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Pathogen profile

The *Fusarium solani* species complex: ubiquitous pathogens of agricultural importance

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SUMMARY

Members of the *Fusarium solani* species complex (FSSC) are capable of causing disease in many agriculturally important crops. The genomes of some of these fungi include supernumerary chromosomes that are dispensable and encode host-specific virulence factors. In addition to genomics, this review summarizes the known molecular mechanisms utilized by members of the FSSC in establishing disease.

Taxonomy: Kingdom Fungi; Phylum Ascomycota; Class Sordariomycetes; Order Hypocreales; Family Nectriaceae; Genus *Fusarium*.

Host range: Members of the FSSC collectively have a very broad host range, and have been subdivided previously into *formae speciales*. Recent phylogenetic analysis has revealed that *formae speciales* correspond to biologically and phylogenetically distinct species.

Disease symptoms: Typically, FSSC causes foot and/or root rot of the infected host plant, and the degree of necrosis correlates with the severity of the disease. Symptoms on above-ground portions of the plant can vary greatly depending on the specific FSSC pathogen and host plant, and the disease may manifest as wilting, stunting and chlorosis or lesions on the stem and/or leaves.

Control: Implementation of agricultural management practices, such as crop rotation and timing of planting, can reduce the risk of crop loss caused by FSSC. If available, the use of resistant varieties is another means to control disease in the field.

Useful websites: http://genome.jgi-psf.org/Necha2/Necha2.home.html

Keywords: cell wall-degrading enzyme, conditionally dispensable, *Fusarium solani*, horizontal gene/chromosome transfer, phytoalexin tolerance, sudden death syndrome, supernumerary chromosome.

INTRODUCTION

The genus *Fusarium* constitutes a large monophyletic group of several hundred species that, with the exception of a few, produce characteristic fusoid multiseptate macroconidia. The genus includes agriculturally important plant pathogens, endophytes and saprophytes capable of metabolizing diverse substrates, and emerging pathogens of clinical importance. Molecular phylogenetic analysis of this genus has revealed that it comprises at least 20 clades or 'species complexes' and that it originated in the middle Cretaceous period approximately 91.3 million years ago (O'Donnell *et al*., 2013).

The *Fusarium solani* species complex (FSSC) is a group currently estimated to contain at least 60 phylogenetically distinct species (Nalim *et al*., 2011; O'Donnell, 2000; O'Donnell *et al*., 2008; Zhang *et al*., 2006). As defined by Snyder and Hansen (1941), *F. solani* was the only species in what is now recognized as the FSSC. As members of the FSSC have been most extensively studied as plant pathogens, these fungi have been further subdivided into *formae speciales* (f. sp.) based on host specificity. Members of the FSSC represent a diverse set of self-fertile (homothallic) and self-sterile (heterothallic) species, although sexual cycles are only known for approximately one-third of the species (O'Donnell, 2000; O'Donnell *et al*., 2008). Seven of the heterothallic species were originally classified as mating populations (MPs I–VII) of *Nectria haematococca* because of their ability to mate with other members of the same biological species (Matuo and Snyder, 1973); however, each mating population has been shown to represent a phylogenetically distinct species (O'Donnell, 2000). On the basis of molecular phylogenetic relationships among isolates, the FSSC comprises three major clades, where Clade 3 encompasses all of the isolates formerly characterized as *N. haematococca* (O'Donnell, 2000; Zhang *et al*., 2006). In addition to *N. haematococca* (Booth, 1971), several other teleomorphic names, including '*Hypomyces solani*' (Reichle *et al*., 1964) and '*Haematonectria haematococca*' (Rossman *et al*., 1999), have been applied to these seven mating populations. Recently, '*Neocosmospora haematococca*' has been proposed for the teleomorph of these fungi, as it predates (from 1899) the traditionally used names (Lombard *et al*., 2015). Although previous nomenclatural rules dictated that the teleomorphic name of a fungus took preference over the anamorphic name, as of 2013 this stipulation no longer applies (Geiser *et al*., 2013). The abandonment of the teleomorphic names, the use of the name *F. solani* for only one species within the FSSC and the discovery of many phylogenetically distinct species have created a situation in which most species are unnamed. In lieu of Latin binomial names, a three-locus haplotype nomenclatural

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Table 1 Taxonomy of the *Fusarium solani* species complex (FSSC) species of relevance to this review.

