susceptible and moderately resistant potato cultivars (**Figures 3.5, 3.6**). The plant height and vascular discoloration followed the same trend in both cultivars, with no significant difference between the mutants and the wild type. In addition to potato, the wild type and *icsh1* mutants were tested on sunflower and tomato plants. In sunflower, both wild type and *icsh1* mutant were able to induce disease, but there were no significant differences between wild type and mutants in the total AUDPC of either infection or disease severity (**Figure 3.7**). In tomato plants, there were no significant differences observed in any of the tested parameters for both pathogen isolates (**Figure 3.8**). In conclusion, the *ICSH1* is important for virulence of *V. dahliae* on potato but not on sunflower and tomato.

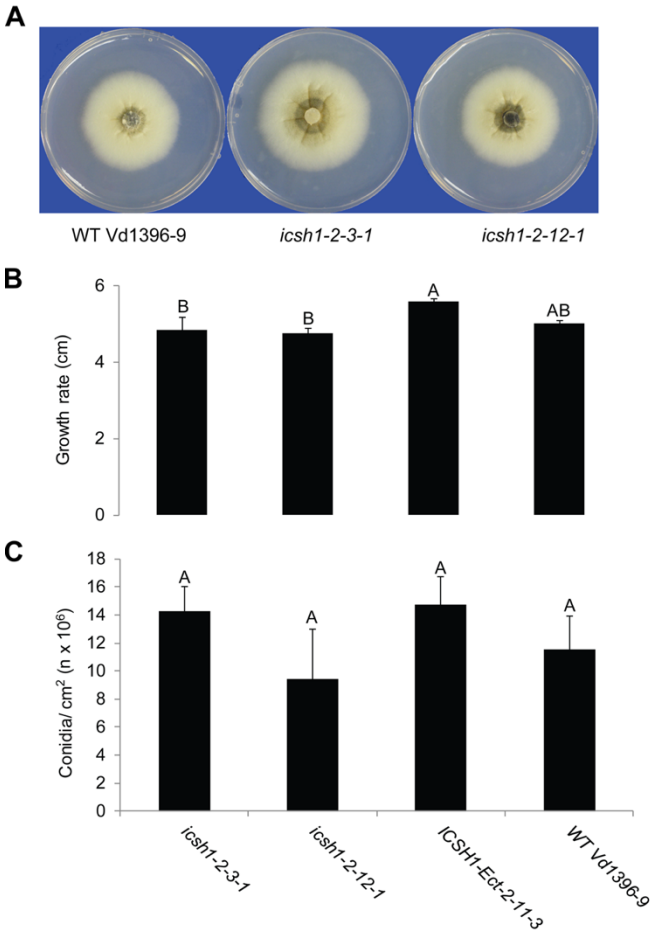


Figure 3.4 Morphology, conidiation and grwoth rate of *icsh1* mutants.
(A) The colony phenotype of *icsh1* mutants (*icsh1-2-3-1* and *icsh1-2-12-1*), ectopic control: *ICSH1-Ect-2-11-3* and wild type (Vd1396-9); (B) The growth rate of *V. dahliae icsh1* mutants, ectopic control: *ICSH1-Ect-2-11-3* and wild type (Vd1396-9). The growth rates were determined by the colony diameter; (C) The conidiation of *icsh1* mutant. The conidia of each isolate were collected from PDA plates, and their concentration determined using a hemocytometer. Bars represented by mean values (n = 4) sharing the same letter are not significantly different from each other ($P < 0.05$).

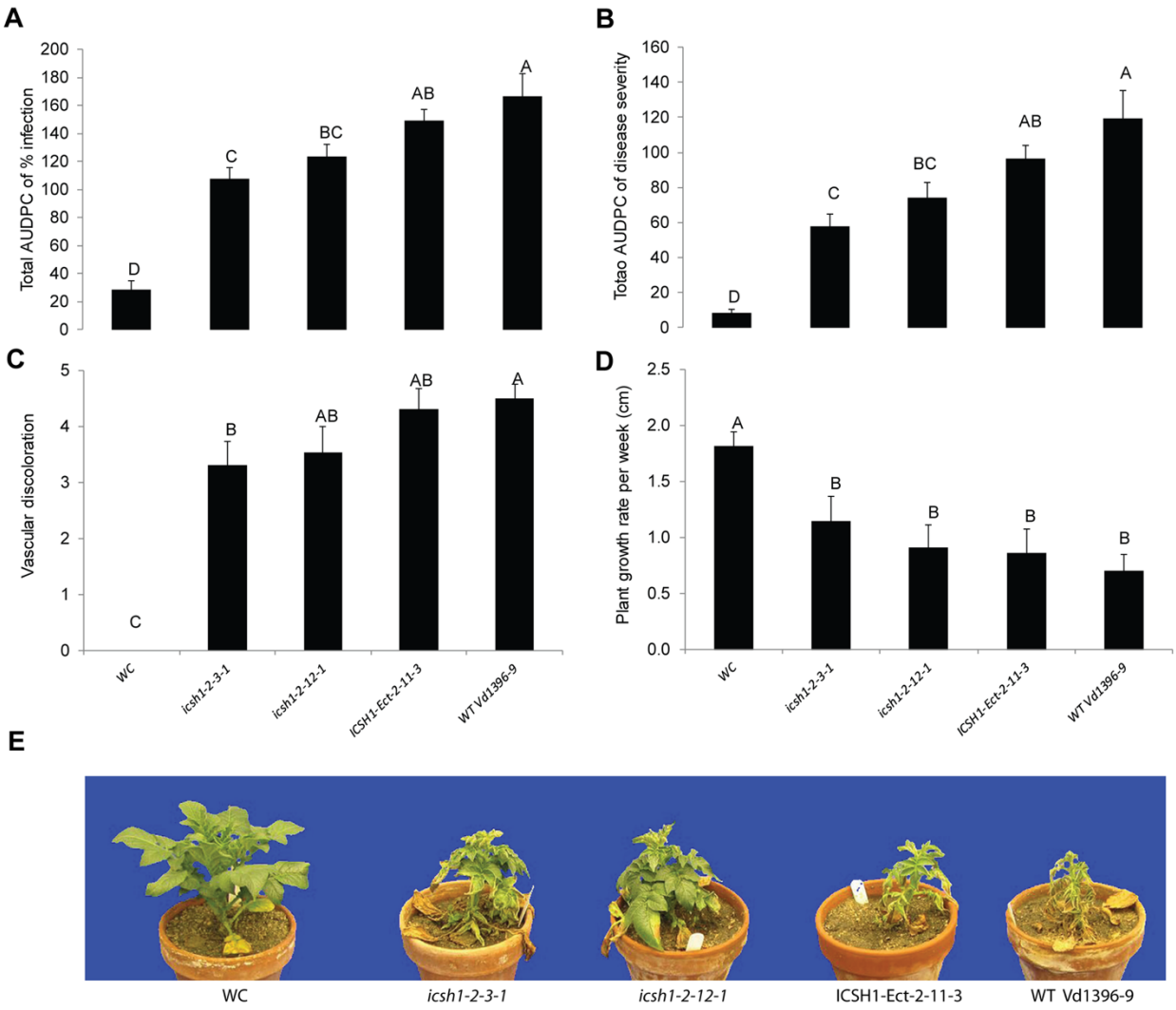


Figure 3.5 Pathogenicity of *icsh1* mutants on susceptible potato cultivar (Kennebec). The percentage of infection, disease severity and plant height were recorded every week. Vascular discoloration of the stem cross-sections was rated at 5 weeks after inoculation with *icsh1-2-3-1* and *icsh1-2-12-1*; ectopic control: *ICSH1-Ect-2-11-3*; wild type: Vd1396-9; WC: water control. (A) Total AUDPC of percentage of infection; (B) Total AUDPC of disease severity; (C) Vascular discoloration; (D) Growth rate of susceptible potato; (E) Kennebec plants infected by *icsh1* mutants at 5 weeks after infection. Bars represented by mean values (n = 12) sharing the same letter are not significantly different from each other ($P < 0.05$). This experiment was repeated three times with similar results.

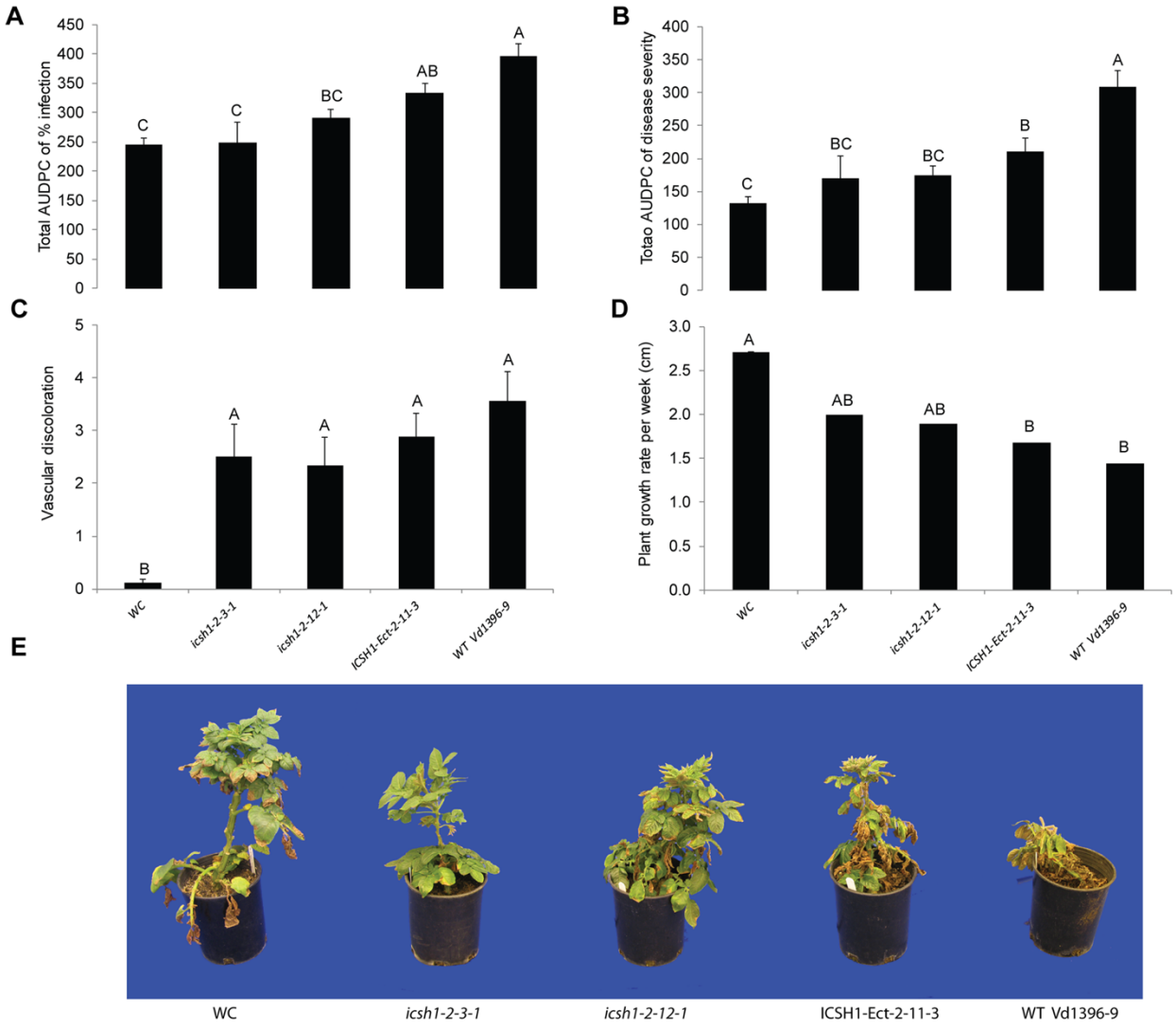


Figure 3.6 Pathogenicity of *icsh1* mutants on moderately resistant potato cultivar (Ranger Russet).

The percentage of infection, disease severity and plant height were recorded every week. vascular discoloration of the stem cross-sections was rated at 8 weeks after inoculation with *icsh1-2-3-1* and *icsh1-2-12-1*; ectopic control: *ICSH1-Ect-2-11-3*; wild type: Vd1396-9; WC: water control. (A) Total AUDPC of percentage of infection; (B) Total AUDPC of disease severity; (C) Vascular discoloration; (D) Growth rate of moderately resistant potato; (E) Ranger Russet plants infected by *icsh1* mutants at 8 weeks after infection. Bars represented by mean values (n=6) sharing the same letter are not significantly different from each other ($P < 0.05$).

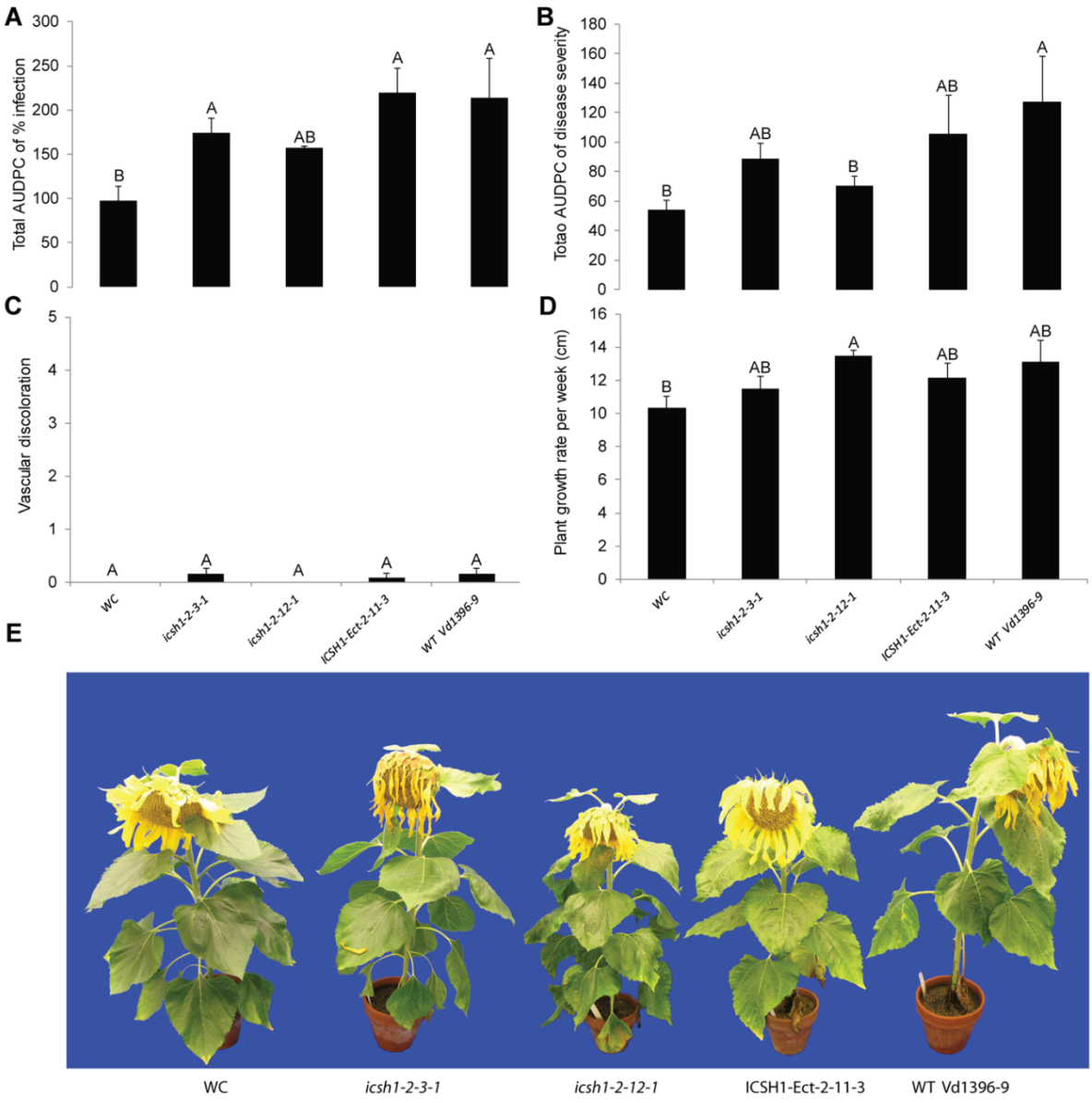


Figure 3.7 Pathogenicity of *icsh1* mutant on susceptible sunflower line IS8048.
Roots of the susceptible sunflower line IS8048 were washed and placed in a conidial suspension of *V. dahliae*. The percentage of infection, disease severity and plant height were recorded every week. The vascular discoloration of the stem cross-sections were rated at 8 weeks after inoculation, with *icsh1-2-3-1* or *icsh1-2-12-1*; ectopic control: *ICSH1-Ect-2-11-3*; wild type: Vd1396-9; WC: water control. (A) Total AUDPC of percentage of infection; (B) Total AUDPC of disease severity; (C) Vascular discoloration; (D) Growth rate of susceptible sunflower; (E) Sunflower line IS8048 infected by *icsh1* mutants at 8 weeks after infection. Bars represented by mean values (n=4) sharing the same letter are not significantly different from each other ($P < 0.05$). This experiment was repeated twice.

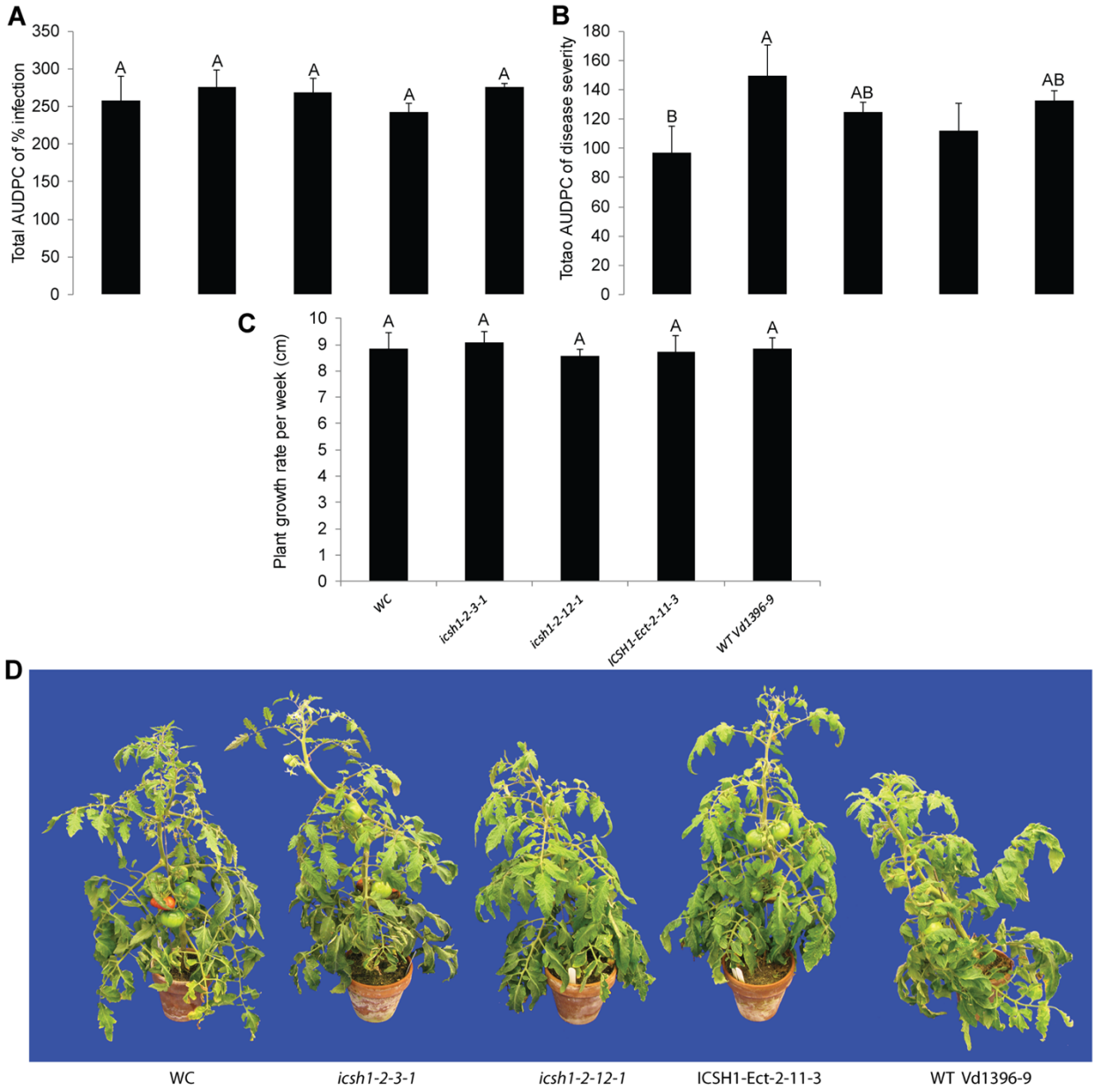


Figure 3.8 Pathogenicity of the *icsh1* mutant on susceptible tomato variety Bonny Best. Roots of the susceptible tomato variety Bonny Best, were washed and placed in a conidial suspension of *V. dahliae*. The percentage of infection, disease severity and plant height were recorded for each week after inoculation with *icsh1-2-3-1* or *icsh1-2-12-1*; ectopic control: *ICSH1-Ect-2-11-3*; wild type: Vd1396-9; WC: water control. (A) Total AUDPC of percentage of infection; (B) Total AUDPC of disease severity; (C) Growth rate of susceptible tomato; (D) Tomato variety Bonny Best infected by *icsh1* mutants at 8 weeks after infection. Bars represented by mean values (n=6) sharing the same letter are not significantly different from each other ($P < 0.05$). This experiment was repeated twice.

3.3.6 Expression of other members of the isochorismatase family in *V. dahliae* under induction with plant extracts

To determine potential roles of other genes from the isochorismatase family in presence and absence of *ICSH1*, the expression of five genes from the isochorismatase family in *V. dahliae* (Accession # VDAG_06346, VDAG_03530, VDAG_06170, VDAG_06688, and VDAG_08870) was tested by QRT-PCR in a highly and a weakly aggressive isolates, and in the isch1 mutant (*icsh1-2-12-1*) under induction with potato extracts. The same volume of water was added in the medium for each strain as a control to potato extracts. All data was analyzed using the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001). In both strains, the expression level of VDAG_06688 and VDAG_08870 was too low to be detected. The expression of VDAG_03530 increased in both the highly and weekly aggressive isolates in response to all types of extracts, without significant differences between the isolates (**Figure 3.9A**). The expression of VDAG_06170 increased in both the highly and weekly aggressive isolates in response to all types of extracts (**Figure 3.9B**), with a higher level in the weakly aggressive isolate in response to potato leaf and root extracts. In response to potato leaf extracts, the expression of VDAG_06346 increased more in the weakly aggressive isolate than in the highly aggressive one but such increase was similar in the two isolates in response to both stem and root extracts (**Figure 3.9C**).

Comparison of the expression of the above-cited genes in the *isch1* mutant and the wild type Vd1396-9 under treatments with different extracts revealed the expression levels of VDAG_03530 and VDAG_06170 to be significantly higher in the *isch1* mutant than those in the wild type isolate Vd1396-9 (**Figures 3.10, 3.11**). To make these comparisons more clear, the data were shown in terms of expression of these genes in *icsh1* relative to the wild type strain under each treatment (**Figure 3.10**). Both stem and root extracts induced a higher expression of

VDAG_03530 and VDAG_06170 in the mutant compared with the wild type (Figures 3.10A & B). Moreover, compared with the wild type with no treatment, the expression of VDAG_03530 in isch1 mutant was about eight-fold higher in response to water (control), stem extracts, and root extracts (**Figure 3.11A**). Similarly, the expression of VDAG_06170 in isch1 mutant in response to water (control), stem extracts and root extracts was about 7- fold, 5-fold, and 4-fold respectively to that of the wild type with no treatment (**Figure 3.11B**). Inversely, the expression of VDAG_06346 was higher in the wild type isolate compared to the *icsh1* mutant in response to root extract (**Figure 3.11C**). In conclusion, the expression of VDAG_03530 and VDAG_06170 in the mutants compensate that of *ICSH1* transcripts, but this compensation is not apparent in presence of potato leaf extracts (**Figures 3.10, 3.11**).

