CHARACTERIZATION OF EFFECTOR ENCODING GENES FROM THE NOVEL SUGAR BEET PATHOGEN FUSARIUM SECORUM

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Characterization of effector encoding genes from the novel sugar beet pathogen *Fusarium secorum*

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ABSTRACT

A new disease of sugar beet, named Fusarium yellowing decline, was recently found in in the Red River Valley of MN and ND. This disease is caused by a novel pathogen named *Fusarium secorum*. Pathogens such as *F. secorum* secrete proteins during infection called 'effectors' that help establish disease. Since pathogenicity and disease development may depend on effector proteins produced by *F. secorum* during infection, effector protein identification furthers our understanding of the biology of this important pathogen. A list of 11 candidate effectors was generated previously. In this study, to characterize putative effectors, we developed a transformation system using polyethylene glycol–mediated transformation. Several mutant lines were created with an effector deleted from the genome using a split-marker knock-out strategy. To explore their role in pathogenicity, mutant strains have been inoculated to sugarbeet and compared to WT *F. secorum*.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Figure Page

CHAPTER I. LITERATURE REVIEW

Sugarbeet history

Sucrose is a carbohydrate naturally occurring in many plants. The molecular structure of sucrose is a non-reducing disaccharide ($C_{12}H_{22}O_{11}$) derived from the combination of monosaccharides glucose and fructose, which are linked via their anomeric carbons (Figure 1). Sucrose is typically produced and stored in plant roots, fruits and nectars primarily as a product of photosynthesis (Lunn, 2008). After extraction, sucrose is one of the most frequently consumed sweeteners in the United States (Food and Agriculture Organization, 2009).

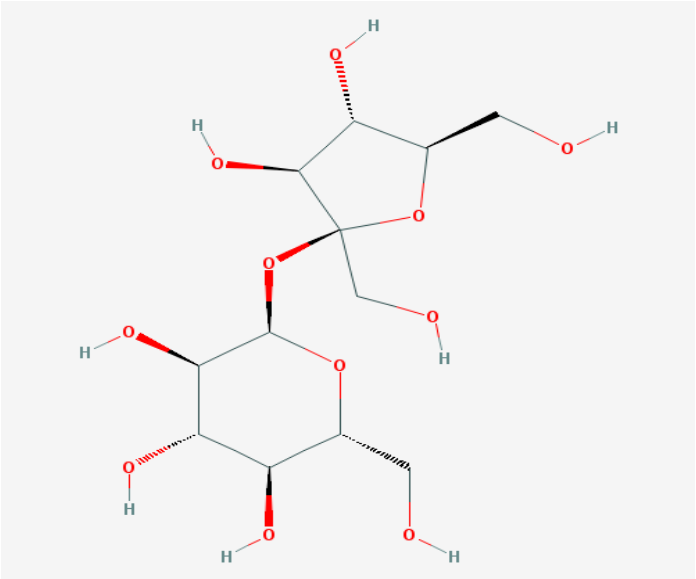

Figure 1-1. Two-dimensional structure of sucrose.

Two plants that produce large amounts of sucrose are sugarcane (*Saccharum officinarum* L.) and sugarbeet (*Beta vulgaris* L.). Worldwide, 80% of sucrose is derived from sugarcane and 20% from sugarbeet (Commission, 2013). Sugarcane is cultivated in tropical and sub-tropical regions for sucrose found in its stems. By contrast, sugarbeet is cultivated in temperate climate countries

mainly in Europe, United States, China, Japan and also in Chile, Morocco and Egypt (Food and Agriculture Organization, 2009) for sucrose found in the root.

The probable ancestor of cultivated sugarbeet, wild sea beet (*B. maritima*), grows in the UK, mainland Europe and North Africa. Initially, people gathered leaves of sea beet to eat as a vegetable (Mabey, 1997). As time went on, different varieties of beet were gradually cultivated as garden vegetables. Beet crops were not cultivated regularly in the field until the seventeenth century, which started the domestication of sugarbeet (Draycott, 2006). Andreas Sigismund Marggraf, who demonstrated that the sweet tasting crystals derived from sugarbeet were the same as from sugarcane, made a great contribution to the modern sugarbeet industry. Marggraf's student Franz Carl Achard, who is now recognized as 'father of the sugarbeet industry,' continued research on sugarbeet and developed the first beet sugar factory. Achard's efforts opened the way to industrial sugar production from sugarbeet (Ministry of Agriculture and Fisheries, 1931).

Napoleon I developed interest on Achard's new ideas about sugar production. Laws were established to allocate money to build factories in France and restrict the importation of sugar from sugarcane, which facilitated and accelerated the development of sugarbeet industry in Europe (Rolph 1917; Draycott 2006). While sugar factories were totally destroyed as consequences of slavery and the fall of Napoleon, new sugarbeet factories were established in France, Germany, Austria, Russia, Germany and UK in late 1840s (Harveson and Rush 1994). Laws were passed by European countries for the purpose of building up and protecting the sugarbeet industry, which resulted in the exportation of sugar since increased production was more than sufficient to maintain domestic demand. France was in the leading position in

exportation of sugarbeet until Germany exceeded it in 1880. Currently, Germany is the leading exporter of sugar worldwide (Draycott, 2006).

Refining sugar from sugarbeet in United States began with a company led by John Vaughn and James Ronaldson when they built a small factory in Philadelphia in 1830. However, the company ultimately failed due to lacking of knowledge of sugarbeet culture and manufacturing techniques (Rolph, 1917). Soon after in Northampton, Massachusetts, David Lee Child, who studied sugarbeet culture and extraction of sugarbeet in Europe, succeed in extracting sugar from sugarbeet roots in 1830 (Ware 1880; Harris 1919). With continued efforts made on sugarbeet cultivation and sugar extraction procedures, commercial sugar production was finally established in California in 1870. Until the 1950s, sugarbeet has been successfully cultivated in 22 states with the centers of beet production located in Rocky Mountains (From Montana to Texas), California, Great Plains, Ohio, Michigan (Draycott, 2006) and Red River Valley (North Dakota and Minnesota). Sugarbeet production in Minnesota and North Dakota began shortly after World War I in 1926. Minnesota Sugar Company, which was bought by Denver-based American Beet Sugar Company later, was attracted by a field test with sugarbeet conducted by University of Minnesota and constructed a processing plant in East Grand Forks (Norris, 1910). Red River Valley has now become the largest and most dynamic region for sugarbeet production. The area planted in this region in the 2000s averaged 296,000 hectares, which consists of approximately 55 percent of total planted U.S. sugarbeet acreage (McConnell, 2013).

Sugarbeet is grown in more than 50 countries worldwide today. Russian is the largest sugarbeet production country followed by Ukraine, Germany, United States, Germany, France and Turkey. The world production has increased over the last few decades and in 2011,

5,069,362 hectares with an average yield of 48 metric ton haectare^{-1} sugarbeet was grown across the world (Yara UK, 2011).

Sugarbeet diseases

Sugarbeet diseases can be caused by viruses, fungi, bacteria, nematodes, and phytoplasmas as well as parasitic plants. Of these, fungal diseases constitute a major constraint on yield of sugarbeet in most areas. Many fields that have relatively short rotations between sugarbeet crops provide suitable environment for soil-borne fungal pathogens that can cause significant yield losses (Cooke, 1993). Major sugarbeet soil-borne pathogens that have led to considerable yield loss including *Rhizoctonia solani* Kühn, *Aphanomyces cochlioides* Drechs., *Verticillium dahiae* Kleb. and *Fusarium* species (Harveson et al. 2009; Draycott 2006; Cooke 1993).

Rhizoctonia root and crown rot

Rhizoctonia root and crown rot is one of the most important sugarbeet root diseases of the world (Kiewnick et al., 2001). Early symptoms are characterized with chlorosis and a sudden wilting of foliage as well as dark brown to black lesions of petioles. Later, dark brown to blackish rot can be observed in roots and usually beginning at crown and develop down to taproot (Draycott, 2006). The causal agent of Rhizoctonia root and crown rot is *Rhizoctonia solani*. *Rhizoctonia solani* strains are primarily grouped into genetically isolated anastomosis groups (AGs) based on hyphal anastomosis reactions, which can be further subdivided into intraspecific groups (ISGs) (Bolton et al. 2010). The fungus can grow both intra or inter cellularly (Ruppel, 1973) and seedlings are generally more easily infected than older plants (Pierson and Gaskill, 1961). *R. solani* can survives as sclerotia, hyphae or in organic debris in soil for several years. Inoculum can be spread in infested soil by irrigation water, wind or transport of soil (Roberts and Herr, 1979).

Aphanomyces root rot

Aphanomyces root rot can lead to foliar chlorosis, wilting, and dull green foliage. Root lesions appear water-soaked yellow-brown and then turn dark brown to black. Plants with latent infections have distorted, stunted roots and scabby lesions on root surface (Schneider, 1965). The causal agent *Aphanomyces cochlioides* is in the class Oomycetes. It can survive in soil or infected plant debris for long periods and spread in infested soil (Tahara and Mizutani, 1999). Although the host range of this pathogen is limited, *Aphanomyces cochlioides*-induced disease has been reported as a serious sugarbeet problem in different countries including Chile, Japan, USA, and Europe (Draycott, 2006).

Fusarium yellows

Fusarium Yellows can cause significant reduction in plant quality, root yield, juice purity and sucrose concentration (Schneider and Whitney, 1986). This disease is found in several countries including Netherlands, India, Belgium, Germany and Red River Valley (Bennett, 1960). Disease is characterized by chlorosis between larger veins at the early stage of disease (Khan et al., 2013). As disease progresses, older leaves may turn necrotic and younger leaves start to show chlorosis and yellowing (Harveson et al., 2009). The foliage usually wilts during the day but regains turgor overnight in the early stages of disease. Eventually leaves collapse around crown but remain attached to the plant after dying (Khan et al., 2013).

The causal agent of Fusarium yellows is *Fusarium oxysporum* f. sp. *betae* (Harveson and Rush, 1998). *F. acuminatum* has been reported to result in Fusarium yellows-like symptoms (Ruppel, 1991). In addition, some isolates of *F. avenaceum* and *F. verticillioides* also have been associated with Fusarium yellows-like symptoms but with less importance because they are not widespread (Hanson and Hill, 2004). While rotation with other crops can reduce inoculum

buildup in infested soil, this management method is of limited value because the pathogens have a wide host range and can survive for long periods (Khan et al., 2013). Since genetic resistance to Fusarium yellows has been developed (Bockstahler, 1940), replacement of susceptible sugarbeet varieties with Fusarium yellows-resistant varieties is the major method to manage the disease (Khan et al., 2013).

Fusarium yellowing decline

Disease history

A disease was present in sugarbeet fields located in central and southwest Minnesota in 2005 to 2007. This disease caused symptoms similar to Fusarium yellows on sugarbeet cultivars resistant to Fusarium yellows, which attracted attention from the local sugarbeet industry. Unlike other sugarbeet pathogenic *Fusarium* species isolated from sugarbeet taproots, this novel *Fusarium* could be isolated from petioles (Rivera et al., 2008). To differentiate it from Fusarium yellow disease, this new disease was named Fusarium yellowing decline (Rivera et al., 2008).

Symptoms

The novel *Fusarium* species was shown to be more aggressive compared to other yellows pathogens (Burlakoti et al., 2012). It can cause interveinal chlorosis on half of the leaves of infected plants two weeks after inoculation. Symptoms start to show on only one side of older leaves at early stages of disease development. Three weeks after inoculation, roots and petiole tissue of infected plants showed vascular discoloration. Eventually, leaves wilt and plants may die (Figure 2) (Secor et al., 2014). Fusarium yellowing decline is differentiated from Fusarium yellows by causing petiole elements discoloration, seedling infection as well as rapid death earlier in the season (Rivera et al. 2008; Secor et al. 2014).

Figure 1-2. Fusarium yellowing decline disease symptoms in sugarbeet cv. VDH 46177. (A) Typical general chlorosis in older leaves, including half- to full-leaf yellowing, 3 weeks after inoculation with Fusarium secorum. (B) Below- and above- ground symptoms of Fusarium yellowing decline 4 weeks after inoculation. (C) Characteristic half-leaf chlorosis and necrosis 2 weeks post inoculation. (D) Root and (E) petiole cross-sections exhibiting vascular discoloration. (Secor et al., 2014).

The causal agent, *Fusarium secorum*

Translation elongation factor 1-α (TEF), calmodulin (CAL) and mitochondrial small subunit (mtSSU) rDNA sequences were amplified from novel *Fusarium* species for BLAST and phylogenetic analysis (Secor et al., 2014). Results strongly supported the monophyly of this novel *Fusarium* species, which has been named *Fusarium secorum* (Figure 3) (Secor et al., 2014). While *F. secorum* can cause similar disease symptoms on sugarbeet as *F. oxysporum* f. sp. *betae*, they only have a distant relationship based on phylogenetic analysis. Phylogenetic analysis showed that *F. secorum* has the closest relationship with *F. acutatum*, which both belong to the *Fusarium fujikuroi* species complex (Secor et al., 2014). *F. acutatum* was first recovered from *Cajanus* sp. and from aphids from wheat in Pakistan and India (Nirenberg and O'Donnell, 1998). However, sugarbeet inoculated with *F. acutatum* did not result in any disease symptoms (Secor et al., 2014).

Colonies of *F. secorum* on potato dextrose agar (PDA) exhibit radial mycelial growth of 5.1 mm d^{-1} on average with entire white colony color in the dark without distinctive odor. When grown under light, the colony color will turn light orange or pink in the center and white on the edge (Figure 4) (Secor et al., 2014). *F. secorum* produces coiled hyphae abundant microconidia, chlamydospores and sparse macroconidia (Figure 5) (Secor et al., 2014). While microcondia are produced in young colonies, macroconidia usually will be produced in older colonies more than five weeks old. Mature colonies can also produce chlamydospores, most of which are subglobose (Secor et al., 2014).