Phylogenetic species	Current scientific name	Traditional name(s)	Reference(s)
FSSC 1	*F. petroliphilum*	*Nectria haematococca* MP V; *F. solani* f. sp. *cucurbitae* race 2	Short *et al*. (2013)
FSSC 2	*F. keratoplasticum*		Short *et al*. (2013)
FSSC 3+4	*F. falcilforme*	*Cylindrocarpon lichenicola*; *Acremonium falciforme*	Summerbell and Schroers (2002)
FSSC 8	*F. neocosmosporiellum*	*Neocosmospora vasinfectum*	O'Donnell *et al*. (2013)
FSSC 10	Unnamed	*N. haematococca* MP I; *F. solani* f. sp. *cucurbitae* race 1	
FSSC 11	Unnamed	*N. haematococca* MP VI; *F. solani* f. sp. *pisi*	
FSSC 13	Unnamed	*N. haematococca* MP VII; *F. solani* f. sp. *robiniae*	
FSSC 17	Unnamed	*N. haematococca* MP III; *F. solani* f. sp. *mori*	
FSSC 22	Unnamed	*N. haematococca* MP IV; *F. solani* f. sp. *xanthoxyli*	
FSSC 23	Unnamed	*N. haematococca* MP II; *F. solani* f. sp. *batatas*	
na	*F. phaseoli*	*F. solani* f. sp. *phaseoli*	
na	*F. virguliforme* *F. tucumaniae* *F. brasiliense* *F. cuneirostrum*	*F. solani* f. sp. *glycines*	Aoki *et al*. (2003)

MP, mating population. Phylogenetically distinct species within Clade 3 of the FSSC are distinguished by Arabic numerals (see O'Donnell *et al*., 2008).
na, not applicable.

system was developed for the members of Clade 3 of the FSSC based on polymorphisms in DNA sequences of translation elongation factor 1α (*EF-1α*), the second largest subunit of RNA polymerase II (*RPB2*), and the nuclear ribosomal internal transcribed spacer region and domains D1 and D2 in the nuclear large subunit (Chang *et al*., 2006; O'Donnell *et al*., 2008). For the purpose of this review, the FSSC species will be referred to by the multilocus haplotype number and any traditionally used name when first introduced (Table 1).

Members of the FSSC are important pathogens of a number of agriculturally important crops. The diverse host range exists not only throughout the species complex, but also within a single species. For example, isolates of FSSC 11 (*N. haematococca* MP VI; *F. solani* f. sp. *pisi*) are best known for causing root rot of garden pea (*Pisum sativum* L.); however, they have a diverse habitat range and have been confirmed to be pathogenic on at least 10 other host plants (VanEtten, 1978). The four pathogens causing soybean sudden death syndrome (SDS), *F. virguliforme*, *F. tucumaniae*, *F. brasiliense* and *F. cuneirostrum*, are members of the FSSC (Aoki *et al*., 2003, 2005). Although traditionally thought to be only a pathogen on soybean, the host range of *F. virguliforme* is more diverse as it is able to cause root necrosis on alfalfa, pinto and navy bean, white and red clover, pea and Canadian milk vetch, and is asymptomatic on a number of other plants (Kolander *et al*., 2012). Investigation of the host range of *F. solani* f. sp. *eumartii* uncovered additional host plants besides potato, including other plants in the Solanaceae, such as tomato and pepper (Romberg and Davis, 2007). In addition to being plant pathogens, members of the FSSC are responsible for the majority of systemic fusarial infections in immunocompromised humans and animals (Muhammed *et al*., 2013; O'Donnell *et al*., 2008; Zhang *et al*., 2006). In immunocompetent individuals, diseases caused by FSSC isolates may manifest as localized, less invasive infections, including abscesses, onychomycosis and/or keratitis (Muhammed *et al*., 2013); for example, the 2005–2006 outbreaks of keratitis associated with a contact lens solution in North America and Asia were caused predominantly by members of the FSSC (Chang *et al*., 2006; Khor *et al*., 2006; O'Donnell *et al*., 2007).

Despite the agricultural importance and broad host range of members of the FSSC, few studies have focused on the identification of virulence factors in this species complex. The interaction between garden pea and isolates of FSSC 11 has been extensively utilized as a model system to study fungal virulence factors and plant defence responses at a molecular level (Hadwiger, 2008). This review focuses on the current understanding of the infection process and virulence factors for this fungus and other closely related members of the FSSC (Table 2).