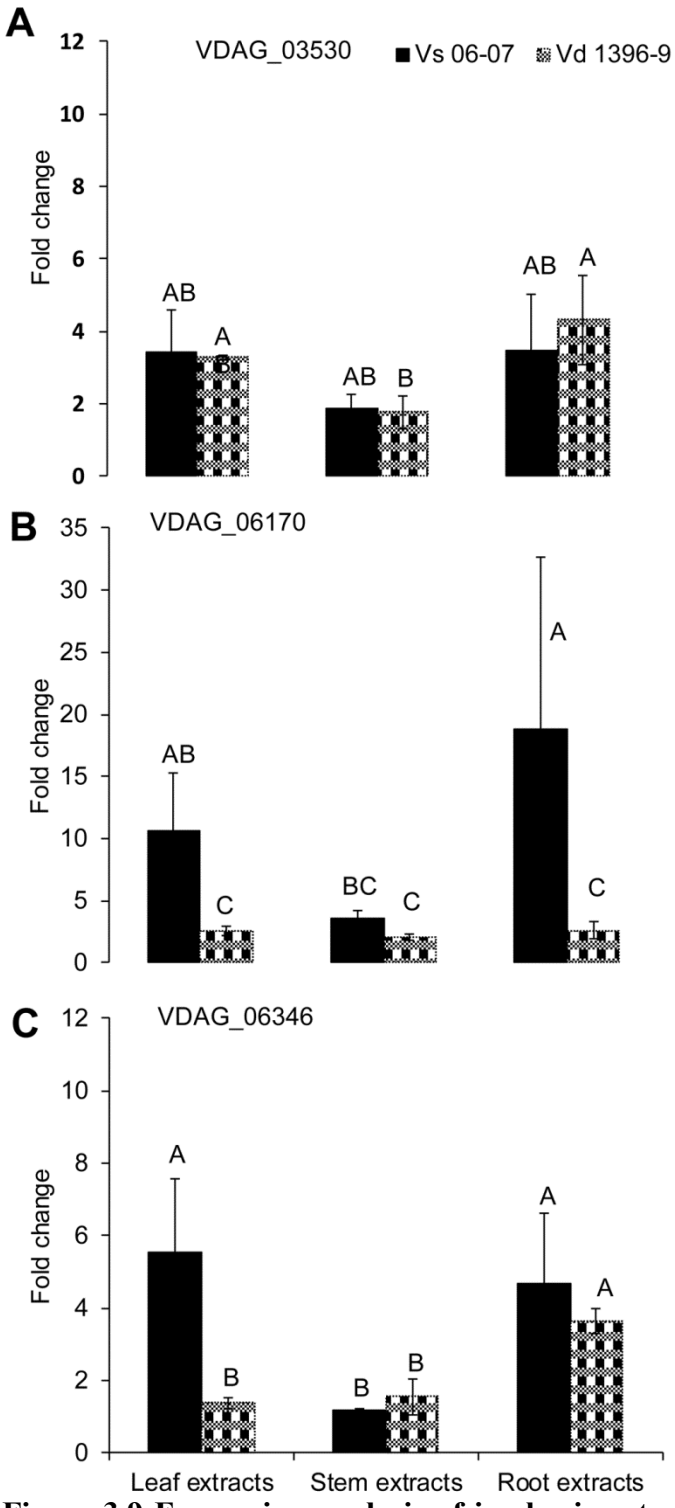


Figure 3.9 Expression analysis of isochorismatase family members in *V. dahliae* wild type isolates under elicitation with different potato tissue extracts.

V. dahliae highly aggressive isolate Vd1396-9 and weakly aggressive isolate Vs06-07 were elicited in liquid media by different potato tissue extracts. Sterilized distilled water was used as a control treatment. QRT-PCR data was normalized using *V. dahliae Histone H3*. (A) VDAG_03530; (B) VDAG_06170; (C) VDAG_06346. Each gene's expression data obtained from both isolates under

different treatments were analyzed using the $2^{-\Delta\Delta C_T}$ method relative to its corresponding isolate cultured in CDB medium with water. Bars represented by mean values (n = 3, with two technical replications) sharing the same letter are not significantly different from each other ($P < 0.05$).

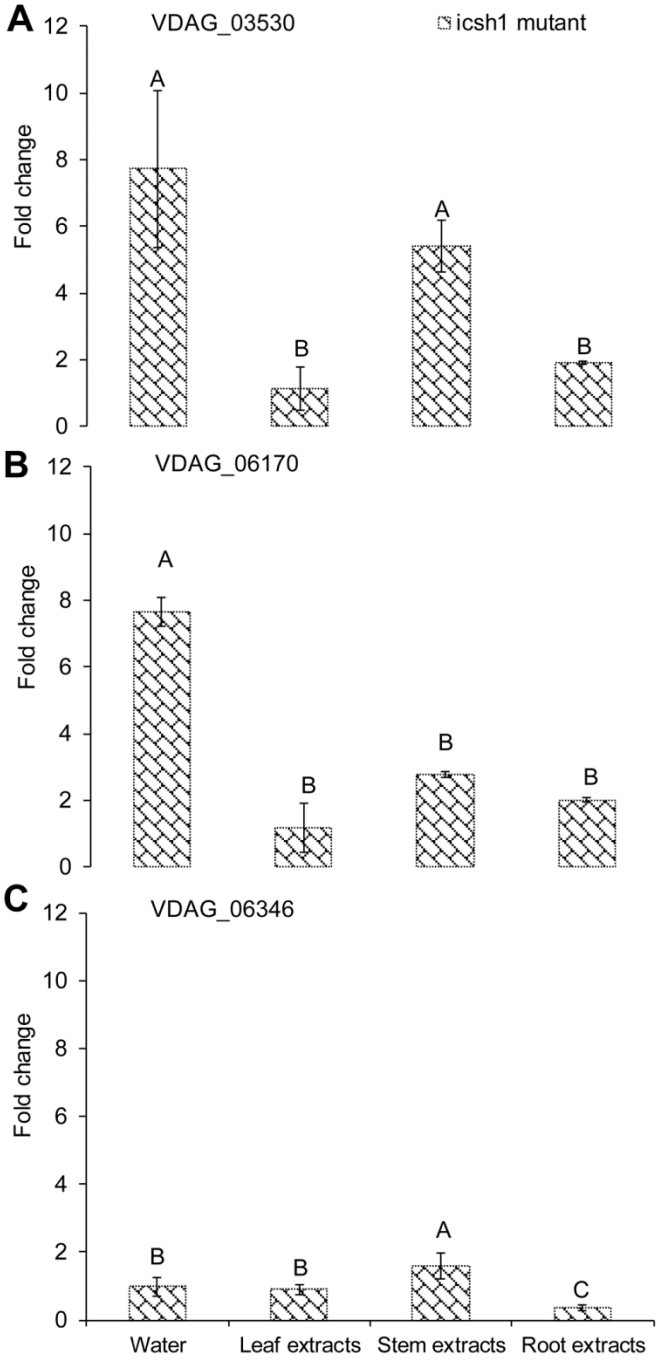


Figure 3.10 Expression of isochorismatase family members in *V. dahliae icsh1* mutant under elicitation with different potato tissue extracts.

V. dahliae wild type strain Vd1396-9 and mutant *icsh1-2-12-1* were elicited in liquid media by different potato tissue extracts. Sterilized distilled water was used as a control treatment. QRT-PCR data was normalized using *V. dahliae Histone H3*. (A) VDAG_03530; (B) VDAG_06170; (C) VDAG_06346. Each gene's expression data obtained with the *icsh1* mutant in CDB with water

control were analyzed using the $2^{-\Delta\Delta C_T}$ method relative to that of wild type Vd1396-9 cultured in CDB medium with water. Each gene's expression data obtained for the *icsh1* mutant under each treatment were analyzed using the $2^{-\Delta\Delta C_T}$ method relative to that of wild type Vd1396-9 cultured in CDB medium with the same treatment. Bars represented by mean values (n = 3, with two technical replications) sharing the same letter are not significantly different from each other ($P < 0.05$).

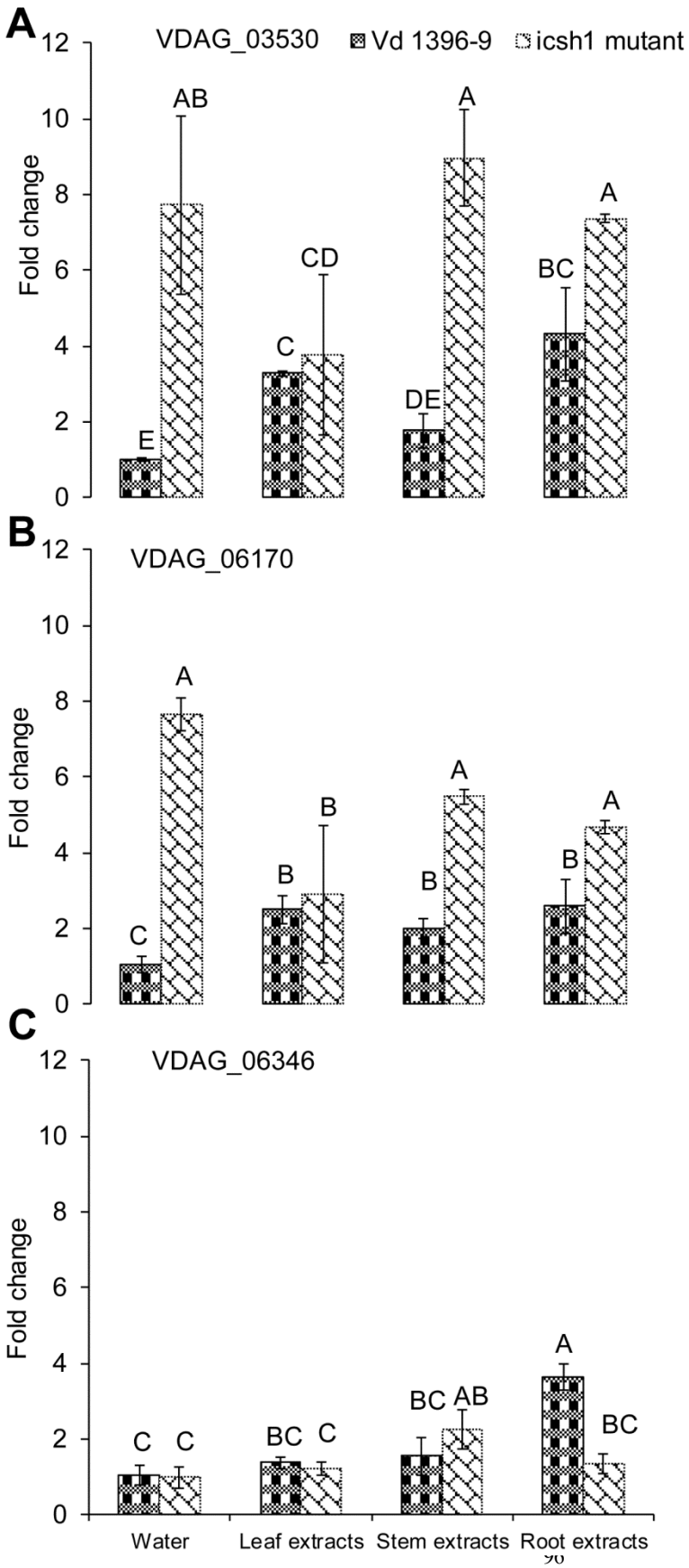

A
VDAG_03530
Vd 1396-9
icsh1 mutant
Fold change
12
10
8
6
4
2
0
AB
E
CD
C
A
DE
A
BC
B
VDAG_06170
Fold change
A
C
B
B
A
B
A
B
C
VDAG_06346
Fold change
C C
BC C
BC AB
A
BC
Water
Leaf extracts
Stem extracts
Root extracts

Figure 3.11 Expression of isochorismatase family members in the *V. dahliae icsh1* mutant and wild type strain Vd1396-9 under elicitation with different potato tissue extracts.
V. dahliae wild type strain Vd1396-9 and *icsh1-2-12-1* were induced in liquid media by different potato tissue extracts. Sterilized distilled water was used as a control treatment. QRT-PCR data was normalized using *V. dahliae Histone H3*. (A) VDAG_03530; (B) VDAG_06170; (C) VDAG_06346. Each gene's expression data obtained from the *icsh1* mutant and wild type strain Vd1396-9 under all treatments and control were analyzed using the $2^{-\Delta\Delta C}{}_{T}$ method relative to that of wild type Vd1396-9 cultured in CDB medium with water. Bars represented by mean values (n=3, with two technical replications) sharing the same letter are not significantly different from each other ($P < 0.05$).

3.3.7 SA and JA Quantification in Potato Plants under Infection

To determine the role of ICSH1 in altering the accumulation of SA and possibly JA, the *icsh1* mutant (*icsh1-2-12-1*) and wild type strain Vd1396-9 were selected for this experiment. The susceptible potato cultivar Kennebec was inoculated by root dip in a conidial suspension, while plant roots dipped in sterilized water were used as a control. The plant tissues were sampled at 4, 9, 14, 21, and 35 days after infection. Quantification of SA in different parts of potato plants after inoculation with *V. dahliae* wild type strain and the *icsh1* mutant was done using HPLC-Fluorescence. SA levels were significantly higher in all infected plant parts with both wild type and the *icsh1* mutant.

In the roots, the level of free SA in response to inoculation went higher at 14 DAI thereafter decreasing compared to the water control (**Figure 3.12C**). At early stages of leaf infection (9 DAI), bound SA accumulation was significantly higher in response to the *icsh1* mutant, compared to the wild type, whereas the opposite happened in the roots (**Figures 3.12D,F**). In stems, there were no significant differences between the SA accumulation in response to *V. dahliae* inoculation and water control until 35 DAI (**Figures 3.12B,E**). In leaves, SA levels were significantly higher in response to the wild type, compared to the *icsh1* mutant, starting from 21 DAI for the free SA and 35 DAI for the bound-SA (**Figures 3.12A,D**). JA was also quantified in leaf, stem and root tissues

of cultivar Kennebec after inoculation with *V. dahliae* wild type and *icsh1* mutant using UPLC-MSMS. There were no significant differences between plants inoculated with the wild type and the mutant in terms of JA induction in the stems and roots, which was less than in the wounded control (**Figures 3.12H,I**). In the leaves, however, both the mutant and wild type induced more JA than the control at 21 DAI. At 35 DAI JA levels in the wild type were significantly higher than the *icsh1* mutant or water control (**Figure 3.12G**).

In conclusion, potato accumulated SA in the roots at early infection stages and in the stems in both early and later stages, while the accumulation of SA and JA occurred in the leaves at later stages in response to *V. dahliae* infection. The SA and JA accumulation followed different trends in the roots and stems at the early stages, and similar trends in the leaves at later stages. The *icsh1* mutant induced a high level of bound-SA in the leaves only at 9 DAI, and thereafter decreased. Taken together, the results indicate that the wild type *V. dahliae* isolate induced more free-SA than the mutant in the leaves, and more bound-SA in the roots. However, in absolute value, the total SA induced by the mutant in the leaves, at 9 DAI, is higher than the free SA induced by the wild type in the roots, stems, and leaves, combined (**Figure 3.12**).

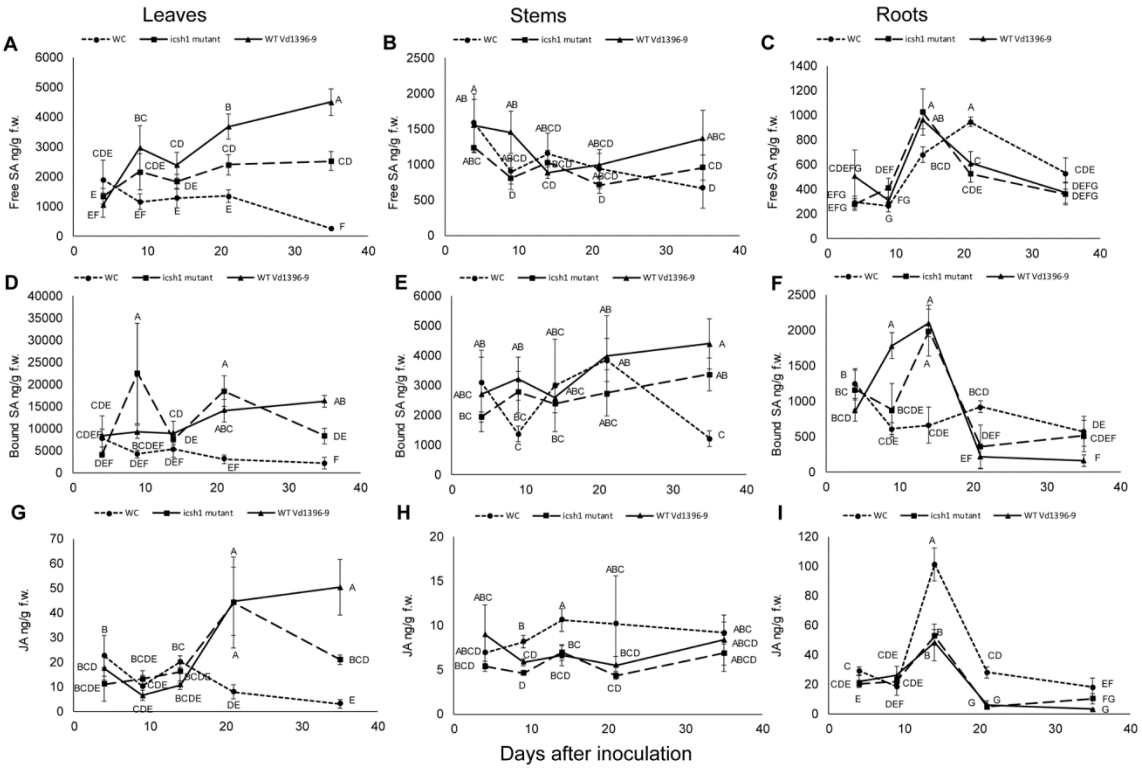


Figure 3.12 Quantification of SA and JA concentration in potato during infection. Susceptible potato cultivar Kennebec was inoculated with conidial suspensions from *icsh1*-2-12-1 and wild type strain Vd1396-9; sterilized water used as a control treatment (WC: water control). (A) Free SA in leaves; (B) Free SA in stems; (C) Free SA in roots; (D) Bound SA in leaves; (E) Bound SA in stems; (F) Bound SA in roots; (G) JA in leaves; (H) JA in stems; (I) JA in roots. Point values represented by mean values (n = 3) sharing the same letter are not significantly different from each other ($P < 0.05$).

3.4 Discussion

In the current study, *Vdicsh1* mutants of *V. dahliae* were generated and functionally characterized on potato and other host species. As we initially predicted the possible role of this gene as a virulence factor using proteomics studies (El-Bebany et al., 2010), *V. dahliae* ICSH1 was found to reduce SA synthesis in cotton (Liu et al., 2014), and in potato tissues in the present study. However, the intricate complexity of *V. dahliae*'s interaction with its hosts raises many questions regarding the mechanisms, and the spatio-temporal unfolding of the effects of ICSH1 during infection and colonization of the host.

The differential expression of *ICSH1* in highly vs. weakly aggressive *V. dahliae* isolates in culture as well as in detached potato leaves inoculated with this pathogen demonstrated its role as a pathogenicity factor on this host plant. The highest induction of *ICSH1* in response to extracts from the roots, compared to leaves and stems suggests the importance of this gene in establishing infection, since *V. dahliae* is a soilborne pathogen and germination of its microsclerotia is known to be stimulated by root exudates (Klosterman et al., 2009). Potato tissues responded to inoculation by activating the isochorismate synthase (ICS) encoding the key enzyme in the ICS pathway. The expression level of potato ICS was upregulated in the leaves but was not associated with the level of the pathogen aggressiveness (**Figure 3.1C**). Both PAL and ICS pathways are involved in SA synthesis (Pasqualini et al., 2003; Wildermuth et al., 2001). PAL mediated SA signaling pathway is arguably involved in potato early response to wounding and infection by *V. dahliae*, with *PAL1* expressed higher in moderately resistant potato Ranger Russet than in susceptible potato Kennebec in both leaves and roots and PAL2 only in the leaves (Derksen et al., 2013a). The activity of ICS, which would also result in SA accumulation, was maintained over time, but was higher 8 DAI (**Figure 3.1C**).

The reduced total AUDPC of percent infection and disease severity as a result of *icsh1* mutation seems to be associated with the reduction of *ICSH1* activity. Similar results were observed in a study on isochorismatase hydrolase (*icsh1*) mutants from *V. dahliae* on cotton and *Phytophthora sojae* on soybean, respectively. Silencing the expression of *V. dahliae VdIcs1* and *P. sojae PsIcs1* resulted in a change in the virulence of these pathogens (Liu et al., 2014). Together, these results demonstrate the importance of ICSH1 as a virulence factor in *Verticillium* and possibly other filamentous fungi. Unlike other virulence factors, ICSH1 protein lacks the transit peptide and the secretion route of this protein is still unknown (Bendtsen et al., 2004; Liu et al.,

2014). Proteomic analysis of several phytopathogenic fungi showed the presence of isochorismatase motif proteins in the secretome of phytopathogenic but not in non-pathogenic filamentous ascomycetes (Soanes et al., 2008). Several members of the ILH superfamily were also identified in bacteria; i.e., *Pseudomonas aeruginosa PchB* possesses IPL and CM, which catalyze the conversion of isochorismate into salicylate and pyruvate (Gehring et al., 1997; Künzler et al., 2005; Maruyama and Hamano, 2009; Parsons et al., 2003).

Plants respond to pathogens by various defense mechanisms. In response to *V. dahliae*, SA accumulates using both the PAL (Derksen et al., 2013a) and ICS pathways. The timing difference in PAL and ICS expression, along with the timing in SA accumulation in the roots vs. leaves is in agreement with the suggestion that the ICS pathway is associated with SAR, while the PAL pathway is essential for initiating cell death in the infection site (Gaffney et al., 1993; Holuigue et al., 2007; Pasqualini et al., 2003; Rairdan and Delaney, 2002). A study on *A. thaliana* has also shown that jasmonates play a central role in early SAR signaling before systemic SA accumulation (Truman et al., 2007). The JA pathway may also play a role in potato SAR response to *V. dahliae* invasion.

Under *V. dahliae* infection, potato roots accumulated higher amounts of free and bound SA than the water control at early stages, while JA was detected at lower levels in the infected potato roots, compared to wounded non-inoculated control. This indicated that potato roots may respond to *V. dahliae* by accumulating more SA and less JA at early stages of infection. However, it is not clear whether the pathogen is the one suppressing JA accumulation (**Figure 3.12I**). Potato stems also responded to *V. dahliae* by accumulating more SA and less JA. At later stages, the potato leaves responded to *V. dahliae* infection by accumulating both SA and JA. The antagonistic effect between JA and SA is known (Davies, 2010; Pieterse et al., 2009). If such antagonism occurred in

our experiments, that would explain the levels of SA and JA in the potato roots and stems in response to *V. dahliae* at early stages of infection. In potato leaves, at later stages, the response to *V. dahliae* involved both SA and JA accumulation.