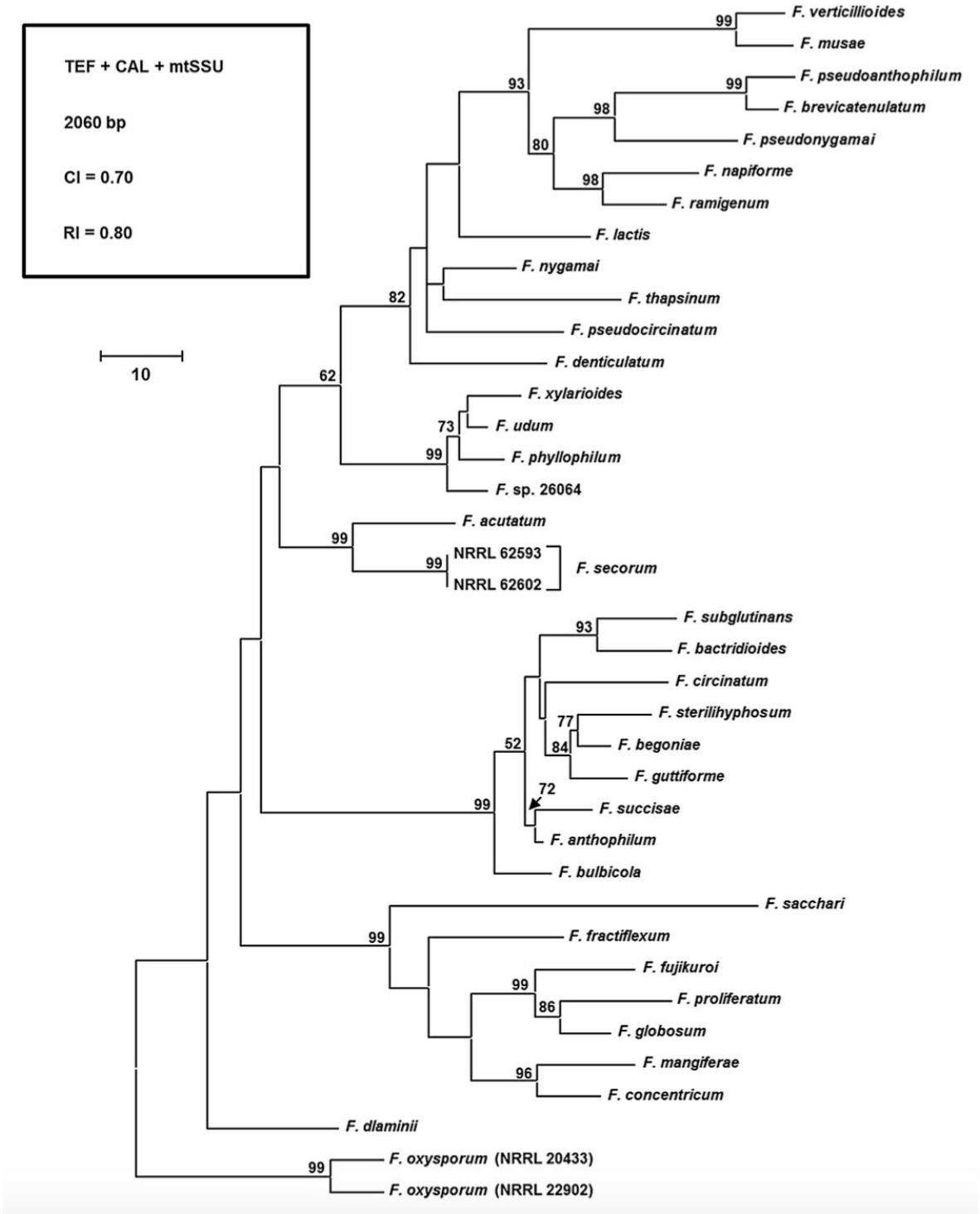

Figure 1-3. Phylogenetic analysis conducted with the partial TEF, CAL and mtSSU rDNA sequence datasets. *F. secorum* is showed to be a new, unique specie next to *F. acutatum* in the phylogenetic tree (Secor et al., 2014).

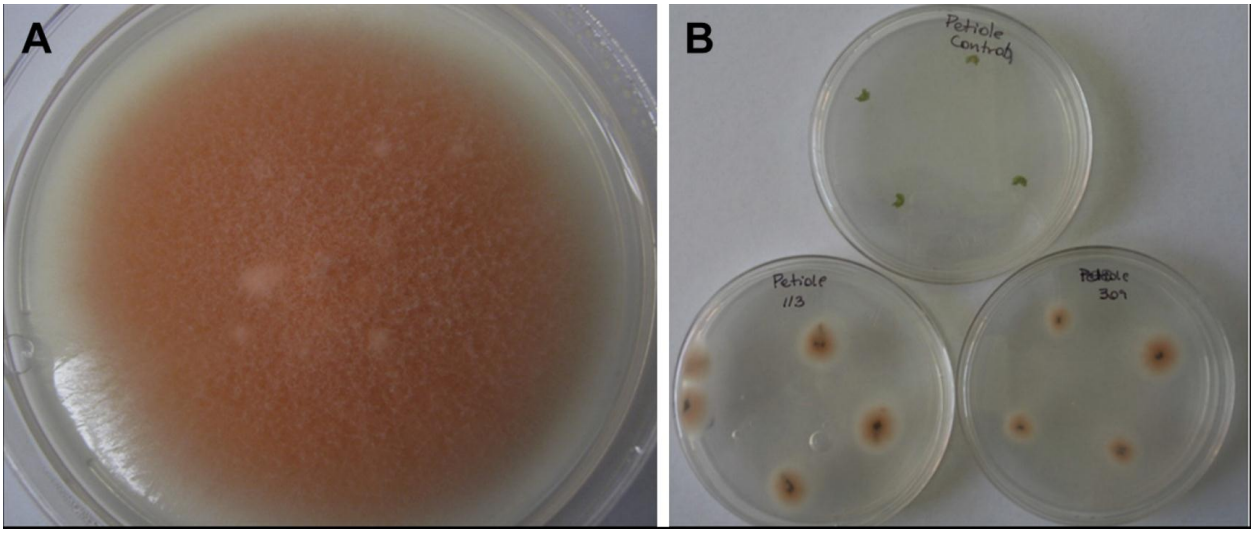

Figure 1-4. Colony morphology of F. secorum on potato dextrose agar (PDA) after growing for two weeks under constant fluorescent light (Secor et al., 2014).

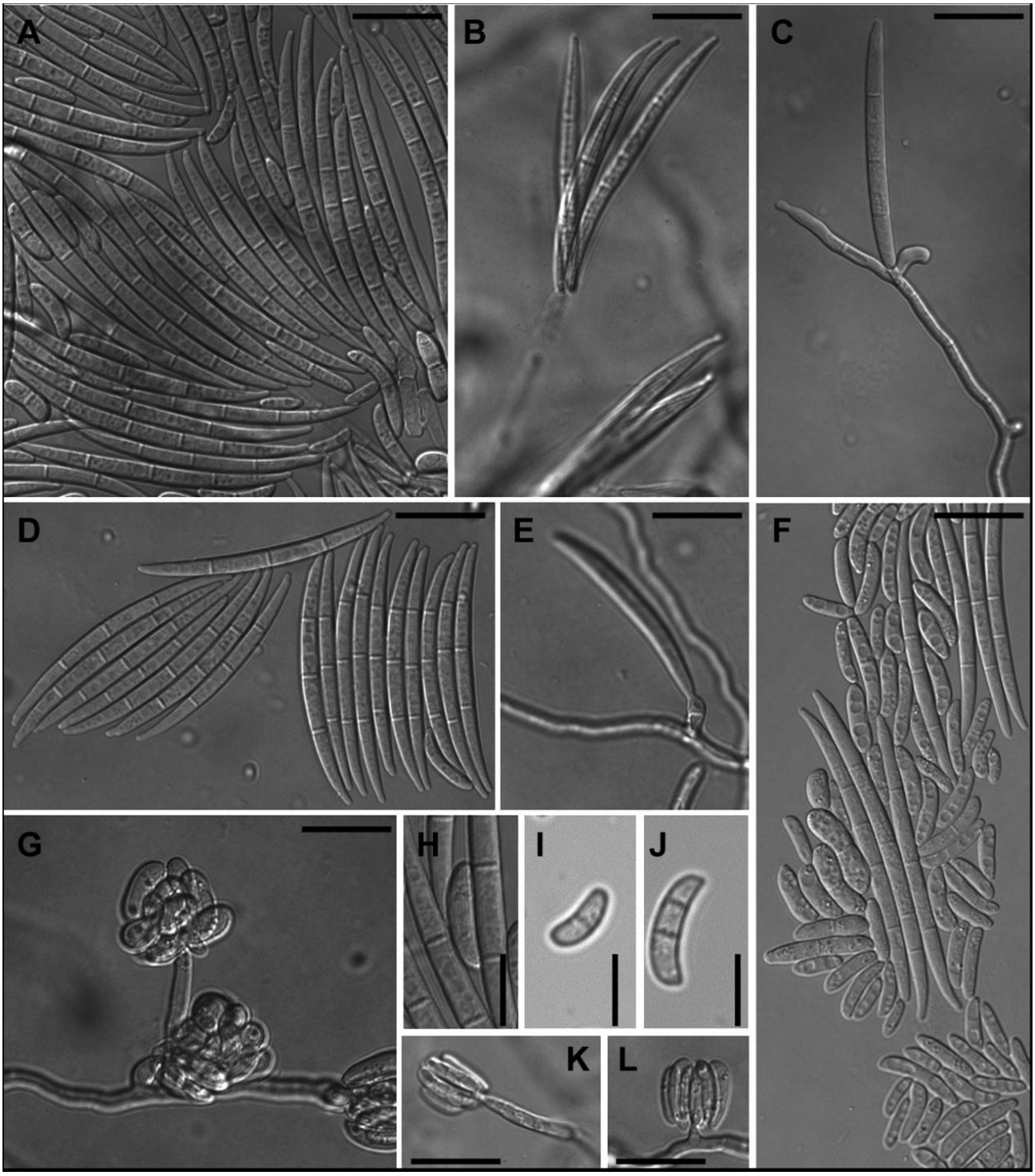

Figure 1-5. Diagnostic morphology of Fusarium secorum. (A and D) Multiseptate sporodochial conidia with papillate basal and apical cells formed on the agar surface. (B, C, and E) Sporodochial conidia formed from monophialides. (F) Zero- and one- septate aerial conidia and multiseptate sporodochial conidia. (G, K, and L) Aerial conidiophores forming conidia from monophialides. (H-J) Zero-, one-, and two- septate reniform aerial conidia, respectively. Scale bars: A-G, 20 mm; H-L, 10 mm (Secor et al., 2014).

Fungal pathogen effectors

Plants have two different types of defense. The first one is called pathogen-associated molecular patterns (PAMPs) triggered immunity (PTI) (Chisholm et al., 2006). It is induced by the recognition of conserved microbial features called PAMPs such as flagellin peptide flg22 by PAMP receptors (Guan et al., 2013) (De Wit, 2007). However, pathogens have evolved effectors that can help suppress PTI. These effectors usually contribute to the pathogenicity or/and virulence of pathogens. Meanwhile, plants have likewise-evolved resistance genes to recognize effector gene products, which activates so-called effector-triggered immunity (ETI) (Jones and Dangl, 2006).

Fungal pathogen effector proteins have been roughly separated into two different groups, extracellular effectors and cytoplasmic effectors (Stergiopoulos and de Wit, 2009). One of the most well-known extracellular fungal pathogens is *Cladosporium fulvum*, which is a pathogen of tomato (De Wit et al., 1997). Four avirulence genes, *Avr2*, *Avr4*, *Avr9* and *Avr4E* have been cloned from *C. fulvum* and confirmed to encode proteins that are secreted by *C. fulvun* during infection (Joosten and Wit, 1999). Six more extracellular proteins (Ecps), Ecp1, Ecp2, Ecp4, Ecp5, Ecp6, Ecp7 were also characterized and their corresponding encoding genes have been cloned from *C. fulvum* in addition to the four Avr effectors (Bolton et al., 2008) (Ackerveken and Wit, 1993). During infection, those pathogen effectors play different roles including inhibition of cysteine proteases and interference with defense signaling pathways so as to suppress hose defense response (Rooney et al., 2005).

Since it is possible that pathogenicity and disease development of *F. secorum* may depend on effector proteins produced during infection, the identification of effector protein can further our understanding of the biology of this pathogen. Moreover, effectors have been shown to be useful tools to identify new resistance genes in host (Vleeshouwers and Oliver, 2014). A better

understanding of effector biology will be necessary to pursue host resistance as a long-term means of managing Fusarium yellowing decline.

Functional genomics of fungi

Different strategies have been applied to identify effector genes from plant pathogens. The most popular methods are map-based cloning and fungal secretome analysis based on biochemical and genetic approaches. With the development of next generation sequencing technology, whole genome sequencing of fungal pathogens has also been used and made great contribution to the identification of candidate effector genes (Stergiopoulos and de Wit, 2009). It is important to study the function of putative effector genes in more detail. A commonly used strategy to study gene function is to disrupt gene expression by homologous recombination followed by phenotypic characterization of the generated mutants. Different strategies have been developed for gene disruption and replacement. For example, the homologous recombination method requires development of constructs with relatively large stretches of homologous sequence flanking the gene of interest (Shafran et al., 2008). A disruption cassette containing a selectable marker gene and sequences from the target gene flank is transformed into fungus to conduct the gene disruption or replacement. Gene disruption/replacement by homologous recombination can also be achieved with creation of homologous sequences through PCR (Weld et al., 2006).