GENOME ORGANIZATION AND SUPERNUMERARY CHROMOSOMES

Genome organization in members of the FSSC has been shown to be very dynamic. Pulse field gel electrophoretic profiles of members of the FSSC have revealed a high degree of variability in the size and number of small chromosomes between species and within a single species (Mahmoud and Taga, 2012; Miao *et al*., 1991a,b; Suga *et al*., 2002). These small chromosomes have been most extensively studied in FSSC 11, where many of them have been shown to be dispensable supernumerary chromosomes (Covert, 1998; Covert *et al*., 1996; Enkerli *et al*., 1998; Funnell and VanEtten, 2002; Miao *et al*., 1991a; VanEtten *et al*., 1998; Wasmann and VanEtten, 1996).

In 2009, genome analysis of FSSC 11 isolate 77-13-4 (FGSC 9596; NRRL 45880) was completed, the first genome for a member of the FSSC (Coleman *et al*., 2009). An optical map identified 17 chromosomes that ranged in size from 530 kb to 6.52 Mb (Fig. 1). The 54.43-Mb genome encoding 15 707 predicted genes was sig-

Table 2 Gene deletions and disruptions in members of the *Fusarium solani* species complex (FSSC) and their phenotypic effects.

FSSC species	Gene	Product/function	Effect of gene inactivation/deletion	Reference
FSSC 11	*cut1*	Secreted cutinase	WT pathogenicity on pea; reduced virulence on pea	Stahl and Schäfer (1992); Rogers *et al*. (1994)
FSSC 1	*cutA*	Cutinase	WT pathogenicity on cucurbits	Crowhurst *et al*. (1997)
FSSC 11	*dhc*1	Cytoplasmic dynein	Reduced growth	Inoue *et al*. (1998a)
F. virguliforme	*fvtox*1	Secreted acidic protein	Reduced virulence on soybean	Pudake *et al*. (2013)
FSSC 11	*MAK*1	FAD mono-oxygenase	Reduced virulence on chickpea; increased sensitivity to maackiain	Enkerli *et al*. (1998)
FSSC 11	*NhABC*1	ABC transporter	Reduced virulence on pea; increased sensitivity to pisatin and rishitin	Coleman *et al*. (2011)
FSSC 11	*Nhkin*1	Kinesin	Reduced growth	Wu *et al*. (1998)
FSSC 11	*PDA1*	Cytochrome P450	Reduced virulence on pea; increased sensitivity to pisatin	Wasmann and VanEtten (1996)
FSSC 11	*pel*A	Pectate lyase	Reduced virulence on pea	Rogers *et al*. (2000)
FSSC 11	*pel*D	Pectate lyase	Reduced virulence on pea	Rogers *et al*. (2000)

FAD, flavin adenine dinucleotide; WT, wild-type.

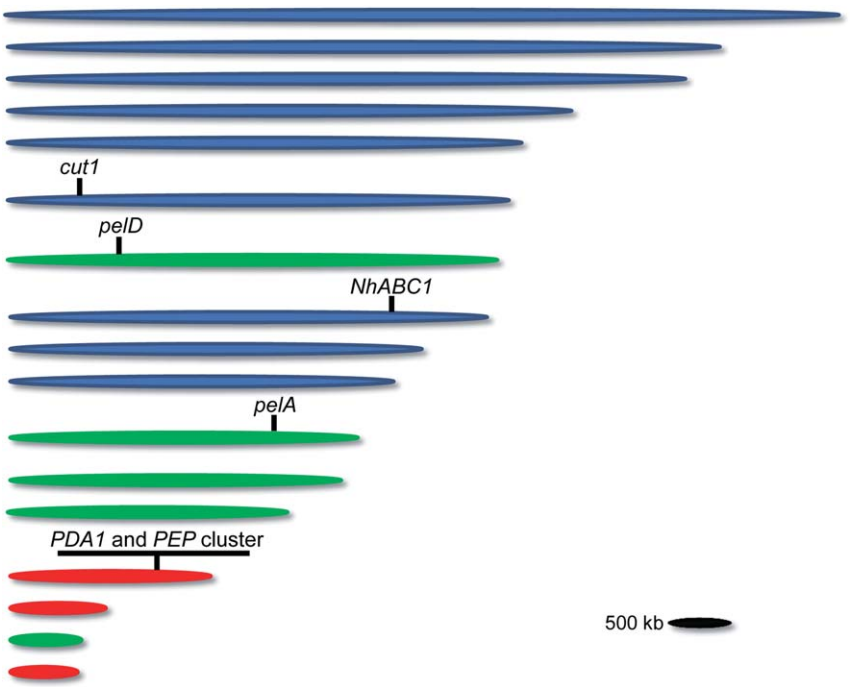

Fig. 1 Profile and characteristics of chromosomes of the *Fusarium solani* species complex (FSSC) 11 77-13-4 isolate genome. Chromosomes that contain a large number of 'unique' genes and in which >10% of the total number of genes were identified as potential pseudoparalogues are coloured green and red; red chromosomes have been confirmed experimentally to be dispensable. Approximate locations of genes involved in virulence are indicated.