Generally, the *icsh1* mutant induced a high level of accumulation of bound-SA in the leaves at 9 DAI. In *A. thaliana*, the SA is glucosylated by UDP-glucosyltransferases UGT74F1 and UGT74F2 into bound-SA in the cytosol (Dean and Delaney, 2008; Dempsey et al., 2011; Lim et al., 2002; Song, 2006). Bound-SA would then be transported and stored in the vacuole (Dempsey et al., 2011). Bound-SA could be hydrolyzed into SA in planta, but it is unclear whether bound-SA is biologically active as such (Dempsey et al., 2011). In tobacco, the hydrolysis of bound-SA into SA by extracellular glucosidases, followed by its injection into tobacco leaves induced the expression of PR-1 (Hennig et al., 1993). The bound-SA in potatoes may also be very important for plant defense, because most fungal plant pathogens are capable of producing hydrolases as part of their pathogenesis processes (Chen et al., 2008; Colen et al., 2006; Kawai et al., 2004; Williams and Orpin, 1987). The increase of bound- SA in the leaves inoculated with the *icsh1* mutant at 9 DAI may also indicate that bound-SA can be an important element for plant defense and needs to be overcome by *V. dahliae* for successful infection. Later, however, SA accumulated at lower levels in potato leaf tissues infected by *icsh1* mutant than those infected by wild type *V. dahliae* strain Vd1396-9. In potato roots, SA accrued in a similar manner in those infected by *V. dahliae* wild type strain Vd1396-9 and those infected by the *icsh1* mutant. This suggested that potato plants infected either by wild type strain or the *icsh1* mutant may compensate the depletion of the isochorismate hydrolyzed by the pathogen's ICSH. This strategy would take place partly by increasing export of chorismate from the plastids into the cytosol (Djamei et al., 2011), where chorismate is converted into isochorismate by ICS (Pasqualini et al., 2003). This may help to retain

the cellular homeostasis of isochorismate. This hypothesis also matches the fact that both ICS and PAL are activated during infection, because chorismate is also a precursor of the PAL pathway (Pasqualini et al., 2003). On the other hand, increasing the expression of other genes from isochorismatase family such as VDAG_03530 and VDAG_06170 in the *icsh1* mutant may compensate for the lack of the ICSH1 activity, but this compensation seems inhibited in presence of the potato leaf extracts, and this may also explain that the *icsh1* mutant induced a high level of accumulation of bound-SA in the leaves at 9 DAI. Oppositely, in the *V. dahliae* cotton and *P. sojae*-soybean pathosystems, SA accumulated at a significantly higher level in the plant roots after infection with *VdIcs1* and *PsIcs1* mutants. This may be due to the lack of activity compensation for the isochorismatase hydrolase, which hydrolyzes the isochorismate and suppresses SA biosynthesis (Liu et al., 2014). In the present study, it is intriguing that the effects of *icsh1* mutation were observed on potato, as the host of origin of the highly aggressive wild type tested strain, but not on sunflowers or tomatoes. This calls for more studies on such associations and the level of specificity observed in these responses.

The present study offers a more dissected analysis of the potential roles of ICSH1 in *V. dahliae*'s pathogenesis processes and sheds more light into its effect on the complex potato signaling in response to this important wilt pathogen.

CHAPTER 4

NOX FAMILY GENES ARE IMPORTANT FOR *VERTICILLIUM DAHLIAE*'S PENETRATION ABILITY AND VIRULENCE IN HOST

Abstract

NADPH oxidase (Nox) genes are responsible for ROS production in living organisms such as plants, animals, and fungi, where ROS exert different functions. ROS are critical for sexual development and cellular differentiation in fungi. Two genes encoding thioredoxin and NADH-ubiquinone oxidoreductase involved in maintaining ROS balance were previously found to be remarkably induced in a highly aggressive *V. dahliae* isolate compared to a weakly aggressive one. This suggested a potentially important role of ROS in the virulence of *V. dahliae*. Three Nox (NADPH oxidase) family genes (*NoxA*, *NoxB*, and *NoxC*) were identified in *Verticillium dahliae*'s genome. We compared *in vitro* expression of *NoxA*, *NoxB*, and *NoxC* genes in highly and weakly aggressive isolates of *V. dahliae* after elicitation with extracts from different potato tissues. *NoxB* expression was significantly higher in the highly than the weakly aggressive isolate in response to potato leaf and root extracts, whereas *NoxA* expression was more induced in the weakly aggressive isolate than in the highly aggressive one in response to leaf and stem extracts. *NoxC* expression responded more strongly in the highly aggressive isolate to elicitation with stem extracts, whereas elicitation with root extracts induced a higher *NoxC* expression in the weakly aggressive isolate. After inoculation of detached leaves from a susceptible potato cultivar with these two *V. dahliae* isolates, both *NoxA* and *NoxB*, but not NoxC, were drastically up-regulated in the highly aggressive isolate compared to the weakly aggressive one. Furthermore, we generated the single gene disruption mutant for each of these genes. Pathogenicity tests showed that both *noxa* and *noxb* mutants had significantly reduced virulence, indicating important roles of both genes in *V. dahliae* pathogenesis on potato. This is consistent with significantly reduced penetration ability of *noxa* and *noxb* mutants tested on cellophane membrane compared to the wild type. The cell wall integrity was also impaired in the *noxb* mutants, compared with the wild type. Interestingly, *noxb*

mutants displayed more resistance to oxidative stress in solid medium.

4.1 Introduction

Potato early dying (PED) is a common problem in potato (*Solanum tuberosum*) production (Powelson and Rowe, 1993). The yield loss caused by PED can be up to 30-50% of total production (Cappaert et al., 1992; Davis, 1981; Nnodu and Harrison, 1979). The primary causal agent of PED are two different *Verticillium spp.*, *Verticillium dahliae* Kleb and *Verticillium albo-atrum* Reinke & Berthold. *V. albo-atrum* was first identified on potato by Reinke and Berthold in 1879 and *V. dahliae* was firstly identified on dahlia (*Asteraceae* family) by Klebahn in 1913 (Barbara and Clewes, 2003). *V. dahliae* can produce resting structure-microsclerotia that can keep viability in the soil for 10-15 years (Rowe and Powelson, 2002; Wilhelm, 1955). *V. dahliae* interacts with the root-lesion nematode *Pratylenchus penetrans* (Cobb) Filipjev & Schuur. Stekh., which has been shown to facilitate PED in North America (Hanmer, 1995; Martin et al., 1982; Rowe and Powelson, 2002). Root-lesion nematodes cause an increase in root branching and enhance *V. dahliae* and root contact to aid in vascular colonization (Bowers et al., 1996; Rowe and Powelson, 2002). Plants inoculated with both *V. dahliae* and *P. penetrans* showed a higher percentage of root tip infection than those inoculated with *V. dahliae* alone, indicating that *V. dahliae* and *P. penetrans* may interact to affect host physiology and plant defense responses (Bowers et al., 1996).

One of the key factors in controlling PED is to reduce the primary inoculum, by preventing the germination of microsclerotia and decreasing their production in soil (Molina et al., 2014). Crop rotation has been shown to be an effective control management practice for microbial pathogens in many other economic crops (An et al., 1993; Erbs et al., 2010; Havlin et al., 1990; Lupwayi et al., 1998; Martin-Rueda et al., 2007). However, *V. dahliae* infects more than 200 dicotyledonous plant hosts (Agrios, 2005; Pegg and Brady, 2002), including many economically

valuated crops such as potato, tomato (*Lycopersicon esculentum*), cabbage (*Brassica oleracea*), eggplant (*Solanum melongena*), cauliflower (*Brassica oleracea*), cotton (*Gossypium hirsutum*), bell pepper (*Capsicum annuum*), chili pepper (*Capsicum annuum*), and lettuce (Lactuca sativa) (Bhat and Subbarao, 1999), which makes crop rotation not an effective method to control this disease.

To date, there is no known treatment that can completely inhibit the disease or help recover the yield of affected crops (Klosterman et al., 2009). Green manures such as Austrian winter pea (*Pisum sativum*), broccoli, Sudan grass (*Sorghum vulgare*) and corn (*Zea mays*), could suppress symptoms by 60-70% and partly recover potato yields (Davis et al., 2010; Ochiai et al., 2008). Chitin and chitosan, which originate from marine crustaceans, also help protect plants from pathogen infections via activation of the host defense (El Hadrami et al., 2010). However, the effect of green manure is usually unpredictable and inconsistent, and the mechanisms of suppression of *V. dahliae* microsclerotia is different under various conditions (Molina et al., 2014). Moreover, green manures do not decrease and may even raise the amount of *V. dahliae* microsclerotia in the soil (Collins et al., 2006). Broccoli (*Brassica oleracea* Italica group) residues suppress *V. dahliae* and reduce both the amount of microsclerotia in soil (Shetty et al., 2000; Subbarao et al., 2007), and wilt symptoms of cauliflower (Subbarao et al., 1999). Glucosinolates, phenolic compounds, and lignin in broccoli may be critical for the suppression of *V. dahliae*, which however may not exhibit the same effect on other crops (Klosterman et al., 2009; Matthiessen and Kirkegaard, 2006). Many biocontrol agents can reduce microsclerotia and Verticillium wilt, but yields of infected crops were only partly recovered compared to healthy plants (Antonopoulos et al., 2008; Daayf, 2015; Tjamos et al., 2004; Uppal et al., 2008). In tomato, a *Ve*-gene that modulates resistance to race 1 of *V. dahliae* and *V. albo-atrum*, was identified and introduced into other tomato

cultivars (Fradin et al., 2009; Kawchuk et al., 2001). Introduction of the wild relative eggplant (*Solanum torvum*) *StVe1* gene into potato partially increased resistance to *V. dahliae* (Liu et al., 2012). In potato, a *StVe1* locus was identified in chromosome 9, however this quantitative trait locus contains multiple genes (at least 11 genes) and it is still not clear if a single gene or multiple genes provide resistance to *V. dahliae* and *V. albo-atrum* (Simko et al., 2004a; Simko et al., 2004b). Soil fumigation has been reported to be the most effective strategy for controlling *V. dahliae* (Duniway, 2002; Rowe and Powelson, 2002; Tsror et al., 2005; Wilhelm and Ferguson, 1953; Wilhelm et al., 1961). The high cost along with environmental and health problems associated with some fumigants have made it necessary to find alternative methods to control the disease. (Ajwa et al., 2002; Davis et al., 1996; Martin, 2003; Rowe and Powelson, 2002).

Since none of the current control methods represents a perfect choice for controlling Verticillium wilt, it is important to find a strategy to reduce the amount and germination of microsclerotia so as to reduce penetration and inhibit host colonization by *V. dahliae*. In the past 14 years, research has shown the important functions of reactive oxygen species (ROS) in 1) fungal pathogen penetration and host colonization, 2) normal spore germination, 3) mycelium polarized growth and differentiation, 4) sexual development and fruiting body formation, 5) nutrition transformation under starvation conditions, and 6) germination of the pathogen resting structure, such as sclerotia in *Sclerotinia sclerotiorum* (Cano-Domínguez et al., 2008; Eaton et al., 2011; Egan et al., 2007; Giesbert et al., 2008; Kim et al., 2011; Malagnac et al., 2004; Rolke and Tudzynski, 2008; Wang et al., 2014; Zhang et al., 2016). ROS can be generated by both non-enzymatic and enzymatic systems (Heller and Tudzynski, 2011). Mitochondria are the primary source of non-enzymatic ROS production (Heller and Tudzynski, 2011). The activity of NADPH oxidase (Nox) is the main source of enzymatic ROS production (Tudzynski et al., 2012). In various

organisms, the Nox protein with $FADH_2$ and heme as cofactors can transport the electrons from NADPH to oxygen to produce ROS (Bedard et al., 2007; Tudzynski et al., 2012). Fungi contain one or more of three types of Nox homologues: NoxA, NoxB, NoxC (Takemoto et al., 2007). The structures of NoxA and NoxB are similar to mammalian gp^{91phox} (Lara-Ortíz et al., 2003; Malagnac et al., 2004; Tanaka et al., 2006), besides an extra 40 amino acids motif at the N-termini of NoxB (Malagnac et al., 2004; Tanaka et al., 2006). The structure of fungal NoxC is similar to mammalian Nox5 (Lewit-Bentley and Réty, 2000). The full function of NoxA and NoxB requires formation of a Nox complex for activation. However, there is no evidence to show that NoxA or NoxB could synchronously interact with all the regulatory subunit in fungi (Tudzynski et al., 2012).

In *Magnaporthe oryzae*, Nox-producing ROS is essential for full development of the infectious cell, called an appressorium, and pathogenicity on rice (Egan et al., 2007). In *Podospora anserina* and *Neurospora crassa*, the *nox1* mutants cannot differentiate the fruiting bodies properly and produce significantly less ascospores than the wild type (Malagnac et al., 2004), while *nox2* mutants can produce ascospores but none of them can germinate (Cano-Domínguez et al., 2008; Malagnac et al., 2004). In *Sclerotinia sclerotiorum*, SsNox1 and SsNox2 have been identified and are responsible for ROS production. Both SsNOX1 and SsNOX2 are required for sclerotial formation, while SsNOX1 is also essential for virulence (Kim et al., 2011). In *Fusarium graminearum*, NoxA is critical for ROS production during perithecia development and ascospore production, pathogenic development on wheat (Wang et al., 2014). In *Claviceps purpurea*, Nox1 is essential for conidial germination, mature sclerotia development and virulence (Giesbert et al., 2008). In *Botrytis cinerea*, functional characterization of *BcNOXA* and *BcNOXB* showed that ROS generated by both NoxA and NoxB is essential for virulence and development of sclerotia (Segmüller et al., 2008). In *Aspergillius nidulans*, deletion of *NoxA* affected the sexual

development by blocking the formation of mature cleitothecia fruiting bodies (Lara‐Ortíz et al., 2003). In *Epichloë festucae,* NoxA as well as its signal regulator RacA, and NoxR play critical roles in controlling mutualistic symbiotic interaction between *E. festucae* and the host perennial ryegrass (Eaton et al., 2011). Recent studies showed VdNoxB, identified in a *V. dahliae* cotton isolate, was required for Ca^{2+} accumulation in hyphopodia via NoxB-produced ROS and activity regulation of the transcription factor VdCrz1 in the control of the penetration peg development on cotton (Zhao et al., 2016).

Taken together, Nox enzyme-producing ROS in various fungal species play important roles in penetration, colonization, and pathogenic development in the host, as well as development of resting or over-wintering structures. The management of PED on potato relies on the control of the primary inoculum and reduction of microsclerotia in the soil. Therefore, we speculate that ROS generated by the Nox family is important for the interaction with potato. El-Bebany et al. (2010) identified two proteins, Thioredoxin and NADH-ubiquinone oxidoreductase, which function in maintenance of ROS balance in the cell (Bazil et al., 2014; Huang et al., 2015; Kussmaul and Hirst, 2006). In a proteomic analysis, both proteins were only detected in a highly aggressive isolate of *V. dahliae* but not in a weakly aggressive one (El-Bebany et al., 2010). According to our unpublished data, NADPH oxidase (NOX) was also involved in the pathogenicity-related pathway in *V. dahliae*. All of this indicates that ROS in *V. dahliae* may be critical for pathogenicity-related processes. Even though NoxB has been proven to be involved in penetration peg formation in a cotton isolate (Zhao et al., 2016), the interaction with different host species may vary (Klosterman et al., 2009; Zhu et al., 2017). In the present study, we aimed to investigate the function of *Nox* gene family members in the highly aggressive *V. dahliae* isolate Vd1396-9 during potato infection. According to Klosterman et al. (2011), *V. dahliae* contains three *Nox* isoforms: *NoxA*

(VDAG_06812.1), *NoxB* (VDAG_09930.1) and *NoxC* (VDAG_0032.1). The objectives for this study are to: 1) investigate the transcriptional activity of *V. dahliae*'s *Nox* gene family members (*NoxA, NoxB,* and *NoxC*) during both elicitation with host plant tissue extracts and during infection; 2) generate individual gene disruption mutants for the three *Nox* genes in *V. dahliae* and analyze their phenotypes; 3) assess the roles of three *Nox* genes in pathogen virulence and during the interaction with potato; and 4) determine their roles in cell wall biosynthesis and response to oxidative and osmotic stress.

4.2 Materials and methods

4.2.1 *V. dahliae* isolates and plant materials

Vd1396-9 and Vs06-07 have been identified as highly and weakly aggressive *V. dahliae* isolates, respectively (Alkher et al., 2009; Uppal et al., 2007). They were isolated from a potato tuber and sunflower stem tissue, respectively. Both isolates were grown on potato dextrose agar (PDA) media at 23±1°C for 21 days. Culture plates were flooded with sterilized water, and then spores were collected for each isolate and counted using a hemacytometer counting chamber (Fisher Scientific), followed by concentration adjustment according to different experimental requirements.

The potato cultivar Kennebec, which is susceptible to Verticillium wilt, was used in this study (Alkher et al., 2009). Plants were grown in a mix of sand, soil and peat moss (12:4:1) in a greenhouse growth room at a 22/18 °C day/night temperature regimen with a 16/8 h light/dark photoperiod.

4.2.2 *Nox family* genes expression in response to infection and during elicitation with potato

tissue extracts

NoxA/ NoxB/ NoxC expression in both Vd1396-9 and Vs06-07 were measured during infection on detached Kennebec potato leaves following the method descripted by Zhu et al. (2017). Briefly, conidia of Vd1396-9 and Vs06-07 were washed from PDA plates then adjusted to the concentration of 3×10^7 conidia/mL, and then inoculated on detached susceptible potato leaves (Kennebec). Samples of detached leaves were taken at 1, 3, 5 and 8 days after inoculation (DAI). Additionally, gene expression in both isolates under elicitation of Kennebec potato leaves, stems, or roots extracts was determined by qRT-PCR following the method described by Zhu et al. (2017). Briefly, 10^8 conidia were cultured in Czapek-Dox Broth (CDB) media (Difco Laboratories, Sparks, MD, USA) for one week. Each isolate was then treated with the addition of 1 ml of potato leaf, stem, or root extract for one week. Samples of fungal mycelium were collected and subjected to RNA extraction and Real time PCR analysis following recommended protocols of Omega Fungal RNA kit (Omega Bio-Tek, Inc., Norcross, GA, USA) and SsoFast EvaGreen Super mix (Bio-Rad Lab, Philadelphia, PA, USA).

4.2.3 Gene disruption of *V. dahliae*

The *NoxA* (VDAG_06812.1), *NoxB* (VDAG_09930.1) and *NoxC* (VDAG_0032.1) gene T-DNA insertion constructs were created based on pDHT vector (Mullins et al., 2001) following the description specified by Zhu et al. (2017), with primers listed in (**Table 4.1**). The constructs were transformed into Vd1396-9 conidia mediated by *Agrobacterium tumefaciens* following the description by Zhu et al. (2017) and Dobinson et al. (2004). Transformants were selected according to the method described by Zhu et al. (2016) and Zhu et al. (2017) in PDA media containing hygromycin B. The positive gene insertion mutants were confirmed by PCR with primer pairs

NoxA/ NoxB/ NoxC-UA-F and NoxA/ NoxB/ NoxC-HindIII-R (**Table 4.1**).

The single locus insertion in the *V. dahliae* genome of the *noxa/noxb/noxc* mutants was confirmed by southern blot with specific probe and restriction enzyme show in (**Table 4.2**). The gene duplication of the NoxA/ NoxB/ NoxC in the *V. dahliae* genome was also checked by Southern blot with a specific probe and restriction enzyme as show in (**Table 4.2**). For DNA extraction, the *V. dahliae* mycelia were collected after one-week culture in CDB liquid media. The DNA was extracted according to the protocol descripted by Al-Samarrai and Schmid (2000). The southern blot, probe hybridization, detection and signal visualization were processed following the description by Zhu et al. (2017) and Maruthachalam et al. (2011).

Table 4.1 Primers used in generating mutants for Nox family genes

Primer's name	Primer sequence	Tm (°C)	Accession number
NoxA-HindIII-F	CCCAAGCTTATGCCTCTCGCCAACCTTT	59.5	VDAG_06812
NoxA-IHindIII-R	CCCAAGCTTTCAGAAATGCTCCTTCCAGAAA	59.3	VDAG_06812
NoxA-UA-F	CCCTCGCCTGACGGGATT	62.7	VDAG_06812
NoxB-HindIII-F	AAGCTTATGGACTACTACTCGATGGGCTC	61.5	VDAG_09930
NoxB-IHindIII-R	AAGCTTCTAGAAGTTCTCCTTGCCCCATT	60.1	VDAG_09930
NoxB-UA-F	CGATAGCCGCTTACGAT	51.6	VDAG_09930
NoxB-probe-F	ACATCCGTTCTGAGCGAC	53.1	VDAG_09930
NoxB-probe-R	CGTCCAGGCTAGGTAGAGTT	53.8	VDAG_09930
NoxC-HindIII-F	CCCAAGCTTATGGCTGAACACCCCGT	59.5	VDAG_00032
NoxC-IHindIII-R	CCCAAGCTTCAATCTTGCTGCCGTCAT	59.3	VDAG_00032
NoxC-UA-F	CGCCGTACCGTTGGAGACT	60.7	VDAG_00032
Hph-YG-F (Pasquali et al., 2013)	GATGTAGGAGGGCGTGGATATGTCCT	61.5	Hph gene
Hph-F (Maruthachalam et al., 2011)	TCAGCTTCGATGTAGGAGGG	55.6	Hph gene
Hph-R (Maruthachalam et al., 2011)	TTCTACACAGCCATCGGTCC	56.5	Hph gene

Note: *Hph* gene: hygromycin resistant gene

Table 4.2 Primers used in southern blot for identified the mutants of Nox family genes

Objectives	target gene	primers	Restriction enzyme
Determine the number of hygromycin resistant gene insertion	NoxA	NoxA-HindIII-F+Hph-YG-F	XhoI
	NoxB	Hph-F/R	EcoRI
	NoxC	NoxC-ORF1-R+Hph-YG-F	XhoI
Determine gene duplication of target gene in the *V. dahliae* genome	NoxA	NoxA-HindIII-F+Hph-YG-F	XhoI
	NoxB	NoxB-probe-F/R	EcoRI
	NoxC	NoxC-ORF1-R+Hph-YG-F	XhoI

4.2.4 Growth rate and conidiation of *nox* mutants

The *V. dahliae* mutants for *NoxA* gene (*noxa-im-1*, *noxa-im-5*, and *noxa-im-7*), the ectopic insertion strains for *NoxA* gene (*NoxA-Ect-3*) (randomly inserting in *V. dahliae* genome but without replacing the original *NoxA* ORF), mutants for *NoxB* gene (*noxb-im-1*, *noxb-im-2*, and *noxb-im-5*), mutants for *NoxC* gene (*noxc-im-3-1*, *noxc-im-3-3*, and *noxc-im-7-1*), the ectopic insertion strains for *NoxC* gene (*NoxC-Ect-3-13*), and the empty vector control insertion strain (an empty pDHt vector, instead of mutation vector, was transformed into Vd1396-9) together with the wild type Vd1396-9, were grown on PDA for 14 days. The growth rate of the colony and the conidia concentration were determined according to the description of Zhu et al. (2017).

4.2.5 The pathogenicity analysis of *nox* family genes mutants

Potato plants (cv. Kennebec) were grown in soil-less mix (LA4 - SunGro Horticulture, Agawam, MA 01001, USA) for one week, and then plants were gently uprooted and approximately one cm long root tips were trimmed and immediately placed in conidial suspensions at concentration of 10^6 conidia/mL using the following mutants, *noxa-im-1*, *noxa-im-5*, *noxa-im-7, NoxA-Ect-3, noxb-im-1*, *noxb-im-2*, *noxb-im-5, noxc-im-3-1*, *noxc-im-3-3*, *noxc-im-7-1, NoxC-Ect-3-13* and the wild type. Sterile water was used as a control treatment. After a 30-seconds inoculation treatment with a conidial suspension, infected plants were re-planted in a pasteurized sand, soil and peat moss mixture with a ratio of 16:4:1. Each treatment contained five biological replicates. The total area under disease progress curve (AUDPC) of percentage infection and disease severity, together with plant growth rate were determined according to Zhu et al. (2017). The stem vascular discoloration was recorded in the last week of assessment according to Alkher

et al. (2009).

4.2.6 Cell wall biosynthesis and response to stress conditions

Calcofluor white can be used as a specific fungal chitin marker, which combines with fungal polysaccharides and changes the assembly of chitin fibrils in the fungal cell wall (Elorza et al., 1983). To assess the role of *Nox* family genes in cell wall biosynthesis, *noxa, noxb*, *noxc* mutants and wild type *V. dahliae* were cultured on solid CDB medium containing calcofluor white with concentration at 0, 50, 90, or 150 $\mu g.ml^{-1}$. Oxidative stress resistance was determined on the mutants and wild type by culturing strains on solid CDB medium with H_2O_2 concentrations at 10 mM, 20 mM, and 30 mM. To determine the response to osmotic stress, mutants and wild type were cultured on solid CDB medium containing 0.8 M NaCl. The strain diameter on various treatments were measured after culturing for 10 days to estimate the inhibition rate under various stress levels following the description of Guo et al. (2011).