Objective

1) Develop a transformation system for *F. secorum*

    1a. Develop polyethylene glycol (PEG)-mediated transformation system

    1b. Confirm mutants

2) Monitor development of *F. secorum* on *Fusarium*-susceptible sugarbeet variety

3) Identification and characterization of *F. secorum* candidate effectors

3a. Knock-out candidate genes to generate deletion mutants

3b. Pathogenicity test of deletion mutants on sugarbeet

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CHAPTER II. DEVELOPMENT OF A TRANSFORMANTION SYSTEM FOR *FUSARIUM SECORUM*

Introduction

Whole genome sequencing of fungal pathogens has made a great contribution to the identification of candidate effector genes (Stergiopoulos and de Wit, 2009). However, confirming the role of candidate effectors is a major limitation to better understanding fungal biology (Shafran et al., 2008). One of the best strategies to facilitate functional genetics is removing the gene of interest through genetic transformation followed by testing the pathogenicity of the resulting mutant. To date, different transformation systems have been developed such as electroporation (Hazell et al., 2000), *Agrobacterium*-mediated transformation (Michielse et al., 2005), biolistics (Davidson et al., 2000) and $CaCl_2$/polyethylene glycol- (PEG-) mediated transformation (Fitzgerald et al., 2003).

PEG-mediated transformation is one of the most commonly used methods for integrating DNA into plant protoplasts (Mathur and Koncz, 1998). PEG-mediated transformation was first demonstrated in fungal species *Aspergillus nidulans*. The basic protocol was then adapted to different fungi as well as fungal-like species (Fincham, 1989). While PEG-mediated methods require a relatively high concentrations of protoplasts, sometimes exhibits low transformation efficiency, and may generate multiple loci integrations, its simplicity in equipment required and technical operation make this method one of the most popular methods for genetic transformation (Ruiz-Díez, 2002). PEG-mediated transformation involves three main procedures: preparation of protoplasts, DNA uptake, and regeneration on selective media (Liu and Friesen,

2012). Despite the reliance on PEG-mediated transformation for genetic manipulation in many fungal species, the underlining mechanism is still unknown (Fincham, 1989).

Since *Fusarium secorum* is a novel sugarbeet pathogen (Secor et al., 2014), there are no published procedures for genetic transformation of this pathogen. Thus, in this study we developed a PEG-mediated transformation system of *F. secorum*. With the developed transformation system, studies related to functional genetics of *F. secorum* can be conducted to facilitate a better understanding of how the pathogen interacts with sugarbeet.

Objective

Develop a transformation system for *F. secorum*

a. Develop polyethylene glycol (PEG)-mediated transformation system

b. Confirm mutants

Materials and methods

Fungal isolate and growth conditions

F. secorum isolate 670-10 and transformants derived from this isolate were grown on potato dextrose agar (PDA; Becton Dickinson and Company, Sparks, MD; 39% [wt/vol] PDA) under constant light. For long-term storage of *F. secorum* and transformants, we employed glycerol (10%, vol/vol) and dimethyl sulfoxide (DMSO) (5%, vol/vol) as cryoprotective agents and stored at -80°C (Crespo et al., 2000).

Hygromycin B concentration test

To determine the proper concentration of Hygromycin B used for selection of hygromycin-resistant transformants, we conducted a Hygromycin B concentration test with *F. secorum*. Plugs with *F. secorum* mycelia were put in the center of PDA plate with Hygromycin B concentrations

of 30, 60, 90, and 120 μg ml^{-1}. PDA plates without hygromycin B was regarded as a positive control. Plates were incubated under constant light at 22°C for seven days.

Preparation of protoplasts

A small piece of media agar with mycelia was cut from a PDA plate grown with *F. secorum* isolate 670-10 and transferred to 100 ml potato dextrose broth (PDB; Becton Dickinson and Company, Sparks, MD; 24% [wt/vol] PDB). The PDB liquid culture was kept under constant light for 3 days at 22°C with shaking at 140 rpm. One ml from the liquid culture was spread on the surface of a PDA plate. The plate was allowed to dry for 10 min before incubation under constant light for seven days at 22°C. After seven days, three ml of distilled water was added to the plate followed by light shaking to dislodge microconidia. The microconidia were then collected and transferred to Fries medium (5% [wt/vol] ammonium tartrate, 1% [wt/vol] ammonium nitrate, 0.5% [wt/vol] magnesium sulfate, 1.3% [wt/vol] KH_2PO_4, 2.6% [wt/vol] K_2HPO_4, 30% [wt/vol] sucrose, 1% [wt/vol] yeast extract, 2% [vol/vol] trace element stock solutions (0.167% [wt/vol] LiCl, 0.107 [wt/vol] $CuCl_2 \cdot 2H_2O$, 0.034% [wt/vol] H_2MoO_4, 0.072% [wt/vol] $MnCl_2 \cdot 4H_2O$, 0.08% [wt/vol] $CoCl_2 \cdot 4H_2O$)) and grown under constant light at 22°C for 48 hours with shaking at 140 rpm to produce fresh mycelium.

Protoplast preparation was conducted based on Liu and Friesen (2012). Briefly, fresh fungal mycelia were poured into a beaker covered with two layers of Miracloth (EMD Millipore Crop., Billerica, MA) to collect mycelium. The mycelium was then washed two times with 50 ml sterilized water and once with 50 ml of mycelial wash solution (MWS; 0.7 M KCl and 10 mM $CaCl_2$). 40 ml of MWS-based cell wall degrading enzymes (400 mg β-1,3 glucanase, 200 mg driselase and 400ml MWS, stir for 10 min, centrifuged at 3,700×*g* at 22°C for 10 min, filter by 0.45 μm, CA membrane) was used to resuspend the washed mycelia in a 100×20 mm petri dish.

The mixture was incubated at 32°C with light shaking for 90 min. After that, the solution was gently poured through four layers of Miracloth into a beaker and then transferred to a 50 ml centrifuge tube. A hemocytometer was used to count protoplasts. Protoplasts were then centrifuged for 10 min at 2,200×*g* at 4°C. The supernatant were gently removed followed by addition of STC buffer to obtain a concentration of 1×10^8 protoplasts ml^{-1}.

Preparation of plasmid DNA

The pDAN plasmid vector, derived from cloning a *cpc*-1::hygromycin-resistance gene (*hph*) from pLP605KO[28] into the SalI site of pBluescript (Friesen et al., 2006), was used in this study. 20 µg of pDAN was linearized with the *Eco*R V enzyme at 37°C for 4 hours in a 20 µl restriction enzyme digestion reaction volume. The mixture was heat inactivated by incubating at 80°C for 20 min and then used for the PEG-mediated transformation.

Fungal transformation

PEG-mediated fungal transformation was developed based on Liu and Friesen (2012). Briefly, 100 µl of freshly prepared protoplasts were gently pipetted into 15 ml centrifuge tubes. Subsequently, 20 µl of linearized pDAN was added to each tube, mixed by gently shaking, and incubated on ice for 20 min. 100 µl of 50% PEG solution (50% [wt/vol] PEG 3500, 10 mM $CaCl_2$, 10 mM Tris-HCl, pH 7.5) was added into the mixture followed by adding two volumes of 450 µl PEG solution with mixing gently between each addition of PEG and then incubated at 22°C for 20 min. After the incubation, one ml STC buffer (1.2 M sorbitol, 10 mM Tris-HCl, pH 7.5, 10 mM $CaCl_2$) was added into the mixture followed by adding two volumes of 3.5 ml STC buffer with mixing gently between each addition of STC. The tubes were then centrifuged at 22°C for 10 min at 3,300×*g*. The pellet was resuspended with one ml regeneration media (1.0 M

sucrose, 0.1% [wt/vol] yeast extract, 0.1% [wt/vol] tryptone) followed by addition of 14 ml warm regeneration media agar (1.0 M sucrose, 0.1% [wt/vol] yeast extract, 0.1% [wt/vol] tryptone, 1.5% [wt/vol] agar) with mixing gently and poured into 100×15 mm Petri dishes and kept in dark at 22℃ overnight. After 16-18 h, 15 ml of warm regeneration media agar containing 120 μg ml^{-1} Hygromycin B was poured onto the surface of the plates. For the positive control, regeneration media agar without Hygromycin B was used. Plates of untreated protoplasts covered with regeneration media containing 120 μg ml^{-1} Hygromycin B were used as negative control. All plates were then kept in dark at 22℃ for 4-7 days to allow transformants to become visible. Transformants were then transferred to fresh PDA plate containing 120 μg ml^{-1} Hygromycin B and incubated at 22℃.

Confirmatoion of *hph* integration in *F. secorum*.

Genomic DNA was isolated from transformants using a CTAB method (Gontia et al., 2014). To detect the integration of *hph*, a PCR reaction using the primer pair M13F (GACGTTGTAAAACGACGGCCAGTG) and M13R (CACAGGAAACAGCTATGACCATGA) was conducted. The PCR reaction (25μl) was conducted using GoTaq Green PCR kit (Promega Corp., Madison, WI) following the manufacturer's suggested protocol. PCR was carried out using a PTC-200 thermal cycler (MJ Research, Hercules, CA) as follows: initial denaturation (94℃, 3 min), followed by 30 cycles of denaturation (94℃, 30 s), annealing (55℃, 30 s), extension (72℃, 1 min), and then one final cycle of extension (72℃, 5 min).

Results

Protoplasts generation

We tested different enzyme concentrations, digestion temperatures, and incubation periods for digestion to identify the most efficient way to digest *F. secorum* cell wall. After optimizing the basic PEG-mediated transformation protocol, protoplasts of *F. secorum* were released from mycelia digested by cell wall degrading enzymes after one hour incubation at 32°C (Figure 2-1).

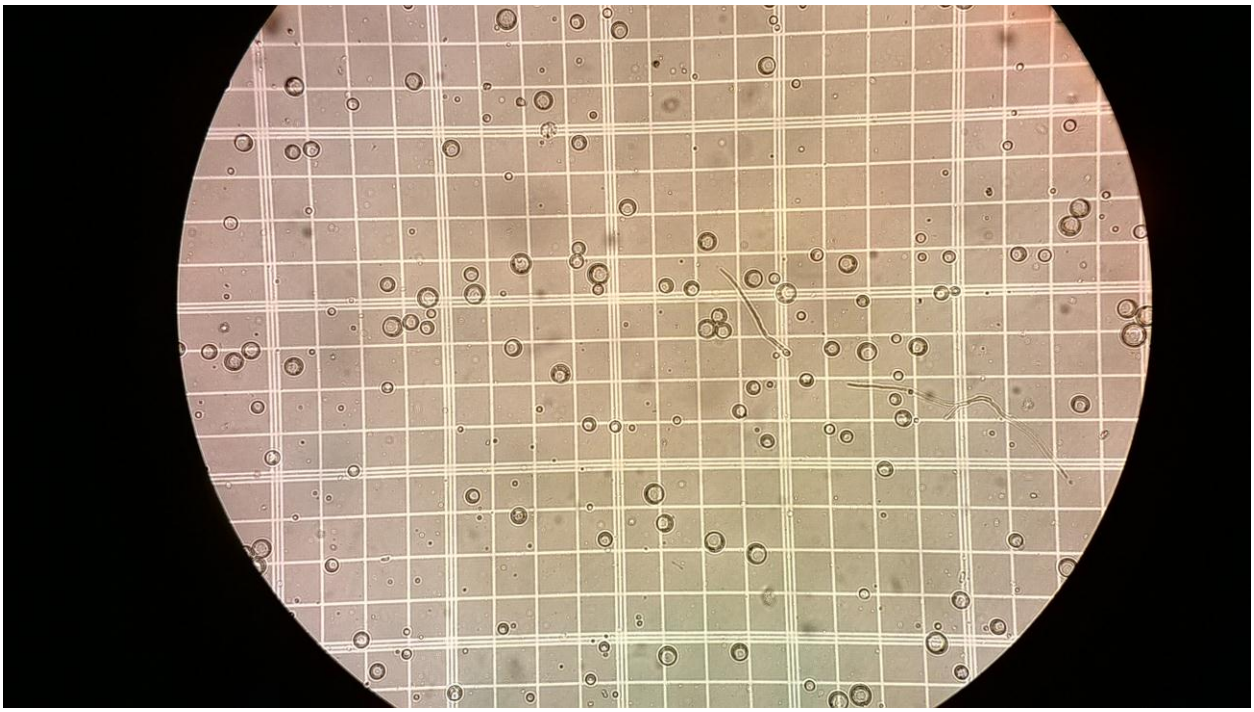

Figure 2-1. *F. secorum* protoplasts released by mycelia after one hour incubation at 32^{O}C.

Hygromycin B concentration test

The concentration test showed that growth of *F. secorum* decreased as the concentration of Hygromycin B increased (Figure 2-2). As the concentration increased to 120 µg ml^{-1}, no fungal growth was observed on the plate (Figure 2-2). Therefore, 120 µg ml^{-1} was chosen as the optimum concentration of Hygromycin B used to select transformants in PEG-mediated transformation of *F. secorum*.