4.2.7 Penetration and germination abilities of *Nox* gene family mutants on cellophane membrane

The fungal penetration assay on cellophane membrane was conducted following the method described by Wang et al. (2014). All mutants and wild type *V. dahliae* strains conidia suspension (10^5 conidia/ml) were cultured on cellophane membranes placed on solid CDB media. After culturing for either 5 or 21 days, the cellophane membranes were removed and the cultured plates were placed in 23±1°C for an additional 4 days. Isolates that successfully penetrated mycelium exhibited growth on the solid CDB medium.

The germination ability of all *nox* mutants and of the wild type strain were observed under

microscopy at 24h, after a conidia suspension (10^5 conidia/ml) of each isolate was placed on cellophane membranes laying on top of solid CDB medium.

The ability of all isolates to form penetration pegs were also observed under microscopy, 72h after a conidial suspension was placed on cellophane membranes on top of solid CDB medium.

4.2.8 Formation of conidiophores of *Nox* gene family mutants

All *nox* mutants and the wild type *V. dahliae* strain were cultured on PDA media for 2 weeks, after which a hole (1cm) was cut and observed following 48h culturing under the same conditions. The conidiophores of each isolate were observed on the edge of the hole under microscopy.

4.2.9 Statistical Analysis

SAS Statistical Analysis Software (SAS Institute, Cary, NC, USA; release 9.1 for Windows) with PROC MIXED program was used for statistical analysis of all data in this study. All data qualified for normal distribution with Shapiro–Wilk test (>0.9) determined by the PROC UNIVARIATE program. They also qualified for homogeneity established on comparison between residuals and studentized residual critical values (Lund, 1975). Some series of data were treated with Log^{10} transformation before analysis when necessary. Mean values were separated according to least squared means and results were assigned a group of letters by the macro PDMIX800.sas (Saxton, 1998) with $\alpha = 0.05$. Results assigned with different letters indicate significant differences between tests ($P<0.05$).

4.3 Results

4.3.1 Expression of *Nox* gene family members in *V. dahliae* in response to potato extracts and infection

ROS plays an important role in the virulence development of several phytopathogens. The role of ROS produced by *Nox* genes (*NoxA*, *NoxB*, and *NoxC*) was investigated through their expression in both *V. dahliae* highly and weakly aggressive isolate Vd1396-9 and Vs06-07 during the infection on potato or under elicitation with potato tissue extracts. The expression of *NoxA* was higher in the weakly aggressive isolate Vs06-07 under the elicitation of leaf and stem extracts (**Figure 4.1A**), whereas the expression of *NoxB* was higher in the highly aggressive isolate Vd1396-9 in response to leaf and root extracts (**Figure 4.1C**). *NoxC* transcriptionally responded more in the highly aggressive isolate Vd1396-9 to stem extracts, but showed the opposite expression pattern under elicitation of root extracts (Figure 1E). During the infection on detached potato leaves, both *NoxA* and *NoxB* were significantly induced in the highly aggressive isolate compared to that in the weakly aggressive one (**Figures 4.1B & D**). *NoxC* transcriptionally responded similarly to infection on detached leaves in both isolates (**Figure 4.1E**).

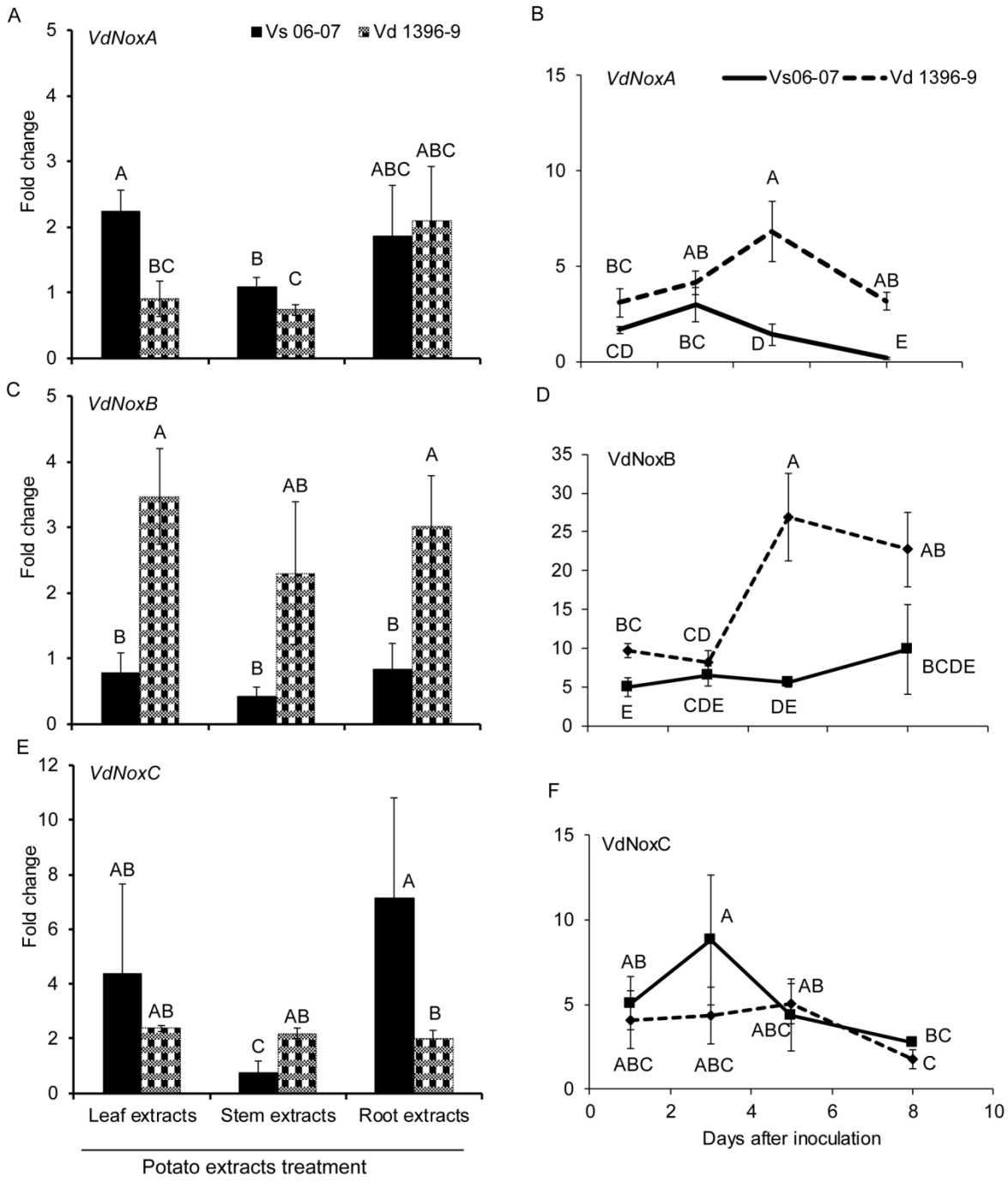


Figure 4.1 Expression of *Nox* gene family members in *V. dahliae* under elicitation or during the infection.

(A, C and E) Expression of *NoxA* (A), *NoxB* (C) and *NoxC* (E) in response to potato leaves, stems and roots extracts. The highly (Vd1396-9) and weakly (Vs06-07) aggressive isolate were cultured in liquid CDB medium added with potato leaves, stems or roots extracts. Sterilized distilled water was added into culture medium as a control treatment. Both Vd1396-9 and Vs06-07 cultured in the CDB medium with water were used as calibrators. The *V. dahliae* Histone H3 gene was employed as the internal control for normalizing all qRT-PCR data. The expression data for each

gene in selected isolate in response to treatments were analyzed with $2^{-\Delta\Delta C_T}$ method, in relation to water treatment as the control group. The bars showed as mean values (n = 3) with different letters were significantly different between treatments (P<0.05). Error bars refer to standard error. (B, D and F) Expression of NoxA (B) or *NoxB* (D) or *NoxC* (F) during the infection on detached Kennebec potato leaves. Four to six pieces of 4-weeks-old Kennebec potato detached leaves from different individual plant were combined as one sample after inoculation by highly (Vd1396-9) or weakly (Vs06-07) aggressive isolate using 10^8 conidia/ml. Sterilized distilled water was mocked to inoculate the detached leaves as the control treatment. Three combining samples were prepared for each treatment at each time point (1, 3, 5 and 8 days after inoculation, DAI). Both Vd1396-9 and Vs06-07 cultured in CDB medium were used as the calibrators. The *V. dahliae* Histone H3 gene was employed as the internal control for normalizing all qRT-PCR data. The expression data for each gene during the infection were analyzed with $2^{-\Delta\Delta C_T}$ method, in relation to isolate cultured in CDB medium as the control group. The point values showed as mean values (n = 3) with different letters were significantly different between treatments (P<0.05). Error bars refer to standard error.

4.3.2 Generation of gene insertion mutants for *Nox* family members

To investigate the functions of *Nox* gene family members in *V. dahliae* during its interaction with potato, individual gene insertion mutants were generated, respectively. The sequencing data showed that the insertion events occurred at No. 789bp of *NoxA* ORF, No. 814 bp of *NoxB* ORF, and No. 448bp or No. 403bp of *NoxC* ORF (note: two mutation vectors were generated for *NoxC* insertion) in the gene disruption mutants. Transformants for each gene were firstly screened by PCR, which identified 13 positive transformants for *noxa* gene mutants (**Figures 4.2. A & B**), ten positive transformants for *noxb* gene mutants (**Figures 4.2. C & D**) and 10 positive transformants for *noxc* gene mutants (**Figures 4.2. E & F**). To determine the insertion number of DNA cassette containing the hygromycin resistant gene (*hph*) and gene duplication of *Nox* family members in the *V. dahliae* genome, the positive transformants for each gene were randomly selected for Southern blot. The number of DNA cassette insertion and gene duplication of *NoxA* and *NoxC* were determined using the same probe containing part of DNA fragment of *NoxA* or *NoxC* ORF and part of DNA fragment of the hygromycin resistant gene. Our results showed that all of 7 selected transformants of *noxa* mutants (**Figure 4.2G**) and 9 transformants of *noxc* mutants

(**Figure 4.2J**) were single-insertion mutants for the corresponding gene, and both *NoxA* and *NoxC* genes were in single copy in the *V. dahliae* genome (**Figures 4.2 G & J**). The number of DNA cassette insertion of in *Noxb* transformants were identified with a probe amplified from the hygromycin resistant gene which showed all of 9 selected transformants were all single-insertion mutants (**Figure 4.2H**). The number of *NoxB* gene duplication was identified with a probe amplified from the *NoxB* ORF and showed there was only one *NoxB* gene copy in the *V. dahliae* genome (**Figure 4.2I**).

All Nox gene mutants confirmed by sequencing and Southern blot analysis made up the population from which individual mutants were randomly selected for pathogenicity tests.

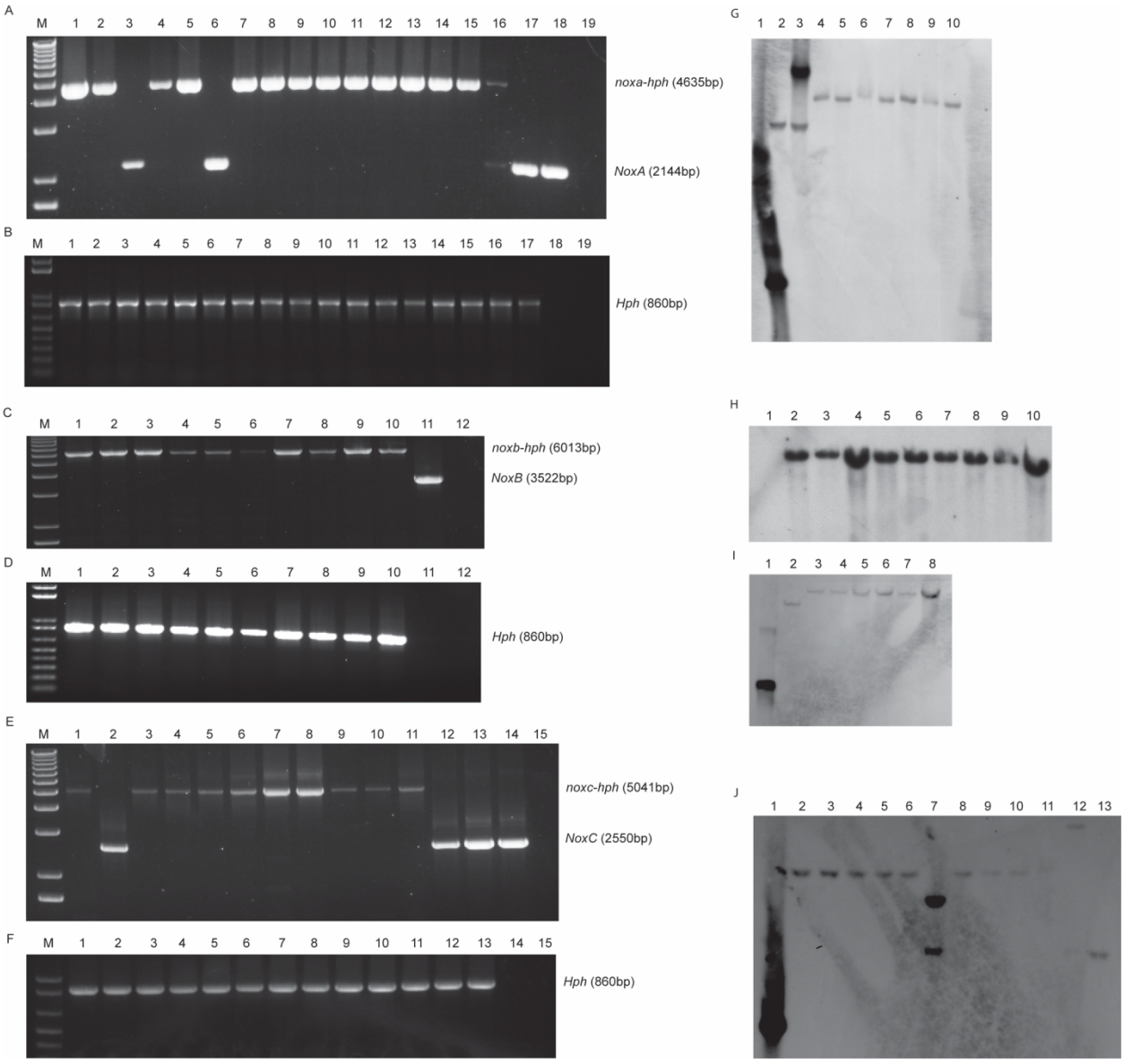


Figure 4.2 Identification of Nox family genes mutants by PCR and southern blot.
(A and B) PCR analysis of transformants for *NoxA* gene insertion. Lane M represents the markers, lane 1 to 17 represent the transformants, lane 18 represents the genomic DNA of wild type strain Vd1396-9, and lane 19 represents the negative water control for PCR. *noxa-hph*: *NoxA* gene disrupted by inserting a DNA cassette containing both a chloramphenicol resistance gene and a hygromycin phosphotransferase gene in the original *NoxA* ORF region; *Hph*: Hygromycin phosphotransferase gene. (C and D) PCR analysis of transformants for *NoxB* gene insertion. Lane M represents the markers, lane 1 to 10 represent the transformants for *NoxB* gene insertion, lane 11 represents the genomic DNA of wild type strain Vd1396-9, lane 12 represents the negative water control for PCR. *noxb-hph*: *NoxB* gene disrupted by inserting a DNA cassette containing both a chloramphenicol resistance gene and a hygromycin phosphotransferase gene in the original *NoxB* ORF region. (E and F) PCR analysis of transformants for *NoxC* gene insertion. Lane M represent the markers, lane 1 to 13 represent the transformants of noxa mutants, lane 14 represent

the genomic DNA of wild type strain Vd1396-9, lane 15 represent the negative control for PCR; *noxc-hph*: NoxC disrupted by inserting a DNA cassette containing both a chloramphenicol resistance gene and a hygromycin phosphotransferase gene in the original *NoxC* ORF region. (G) Southern blot analysis of positive transformants of *noxa* mutants. Lane 1 represent wild type strain Vd1396-9, lane 3 represents ectopic control of *NoxA* insertion, and lane 4 to 10 represent positive transformants of *noxa* mutants. (H) Southern blot analysis of positive transformants of *noxb* mutants by *hph* probe. Lane 1 represents wild type strain Vd1396-9, lane 2 to 10 represent positive transformants of *noxb* mutants. (I) Southern blot analysis of positive transformants of *noxb* mutants by *NoxB* probe. Lane 1 represents the southern blot probe derived from *NoxB* gene, lane 2 represents wild type strain Vd1396-9, lane 3 to 8 represent positive transformants of *noxb* mutants. (J) Southern blot analysis of positive transformants of *noxc* mutants. Lane 1 represents the southern blot probe for *NoxC* gene (containing the joint fragment of *NoxC* ORF and the hygromycin phosphotransferase gene), lane 2 to 6 and lane 8 to 11 represent positive transformants of *noxc* mutants, lane 7 and 12 represent the ectopic control of *NoxC* insertion, and lane 13 represents wild type strain Vd1396-9. M: DNA marker (1Kb Plus DNA Ladder, Invitrogen, USA)

4.3.3 Phenotype analysis of *Nox* gene mutants

The growth rate and spore production of the *nox* gene mutants were assessed on PDA medium. The pathogenicity was tested on the susceptible potato cultivar Kennebec. The growth rate, colony morphology, spore production, and microsclerotia formation of *noxa* mutants (*noxa-im-1*, *noxa-im-5*, and *noxa-im-7*) were not significantly different from the ectopic control *NoxA-Ect-3*, empty vector control (EVC), and wild type Vd1396-9 (**Figure 4.3**). Similar results were evident in the *noxb* mutants (*noxb-im-1*, *noxb-im-2*, and *noxb-im-5*) and *noxc* mutants (*noxc-im-3-1*, *noxc-im-3-3*, and *noxc-im-7-1*) (**Figure 4.3**). The total AUDPC of infection and disease severity, as well as the vascular discoloration rate of infected potato stems caused by *noxa* mutants (*noxa-im-1*, *noxa-im-5*, and *noxa-im-7*) and *noxb* mutants (*noxb-im-1*, *noxb-im-2*, and *noxb-im-5*) were dramatically reduced on three sets of experiments conducted from 2016 to 2018 (**Figures 4.4, 4.5, & 4.6**). The growth rate of the potato plants inoculated with the mutants was similar to that of the water control treatment, but significantly higher than that of those inoculated with wild type Vd1396-9 and EVC (**Figures 4.4C, 4.5C, & 4.6C**). The total AUDPC of infection and disease severity, the potato plant growth rate, and vascular discoloration of potato stems infected by *noxc*

mutants were similar to the wild type, EVC and the ectopic control (**Figure 4.4**). These results indicate that either disruption of the *NoxA* or *NoxB* gene, but not the *NoxC* gene, can significantly reduce the virulence of *V. dahliae* on the potato cultivar.

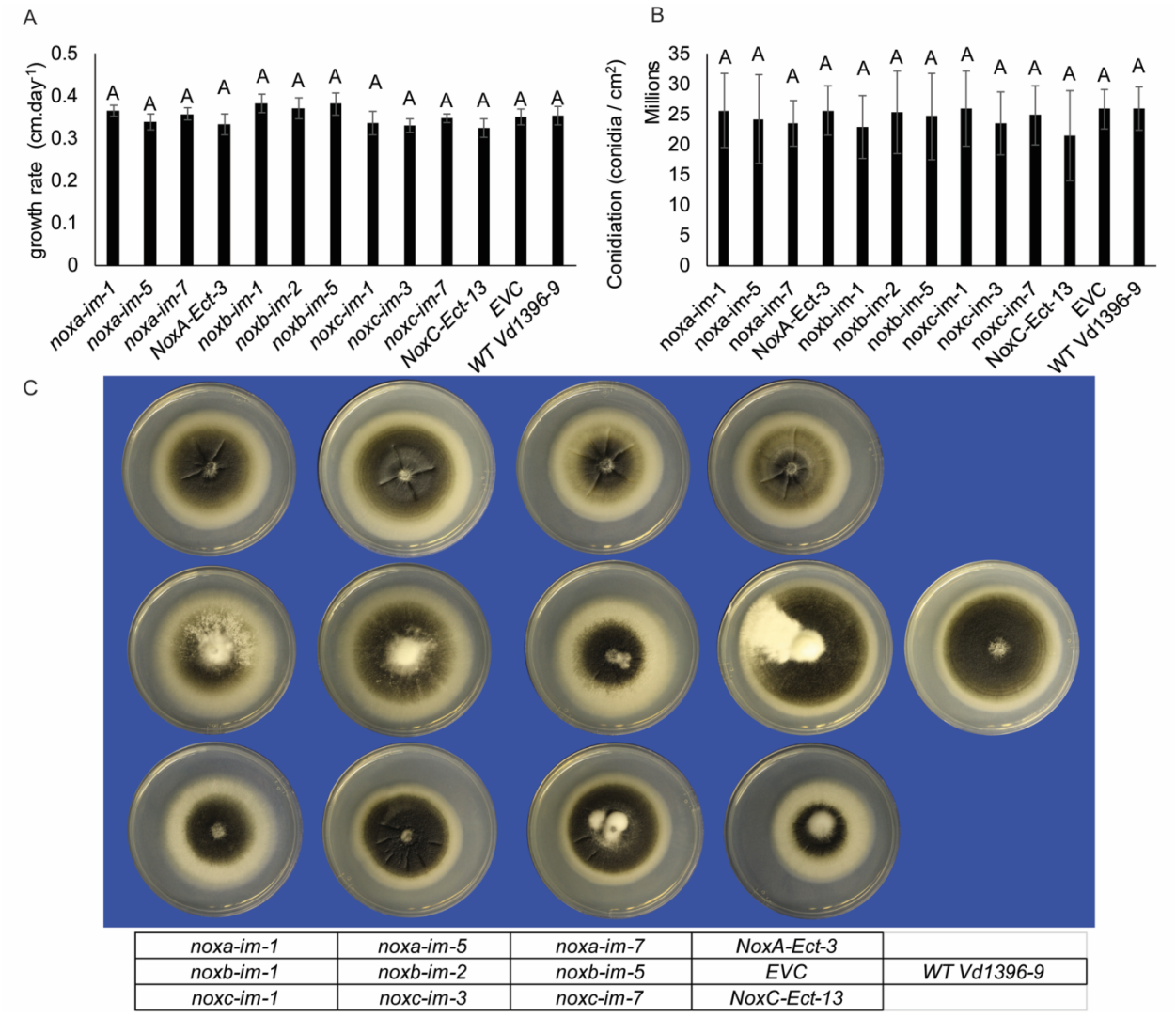


Figure 4.3 Phenotypic analysis of *nox* family gene mutants on PDA medium.
(A) The growth rate of *nox* family gene mutants. (B) The conidiation of *nox* family gene mutants. (C) The colony phenotype of *nox* family gene mutants. The bars showed as mean values (n = 8) for growth rate experiment and (n=5) for conidiation experiment with different letters representing significant difference from each other (P<0.05). Error bars refer to standard error.