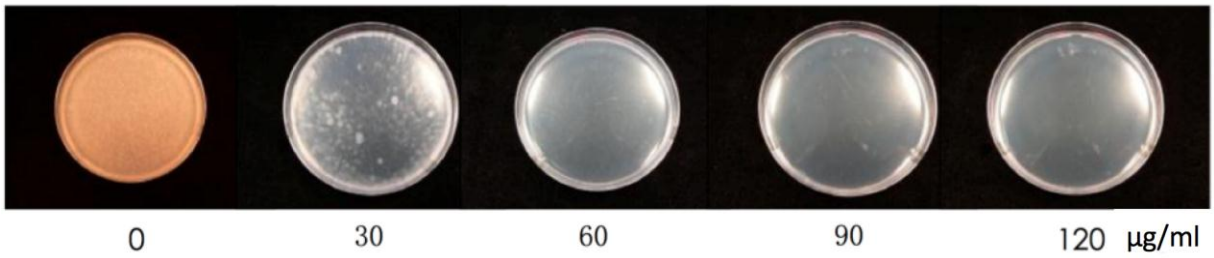

Figure 2-2. Hygromycin B concentration test of F. secorum. No fungal growth was observed at a concentration of 120 μg ml^{-1}.

hph* insertion transformants of *F. secorum

After incubation at 22°C under constant light for 5 days, transformants started to grow on the surface of regeneration plates containing 120 μg ml^{-1} Hygromycin B (Figure 2-3). PCR was conducted to confirm the integration of *hph* (Figure 2-4).

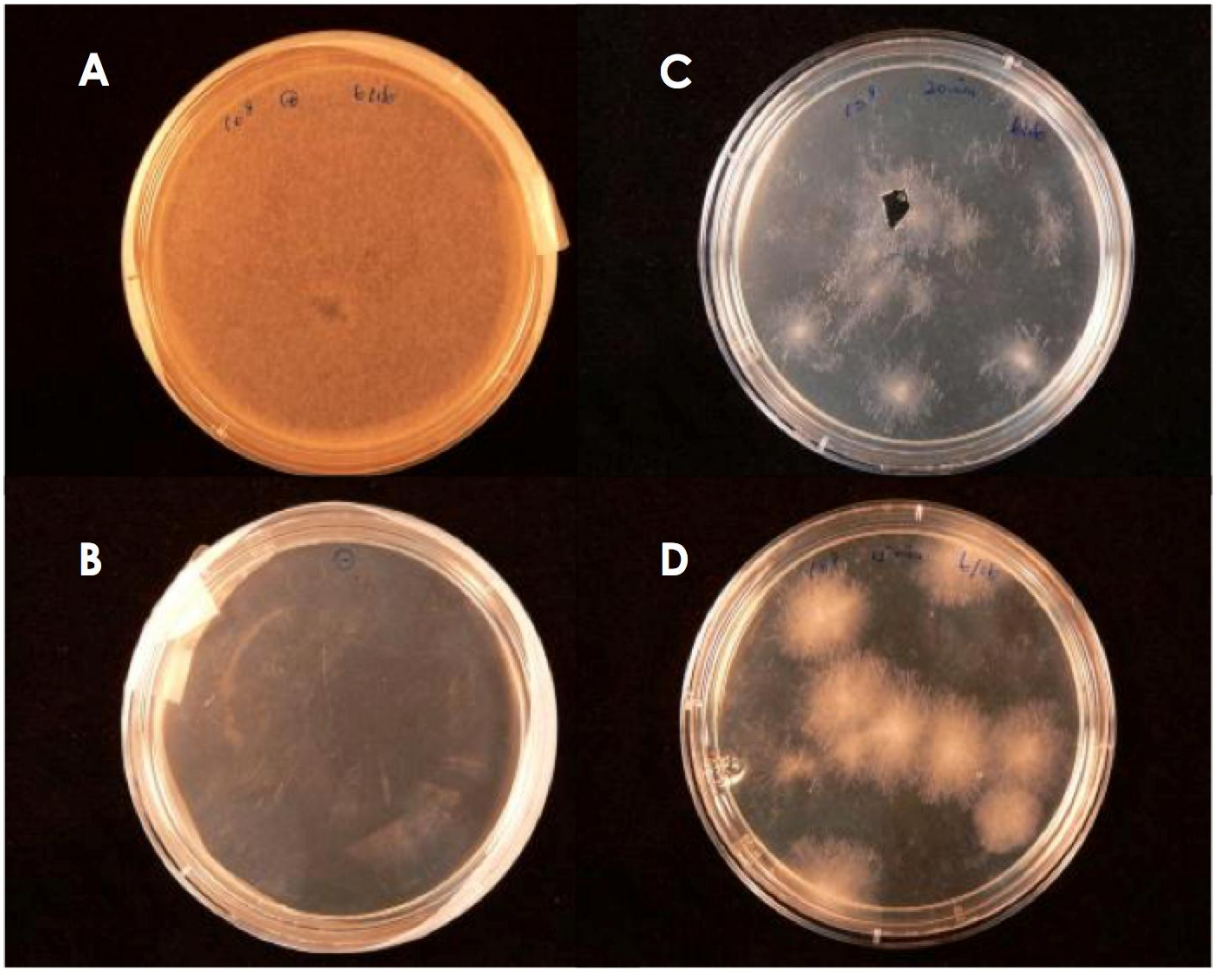

Figure 2-3. Growth of F. secorum and hph insertion transformants on regeneration media plates with or without Hygromycin B. (a) positive control: F. secorum without transformation grow normally on plate free of Hygromycin B. (b) negative control: No fungal growth was observed on plate containing 120 µg µl-1 Hygromycin B. (c,d) hph insertion transformants grown on plates containing 120 µg µl-1 Hygromycin B.

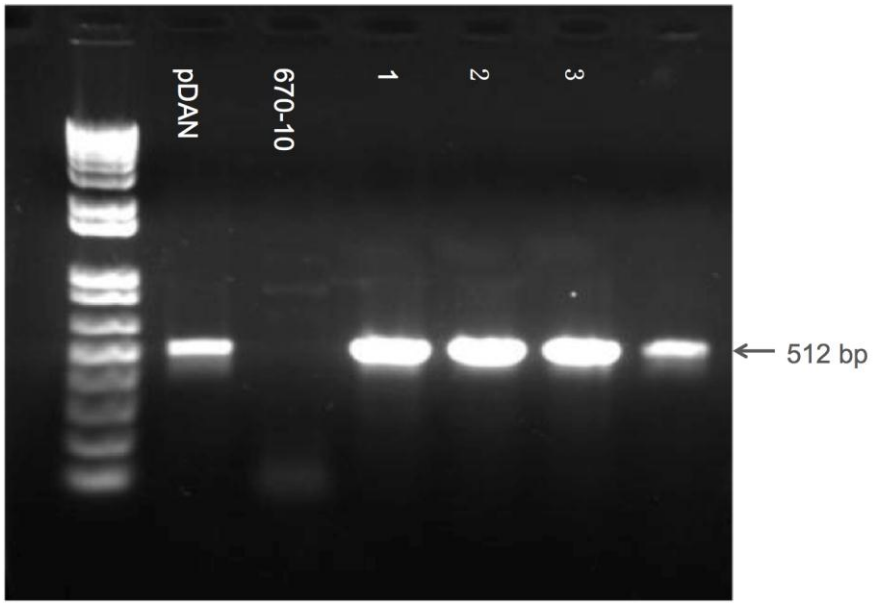

Figure 2-4. PCR detection of hph insertion transformants. 670-10 is wild type F. secorum. 1-3 are three different transformants grown on the plates. Only pDAN plasmid and those transformants amplified the targeted fragment.

Discussion

The first successful transformation using protoplasts was reported in *Saccharomyces cerevisiae* in 1975 (Hinnen, 1978). Since then, different methods and techniques for genetic transformation have been developed using the model filamentous fungi *Aspergillus nidulans* (Yelton et al., 1984) and *Neurospora crassa* (Case et al., 1979). To date, genetic transformation has become an important method that facilitates gene identification and the study of functional genomics of all major groups of filamentous fungi (Hou et al., 2013).

PEG-mediated transformation is one of the most commonly used methods for genetic transformation. This method require relatively high concentrations of protoplasts, which is a key factor for successful transformation (Moradi et al., 2013). Young and fresh mycelium is critical for the generation of high quality protoplasts in relatively short digestion time. However, *F. secorum* has a slow growth rate in liquid culture and typically the liquid culture needs to grow for four days until we can collect enough mycelium for cell wall digestion. As a result, culturing

F. secorum for four days lead to a significant amount of old mycelium, which is difficult to digest by cell wall degrading enzymes. Use of blender to break mycelium and incubate before digestion has been shown to produce fresh mycelium (Anunciação et al., 1990), but it did not work well with *F. secorum*. In this study, fresh mycelium derived from germinated conidia could be easily digested by cell wall degrading enzymes in a relatively short incubation period. Different media have been used for conidia production of *Fusarium* species such as V8 juice (Proctor et al., 2002) and GYEP (McCormick et al., 1999). However, *F. secorum* did not produce conidia or produced very limit amount of conidia with those media. Our results showed that half-strength PDA media facilitated the best production of *F. secorum* conidia. Instead of inoculating fungal plugs into liquid media and centrifugation to gather conidia after a suitable incubation time, we spread PDB media containing *F. secorum* mycelium on half-strength PDA media and allowed the plates to dry before incubating for seven days under constant light to produce conidia. Using this method, microconidia were produced after seven days of incubation and macroconidia were produced if incubated more than seven days.

It was reported in some other studies that mycelium of different ages from 8-18h have been used (Proctor et al. 1997; Lee et al. 2002; Watson et al. 2008). Our study showed that conidia derived from mycelium at 48 h could be easily digested in 1.5 h, resulting in nearly 100% protoplast formation. With the recommended amount of cell wall degrading enzymes (Silva et al., 2009), only half of the incubation time was necessary to produce protoplasts. Different osmolites such as glucose, sorbitol and sucrose have been recommended for protoplast regeneration media, among which sorbitol is the most commonly used osmotic stabilizer (Fincham, 1989). In our study, regeneration frequencies were much higher with regeneration media containing sucrose.

Colonies that grew for five days in hygromycin-amended media were transferred to fresh regeneration media. PCR reaction was used to demonstrate the integration of *hph*.

In summary we have successfully developed a PEG-mediated transformation system of the novel sugarbeet pathogen *F. secorum*. This modified system is rapid, inexpensive and reliable, which facilitate the functional genetics as well as other different kinds of studies of the novel sugarbeet pathogen *F. secorum*.

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CHAPTER III. PATHOGENICITY PROFILIING OF *FUSARIUM SECORUM*

Introduction

Sugar is one of the most frequently consumed sweeteners in the United States. Two plants that produce large amounts of sugar are sugarcane (*Saccharum officinarum*) and sugarbeet (*Beta vulgaris* L.). Sugarbeet, which is originally from north-west Europe (Bock, 1986), has an important economic impact on sugar industry in the United States (Draycott, 2006). Sugarbeet growing areas in ND and MN constitute the largest sugarbeet production area in the United States. *Fusarium* yellows caused by *Fusarium oxysporum f. sp. betae* has been a sugarbeet disease typically associated with western United States (Harveson, 2008). This disease is characterized by wilting of foliage, interveinal chlorosis, and vascular discoloration of the taproot, often leading to plant death (Khan et al., 2013). A new sugarbeet disease, which is caused by a novel sugarbeet pathogen *Fusarium secorum*, was recently found in Red River Valley of MN and ND that caused *Fusarium* yellows-like symptoms (Rivera et al., 2008). This new disease has been named *Fusarium* yellowing decline (Secor et al., 2014). *Fusarium* yellowing decline is differentiated from *Fusarium* yellows by causing discoloration of petiole vascular elements as well as seedling infection and rapid death of plants earlier in the season (Secor et al., 2014). Since *F. secorum* is a novel sugarbeet pathogen and not closely related to other *Fusarium* pathogens of sugarbeet, very limit research has been done. While some studies have tested the optimum growth conditions and host range of *F. secorum* (Villamizar-Ruiz, 2013), there is no information of pathogen development in plant tissue.

Relative quantification and absolute quantification are two different types of quantification methods utilizing quantitative PCR. Absolute quantification requires a highly validated methodology as well as identical amplification efficiencies for standard samples and target DNA, which is time consuming and technically complex (Morrison et al., 1998). However, relative quantification is based on the relative abundance of a target gene in comparison to a reference gene which is sufficient for most research purposes (Livak and Schmittgen, 2001). A mathematical model for relative quantification of a target gene was developed by Pfaffl (2001) in which only the crossing point deviation and the quantitative PCR efficiencies of an unknown sample versus a control are used to calculated the relative abundance of genes ratio. In this study, we inoculated *F. secorum* on a *Fusarium*-susceptible sugarbeet variety and collected samples at different time points after inoculation to better understand how this pathogen develops in different host tissues over time. Quantitative PCR was used to measure fungal biomass in infected plant tissues.

Objective

Monitor development of *F. secorum* on *Fusarium* susceptive sugarbeet variety

Materials and methods

Preparation of conidia

A small piece of media agar with mycelia was removed from a potato dextrose agar (PDA; Becton Dickinson and Company, Sparks, MD; 39% [wt/vol] PDA) plate grown with *F. secorum* isolate 670-10 and transferred to 100 ml potato dextrose agar (PDB; Becton Dickinson and Company, Sparks, MD; 24% [wt/vol] PDB). The liquid culture was kept under constant light for 5 days at 22°C with shaking at 140 rpm. From the liquid culture, one ml was spread on the surface of a PDA plate allowed to dry for 10 minutes and incubated under constant light at 22°C

for 10 days. After 10 days, three ml of distilled water was added to the plate and lightly shaken to dislodge conidia. Conidia suspension was subsequently transferred to a clean 50 ml centrifuge tubes using pipette followed by addition of distilled water to obtain a concentration of $1x10^5$ conidia ml^{-1}.