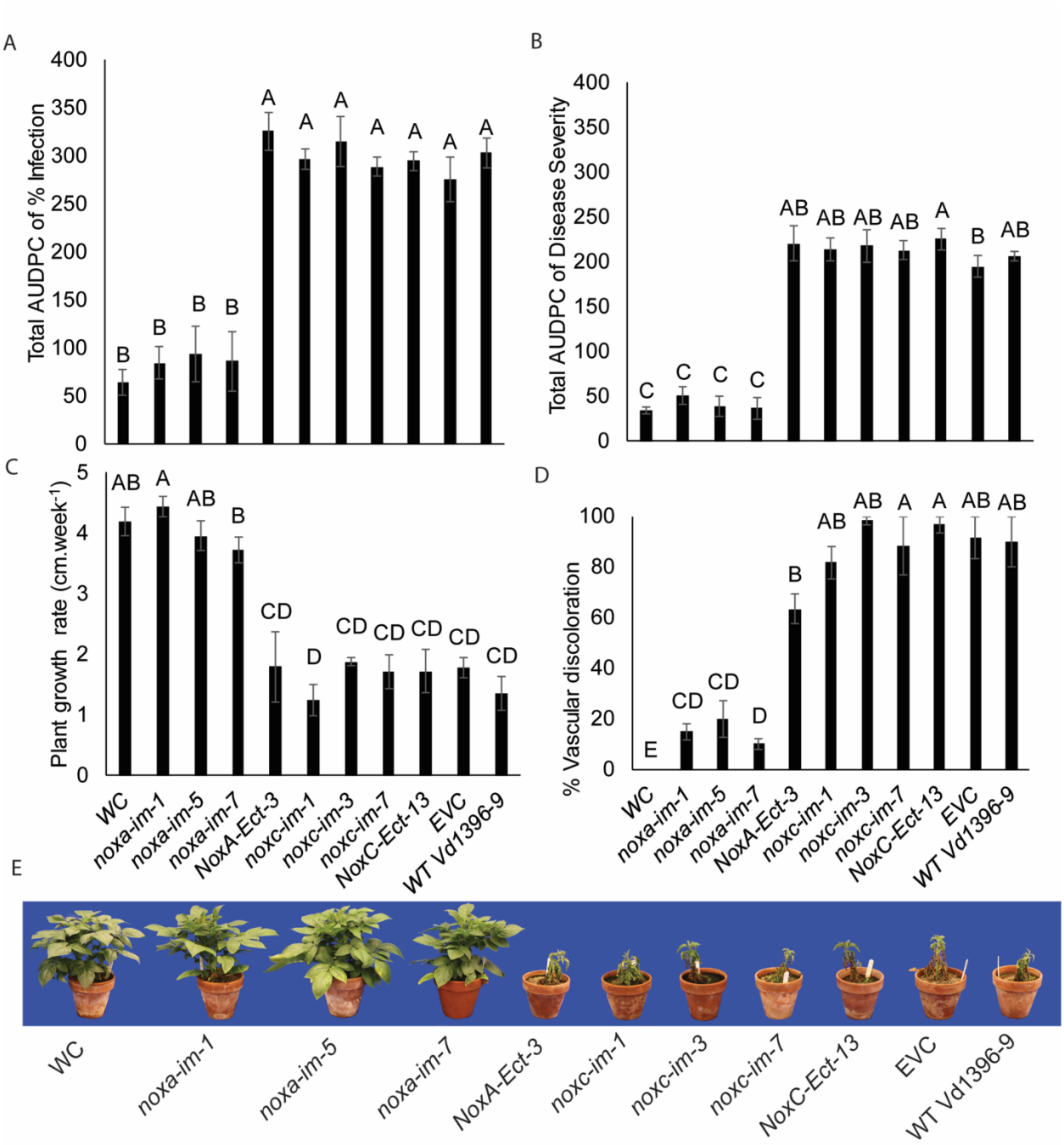


Figure 4.4 Pathogenicity test of *noxa* and *noxc* mutants on susceptible potato cultivar (Kennebec) in 2016.

(A) Total AUDPC of percentage of infection. (B) Total AUDPC of disease severity. (C) Growth rate of potatoes. (C) Percentage of vascular discoloration. (E) Kennebec potatoes infected by *noxa* and *noxc* mutants at 6 weeks after infection. The bars showed as mean values (n = 4) with different letters representing significant difference from each other ($P<0.05$). Error bars refer to standard error.

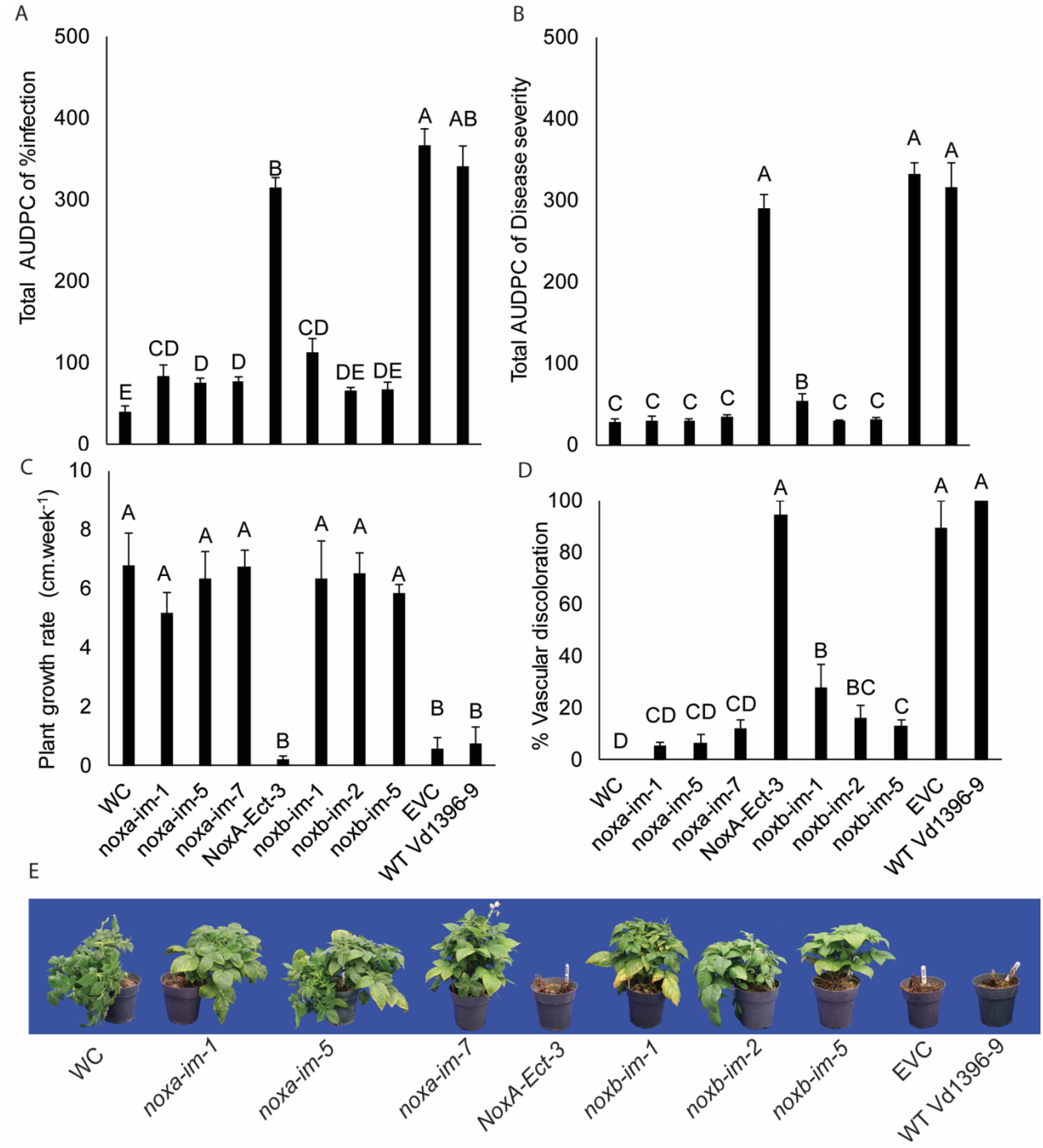


Figure 4.5 Pathogenicity test of *noxa* and *noxb* mutants on susceptible potato cultivar (Kennebec) in 2017.

(A) Total AUDPC of percentage of infection. (B) Total AUDPC of disease severity. (C) Growth rate of potatoes. (C) Percentage of vascular discoloration. (E) Kennebec potatoes infected by *noxa* and *noxb* mutants at 6 weeks after infection. The bars showed by mean values (n = 5) with different letters presenting significant difference from each other ($P<0.05$). Error bars refer to standard error.

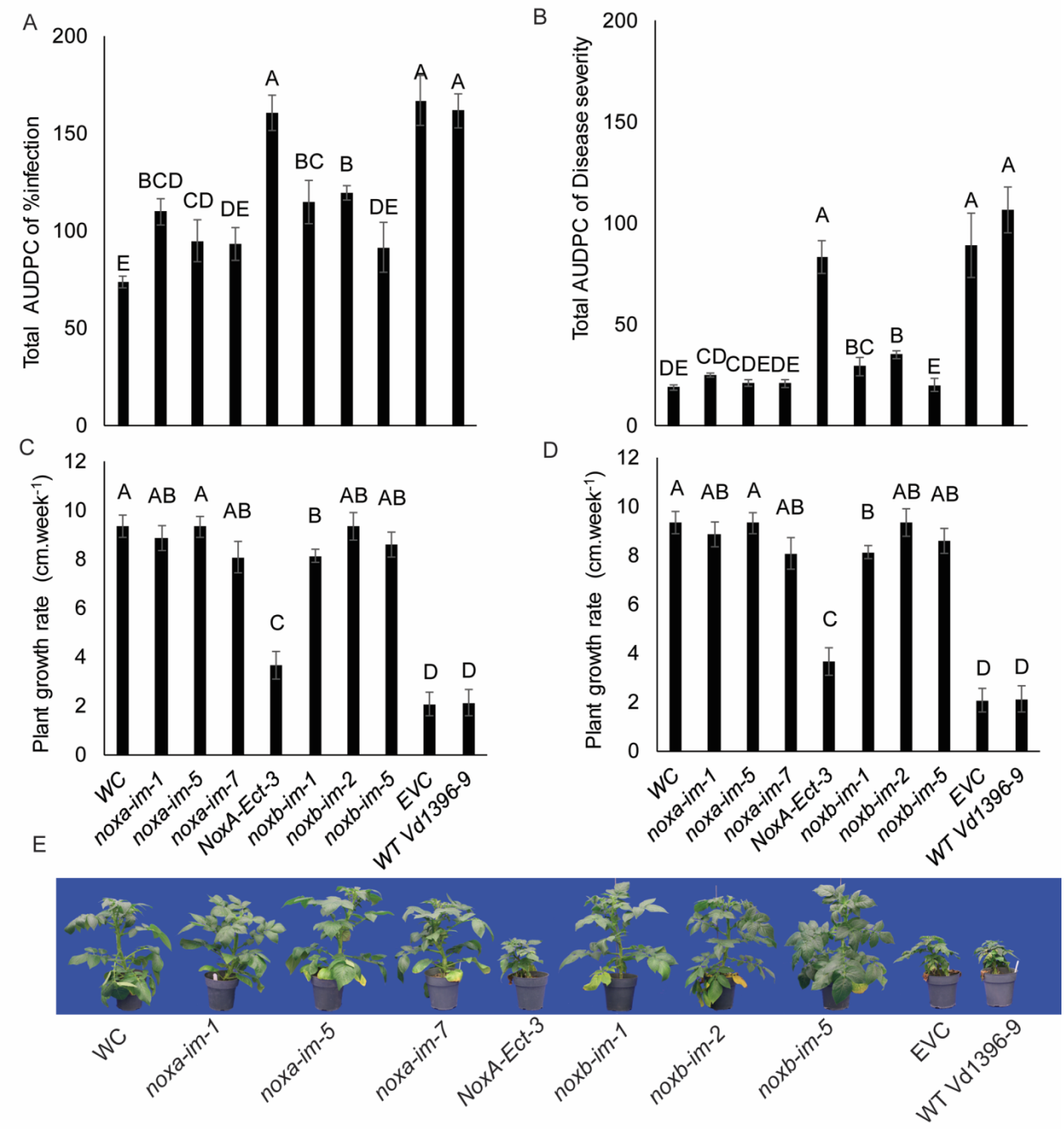


Figure 4.6 Pathogenicity test of *noxa* and *noxb* mutants on susceptible potato cultivar (Kennebec) in 2018.

(A) Total AUDPC of percentage of infection. (B) Total AUDPC of disease severity. (C) Growth rate of potatoes. (C) Percentage of vascular discoloration. (E) Kennebec potatoes infected by *noxa* and *noxb* mutants at 5 weeks after infection. The bars showed as mean values (n = 6) with different letters presenting significant difference from each other ($P<0.05$). Error bars refer to standard error.

4.3.4 *NoxB* is involved in cell wall biosynthesis

There was no significant difference between *noxa* or *noxc* mutants and the wild type in response to Calcofluor white treatment. However, inhibition rate of mycelial growth of *noxb* mutants was significantly reduced in presence of all three tested concentrations of Calcofluor white, compared to the wild type (**Figure 4.7**). This indicates that cell wall biosynthesis was affected in *noxb* mutants.

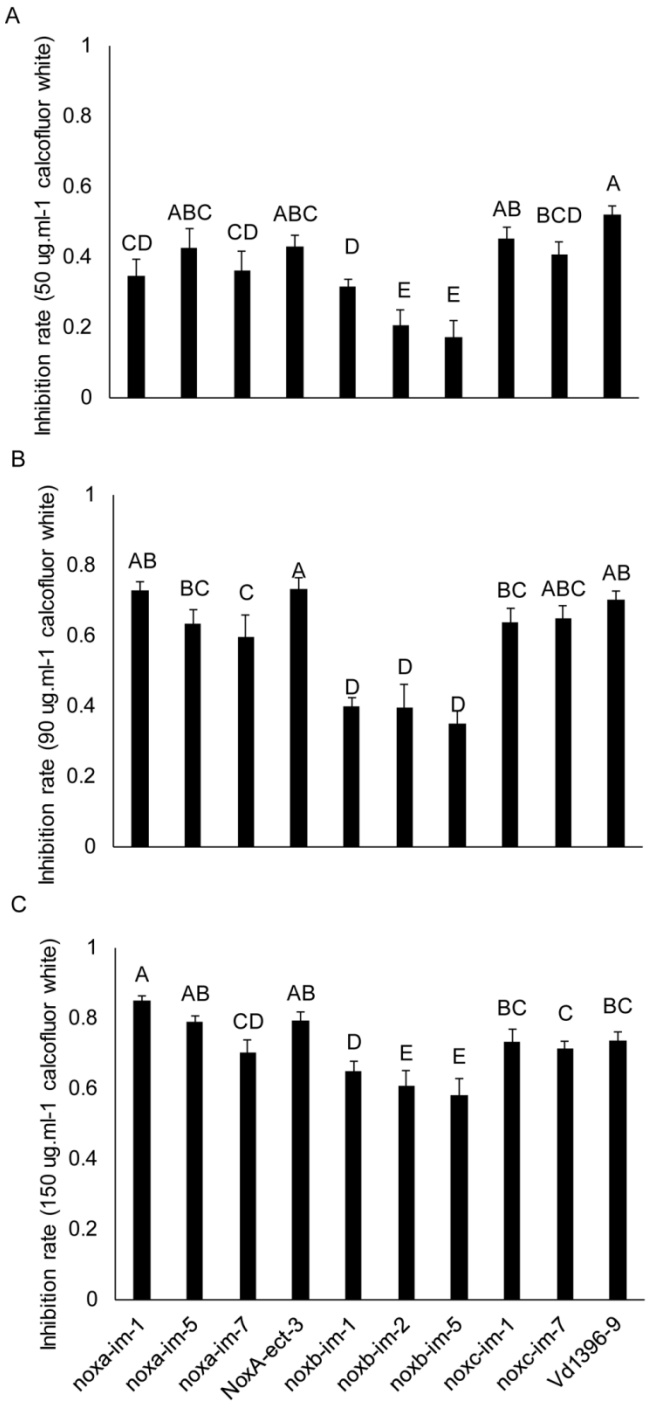


Figure 4.7 Resistance of *nox* mutants to Calcofluor white treatment.
(A) Resistance of *nox* mutants to 50 ug.ml^{-1} Calcofluor white. (B) Resistance of *nox* mutants to 90 ug.ml^{-1} Calcofluor white. (C) Resistance of *nox* mutants to 150 ug.ml^{-1} Calcofluor white. The bars showed as mean values (n = 17) with different letters representing significant difference from each other ($P<0.05$). Error bars refer to standard error.

4.3.5 The *noxb* mutants exhibit more resistance to oxidative stress treatment

To determine the response to oxidative stress and osmotic stress, *noxa, noxb, noxc* mutants and the wild type were cultured on solid CDB medium with varying H_2O_2 concentrations as well as with 0.8 M NaCl. There was no significant difference between *noxa, noxb, noxc* mutants and the wild type with respect to osmotic stress. However, the *noxb* mutants, but not *noxa* or *noxc* mutants, showed more resistance to oxidative stress than the wild type under all three-concentration conditions (**Figure 4.8**).

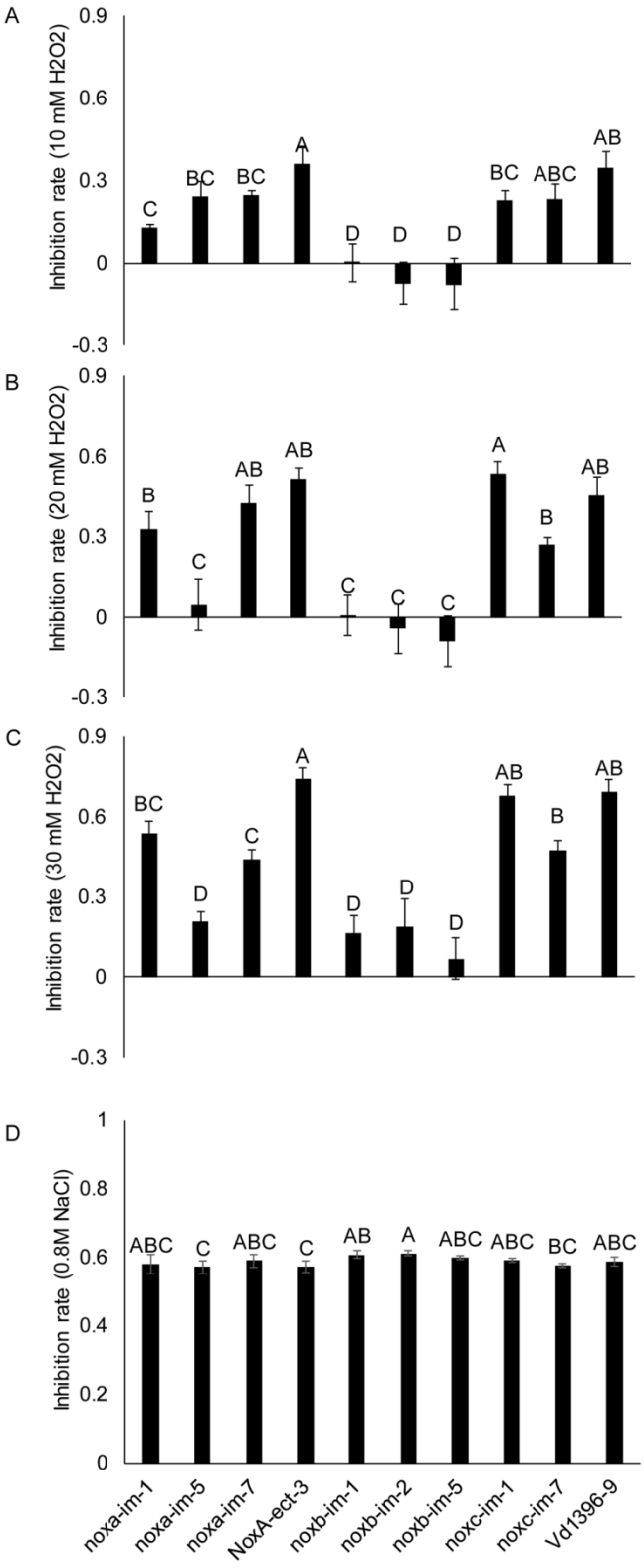


Figure 4.8 Resistance of *nox* mutants to oxidative stress and osmotic stress.
(A) Resistance of *nox* mutants to 10mM H_2O_2. (B) Resistance of *nox* mutants to 20mM H_2O_2. (C)

Resistance of *nox* mutants to 30mM H_2O_2. (D) Resistance of *nox* mutants to 0.8M NaCl. The bars showed as mean values (n = 17) for each H_2O_2 condition and (n=6) for NaCl condition with different letters representing significant difference from each other ($P<0.05$). Error bars refer to standard error.

4.3.6 The penetration ability of the *nox* family gene mutants

To further assess the virulence change in the *noxa* or *noxb* mutants, the penetration ability was determined on a cellophane membrane. After both 5 and 21 days-post-inoculation, neither *noxa* nor *noxb* could penetrate the cellophane membrane whereas the wild type did (**Figure 4.9**). This indicates that the penetration ability was affected in both *noxa* and *noxb* mutants compared to the wild type. Furthermore, the germination ability for both mutants and the wild type strain were observed at 24 hours-post-inoculation (HPI). All tested isolates can normally germinate on cellophane membrane (**Figure 4.10A**). At 72 HPI, all three *noxa* mutants and three *noxb* mutants failed to form the penetration peg in the hyphopodium cell, however, the control strain including NoxA-Ect-3, EVC, the wild type strain Vd1396-9, all formed the penetration peg in the hyphopodium cell (**Figure 4.10B**). Conidiophore morphology was similar between *noxa*, *noxb* and the wild type strain (**Figure 4.10C**).

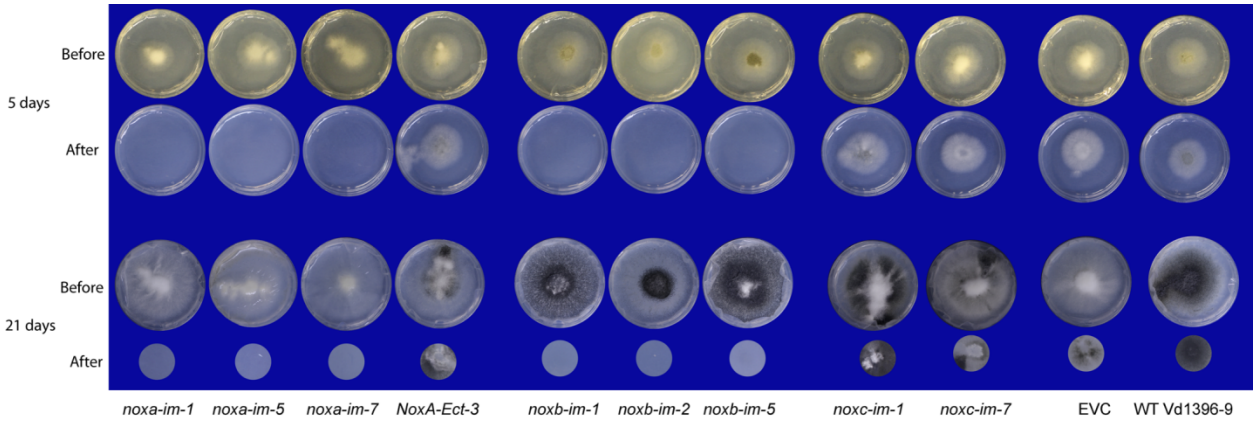
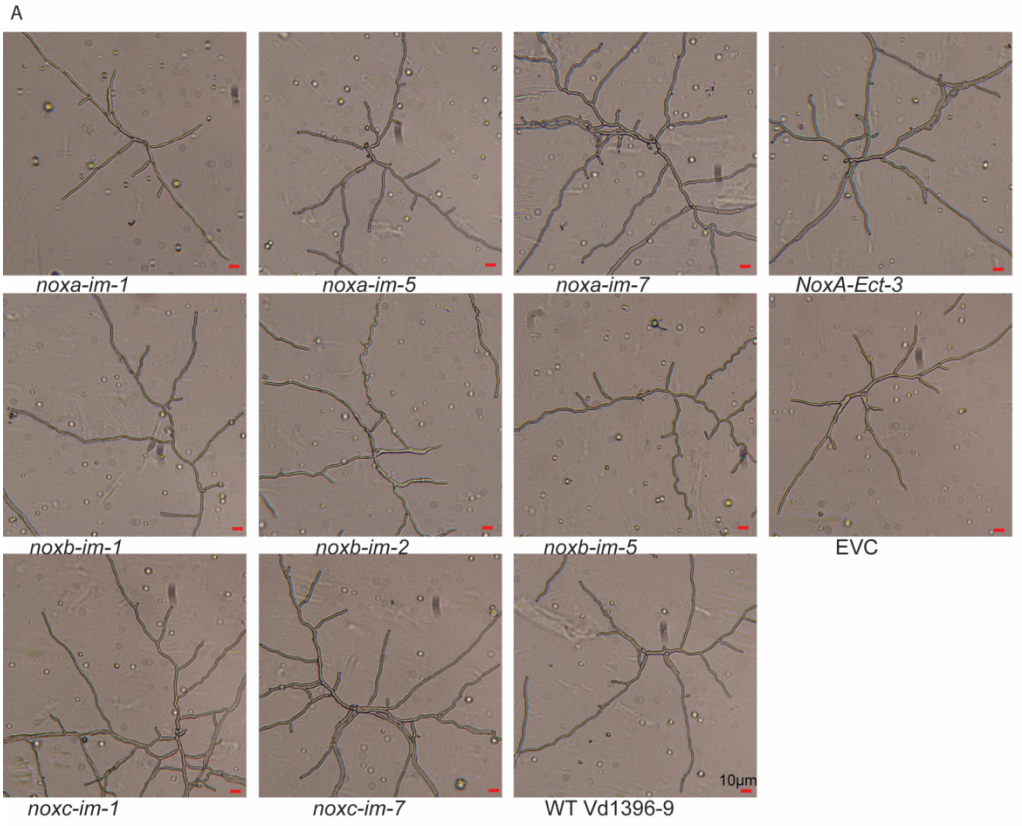
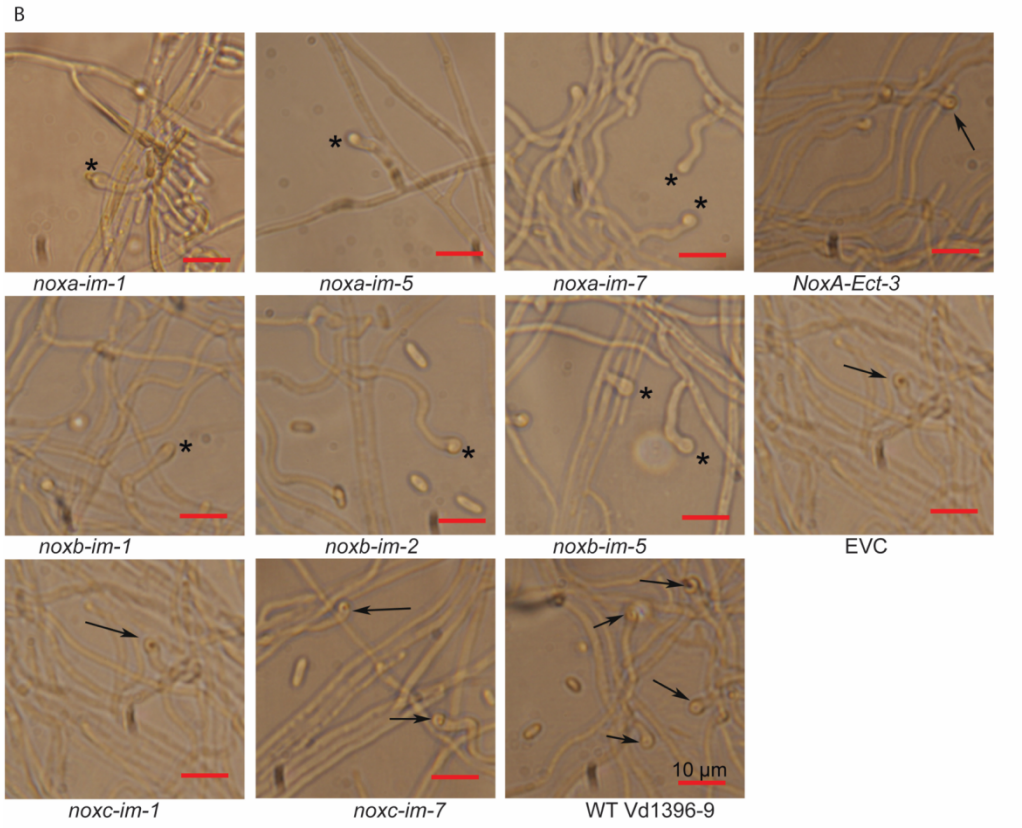


Figure 4.9 The penetration ability test of *nox* mutants on cellophane membrane.
Each isolate was repeated 6 times. All isolates were firstly inoculated on solid CDB media covered with a cellophane membrane for 5 and 21 days (indicated as **Before**) at 23±1°C, following which the cellophane membranes were removed from the media and maintained under the same

conditions for an additional 4 days (indicated as **After**).


A
noxa-im-1
noxa-im-5
noxa-im-7
NoxA-Ect-3
noxb-im-1
noxb-im-2
noxb-im-5
EVC
noxc-im-1
noxc-im-7
WT Vd1396-9
10μm


B
noxa-im-1
noxa-im-5
noxa-im-7
NoxA-Ect-3
noxb-im-1
noxb-im-2
noxb-im-5
EVC
noxc-im-1
noxc-im-7
WT Vd1396-9
10 μm

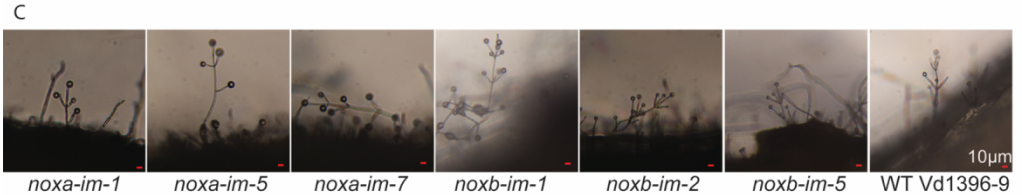

C
noxa-im-1
noxa-im-5
noxa-im-7
noxb-im-1
noxb-im-2
noxb-im-5
WT Vd1396-9
10μm

Figure 4.10 Morphology of *nox* mutants germination, penetration, and formation of conidiophores.
(A) Conidia germination ability test of *nox* mutants on cellophane membrane at 24h. The conidia of all *nox* mutants and the wild type strain (WT Vd1396-9) were cultured on cellophane membrane placed on solid CDB media for 24h, then observed under microscopy.
(B) The formation of penetration peg test of *nox* mutants on cellophane membrane at 72h. The conidia of all *nox* mutants and the wild type strain were cultured on cellophane membrane placed on solid CDB media for 72h, then observed under microscopy. The penetration pegs are shown as a dark spot in the hyphopodium cell. The *noxc* mutants (*noxc-im-1* and *noxc-im-7*), *NoxA-Ect-3*, EVC, and the wild type strain, can both form the penetration peg, which are indicated by arrows. Both the *noxa* mutants (*noxa-im-1*, *noxa-im-5*, and *noxa-im-7*) and *noxb* mutants (*noxb-im-1*, *noxb-im-2*,and *noxb-im-5*) formed the hyphopodium cell without penetration peg, which are indicated by asterisks.
(C) The conidiophore formation test of *noxa* and *noxb* mutants at 48h. All the *noxa* and *noxb* mutants and the wild type strain were cultured on PDA plates, then a hole was punched on each culture. After 48h conidiophores were observed on the edge of the hole under microscopy.
All Bars are equal to 10μm.

4.4 Discussion

V. dahliae causes wilt symptoms in more than 200 dicotyledonous plant species, and in potato it contributes to potato early dying (PED) (Pegg and Brady, 2002; Rowe and Powelson, 2002). The management of this disease depends on crop rotation, green manure, and soil fumigation; however, these methods are either costly or ineffective (Antonopoulos et al., 2008; Bhat and Subbarao, 1999; Molina et al., 2014; Subbarao et al., 1999; Tsror et al., 2005). Tomato plants with *Ve*1-gene show resistance to race 1, not race 2, of *V. dahliae* and *V. albo-atrum* (Fradin et al., 2009; Kawchuk et al., 2001). A quantitative trait locus (QTL) contained at least 11 different homologues (leucine- rich repeat (LLR) protein) of *StVe1* was identified in chromosome 9 in tetraploid potato, but whether single or multiple copies of these homologues provide resistance to *V. dahliae* and *V. albo-atrum* is still not clear (Simko et al., 2004a; Simko et al., 2004b). In past years, the disease resistance cultivars of potato were not applied in a wild range (Johnson and Dung, 2010). Resistance is defined as restricting the development of pathogen or disease symptoms on the host (Johnson and Dung, 2010). In other words, disease resistance does not entail

killing the pathogen completely, but is an active process that could restrict the pathogen in the host (Johnson and Dung, 2010). One of the most effective ways to manage this soil-borne disease, may be by controlling the initial inoculum, both in the soil and during pathogen infection on the host (Johnson and Dung, 2010).

El-Bebany et al. (2010) conducted comparative analyses on two *V. dahliae* isolates differing in virulence, and detected two proteins, Thioredoxin and NADH-ubiquinone oxidoreductase, only in the highly aggressive isolate while they were lacking in the weakly aggressive one, with the former protein playing role in ROS cleavage and the latter functioning in non-enzymatic ROS production in mitochondria (Bazil et al., 2014; Huang et al., 2015; Kussmaul and Hirst, 2006). Knowing that ROS generated by Nox proteins were important for sexual development and host penetration (Bedard et al., 2007; Brun et al., 2009; Tanaka et al., 2008; Wang et al., 2014; Zhao et al., 2016), we investigated the functions of *Nox* family genes in *V. dahliae*.

In response to different potato tissue extracts, three *Nox* family genes transcriptionally expressed in different manners under different conditions. This may indicate multiple functions of *Nox* family genes in different processes of infection or interaction with the host plant. The increasing transcriptional activity of *NoxA* and *NoxB* in the highly aggressive *V. dahliae* is in line with the findings in *C. purpurea* that expression of *CpNOX1* increases during infection in planta and reaches a maximum at a later infection stage (Giesbert et al., 2008). In other fungi, *Nox* family genes usually play roles in different processes of cell differentiation. Although these genes have similar structure and function, they still have different properties in various cellular processes, such as penetration, production and germination of ascospores, and pathogenicity (Brun et al., 2009; Egan et al., 2007; Malagnac et al., 2004). In *V. dahliae NoxA* and *NoxB* must play critical roles during the infection process, which is demonstrated by the fact that both mutants of *NoxA* and

NoxB had much lower virulence than their wild type counterpart. This defect in virulence is similar to *nox1* and *nox2* mutants in *M. oryzae*, *noxa* and *noxb* mutants in *F. graminearum* and *B. cinerea*, and *nox1* mutant in *C. purpurea* (Egan et al., 2007; Giesbert et al., 2008; Segmüller et al., 2008; Wang et al., 2014). However, *NoxC* is not essential for *V. dahliae*'s virulence on potatoes. Homologues of NoxA and NoxB are more similar in structure to mammalian gp^{91phox} (Malagnac et al., 2004), while NoxC is more similar to mammalian Nox5 (Lewit-Bentley and Réty, 2000). This apparently indicates that NoxA and NoxB would play more important roles than NoxC in the pathogenicity of *V. dahliae*. Among *P. anserina*, *N. crassa*, *F. graminearum* and *A. nidulans*, the homologue of Nox1 or NoxA would regulate sexual development (Cano-Domínguez et al., 2008; Lara‐Ortíz et al., 2003; Malagnac et al., 2004; Wang et al., 2014); however, since *V. dahliae* has no known sexual stage, we were unable to study the roles of *NoxA* or *NoxB* in this process. The growth, spore production, and formation of microsclerotia were not affected in the mutants of *NoxA*, *NoxB* or *NoxC*. This is in contrast to the observation on *M. grisea*, *P. anserina*, *N. crassa*, *S. sclerotiorum*, *F. graminearum*, *C. purpurea*, and *B. cinerea* where homologues of mammalian gp^{91phox} regulate spore production and germination, and resting structure development (Cano-Domínguez et al., 2008; Egan et al., 2007; Giesbert et al., 2008; Kim et al., 2011; Malagnac et al., 2004; Segmüller et al., 2008; Wang et al., 2014). *NoxB* in *V. dahliae* also plays an important role in cell wall biosynthesis, which is similar to *Nox1* in *M. oryzae* (Egan et al., 2007). Mutants of *NoxB* gene showed more resistance to oxidative stress than the wild type, with the mechanism remaining to be unraveled in future studies. Both *NoxA* and *NoxB* in *V. dahliae* are important for penetration on cellophane membrane, as *noxa* and *noxb* mutants could not breach the cellophane membrane after 5 and 21-day-inoculation. Furthermore, the reason that both *noxa* and *noxb* mutants lost the penetration ability, is that both mutants can no longer form the penetration peg on

cellophane membrane. In *M. oryzae*, *P. anserina*, *F. graminearum*, and *B. cinerea*, homologues of Nox also regulate penetration on the host (Brun et al., 2009; Egan et al., 2007; Malagnac et al., 2004; Segmüller et al., 2008; Wang et al., 2014). In addition to the loss of the ability to form the penetration peg, another explanation may be that, homologues of Nox may regulate cellulose degradation to control the penetration process in the host. This has been proven by studies on Nox1 and Nox2 in facilitating cellulose degradation in different manners and affecting the penetration ability as well in *P. anserina* (Brun et al., 2009).

In this study, we investigated the functions of Nox gene family members in *V. dahliae* and showed that they are key elements in manipulating the fungal penetration onto host and in facilitating the virulence during the interaction between *V. dahliae* and potato. Further studies will focus on decoding the detailed molecular mechanism for regulating the penetration. In the future, this research may help provide more strategies to prevent the initial infection of *V. dahliae* as part of disease management practices.

CHAPTER 5

GENERATION OF AN EXOPOLYGALACTURONASE MUTANT OF *VERTICILLIUM DAHLIAE* USING A VECTOR–FREE PROTOPLAST TRANSFORMATION METHOD

Abstract

Verticillium dahliae is a hemibiotrophic pathogen that has been responsible for great losses in dicot crop production. Previous research has shown that in general dicot pathogens can produce more pectin-degrading enzymes. An *ExoPG* gene (VDAG_03463) previously identified using subtractive hybridization cDNA-AFLP in our lab, showed much higher expression in the highly related to a weakly aggressive *V. dahliae* isolate. I used a vector–free split-marker recombination method with PEG-mediated protoplast to delete the *ExoPG* gene in *V. dahliae.* This is the first instance of using this method in *V. dahliae* transformation. Only 2 steps of PCR and one step of transformation is required for the procedure, markedly reducing the necessary time for deleting a gene in *V. dahliae*. Six mutants for the *ExoPG* gene, were identified by PCR and southern blot. *ExoPG* responded more in the highly aggressive isolate than the weakly one in response to potato leaf and stem extracts. Its expression increased in both isolates during infection, with a higher level in the highly aggressive isolate at the early infection stage. Unexpectedly, the disruption of *ExoPG* did not influence virulence, nor did it affect total exopolygalacturonase activity in *V. dahliae*. Given that the role of polygalacturonase in the virulence of fungal pathogens, it is probable that the suppression of ExoPG has been compensable by the activity of other genes.

5.1 Introduction

Verticillium dahliae is a hemi-biotrophic pathogen that causes wilt symptoms and result in a great loss in a wide range of hosts (Pegg and Brady, 2002). In economic crops, this disease may cause huge yield loss in crop production (Cappaert et al., 1992). Traditional control management using crop rotation and green manure are not effective in prevent Verticillium wilt (Collins et al., 2006; Molina et al., 2014). Soil fumigation can be an effective management for Verticillium wilt but can also cause negative environmental issues (Ajwa et al., 2002; Davis et al., 1996; Martin, 2003). Even though the tomato which contains the Ve gene shows resistance to *V. dahliae* race 1 (Fradin et al., 2009; Kawchuk et al., 2001), efficient plant resistant cultivars have not been applied to the majority of the crops at risk.

The plant cell wall is a key barrier of protection from pathogen attacks. It consists primarily of cuticular wax, cutin, glycans, cellulose, pectic substances, and cell wall structural proteins (Agrios, 2005). However, many microbes produce various cell wall-degrading enzymes (CWDEs) to degrade these plant cell wall components. CWDEs include cutinases, pectinases, cellulases, glucanases and proteases (Agrios, 2005). Glycosidic bonds of polysaccharides can be cut by enzymes belong to the glycoside hydrolases (GH) family (Kubicek et al., 2014). Polygalacturonases belong to these pectin-degrading enzymes, which include both endopolygalacturonases (EndoPGs) and exopolygalacturonases (ExoPGs). EndoPGs hydrolyze the polysaccharide randomly to produce oligogalacturonides, while ExoPGs hydrolyze the polymer from the non-reducing end to produce a single galacturonic acid (Abbott and Boraston, 2007; Schacht et al., 2011). Both the Endo-PGs and Exo-PGs belong to the GH28 family (Abbott and Boraston, 2007).

The majority of *V. dahliae*'s host range belongs to the dicot family (Pegg and Brady, 2002),

moreover cell walls of the dicot plants contain a large amount of pectin (Kubicek et al., 2014; Varner and Lin, 1989). In parallel, pathogens of dicots contain more pectin-degrading enzymes-encoding genes than that of monocots (Kubicek et al., 2014). However, the function of PGs in pathogenicity seem to be more specific to certain types of disease, rather than the classification of pathogens (Kubicek et al., 2014). *Alternaria citri* and *Alternaria alternata* "rough lemon pathotype" are two pathogens for citrus with similar morphology (Isshiki et al., 2001). *A. citri* causes Alternaria black rot, *A. alternata* causes Alternaria brown spot, both in citrus (Isshiki et al., 2001). The EndoPGs from the two pathogens show a high similarity in nucleotide identity (99.6%) and biochemical properties (Isshiki et al., 2001). Disruption of the *Endo-PG* gene in *A. citri* significantly decreased the magnitude of Alternaria black rot symptoms in citrus and maceration symptoms in potato tissue, while disruption of *Endo-PG* gene in *A. alternata* did not induce any change in virulence (Isshiki et al., 2001). This indicates that pectin-degrading enzyme may play different functions and participate in different processes in various pathogens (Isshiki et al., 2001).

Using a subtractive hybridization cDNA-AFLP method, El-Bebany et al. (2011)found that a *ExoPG* gene (VDAG_03463) was up-regulated in the highly aggressive *V. dahliae* isolate (Vd1396-9) and down-regulated in the weakly aggressive isolate (Vs06-14) in response to root extracts from both moderately resistant and susceptible potato. Most *V. dahliae'*s hosts belong to dicot plants that contain high amount of pectin (Kubicek et al., 2014; Pegg and Brady, 2002; Varner and Lin, 1989), this finding suggested that this *ExoPG* gene (VDAG_03463) may play important roles in the pathogenicity of *V. dahliae*.

A common method to investigate the function of fungal gene is targeted gene disruption. In *V. dahliae*, the most popular method for disrupting the target gene is the vector-based *Agrobacterium*-Mediated T-DNA insertion method (Tian et al., 2015; Zhu et al., 2017). However,

a vector-free split-marker recombination which was developed for gene deletion in *Saccharomyces cerevisiae* and also be applied to *Magnaporthe oryzae* seemed attractive (Catlett et al., 2003; Zhou et al., 2017b). Catlett et al. (2003) developed two DNA fragments, each containing a DNA flanking of the targeting gene with half of the selected marker gene. They were introduced into fungal protoplast cells to obtain the knockout mutant by homologous recombination between the overlapping regions of the half marker gene region in the two DNA fragments, and between the flanking region of the target gene (Catlett et al., 2003). PEG-mediated protoplast transformation is a common gene transfer method for many other fungi and plants (Mathur and Koncz, 1998; Ruiz‐Díez, 2002; Zhou et al., 2017a). However, even though there are a few publications related to protoplast transformation in *V. dahliae* (Dobinson, 1995; Rehman et al., 2016), this easy method is not as popular as *Agrobacterium*-mediated transformation. We are interested in employing a vector–free split-marker recombination method with an optimized PEG-mediated protoplast transformation in *V. dahliae* for gene deletion of VdExoPG.

The objective of this study were: (1) determine the expression of *Exo-PG* gene under induction with potato tissues and during in-planta infection; (2) introduce the vector–free split-marker recombination method to obtain *ExoPG* mutants in *V. dahliae*; (3) determine the pathogenicity of the mutants; and (4) determine the total Exopolygalacturonase activity of the mutants and wildtype.

5.2 Materials and methods

5.2.1 Fungi isolates and Plant material

Highly aggressive *V. dahliae* isolate Vd1396-9 and weakly aggressive one Vs06-07 were used in this study (Alkher et al., 2009; Uppal et al., 2007). Susceptible potato cultivar Kennebec

was employed in this study (Alkher et al., 2009). Kennebec seedlings (germinated for 1.5 weeks) were planted in soil, sand, peat moss mixture with a ratio of 12:4:1, with day/night temperatures of 22/18 °C and 16/8 h photoperiod in growth cabinet.

5.2.2 *Exo-PG* expression in response to potato tissue extracts and on inoculated detached leaves

The expression of *Exo-PG* in the differentially aggressive *V. dahliae* isolates Vd1396-9 and Vs06-07 in response to elicitation with potato leaf, stem, and root extracts were performed as described in the method by Zhu et al. (2017). Briefly, the potato leaf, stem, or root extracts were added into 7-day cultured *V. dahliae* isolates Vd1396-9 and Vs06-07 respectively, and following additional one-week culturing were collected for RNA extraction.

The expression of *Exo-PG* in the same differentially aggressive *V. dahliae* isolates inoculated onto potato detached leaves were also performed as described by Zhu et al. (2017). In brief, *V. dahliae* isolates Vd1396-9 and Vs06-07 were cultured on potato dextrose agar (PDA) for 3 weeks prior to harvesting the conidia by water flooding. Four week old detached leaves were inoculated by immersing the petiole into 1ml of a conidial suspension of Vd1396-9 or Vs06-07, including sterilized water as a control. Four to 6 individual detached leaves from different plants were combined as one samples and three samples were collected for each treatment at each selected time point (12,17 days after inoculation (DAI)). RNA extraction and Quantitative Real-Time RT-PCR were employed here with the protocol from Omega Fungal RNA kit (Omega Bio-Tek, Inc., Norcross, GA, USA) and SsoFast EvaGreen Super mix (Bio-Rad Lab, Philadelphia, PA, USA).

5.2.3 Protoplast preparation and transformation

Preparation of the protoplast cells of *V. dahliae* (highly aggressive isolate Vd1396-9) was conducted following the description of Dobinson (1995) and Yelton et al. (1984) with some modifications and improvements. In brief, *V. dahliae* was cultured in PDB broth for 4 days at 24°C without shaking, after which the mycelium was collected and finely ground by sterile mortar and pestle in the laminar flow hood. The ground mycelium was then re-cultured in fresh PDB broth containing 0.001% thiamine for an additional 14 hours in the shaker with a speed of 120 rpm. Mycelium were collected by miracloth and washed with mycelia buffer (10mM $NaPO_4$ pH7.5; 10mM EDTA pH8.0; 1mM dithiothreitol) 2-3 times. The mycelium was then re-suspended in 30 ml mycelia buffer and shaken for 2 hours at 24°C at 60 rpm. The culture was then centrifuged at 1900g for 10 minutes to collect the mycelium, decanted the supernatant, then incubated in 15 ml OM buffer (1.2 M $MgSO_4$, 10 mM $NaPO_4$ pH5.8) with 10 mg/ml Lysing Enzymes from *Trichoderma harzianum* (Sigma-Aldrich Canada Co.) and shaken overnight at 30°C at 65 rpm. The suspension was finally filtered by miracloth and transferred into 50ml centrifuge tube, overlayed with 10ml ST buffer (0.6M sorbitol; 100mM Tric.HCl pH7.0), then centrifuged at 4°C at 4000g for 20 minutes. A glass pasteur pipet was used to retrieve the 5-10 ml of protoplast cell, which was in the interface between the OM and ST buffer. Two to 4 volumes of STC buffer (1.2M sorbitol; 10mM Tric.Hcl pH7.5; 10mM $CaCl_2$) was added, centrifuged at 4°C, 4000g for 20 minutes. The precipitate was washed 2 times with STC buffer, and finally re-suspended in 0.5ml STC buffer with 8%PEG (PEG3350) or 8% DMSO, yielding a final protoplast concentration ranging from 3 to 5 x 10^7 protoplast/ml.

The transformation protocol followed the description of Dobinson (1995) with some modifications and improvements. Two µg of DNA was added to 200 µl protoplast cell and incubated on ice for 20 minutes, following the gentle addition of 0.625ml PTC buffer (40% PEG

3350 in TSTC buffer (1M Sucrose; 50mM Tris.HCl pH8.0; 50mM $CaCl_2$)). The solution was mixed well following buffer addition and mixing., then incubated at room temperature for 20 minutes, following the addition of 5ml complete media (CM) (Bennett and Lasure, 1991) containing 1M sucrose and shaking at 90 rpm, 24°C for overnight.