Inoculation and sample collection

Fusarium susceptible sugarbeet line 4022RR was used in this study. Sugarbeet seeds were first grown in 10x10x9 cm pots filled with pasteurized soil (Sun Gro Horticulture, Agawam, MA). Plants were kept at a constant temperature at 18°C and in a cycle of 14 hours of light and 10 hours of darkness. Three weeks old seedlings were removed from pots and briefly washed. Plants were inoculated by dipping the root into a spore suspension of $1x10^5$ conidia ml^{-1} for 8 min without agitation. Inoculated plants were then transferred to individual pots. Plants treated with distilled water were considered as negative controls. The inoculation was conducted in three biological replicates. After inoculation, plants were kept in growth chambers with a random order in each biological replicate. Plants were grown at a constant temperature at 18°C, 50% relative humidity (RH), and in a cycle of 14 hours of light and 10 hours of darkness. Plants were watered every 24 hours. Root, petiole and leaf materials were collected at day 2, 5, 8 and 11 after inoculation. For each biological replication, three different pots were randomly chosen for the collection. Plants were carefully removed from soil and briefly washed followed by storing at -80°C.

Relative quantification of fungal biomass in sugarbeet

To detect growth of the fungus *in planta*, quantitative PCR with primers of sbEc-F/sbEc-R for endogenous sugarbeet control (de Coninck et al., 2012) and MDB-1182 (5'-GGCCGTATTGAGACTGGTG-3')/ MDB-1183 (5'-GCATCTCGACGGACTTGAC-3')

designed to amplify *elongation factor 1-alpha* was used. Relative quantification of fungal biomass was carried out using the method of Pfaffl (2001). Genomic DNA was isolated from different samples using a CTAB method (Gontia et al., 2014). All sample DNAs were diluted to 5 ng μl^{-1} and used as template for qPCR. Quantitative PCR was performed with the SYBR Green PCR Master Mix (Promega, Madison, WI) using a PTC-200 thermal cycler (MJ Research, Hercules, CA) outfitted with a Chromo4 Real-Time PCR Detector (Bio-Rad, Hercules, CA) and MJ Opticon Monitor analysis software (version 3.1; Bio-Rad). A master mix was prepared to perform several parallel reactions. The reaction mixture (20 µl) was made using the SYBR® Green PCR Master Mix (Promega, Madison, WI) following the manufacturer's suggestions. Thermal cycling conditions are described as follow: an initial 95°C denaturation step for 2 min followed by annealing for 1 min at 60°C, and extension for one min at 72°C for 40 cycles.

Results

F. secorum inoculation

The phenotypic difference between plants inoculated with *F. secorum* and water control became apparent at the 11 days after the inoculation (Figure 3-1). Chlorosis, yellowing of leaves and scorching of leaves typical of yellowing decline was observed on plants inoculated with *F. secorum* (Figure 3-1).

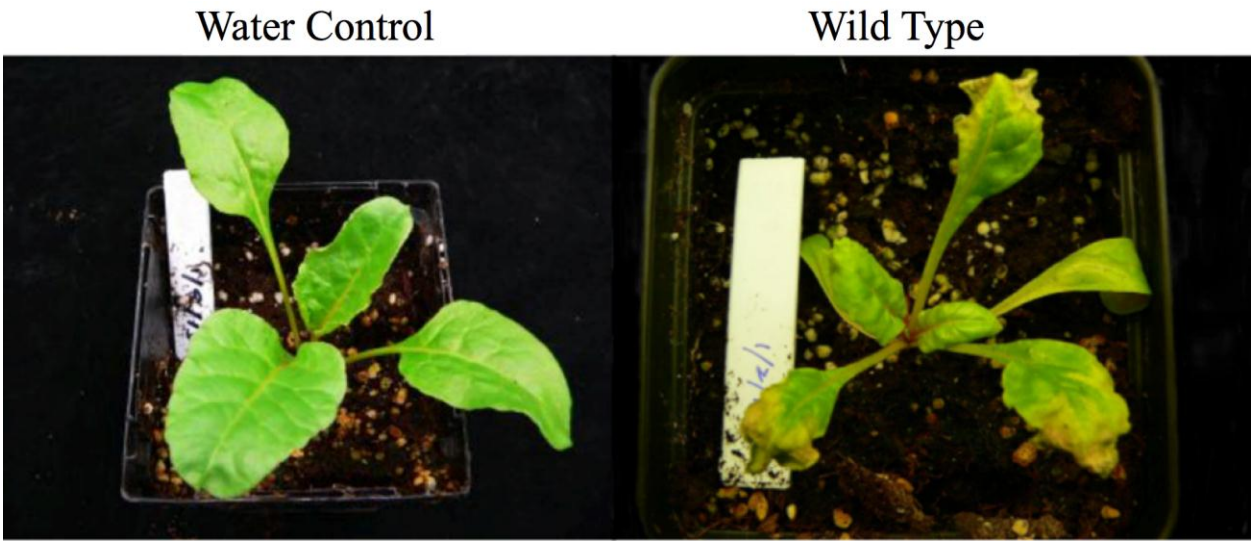

Figure 3-1. Symptoms of sugarbeet plants 4022RR 11 days after inoculation with a water control or *F. secorum*.

Quantitative PCR and relative quantification of pathogen in *planta*

A standard curve of primer pair MDB1182/MDB1183 was generated by the amplification of a 10-fold dilution series of *F. secorum* genomic DNA. Correlation between Ct value and target DNA concentration was high (R^2>0.995), which indicated that the primer set was highly accurate over a linear range of at least four orders of magnitude.

Fungal biomass of *F. secorum* in root, petiole and leaf materials of infected sugarbeet plants at 2, 5, 8 and 11 days after inoculation was calculated (Pfaffl, 2001) (Figure 3-2). Gradual progression of fungal biomass was shown in root material, but remained relatively low when compared to fungal biomass in petiole at the last two time points (Figure 3-2). For petioles, fungal biomass was low at the first two time points but greatly increased at the third and fourth time points. No fungal biomass was detected in leaf material 2 and 5 days after inoculation, but fungal growth began at day 8 after inoculation and fungal biomass in leaf material increased during the infection (Figure 3-2).

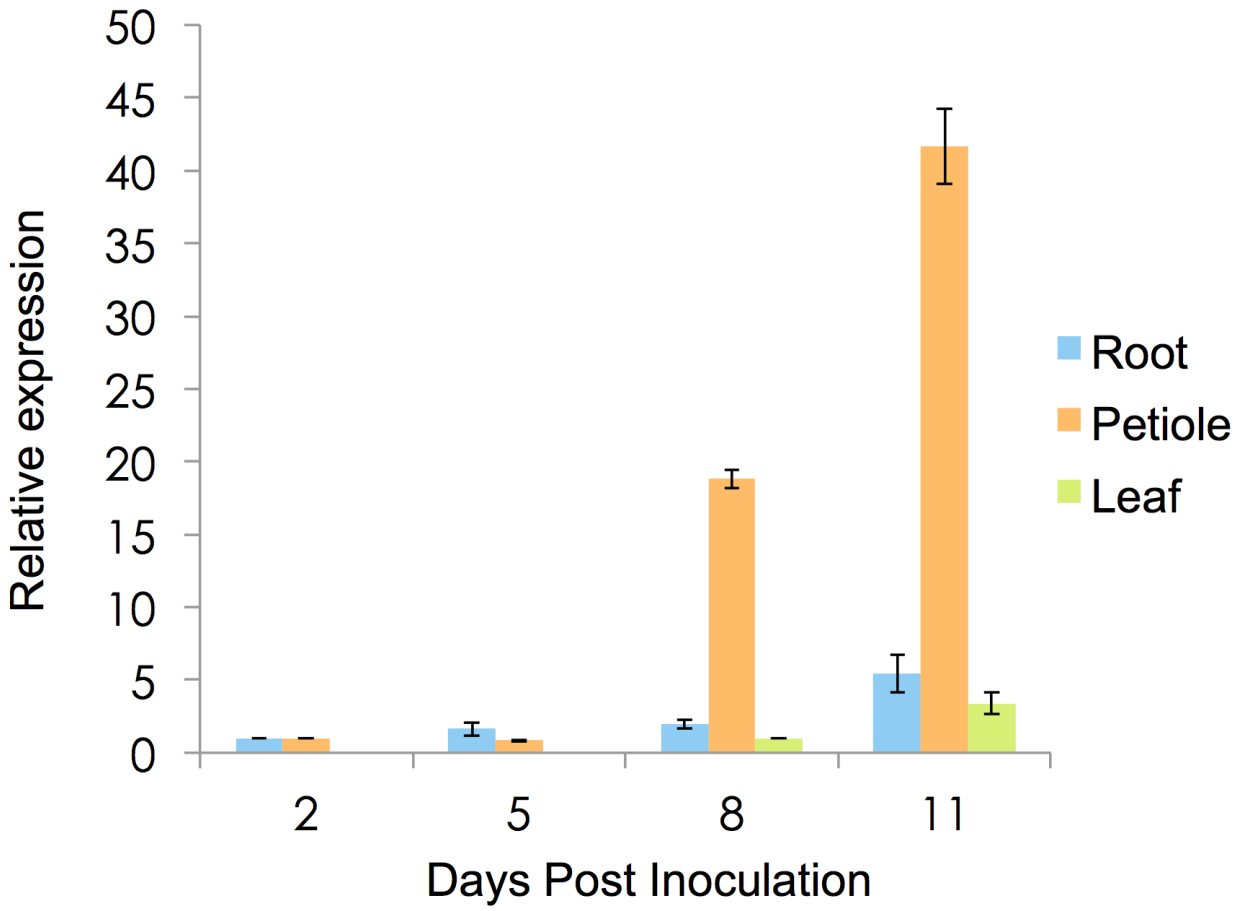

Figure 3-2. Relative quantification of fungal biomass of *F. secorum* in root, petiole and leaf materials of infected sugarbeet plants at 2, 5, 8 and 11 days, after inoculation.

Discussion

A new disease of sugarbeet was reported in Minnesota that caused Fusarium yellows-like symptoms (Rivera et al., 2008). This new disease has been named Fusarium yellowing decline and the pathogen that causes this disease was named *Fusarium secorum* (Secor et al., 2014). This pathogen was shown to be more aggressive than other yellows pathogens and has become a problem for some growers in North Dakota and Minnesota (Secor et al., 2014). Since *F. secorum* is a novel sugarbeet pathogen, very little information is available about pathogen development in host tissues. In this study, we used quantitative PCR to quantify fungal biomass in different

infected tissues and at different time points to monitor the fungal development on sugarbeet infected with *F. secorum*.

A standard curve shows that the primer pair targeting *elongation factor 1-alpha* in *F. secorum* allowed reliable DNA quantification over a very wide range and was highly sensitivity. The results showed that this qPCR-based method allows the assessment of fungal development in sugarbeet root, petiole and leaf tissues during the entire time-course of infection. While it was reported that qPCR-based assays could have high variation between replicate samples (Brouwer et al., 2003), qPCR provides high sensitivity.

We set 11 days after inoculation as the last time point because infected sugarbeet was almost dead at this stage. Fungal biomass significantly increased from the eighth day after inoculation in petioles. This might result from the specific structure of the petiole tissue, which has relatively less plant tissue and may provide more space for the pathogen to develop and accumulate. No fungal biomass was detected in leaf material at the second and fifth days after inoculation but it began to show fungal growth from the eighth day after inoculation. This observation is different from other *Fusarium* sugarbeet pathogens such as *F. acuminatum* and *F. oxysporum* which have leaf symptom but no fungal growth reported on detected in leaf material.

In this study, we successfully developed a quantitative PCR-based method to calculate the fungal biomass of *F. secorum*, which can quickly and reliably monitor disease progression in sugarbeet infected with *F. secorum* from the beginning of infection until very late stages. This technique can also be used to assess the aggressiveness of wild type fungal and mutant strains.

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CHAPTER IV. CHARACTERIZATION OF FUSARIUM SECORUM CANDIDATE EFFECTORS

Introduction

Plants employ different defense strategies when interacting with pathogens to protect themselves from infection (Agrios 1997). Basal line defense involves the recognition of pathogen-derived elicitors known as pathogen-associated molecular patterns (PAMPs), which triggers the host defense called PAMP-triggered immunity (PTI) and restricts pathogen entry as well as infection development process (Bent and Mackey 2007). Fungal cell wall components chitin and glucan (Vega and Kalkum 2012), bacterial components flagellin, and lipopolysaccharides are all regarded as PAMPs which can trigger PTI (Schwessinger and Zipfel 2008). As a result, pathogens evolved effector proteins that suppress or interfere with plant defense response (Jones and Dangl 2006). Plants also evolved corresponding resistance (R) proteins that interact with pathogen effectors and trigger a second layer of defense called effector-triggered immunity (ETI) to suppress disease process (De Wit 2007). Thus, studies on those effectors secreted by pathogens during infection process lead to a better understanding the mechanism underline the interaction between pathogens and host plants.

Due to recent technological advances in sequencing technology, fungal genomes are being sequenced at a rapid pace which provide vital information for the identification of effector proteins. Effector proteins have been identified that play important roles in the interaction between host plants and *Fusarium* species. For example, the proteins Six1, Six2, Six3 and Six4 (for secreted in xylem) were identified in *F. oxysporum* f. sp. *lycopersici* (Fol) and are produced during infection process (Stergiopoulos and de Wit 2009). To date, 11 SIX proteins have been

identified in Fol (Ma et al. 2010). Effector Avr1 contributes avirulence to Fol strains on tomato lines carrying *I* or *I-1* resistance gene (Houterman et al. 2008). In addition, Avr3 was demonstrated to be required for full virulence on tomato (Huang and Lindhout 1997). Since pathogenicity and disease development may depend on effector proteins produced by the novel sugarbeet pathogen *F. secorum* during infection, effector protein identification can further our understanding of the biology of this pathogen.