Twenty-five ml of CM medium (containing 1M sucrose,1% agar, 25 µg/ml Hygromycin B, 200 µg/ml Ampicillin) was added and gently mixed then poured in a 15cm diameter petri dish. After solidifying, the media was overlaid with 50ml CM medium (containing 1M sucrose,1% agar, 75 µg/ml Hygromycin B, 200 µg/ml Ampicillin), incubated at 24°C for 7-14 days until the transformants grew to the surface. The transformants were then separated and transferred into fresh CM medium (1.5 % agar, 25 µg/ml Hygromycin B, 200 µg/ml Ampicillin).

5.2.4 Vector-free split-marker recombination method for knocking out *Exo-PG* in *V. dahliae*

Zhou et al. employed Catlett et al.'s vector-free split-marker recombination method for *Saccharomyces cerevisiae* to delete a gene in *Magnaporthe oryzae* (Catlett et al., 2003; Zhou et al., 2017b). This method knocks out a target gene in fungi requiring only two PCR steps followed by one transformation step. Our aim was to knock out the front 1319 bp fragment of the *ExoPG* open reading frame (ORF) (1836 bp), but due to the proximity of the 3' DNA sequence of *ExoPG* to another gene, we designed the downstream homologous recombination region to not interfere with the ORF of the other gene, but to cover a short part of that region with the *ExoPG* ORF. Briefly, as show in Figure 1, the first step was to amplify the flanked upstream and downstream regions of the selected gene from the *V. dahliae* genome with the primers FS-AF/R and FS-BF/R (**Table 5.1**), then amplify the 5' and 3' segments of the *hygromycin resistance gene* (*Hph*) from pSK846 vector (Dobinson et al., 2004) with the primers Hph-F/HY-R, YG-F/Hph-R (**Table 5.1**),

respectively. Both primer FS-AR and FS-BF were fused with a short sequence of 5'terminator and 3' terminator of the *Hph* gene (**Figure 5.1**). The amplified PCR product of the upstream region of the selected gene from *V. dahliae* and the 5' segment of the *Hph* gene was used as a template for overlapping PCR (**Table 5.2**) in order to get the DNA fragment of the upstream region fused with the 5' segment of *Hph*. Note the annealing temperature for the first 8 cycles of "self-primer" should be low to promote this combination. The downstream region of the selected gene from *V. dahliae* and the 3' segment of the *Hph* gene was used as a template for overlapping PCR to get the DNA fragment of the downstream region fused with the 3' segment of *Hph* (**Figure 5.1**). Finally, both overlapping PCR products of the upstream region fused with 5' segment of *Hph* and downstream region fused with the 3' segment of *Hph* were transformed into protoplast cells of *V. dahliae* wild type Vd1396-9 (**Figure 5.1**). Only the homolog recombination which arose from both upstream and downstream regions of the selected gene, as well as the overlapping 5' segment and 3' segment of the *Hph* gene, could produce positive transformants (**Figure 5.1**). All of the transformants were selected in PDA plates with hygromycin B. The knocked out transformants were screened by PCR with primers ExoPG-ORF-F/R, ExoPG-UAF/Hph-TR (**Table 5.1**). Those positive transformants were then determined by Southern blot following the description of Zhu et al. (2017).

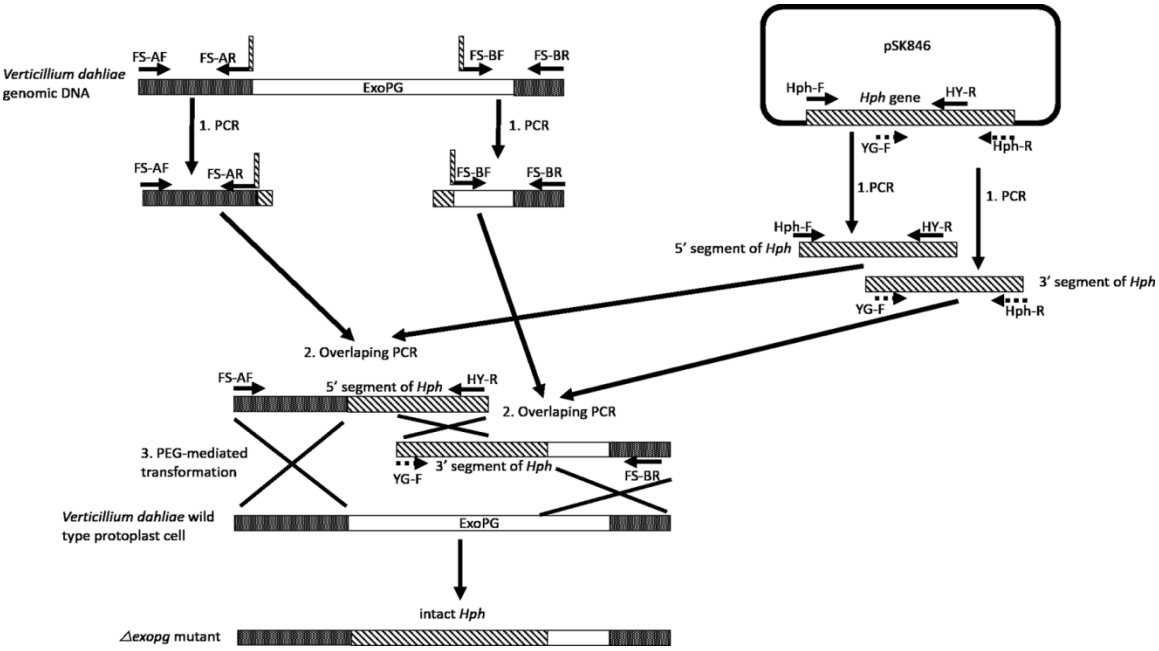


Figure 5.1 The procedure of vector-free based knocking out for the *Exo-PG* gene of *V. dahliae*

Table 5.1 The primers in current study

primer name	5' to 3'	Annealing Temperature (°C)
FS-AF	GCCGTGTCAGTCAGAGGG	56.1
FS-AR	cctccactagctccagccaagcccaaaGGAAGTGTCACGAAACGC	56.1
FS-BF	agcactcgtccgagggcaaaggaatagACAAGACACGCCCAGGAC	57.8
FS-BR	CCAAAGTCGATTGAATGAAAT	57.8
ExoPG-ORF-F	GCACGGAGTACCAAAGG	54
ExoPG-ORF-R	GCAGCCAAGTCAGTAACAA	54
ExoPG-UA-F	TCACCTCACTATTATCCACCTC	55.1
Hph-TR	GCTCCATACAAGCCAACC	55.1
Hph-F	TTTGGGCTTGGCTGGAGCTAGTGGA	55
Hph-R	CTATTCCTTTGCCCTCGGACGAGT	55
YG-F (Ramamoorthy et al., 2007)	GATGTAGGAGGGCGTGGATATGTCCT	55
HY-R (Ramamoorthy et al., 2007)	GTATTGACCGATTCCTTGCGGTCCGAA	55

Table 5.2 The procedure of overlapping PCR

Overlapping PCR	
PCR mix 25ul (final concentration)	procedure
1x HF buffer 0.25 mM DNTPs 2 mM $MgCl_2$ Phusion Taq 1 unit 50 µg of PCR product of upstream/ or downstream region of the selected gene 50 µg of PCR product of 5'/ or 3' segment of *Hph* gene	1). Prepare the PCR mix 2). 98 °C for 30 seconds 3). 98 °C for 10 seconds 4). 49 °C for 90 seconds 5). 72 °C for 120 seconds Repeat step 3 to 5 for 8 cycles 6). 4°C 7). Add primers (FS-AF/HY-R or YG-F/FS-BR) to a final concentration 0.2uM 8). 98 °C for 30 seconds 9). 98 °C for 10 seconds 10). 58 °C for 90 seconds 11). 72 °C for 120 seconds Repeat step 8 to 10 for 30 cycles 12). 72 °C for 10 minutes 13). 4°C

5.2.5 Pathogenicity analysis of ∆*exopg* mutant

The virulence of ∆*exopg* mutants were tested on potato cv. Kennebec following the description of Zhu et al. (2017). Briefly, Kennebec plants were grown in LA4 soil mix (SunGro Horticulture, Agawam, MA 01001, USA) for 21days, when plants were up-rooted, roots were washed and 1cm. trimmed from the root tips , following inoculation with 10^6 conidia/mL suspension of ∆*exopg* mutants (△*exopg*-ko-18, ∆*exopg*-ko-23), wild type, Vd1396-9 and ectopic control, *ExoPG-Ect-13* (Fragment randomly inserted in *V. dahliae* genome without replacing the original *ExoPG* ORF). Plants were trans-planted into 6-inch pots containing a mixture of pasteurized sand, soil and peat moss (16:4:1) and placed back into controlled growth area for 2 weeks. Total area under the disease progress curve (Total AUDPC) of percentage of infection and disease severity was measured weekly according to Zhu et al. (2017). Plant height measurements and vascular discoloration ratings were conducted at 5 weeks post infection.

5.2.6 Growth rate and conidiation of Δ*exopg* mutants

Δ*exopg* mutants (Δ*exopg*-ko-18, △*exopg*-ko-23), wild type Vd1396-9 and ectopic control *ExoPG-Ect-13* are grown on PDA for 14 days, following colony growth and conidia production analysis according to Zhu et al. (2017).

5.2.7 Total exopolygalacturonase activity of Δ*exopg* mutants

The determination of total exopolygalacturonase activity of the Δ*exopg* mutants followed the description of Teixeira et al. (2000) with some modification. Briefly, Δ*exopg* mutant △*exopg*-ko-18, Δ*exopg*-ko-23, with the control Vd1396-9 were cultured in Czapek-Dox Broth (CDB) media (Difco Laboratories, Sparks, MD, USA) with the addition of 1% pectin and shaken at 24°C with a speed of 100rpm. The same isolates cultured in CDB liquid media without pectin were used as a control. All treatments had 3 replicates. Samples of culture fluid (2 ml) were taken at 0, 2,3,4,6,10,14 days after inoculation (DAI) and filtered with miracloth. Samples were prepared by taking 50ul from each culture filtrate and mixed with 450ul reaction mixture (1% pectin in 25 mM sodium acetate buffer (pH 4.5)). The mixture was then incubated at 45°C for 10 minutes, then added 500ul DNS solution (Dinitrosalicylic acid 10 g/L(1%), Phenol 2 g /L(0.2%), Sodium sulfite 0.5 g/L(0.05%), Sodium hydroxide 10 g/L(1%)) (Miller, 1959) following boiling for 5 minutes. Total exopolygalacturonase activity was determined by recording the absorbance at 575 nm, which is measured by the release of galacturonic acid from pectin (Li et al., 2004b).

5.2.8 Statistical Analysis

PROC MIXED program processed by SAS Statistical Analysis Software (SAS Institute,

Cary, NC, USA; release 9.1 for Windows) was using for data analysis in this project. Some sets of data were applied with Log^{10} transformation for statistical analysis when necessary. The PROC UNIVARIATE was using to test the normality, all data qualified for normal distribution with Shapiro–Wilk test >0.9. The test for homogeneity was determined by residuals comparison with studentized residuals critical values (Lund, 1975). Mean values of all data were separated by least squared means, and classified by the macro PDMIX800.sas (Saxton, 1998) with $\alpha = 0.05$ into a bunched letters results. Significant differences between different treatments was shown with totally different letters ($P<0.05$).

5.3 Results

5.3.1 *ExoPG* responds more in a highly than a weakly aggressive isolate when exposed to potato leaf and stem extracts

The *ExoPG* gene's response was measured under treatment with potato leaf, stem and root extracts. As shown in Figure 2, *ExoPG* responded more in the highly aggressive isolate Vd1396-9 to potato leaf, stem extracts, compared to that in the weakly aggressive isolate Vs06-07. However, gene expression in both highly and weakly aggressive isolates were up-regulated in response to root extracts with no significant difference between them.

The expression of the *ExoPG* gene during infection of potato detached leaves was significantly higher in the highly aggressive isolate Vd1396-9 at 3DAI, when compared to the weakly aggressive one (Figure 5.2).

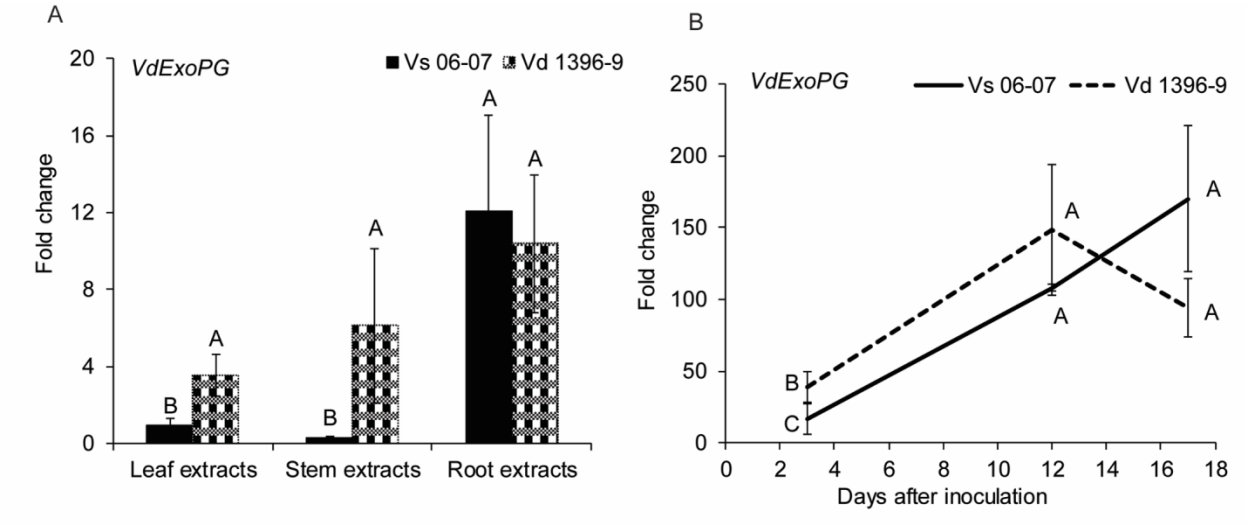


Figure 5.2 Expression of *ExoPG* gene in response to potato extracts and during infection. (A) Expression of *ExoPG* gene in response to potato extracts; (B) Expression of *ExoPG* gene during infection of detached Kennebec potato leaves. All QRT-PCR data were normalized with *V. dahliae* Histone H3. The bars (A) and point values (B) represent mean values (n = 3). Mean values marked by the same letters are not significantly different (P<0.05).

5.3.2 Vector-free split-marker recombination method to generate the △*exopg* mutant

As show in **Figure 5.3**, the 715 bp from the upstream region and the 713 bp from the downstream region of the selected *ExoPG* as well as the 789 bp from the 5' segment and 909 bp from the 3' segment of *Hph* gene were both successful amplified by PCR. Both 5'terminator of primers of FS-AR and FS-BF were flanked with a 27 bp nucleotide sequence from 5' and 3' terminator of the *Hph* gene sequence, respectively. The PCR product of the upstream region of the selected *ExoPG* region and the 5' segment of the *Hph* gene were used as templates to employ the overlapping PCR (**Figure 5.1**; **Table 5.2**). During the first 8 cycles of the overlapping PCR, these two DNA fragments used each other's overlapping regions as a "primer" to elongate a new DNA fragment. The primer (Primer: FS-AF/ HY-R; Table1) can attach to both ends of the new fragment, to enrich the new DNA fragment (**Figure 5.1**; **Table 5.2**). The same procedure was performed with

the PCR product of the downstream region of the selected *ExoPG* region and 3' segment of *Hph* gene with primer YG-F/ FS-BR (**Figure 5.1**; **Table 5.1 & 5.2**). As show in Figure 3, the 1486bp from DNA fusion of the upstream region of the selected *ExoPG* region and the 5' segment of *Hph* gene, the 1622 bp from DNA fusion of the downstream region of the selected *ExoPG* region and the 3' segment of the *Hph* gene, were both successfully amplified. After transforming both fused DNA from overlapping PCR into the protoplast by PEG-mediated transformation (**Figure 5.1**), seven positive knock-out transformants were obtained from 40 transformants identified by PCR (**Figure 5.4**). In addition, six single hygromycin gene replacement mutants of the 7 transformants were identified by southern blot (**Figure 5.4**). The mutants *△exopg-ko-18, and ∆exopg-ko-23* were randomly chose for subsequent experiments.

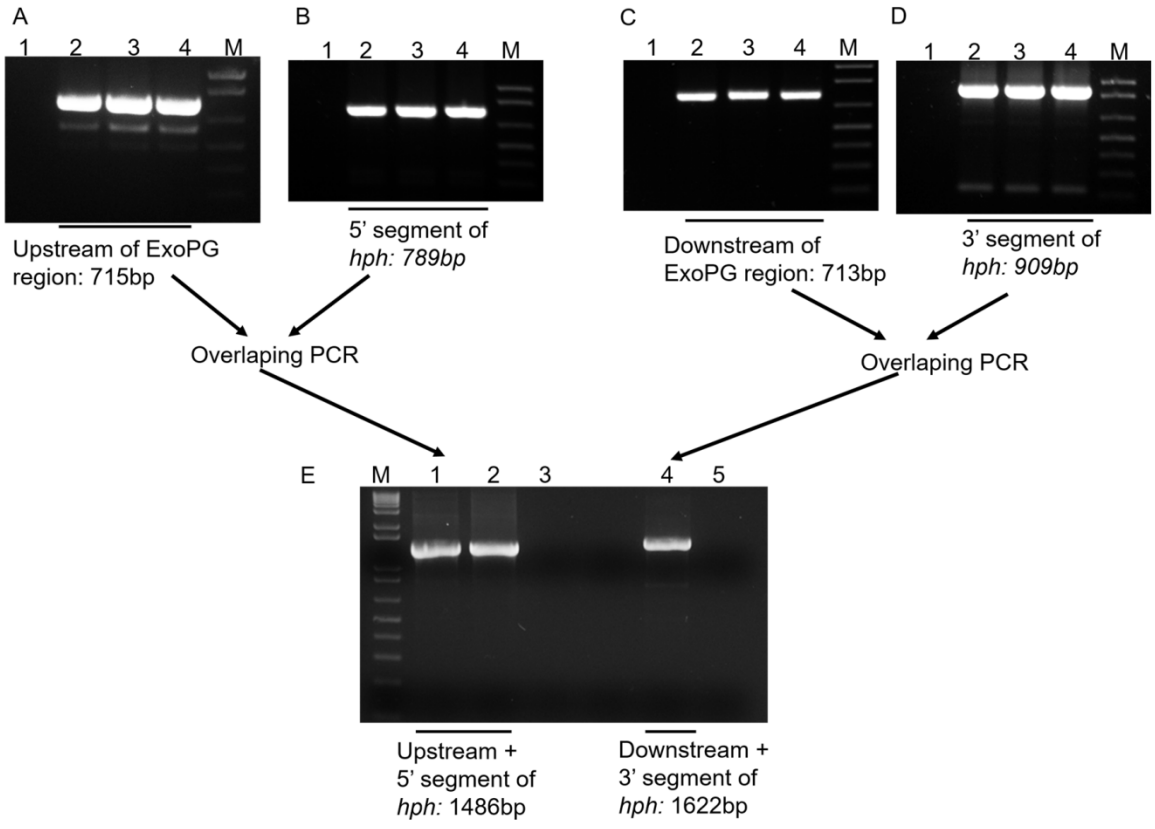


Figure 5.3 Construction of DNA fragment for knocking-out *ExoPG* gene in *V. dahliae* by overlapping PCR.

(A) Amplification of upstream DNA region of *ExoPG* ORF; (B) Amplification of 5' segment of *hph* gene from pSK846 vector; (C) Amplification of downstream DNA region of *ExoPG* ORF; (D) Amplification of posterior-half of *hph* gene from pSK846 vector; Lane 1 represent the negative control for PCR; Lane 2 to 4 represent the PCR products; Lane M represent the DNA marker (1Kb Plus DNA Ladder, Invitrogen, USA); (E) Connecting the PCR fragment by overlapping PCR; Lane 1 and 2 represent the upstream DNA connected with 5' segment of *hph* by overlapping PCR; Lane 3 represent the negative control for PCR; Lane 4 represent the downstream DNA connected with 3' segment of *hph* by overlapping PCR; Lane 5 represent the negative control for PCR; Lane M represent the DNA marker (1Kb Plus DNA Ladder, Invitrogen, USA).

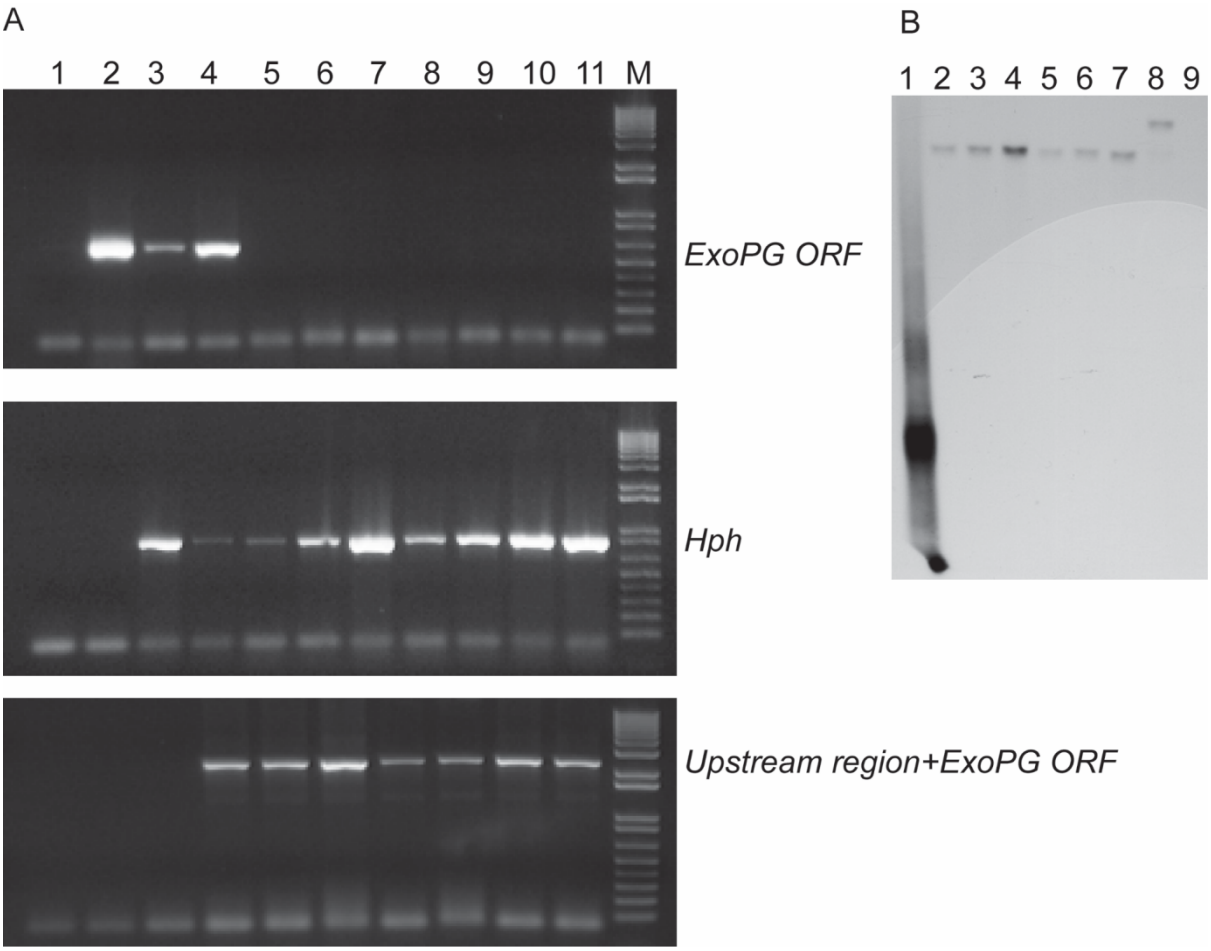


Figure 5.4 Identification of ∆*exopg* knock-out mutants.
(A) PCR analysis of ∆*exopg* knocking-out transformants; Lane 1 represent the negative control for PCR; Lane 2 represent the genomic DNA of wild type Vd1396-9; Lane 3 to 11 represent transformants; Lane M represent the DNA marker (1Kb Plus DNA Ladder, Invitrogen, USA); (B) Southern blot analysis of positive transformants of ∆*exopg* knocking-out transformants; Lane 1 represent the probe amplified from *hph* gene; Lane 2 to 7 represent single hygromycin gene replacement ∆*exopg* mutants; Lane 8 represent ectopic control; Lane 9 represent the wild type Vd1396-9.