The novel sugarbeet pathogen *Fusarium secorum* causes Fusarium yellowing decline (Secor et al. 2014). The *F. secorum* genome has been sequenced, from which 15,872 proteins were predicted (Melvin Bolton and Ronnie de Jonge, unpublished results). Putative *F. secorum* effectors were identified by screening the set of 15,872 predicted proteins for those that have a signal peptide and are predicted to be localized extracellularly. Furthermore, elevated gene expression during sugarbeet colonization was regarded as typical signature for effector genes. Selected candidates were further filtered by protein domain assessment and homology to other proteins. Finally, a list with was generated with 11 candidate effector encoding genes. In this study, a split marker system was used to delete the target candidate gene (Catlett et al. 2002). We assessed their role in pathogenicity by inoculating these mutant lines on *Fusarium* susceptible sugarbeet variety and comparing to the wild type *F. secorum* in both phenotype and fungal biomass in infected tissues using quantitative PCR.

Objective

Identification and characterization of *F. secorum* effectors

a. Knock-out candidate genes to generate deletion mutants

b. Pathogenicity test of deletion mutants on sugarbeet

Materials and methods

Fungal isolate and growth conditions

F. secorum isolate 670-10 was used as recipient for all transformation experiments. *F. secorum* isolate 670-10 and mutants derived from this isolate were grown on potato dextrose agar (PDA; Becton Dickinson and Company, Sparks, MD; 39% [wt/vol] PDA) under constant light at 22 ℃. For DNA isolation, protoplast preparation and conidia preparation, *F. secorum* or mutant isolates of *F. secorum* were grown in 100 ml potato dextrose broth (PDB; Becton Dickinson and Company, Sparks, MD; 24% [wt/vol] PDB) at 22 ℃ with shaking at 150 rpm. For long-term storage of *F. secorum* and transformants, we used glycerol (10%, vol/vol) plus dimethyl sulfoxide (DMSO) (5%, vol/vol) as cryoprotective agents for storage at -80 ℃ (Crespo et al. 2000). The *Fusarium* susceptible sugarbeet line 4022RR will be used for pathogenicity testing.

Targeted gene replacement

We used the split marker method (Catlett et al. 2002) combined with PEG-mediated transformation system developed previously to delete target candidate genes. Briefly, PCR was conducted to amplify two fragments HY and YG, which represent the first two thirds (HY) and the last two thirds (YG) of the hygromycin-resistance gene (*hph*) from the pDAN vector (Friesen et al. 2006) using M13F/HY and M13R/YG primers, respectively. PCR reactions (25μl) were conducted using GoTaq Green PCR （Promega Corp., Madison, WI） kit following the manufacturer's protocol. The PCR reaction was carried out using a PTC-200 thermal cycler (MJ Research, Hercules, CA) and the thermal cycling conditions were described below: initial denaturation (94℃, 3 min), following by 30 cycles of denaturation (94℃, 30 s), annealing (55 ℃, 30 s), extension (72 ℃, 1 min), and then one final cycle of extension (72 ℃, 5 min). 5' and 3'

flanking region of targeted gene were amplified using the primers 1F/2R and 3F/4R of each gene, respectively using PCR conditions described above. Four fragments were purified using PCR clean kit （Qiagen, Valencia, CA) for fusion PCR. Two constructions, 5’ construct and 3’ construct, were developed by fusing the two marker fragments (HY and YG) with 5’ flanking region and 3’ flanking region of target gene using 1F/HY and YG/4R primers respectively through fusion PCR (Figure 4-1). Fusion PCR reaction (50µl) contained 50 ng of each fragment, 1×reaction buffer, 125 mM $MgCl_2$, 15mM dNTP, 10 mM of each primer, and 2.5U of Taq DNA polymerase (Promega, Madison, WI). The PCR reaction was carried out using a PTC-200 thermal cycler and the thermal cycling conditions were as follow: initial denaturation (94℃, 4 min), followed by 42 cycles of denaturation (94℃, 30 s), annealing (60 ℃, 30 s), extension (68 ℃, 2 min), and then one final cycle of extension (68 ℃, 10 min). Equal amount of 5’ and 3’ constructs were then mixed together and purified by ethanol precipitate for PEG-mediated transformation.

Fungal transformation

Transformation was carried out as described in chapter I with following modifications. Instead of mixing 20 µg of pDAN into protoplasts, we added 20 µg of each 5’ and 3’ construct.

Confirmation of deletion of targeted genes

Genomic DNA of fungal transformants was isolated for PCR detection of deleted targeted genes. To confirm the deletion of targeted gene in mutants, we first used YG/HY primers to confirm the integration of *hph* gene. We then use 5p1F forward primer, which starts upstream of the 5’ flanking region and HY-R2 reverse primer, which start inside *hph* gene to confirm the deletion of target gene. YG and 6R reverse primers, which starts downstream of the 3’ flanking

region were also used to confirm the deletion of target gene (Figure 4-1). PCR reactions were carried out using a PTC-200 thermal cycler. The PCR reaction (25μl) was conducted using GoTaq Green PCR kit (Promega Corp., Madison, WI) following the manufacturer's suggestions and thermal cycling conditions were described below: initial denaturation (94°C, 3 min), followed by 30 cycles of denaturation (94 °C, 30 s), annealing (55 °C, 30 s), extension (72 °C, 1 min), and then one final cycle of extension (72 °C, 5 min).

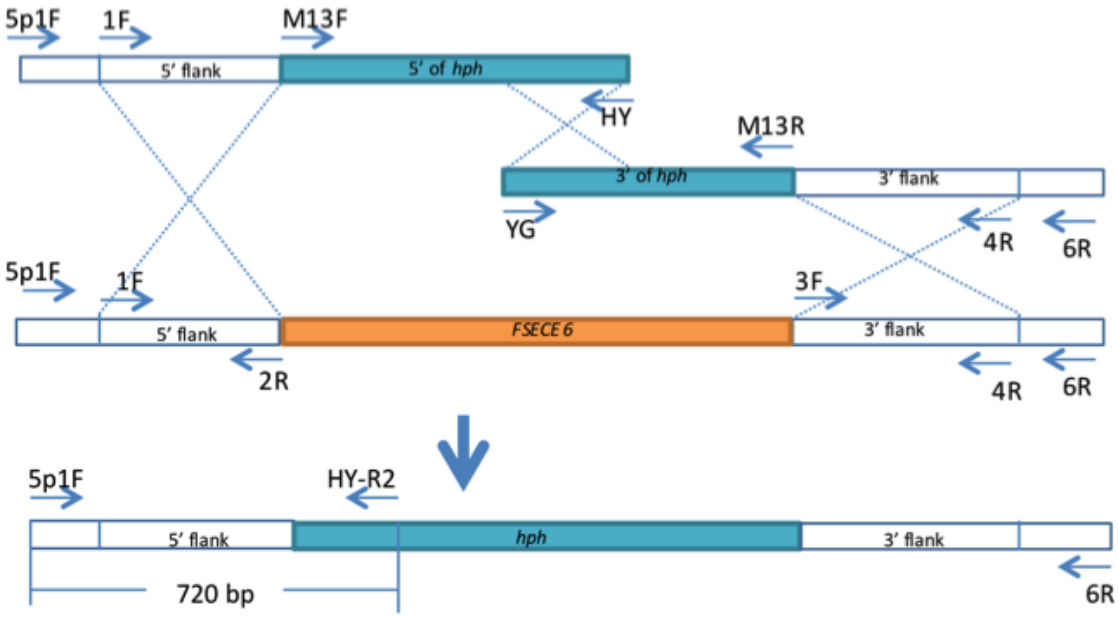

Figure 4-1. Diagram of gene replacement of *FSECE6* gene using split-marker system**.**

Table 1. Primers used in this study.

Primer name	Primer sequence (5'-3')
M13F	GACGTTGTAAAACGACGGCCAGTG
M13R	CACAGGAAACAGCTATGACCATGA
HY	GGATGCCTCCGCTCGAAGTA
YG	CGTTGCAAGACCTGCCTGAA
FSECE3 1F	CACGTCAATACCAAGCCAGA
FSECE3 2R	CACTGGCCGTCGTTTTACAACGTCTGTGAGCAAAGGTTGCTTGA
FSECE3 3F	TCATGGTCATAGCTGTTTCCTGTGACAGCGGGACCAAACCAG
FSECE3 4R	ACAGTACCTGCGAGGCACAC
FSECE5 1F	GGGCCTGTCTATAGTCAACTCAA
FSECE5 2R	CACTGGCCGTCGTTTTACAACGTCTGACGGTTGTTGTTGGGTTA
FSECE5 3F	TCATGGTCATAGCTGTTTCCTGTGACGGCGCAACTCTACGATT
FSECE5 4R	CCTCTATCTGACCCTCCCTCT
FSECE6 1F	AAAGCGCTTCTTTTTGCTTG
FSECE6 2R	CACTGGCCGTCGTTTTACAACGTCTGCTTGAGATGCATGTTGGT
FSECE6 3F	TCATGGTCATAGCTGTTTCCTGTGAGGCTGTGACTCAGACATCG
FSECE6 4R	AGACCGTCAATAATTGGAACAG
FSECE8 1F	GCTAGTCCGGTATGGTGAGC
FSECE8 2F	CACTGGCCGTCGTTTTACAACGTCTGCGAATGCAGAAATGAGAG
FSECE8 3F	TCATGGTCATAGCTGTTTCCTGTGACAAGCCTAACCCGCCTTAT
FSECE8 4R	GGTCAGGTTTCCAATGCTTC
FSECE10 1F	ACGTGCGTCAGAATGTGTTG
FSECE10 2R	CACTGGCCGTCGTTTTACAACGTCGGGGACTAGCAATGCAGCTA
FSECE10 3F	TCATGGTCATAGCTGTTTCCTGTGTTCAGTGGTGGTGCAGGAA
FSECE10 4R	CGCATTCATTTAGCAGTAGATAGA
FSECE11 1F	GCTATCCCTTCGCTCTGTGT
FSECE11 2R	CACTGGCCGTCGTTTTACAACGTCGCCACAATTCAAAACCAACC
FSECE11 3F	TCATGGTCATAGCTGTTTCCTGTGGCCTGGGGTTATGAGGTTG
FSECE11 4R	CATGATCGTTCCCTCACAGA
HY-R2	GGCAGGTAGATGACGACCAT
FSECE3_5p1F	TCGCTCAAATAGAGGCCAGT
FSECE5_5p1F	CGTTACGTCGTGCTGTTCAC
FSECE6_5p1F	AAAGCGTAGCATTGCGGTAT
FSECE8_5p1F	AGTTGACGAGAGGCCCAGTA
FSECE10_6R	AAAGCGTAGCATTGCGGTAT
FSECE11_5p1F	CCTCCTTGCCAAATCGAC

Pathogenicity assay of mutant isolates of *F. secorum*

We inoculated *Fusarium* susceptible sugarbeet variety 4022RR with spores of generated mutant lines of *F. secorum* to assess their pathogenicity. Inoculation procedure was described in chapter II. For each mutant isolate, we selected three different confirmed mutants for pathogenicity assays. Plants inoculated with wild type as well as the ones treated with distilled water were considered as positive and negative control, respectively. Inoculation was conducted in a randomized complete block design (RCBD) with three biological replicates and three plants per treatment. Treated plants were maintain in the growth chamber at a constant temperature at 18°C, 50% relative humidity (RH), and in a cycle of 14 hours of light and 10 hours of darkness. Samples were prepared from sugarbeet roots at five and eleven days after inoculation.

For quantification of fungal biomass, genomic DNA was used. Genomic DNA was isolated from different samples using a CTAB method (Gontia et al. 2014). All sample DNAs were diluted to 5 ng μl^{-1} and used as template for quantitative PCR. Primers of sbEc1-F/sbEc-R for endogenous sugarbeet control (de Coninck et al. 2012) and MDB-1182 (GGCCGTATTGAGACTGGTG)/ MDB-1183 (GCATCTCGACGGACTTGAC) designed to amplify *elongation factor 1-alpha* was used for quantitative PCR. Quantitative PCR was performed with the SYBR Green PCR Master Mix (Promega, Madison, WI) using a PTC-200 thermal cycler (MJ Research, Hercules, CA) outfitted with a Chromo4 Real-Time PCR Detector (Bio-Rad, Hercules, CA) and MJ Opticon Monitor analysis software (version 3.1; Bio-Rad). A master mix was prepared to perform several parallel reactions. The reaction mixture (20 µl) was made followed standard protocol of SYBR® Green PCR Master Mix (Promega, Madison, WI). Thermal cycling conditions are described as follow: an initial 95°C denaturation step for 2 min

followed by annealing for 1 min at 60°C, and extension for one min at 72°C for 40 cycles. For the analysis of fungal biomass, the method described by Pfaffl (2001) was used.

Results

Confirmation of target genes deletion

Five days after transformation of *F. secorum* wild type strain 670-10, colonies of transformants started to grow on the plates containing 120 µg µl^{-1} Hygromycin B. For *FSECE6* mutant, genomic DNA isolated from its transformants was used as template in PCR detection. Figure 4-3 shows the PCR analysis of these transformants. Only two (4 and 9) appeared to be site directed transformants while the remaining eight transformants were confirmed to be ectopic insertions. Transformants of *FSECE3* mutant, *FSECE5* mutant, *FSECE8* mutant, *FSECE10* mutant, and *FSECE11* mutant were also confirmed by PCR using the corresponding primers (Table 1). Finally, we successfully identified at least two separate mutants for each six different genes including *FSECE3* mutant, *FSECE5* mutant, *FSECE6* mutant, *FSECE8* mutant, *FSECE10* mutant, and *FSECE11* mutant (Table 2).