5.3.3 Characterization of Δ*exopg* mutant

As shown in **Figure 5.5**, there were no significant differences in the growth, conidiation, or formation of microsclerotia between the Δ*exopg* mutants (*△exopg-ko-18, Δexopg-ko-23)* and wildtype Vd1396-9.

To determine the difference in virulence between the Δ*exopg* mutants and wildtype (**Figure 5.6**), all isolates were inoculated onto the susceptible potato cultivar Kennebec. There was no dramatic change in total area under disease progress curve (Total AUDPC) for infection or disease severity, or plant height and vascular discoloration measurements.

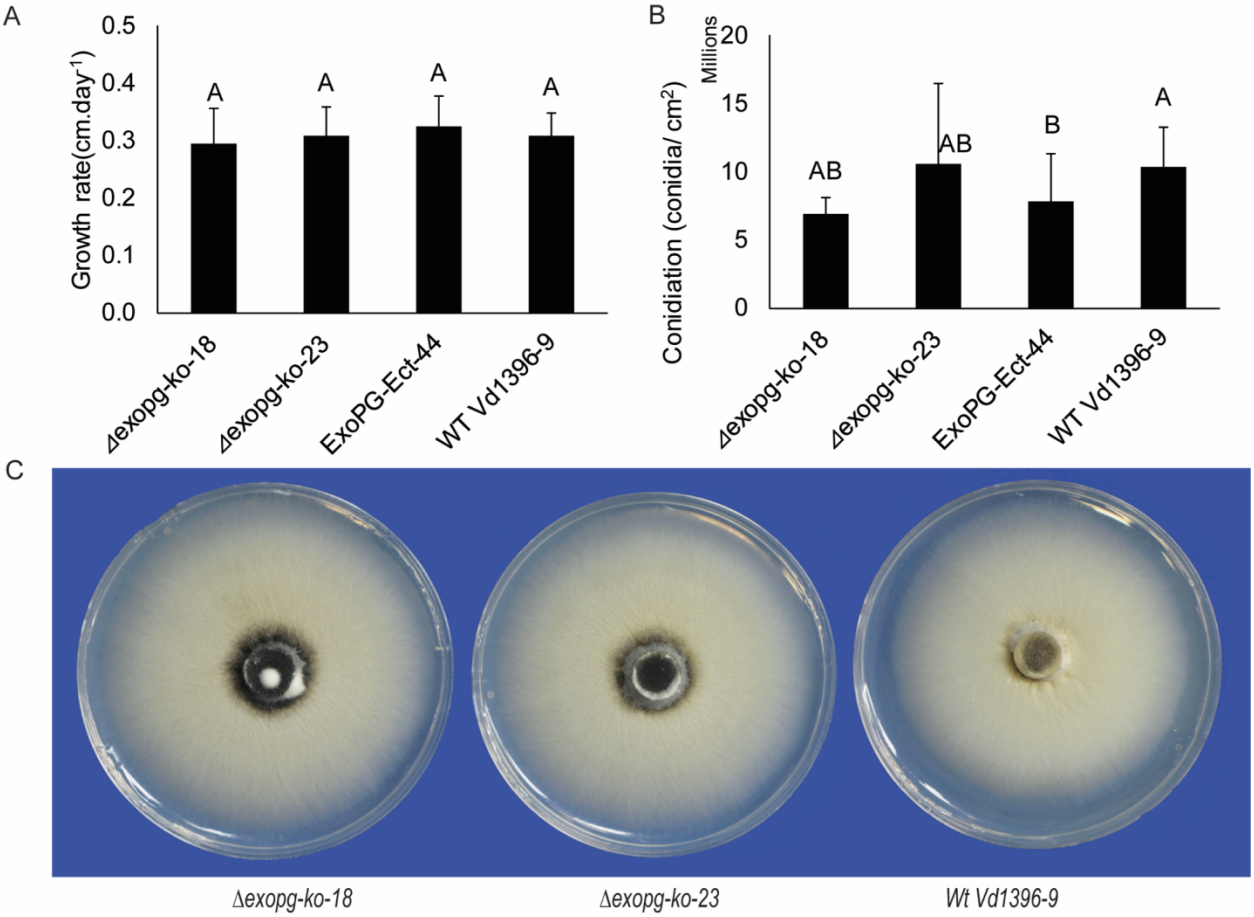


Figure 5.5 The phenotype analysis of Δ*exopg* mutants.
(A) The mycelial growth rate of Δ*exopg* mutants on PDA medium; (B) The conidiation of Δ*exopg* mutants; (C) The colony phenotype of Δ*exopg* mutants. The bar graphs depict mean values (n = 8 for growth rate experiment, and n=5 for conidiation experiment). Error bars refer to standard error.

Mean values marked by the different letters are significantly different (P<0.05).

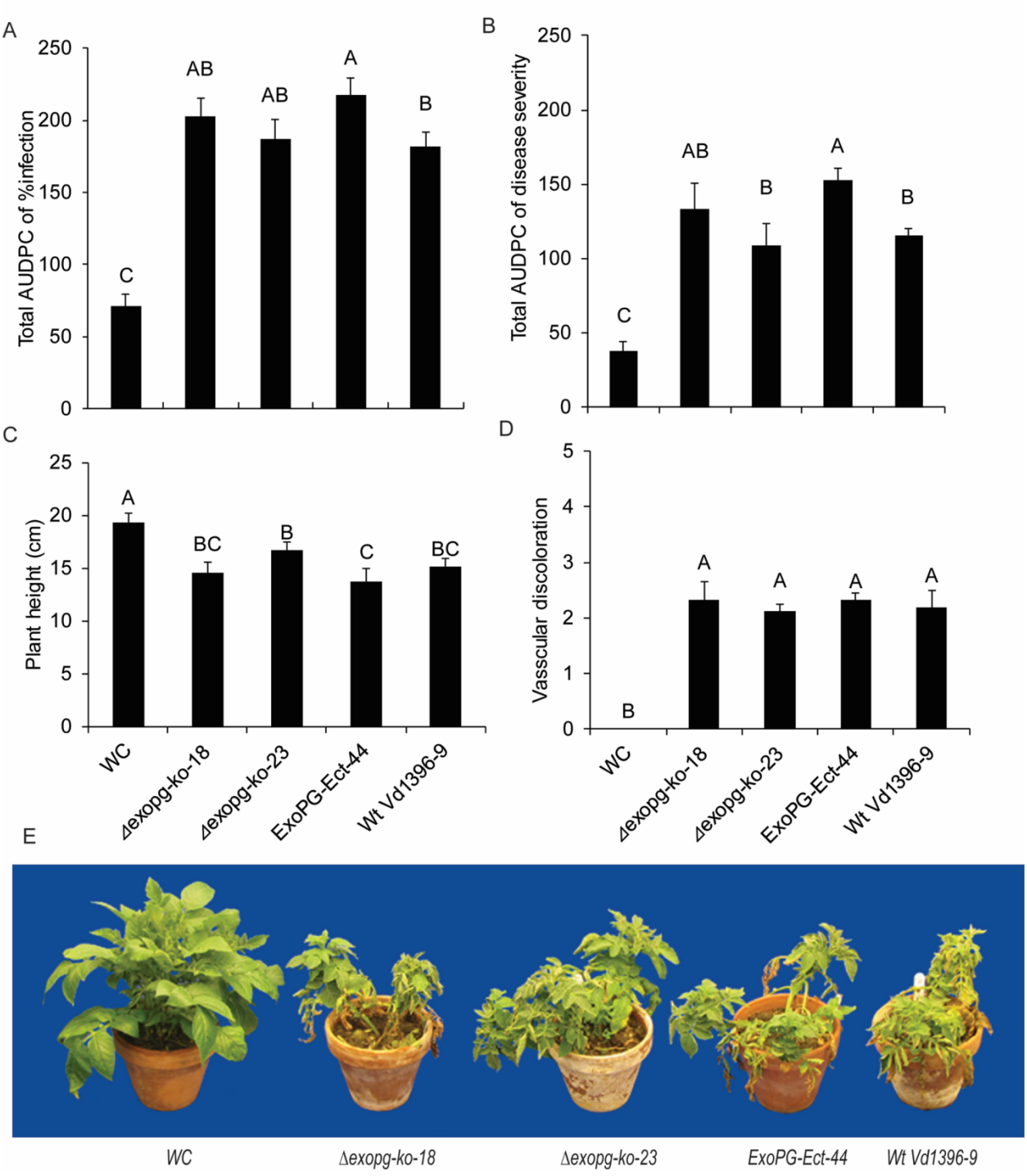


Figure 5.6 Pathogenicity test of Δ*exopg* mutants on potato cv. Kennebec.
(A) Total AUDPC of percentage of infection; (B) Total AUDPC of disease severity; (C) Plant height of potatoes at 5 weeks post-inoculation; (C) Vascular discoloration rate at 5 weeks post-inoculation; (E) Kennebec potatoes infected by Δ*exopg* mutants at 5 weeks after infection. Error bars refer to standard error. The bars represent mean values (n = 6) with different letters

representing significant differences between mean values (P<0.05).

5.3.4 Total exopolygalacturonases activity of ∆*exopg* mutant

To determine the influence of the disruption of *ExoPG* in total exopolygalacturonases activity, the supernatant from cultured isolates (*⊿exopg-ko-18,* ∆ *exopg-ko-23* and wildtype Vd1396-9) were collected over time and assayed for enzyme activity. Pectin was added to the CDB medium to stimulate the production of ExoPG. As show in **Figure 5.7**, the total exopolygalacturonases activity in both isolates under the elicitation of pectin is significantly higher than the control group, however, there is only small differences between the ∆*exopg* mutants and wildtype.

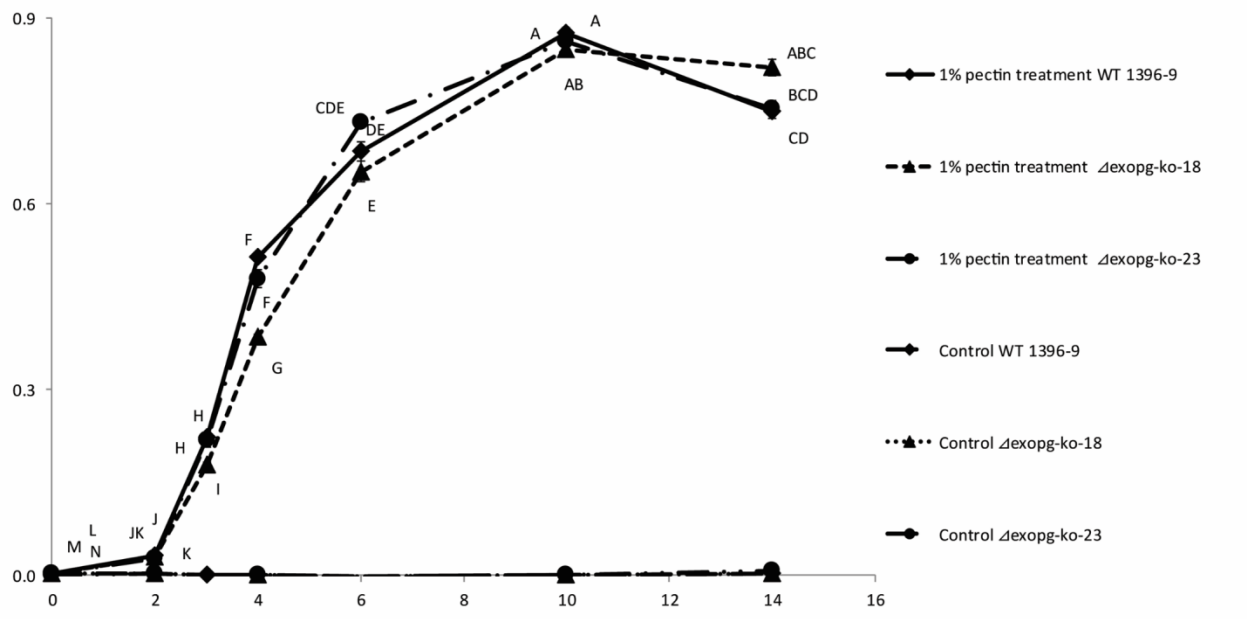


Figure 5.7 Total exopolygalacturonases activity of ∆*exopg* mutant.
Graph points represent mean values (n = 3, with 3 technical replicates for each biological replicate). Means labelled by different letters are significantly different between treatments (P<0.05). Error bars refer to standard error.

5.4 Discussion

The most common transformation method in *V. dahliae* is *Agrobacterium*-mediated

transformation, in both disruption and overexpression of a targeted gene (Dobinson et al., 2004; Gao et al., 2010; Klimes and Dobinson, 2006; Maruthachalam et al., 2011; Tzima et al., 2012; Zhu et al., 2017). However, PEG-mediated protoplast transformation is a useful method in other fungi and plant transformation (Mathur and Koncz, 1998; Ruiz‐Díez, 2002; Zhou et al., 2017a; Zhou et al., 2017b). Here, we successfully employed this method in *V. dahliae*. Using this method, we successfully obtained 40 trans-DNA transformants including ectopic strains and targeted gene mutation strains.

Gene disruption in fungi, by either *Agrobacterium*-mediated transformation or PEG-mediated protoplast transformation, often requires vector construction (Shafran et al., 2008; Zhou et al., 2017a; Zhu et al., 2017; Zhu et al., 2016). In particular, *Agrobacterium*-mediated transformation requires a special vector in the procedure (Lu et al., 2014; Tzima et al., 2012; Weld et al., 2006). Constructing a vector is time-consuming and requires many steps. Here, we employed a vector-free split-marker recombination method for knocking out target genes in *V. dahliae*, as previously done in *S. cerevisiae* (Catlett et al., 2003) and *M. oryzae* (Zhou et al., 2017b). This method can be easily processed by only two steps of PCR and therefore can be typically done in one day. This method can substantially reduce the time and cost when compared to at least one-week requirement for constructing a vector for a targeted gene. Combining both vector-free split-marker recombination method with PEG-mediated protoplast transformation reduces the total time for manipulation including transformation to as little as three days. This is dramatically shorter than the vector-based *Agrobacterium*-mediated transformation. Besides time consideration, this method could reduce the number of transformants that contain randomly inserted exotic DNA in the fungal genome. If each of the two fusion DNA fragments (flanking DNA with half marker gene) were separately inserted into the fungi genome, the transformants cannot grow on the

selection media, because of the non-intact marker gene. Only when the two fusion DNA fragments are inserted in the proper place, the homologous recombination can occur between overlapping regions of the two split-markers. The overlapping PCR in this study is a convenient and quick method to fuse different DNA fragments together, without finding or adding appropriate restriction enzyme sites in the edge of different DNA fragments.

Plant cell walls and cuticle consist of different components, such as pectic substances, cutin and cellulose, to protect the plant from pathogen attacks (Agrios, 2005). However, pathogens can also overcome these strategies by producing cell wall-degrading enzymes (CWDEs) to degrade the plant cell wall, such as pectinases, cutinases, glucanases, and cellulases (Agrios, 2005). *V. dahliae* can cause disease on a great number of dicot plants which have been shown to contain plenty of pectin (Kubicek et al., 2014; Pegg and Brady, 2002; Varner and Lin, 1989). Moreover, pectin-degrading enzymes may play more critical roles in microbes infecting dicot plants. Dicot plant pathogens possess more genes for encoding pectin-degrading enzyme than that of monocots pathogens (Kubicek et al., 2014). In *A. citri*, an *EndoPG* gene is required for pathogenicity (Isshiki et al., 2001). El-Bebany et al. found that when *V. dahliae* responds to root extracts from both moderately resistant and susceptible potato cultivars, the expression of *Exo-PG* (VDAG_03463) was up-regulated in the highly aggressive *V. dahliae* isolate (Vd1396-9) but down-regulated in a weakly one (Vs06-14) (El-Bebany et al., 2011). Bases on these results, we wanted to investigate if the function of this *ExoPG* is involved in pathogenicity or interaction with the host. However, the degree of up or down regulation in the differential aggressive isolates of *V. dahliae* were not provided by El-Bebany et al., since the experiment was done using a subtractive hybridization cDNA-AFLP method (El-Bebany et al., 2011).

Here, we aimed to determine the fold change of *ExoPG* expression in differentially

aggressive *V. dahliae* isolates in response to potato leaf, stem, or root extracts, and during infection. *ExoPG* responded more in the highly aggressive isolate Vd1396-9 to potato leaf and stem extracts, compared to that in the weakly aggressive one. *ExoPG* was up-regulated in both isolates elicited by root extracts. However, *ExoPG* was up-regulated more in the highly aggressive isolate than the weakly one at early infection stages. This may indicate that this gene may be activated by potato extracts in both isolates but at a higher level in the highly aggressive one and therefore may not play a primary role in infection. Subsequent comparison of the Δ*exopg* mutants and wildtype proved that *ExoPG* is not alone necessary for virulence in *V. dahliae*. The total exopolygalacturonases activity in *V. dahliae* did not change by the disruption of the *ExoPG* gene. It is possible that other isoforms of *ExoPG* compensated for the function, and this particular *ExoPG* may not be the primary one in *V. dahliae.*

CHAPTER 6

GENERAL DISCUSSION

The potato crop is a large contributor to the Canadian economy placing the production between 1996 to 2016 to the fourteenth largest in the world ("http://faostat.fao.org/site/339/default.aspx"). However, the yield of potato is greatly influenced by a disease complex named potato early dying (PED), causing production losses ranging from 10% up to 50% (Johnson and Dung, 2010; Johnson et al., 1986; Powelson and Rowe, 1993; Rowe and Powelson, 2002). PED is primarily caused by *V. dahliae* and *V. albo-atrum,* with the former having a far wider host range than the later (Pegg and Brady, 2002). The management of *V. dahliae* including biocontrol agents, soil fumigation, crop rotation, and green manure are sometimes costly and ineffective (Antonopoulos et al., 2008; Bhat and Subbarao, 1999; Molina et al., 2014; Subbarao et al., 1999; Tsror et al., 2005). In Dr.Daayf's lab, previous studies employing two isolates with contrasting pathogenicity, showed several proteins with differential accumulation between a highly and a weakly aggressive isolate of *V. dahliae* (El-Bebany et al., 2010). Another study employed the same isolates to identify genes which were up regulated after elicitation by susceptible / moderately resistant potato root extracts (El-Bebany et al., 2011). In this Ph.D project, we aimed to understand more about these proteins and genes' roles in the interaction between *V. dahliae* and its potato host, and provide more insights to finding better strategies for the management of *V. dahliae*.

6.1 Monitoring expression of potential pathogenicity-related genes in *V. dahliae* by qPCR

We used qPCR to monitor the expression of 15 genes, selected from the previous studies, during infection of detached potato leaves with two isolates of *V. dahliae* (one highly and one weakly aggressive), as well as in response to elicitation with extracts from different types of potato tissues. The higher activities of Ras-GAP like protein, serine/threonine-protein kinase, Ubiquitin-

conjugating enzyme variant MMS2, NADH-ubiquinone oxidoreductase, Thioredoxin, Pyruvate dehydrogenase E1 component subunit beta (VdPDHB), myo-inositol 2-dehydrogenase, and HAD-superfamily hydrolase, in the highly aggressive isolate may partially clarify its virulence in the highly aggressive isolate. These genes that are involved in cell differentiation and proliferation, DNA repair, ROS regulation, cellular metabolism and detoxification, may be critical for virulence. This study provides quantitative insight into the expression of several key genes participating in response to different types of potato extracts and potentially facilitating the virulence in the highly aggressive isolate. Additional characterization of these genes in the future could improve our understanding of fungal pathogenesis and may serve in discovering new systems for controlling Verticillium wilt.

6.2 Isochorismatase hydrolase is involved in the interaction with the salicylate and jasmonate defense signaling pathways in potatoes

V. dahliae's Isochorismatase Hydrolase (*ICSH1*) gene responded more to potato root extracts than leaf and stem extracts, with no significant difference between the highly and weakly aggressive isolates. In constrast, its expression was much higher in the highly than weakly aggressive isolate during infection of detached potato leaves. We hypothesized that ICSH could hydrolyze the isochorismate and therefore hijack the SA defense pathway in potato. To further determine the function of VdICSH1, gene disruption mutants were generated from the highly aggressive isolate. Pathogenicity tests on *icsh1* mutants shows that in *V. dahliae* this gene is required for full virulence on potato. Our results showed that SA and JA accumulation exhibit distinctive tendencies in the stems and roots at early infection stages, and similar tendencies in the leaves at later stages. The *icsh1* mutant induced more bound-SA in the leaves at 9 DAI, compared

to the wild type. The increased expression of the isochorismatase family members (VDAG_03530 and VDAG_06170) in the *icsh1* mutants compensate for the absence of ICSH1, but this compensation is not obvious in company with potato leaf extracts. The wildtype induced higher levels of both free-SA and bound-SA than the mutant in the leaves, but only more bound-SA in the roots. This study highlighted the important role of VdICSH1 in *V. dahliae*'s virulence and alteration of the SA/JA defense signaling networking.

6.3 NoxA and NoxB are important for *V. dahliae's* penetration and virulence on potatoes

Previous studies suggested a potential role of ROS in *V. dahliae*'s pathogenicity. This was proven by two genes that work in sustaining the ROS balance, which was only present in the highly aggressive *V. dahliae* isolate but not in the weakly aggressive one. In *V. dahliae*'s genome, three types of Nox genes responsible for enzymatic ROS production had been identified, NoxA, NoxB, and NoxC. In response to potato leaf and stem extracts, NoxA expression increased more in the weakly than in the highly aggressive isolate of *V. dahliae*. NoxB expression increased more in the highly than in the weakly aggressive isolate in response to root and leaf extracts. However, NoxC responded in the opposite way in response to potato root and stem extracts in these isolates. During inoculation of detached leaves, the expression of NoxA and NoxB, but not NoxC, drastically increased in the highly aggressive isolate compared to the weakly aggressive one. To further determine their roles in virulence, the penetration ability of *noxa* and *noxb* mutants on cellophane membrane were analyzed. The penetration ability of the *noxa* and *noxb* mutants was significantly reduced compared to the wild type. NoxB was also shown to be involved in the cell wall biosynthesis pathway, which was altered in *noxb* mutants compared to the wild type. Also, *noxb* mutants showed more resistance to oxidative stress in solid medium.

6.4 Generation of an exopolygalacturonase mutant in *V. dahliae* with a new vector–free split-marker recombination method

Previous research has shown that, in general, dicot pathogens can produce more pectin-degrading enzymes than their monocot counterparts. By comparing gene expression in a highly and a weakly aggressive *V. dahliae* isolates, after elicitation with both susceptible and moderately resistant potato root extracts, a *ExoPG* gene (VDAG_03463) was identified as apossible contributor to virulence (El-Bebany et al., 2011). Here, we employed a new method derived from *Saccharomyces cerevisiae*, a vector–free split-marker recombination method with PEG-mediated protoplast transformation, to delete the *ExoPG* gene in a highly aggressive *V. dahliae* isolate. This new method is time-saving and easy to operate as it only requires a 2 step PCR and one step transformation, which successfully generated at least 6 mutants for the *ExoPG* gene. The expression of *ExoPG* responded more in both the highly and weakly aggressive isolates during the elicitation with potato root extracts, but it's expression was significantly increased in the highly aggressive isolate when exposed to leaf and stem extracts. During infection of detached leaves, the expression of this gene was elevated in both highly and weakly aggressive isolates, with a significantly higher level in the highly aggressive isolates at early infection stage, but similar in both at later stages. Interestingly, deletion of *ExoPG* does not affect either virulence nor total exopolygalacturonases activity in *V. dahliae*.

In conclusion, in this study, we analyzed a set of selected genes' expression in response to various potato plant part extracts during infection. We analyse these genes' in an integral model. Later, we choose some of the most interesting genes like VdICSH1 and Nox family genes for

further investigation of their roles in the interaction between *V. dahliae* and potato. We also employed a new method for quick generation of *V. dahliae* mutants. In general, these studies added to the knowledge on *V. dahliae*'s mechanisms of interaction with its potato host.

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