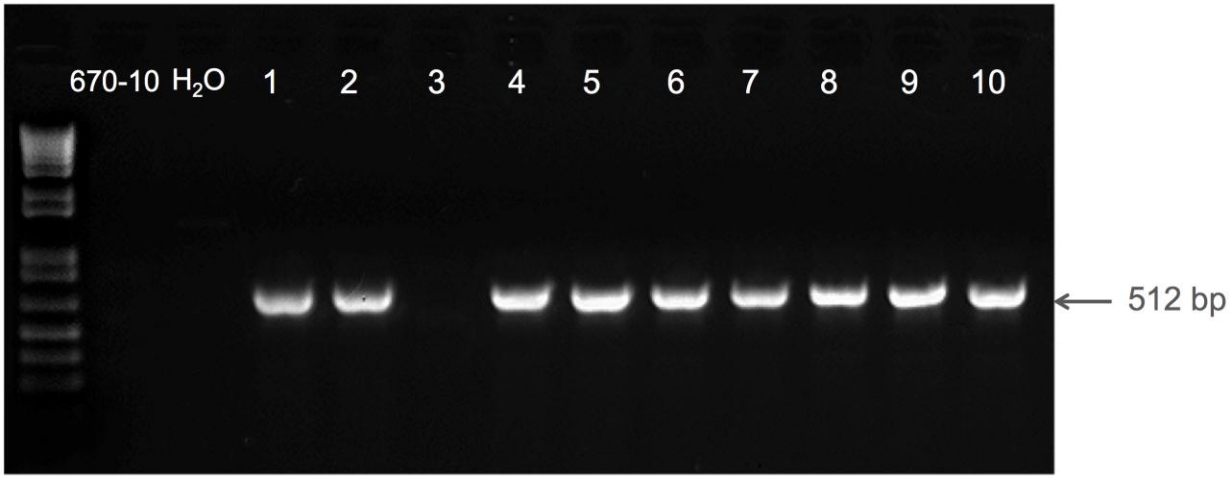

Figure 4-2. Confirmation of *hph* integration in *FSECE6* mutant using primers M13F and M13R. 670-10 is wild type *F. secorum*. 1-10 are transformants of *FSECE6* mutant. Only those transformants have *hph* integration amplified the target fragment.

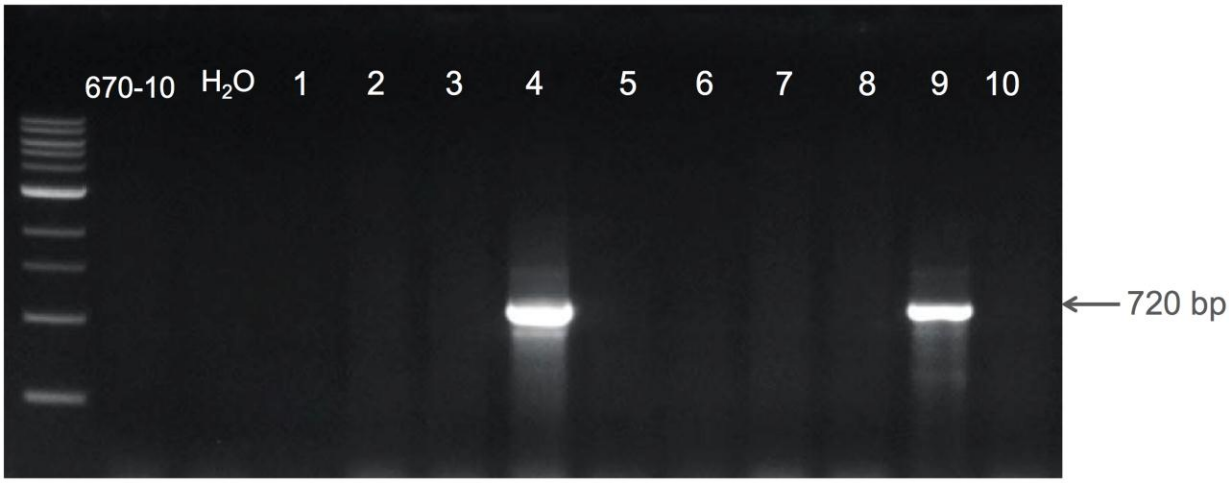

Figure 4-3. Confirmation of target gene deletion in *FSECE6* mutant using primers 5p1F/HY-R2 or YG/6R. 670-10 is wild type *F. secorum*. 1-10 are transformants of *FSECE6* mutant. Wild type and ectopic transformants did not amplify. Only those site-directed transformants amplified the target fragment.

Pathogenicity assay of *FSECE3* mutant, *FSECE5* mutant, FSECE6 mutant, and *FSECE8* mutant

Table 2 shows a summary of mutants we created for different genes of interest and pathogenicity assays we have conducted with created mutants.

We conducted pathogenicity assays of *FSECE8* mutant on *Fusarium* sugarbeet susceptible variety 4022RR. Result showed apparent difference in phenotype between plant inoculated with wild type and *FSECE8* mutant. Inoculation with the wild type caused serious disease symptoms including chlorosis yellowing and wilting of foliage (Figure 4-4). In contrast, disease symptoms were significantly reduced in plants inoculated with *FSECE8* mutant (Figure 4-4).

Quantitative PCR was conducted to relative quantify the fungal biomass of *FSECE8* mutant and *F. secorum* wild type in root material (Figure 4-5). Similar to the observed phenotype, plants inoculated with *FSECE8* mutant showed reduced fungal biomass when compared to those inoculated with wild type (Figure 4-8). All results described above revealed that *FSECE8* mutant exhibits reduced pathogenicity, which in turn indicated that *FSECE8* is a virulence gene

of *F. secorum*. Additionally, restoration to wild-type virulence by complementation of this mutant with *FSECE8* will further confirm that *FSECE8* is a virulence factor of *F. secorum*.

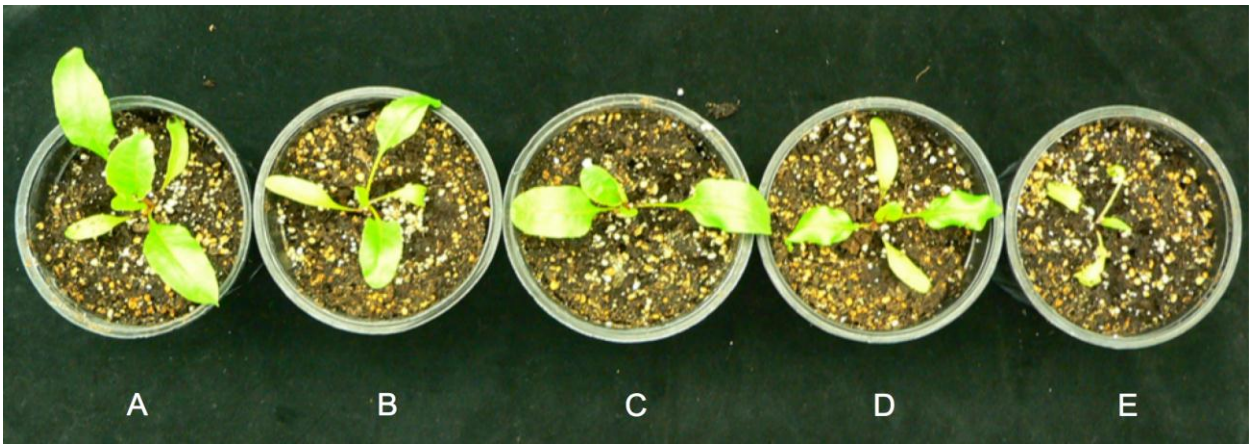

Figure 4-4. Sugarbeet variety 4022RR inoculated with *FSECE8* mutant as well as wild type and distilled water. (A) plants treated with distilled water, (B, C, D) plants inoculated with three different mutants in *FSECE8* showed reduced disease symptoms (E) plants inoculated with wild type showed yellowing and wilting of leaves.

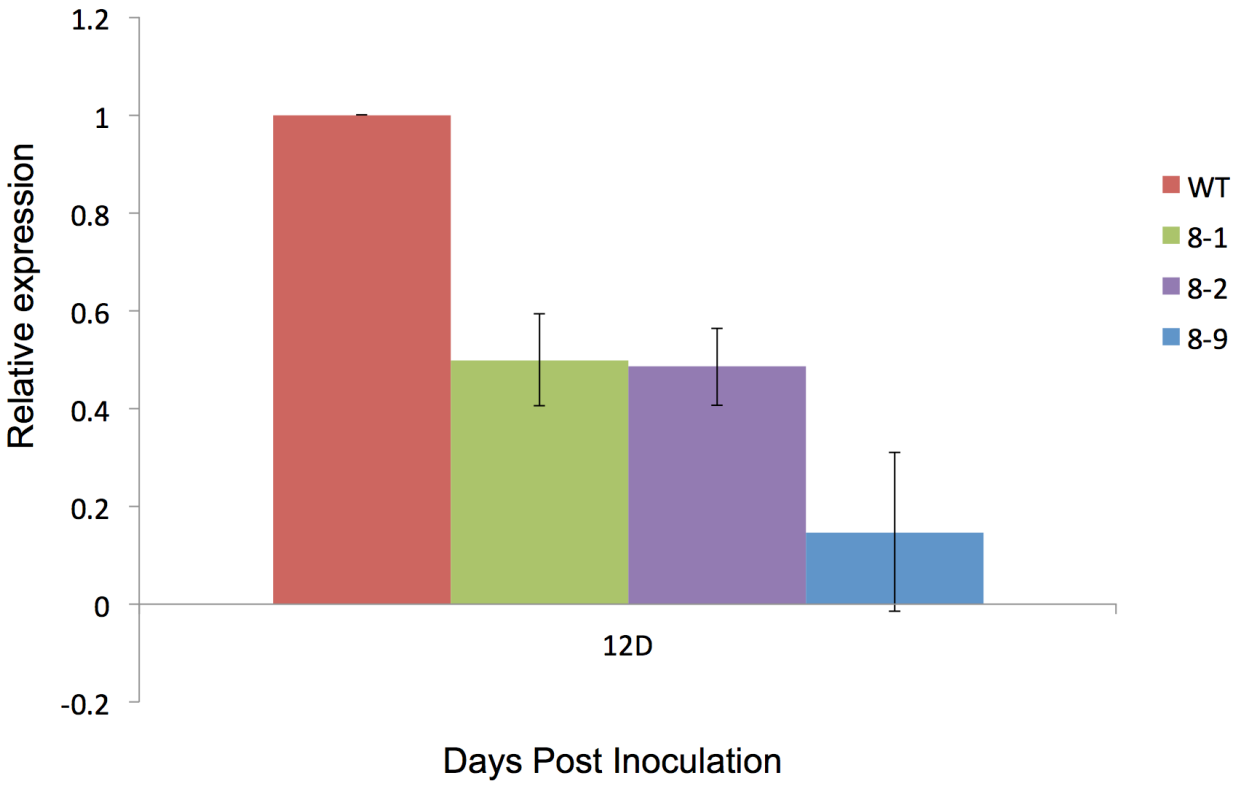

Figure 4-5. Fungal biomass of wild type and *FSECE8* mutant in root material twelve days after inoculation. WT is wild type *F. secorum*; 8-1, 8-2 and 8-9 are three different mutants in *FSECE8*. Error bars indicate standard deviation. Reduced fungal biomass was observed in mutant isolate six when compared to wild type.

No significant phenotypic difference between plant inoculated with wild type and *FSECE3* mutant, *FSECE5* mutant and *FSECE6* mutant was observed. Plants inoculated with wild type and different mutants all showed serious disease symptoms including chlorosis yellowing and wilting of foliage at eleven days after inoculation.

Quantitative PCR was conducted to relative quantify the fungal biomass of different mutant isolates and wild type of *F. secorum* in root materials. No significant difference of fungal biomass between plants inoculated with wild type and *FSECE3* mutant, *FSECE5* mutant, *FSECE6* mutant was observed (Figure 4-6, 4-7, 4-8). This result indicated that *FSECE3*, *FSECE5*, and *FSECE6* might not contribute to the pathogenicity of *F. secorum*.

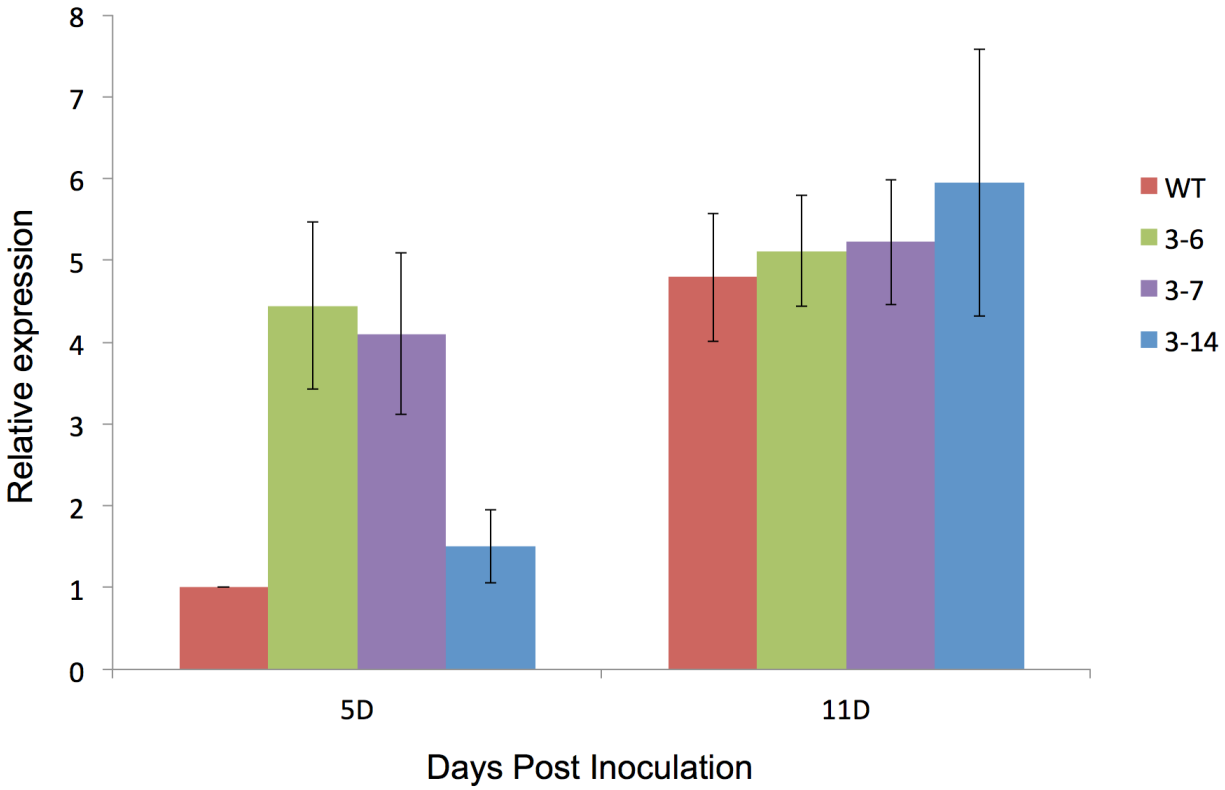

Figure 4-6. Fungal biomass of wild type and mutant isolate three in root material five and eleven days after inoculation. WT is wild type *F. secorum*; 3-6, 3-7 and 3-14 are three different mutants in *FSECE3*. Error bars indicate standard deviation. No significant different between wild type and mutants was observed.

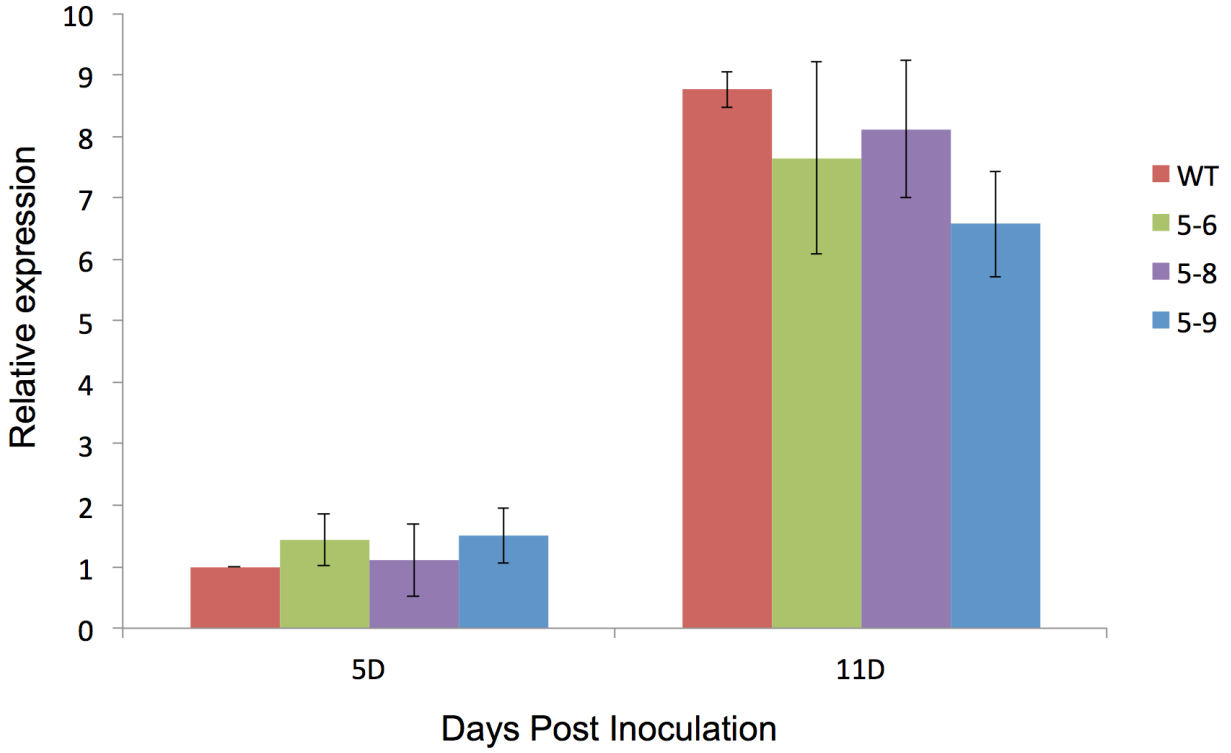

Figure 4-7. Fungal biomass of wild type and *FSECE5* mutant in root material five and eleven days after inoculation. WT is wild type *F. secorum*. 5-6, 5-8 and 5-9 are three different mutants in *FSECE5*. Error bars indicate standard deviation. No significant different between wild type and mutants was observed.

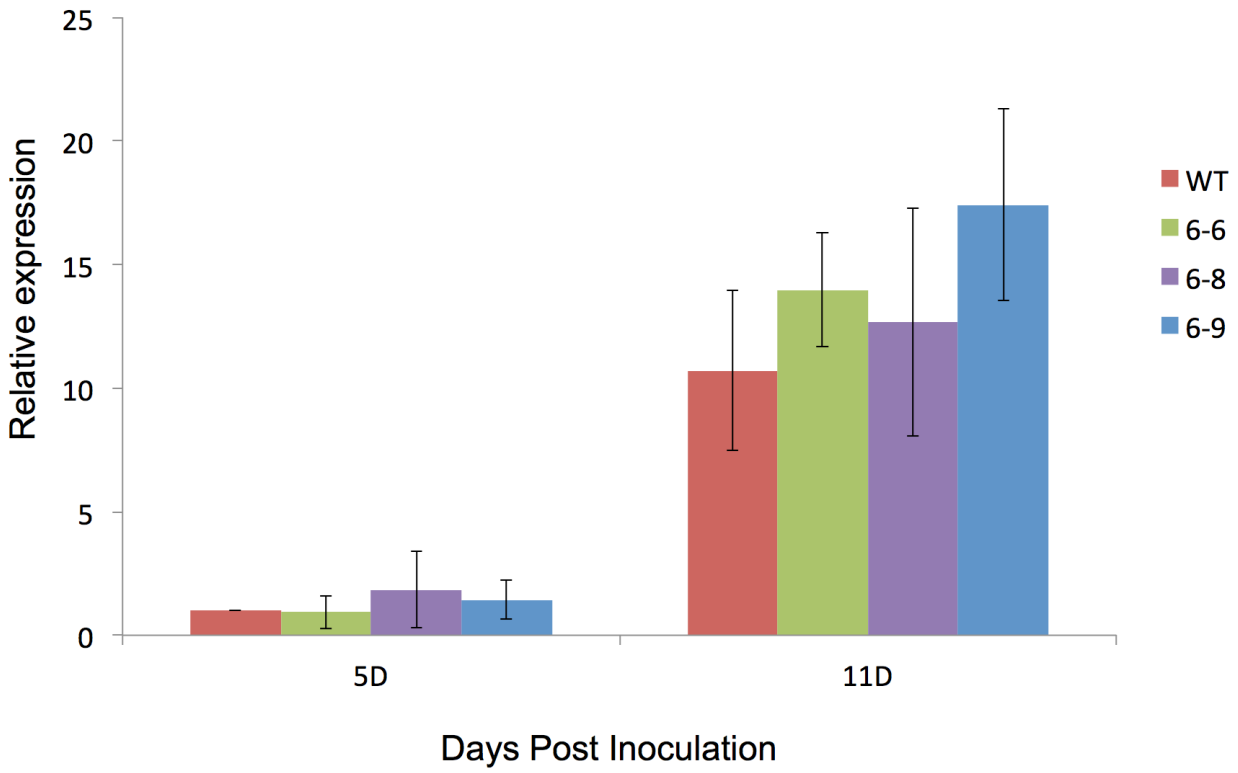

Figure 4-8. Fungal biomass of wild type and *FSECE6* mutant in root material five and eleven days after inoculation. WT is wild type *F. secorum*; 6-6, 6-8 and 6-9 are three different mutants in *FSECE6*. Error bars indicate standard deviation. No significant different between wild type and mutants was observed.

Table 2. Summary of Gene knock out and pathogenicity assay.

Gene	Annotation	Mutant	Inocu
FSECE1	no hits		
FSECE2	conserved hypothetical protein (*Fusarium spp.*)		
FSECE3	hypothetical protein (*Fusarium spp.*)	Yes	Yes
FSECE4	Similarity to m6 protein & AvrLm6 (*L. maculans*) &Six5 (*F. oxysporum* f. sp. *lycopersici*)		
FSECE5	SnodProd1 (Cerato-plantanin)	Yes	Yes
FSECE6	LysM domain-containing protein	Yes	Yes
FSECE7	conserved hypothetical protein		
FSECE8	conserved hypothetical protein	Yes	Yes
FSECE9	putative CFEM domain-containing protein		
FSECE10	SIX6	Yes	
FSECE11	conserved hypothetical protein (limited number)	Yes	

Discussion

Pathogen effectors play important roles in the interaction between plant and pathogens. Those effectors secreted by pathogens during infection can contribute to suppressing host first line defense PTI or trigger host second line defense ETI (Stergiopoulos and de Wit 2009). Thus, research on pathogen effectors can facilitate a better understanding of the mechanism of how pathogens develop and interact with host plants during infection. 11 candidate effector genes of the novel sugarbeet pathogen *F. secorum* have been predicted (Melvin D. Bolton and Ronnie de Jonge, unpublished). Based on former experiments described in chapter I and chapter II, we generated six different mutant isolates of *F. secorum* by deleting target genes using split-marker method (Catlett et al. 2002). Pathogenicity of those generated mutant isolates were then tested by inoculation of a susceptible sugarbeet variety with conidia produced by mutant isolates. Both

phenotype and fungal biomass were observed or calculated to determine whether there is any difference in pathogenicity between mutant isolates and *F. secorum* wild type.

Two different constructs need to be developed by fusion PCR in split-marker approach (Catlett et al. 2002). Construct 1 contained the 5' flanking region and HY region of *hph* while construct 2 consists of YG region of *hph* and the 3' flanking region of the targeted gene. It was observed that with same amount of DNA template, we could generate larger amounts of construct 2 than construct 1 under same PCR reaction condition. This might result from the larger size of HY region, which make fusion PCR of construct 2 more difficult than construct 1. Thus, instead of mixing equal amount of the two constructs for PEG-mediated transformation, combining more of construct 1 with construct 2 can help increase the transformation efficiency. It was reported that the length of the target gene flanking regions is critical in homologous recombination efficiency (You et al. 2009). Usually several hundred to several thousand base pair flanking regions are required for filamentous fungi to generate reasonable numbers of mutants (Meyer 2008). However, homologous recombination efficiency is also influenced by difference in fungal species as well as the targeted loci (Zwiers and De Waard 2001). Same as it was observed in this study, transformation efficiency is different between mutant isolates. Moreover, the site-directed mutant rate is also variable between different mutant isolates. For example, 12 site-directed mutants were confirmed from total 15 transformants in *FSECE8* while only two out of 20 transformants were site-directed mutants in *FSECE10*.

Pathogenicity assay of generated mutant isolates were conducted using a *Fusarium* sugarbeet susceptible variety. For plants inoculated with *FSECE3* mutant, the different fungal biomass at the five days after inoculation (Figure 4-6) might have resulted from a different initial conidia concentration. Except this difference, no big difference in both phenotype and fungal

biomass between plants inoculated with *FSECE3* mutant, *FSECE5* mutant, *FSECE6* mutant and *F. secorum* wild type (Figure 4-6, 4-7, 4-8) was observed. Reduced disease symptoms were observed in plants inoculated with *FSECE8* mutant (Figure 4-4). It also shown relatively less fungal biomass in root tissue of plants inoculated with *FSECE8* mutant when compared to those inoculated with *F. secorum* wild type (Figure 4-5). This result indicates that candidate effector encoding gene *FSECE8* could be a virulence gene of *F. secorum* that influences pathogenicity on sugarbeet. The protein encoded by *FSECE8* was predicted to be a hypothetical protein and based on the BLAST search against NCBI database, no conserved domain was identified. FSECE8 was 96% identical to the protein FOTG_18965 (NCBI access number: EXM12534) of *Fusarium oxysporum* with E value 2e-114 (data not shown). To further confirm the reliability of this result, it is necessary to repeat the inoculation of *FSECE8* mutant but with additional time points and use an ectopic mutant as another control besides *F. secorum* wild type. Additionally, restoration to wild-type virulence by complementation of this mutant with *FSECE8* will further confirm that *FSECE8* is a virulence factor of *F. secorum*.

We attempted to quantify fungal biomass in leaf tissue, however it has proven to be sometimes difficult. As it was shown in *F. secorum* wild type pathogenicity test in chapter II, fungal growth in leaf material was detected in late infection stage and with relative less biomass when compared to root and petiole tissues. There might not be enough fungal biomass in leaf tissue of sugarbeet infected with mutant isolates to be detected by quantitative real-time PCR. Since *F. secorum* can infect sugarbeet plant through wounds on roots and the earliest detection of fungal biomass was in root material, we finally selected root samples as the material to quantify fungal biomass.

In summary, we developed an efficient gene replacement approach for the novel sugarbeet pathogen *F. secorum*, which could facilitate research on dissection of the candidate effector encoding genes. Six different mutant isolates of *F. secorum* were successfully generated and pathogenicity assay were conducted with four of them. It was shown that *FSECE8* is a putative virulence gene of *F. secorum*. Further research will be conducted to further confirm the relationship between this gene and the virulence of *F. secorum*. More research can be designed to explore the role this gene plays in the interaction between pathogen and host plants.

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