nificantly larger than the 36.45-Mb genome of *Fusarium graminearum* (Cuomo *et al*., 2007), the only other *Fusarium* sequenced at the time, and 92.3% of the genome sequence was mapped to one of the 17 chromosomes. Comparative genomics revealed that enlargement of the genome of FSSC 11 was a result of genes not known to be present in *F. graminearum* and the presence of multiple copies of a gene that exists as a single copy in other fungi. The origin of these genes appears to have occurred through gene duplication events or may have been acquired by horizontal gene transfer. Gene families that showed a significant increase in number in the FSSC 11 genome included carbohydrate-active enzymes, oxidoreductases, monooxygenases, dioxygenases and transporters; the increase in these gene families may be indicative of the increased niche colonization ability and adaptation of FSSC 11 when compared with *F. graminearum*.

Based on their loss in otherwise isogenic isolates, three chromosomes (14, 15 and 17) of the sequenced isolate were determined to be supernumerary. Chromosome 14 was previously known to be a supernumerary chromosome that was conditionally dispensable (CD). A CD chromosome is a chromosome that can be lost with no alteration in growth under laboratory conditions; however, it is able to broaden the habitat of the fungus when present (Covert, 1998). Chromosome 14 carries the *PDA1* gene (discussed further below) and is referred to as the *PDA1*-CD chromosome. Genomic analyses of these three supernumerary chromosomes showed that they were enriched for unique, duplicated genes, repeated sequences and had a decreased G + C content when compared with the other FSSC 11 chromosomes. Phylogenetic analysis of genes on the smaller chromosomes showed that they were more closely related to genes from the

aspergilli than to genes within *F. graminearum*. In addition to unique genes on the supernumerary chromosomes, the population of repeated elements was enriched. A number of repeated elements have been characterized in FSSC 11 isolates that are associated with CD chromosomes: *Nht1*, *Nht2* and *Nrs1* (Enkerli *et al*., 1997; Kim *et al*., 1995; Shiflett *et al*., 2002). Analysis of the *pogo*-like transposable elements (*Fot2*, *Fot3*, *Fot4*, *Fot5* and *Fot6*) found 84 of these elements in the genome, with *Fot5* the predominant *pogo*-like transposable element in the FSSC 11 genome (n = 60; Dufresne *et al*., 2011). The chromosomal locations of these *pogo*-like elements were enriched on the supernumerary chromosomes (36%) and unmapped scaffolds (37%). Similar characteristics (enrichment of specific gene families and repeated elements) of the supernumerary chromosomes in FSSC 11 were also found to occur for the lineage-specific supernumerary chromosomes of *Fusarium oxysporum* f. sp. *lycopersici* (Ma *et al*., 2010).

The mitochondrial (mt) genome of FSSC 11 is ~63 kb and encodes 15 genes expected to be in fungal mt genomes based on other analyses (Al-Reedy *et al*., 2012); however, the FSSC 11 mt genome has two unidentified open reading frames (uORFs) and several more group I and II introns when compared with other sequenced fusarial mt genomes (Al-Reedy *et al*., 2012). One of the unidentified ORFs was common to several *Fusarium* mt genomes and was exceptionally large (2013 amino acids in length in the FSSC 11 isolate). This ORF, termed LV-uORF, is speculated to have been acquired by horizontal gene transfer based on a higher G + C content and a bias in codon usage (Al-Reedy *et al*., 2012). Two linear plasmid-like DNAs were identified that exist in the mitochondria of FSSC 10 (formerly *N. haematococca* MP I; *F. solani* f. sp. *cucurbitiae* 'race 1') and were termed pFSC1 (9.2 kb) and pFSC2 (8.3 kb) (Samac and Leong, 1988). When the isolate was cured of these plasmids, there was no significant difference in growth rate or conidiation between the wild-type and plasmid-cured isolates; however, the wild-type isolate was significantly more virulent than the plasmid-cured strains when assayed on a cucurbit using a low concentration of inoculum (Samac and Leong, 1988).

The genome of *F. virguliforme*, a member of the FSSC and one of the species responsible for SDS, has been completed recently (Srivastava *et al*., 2014). The Mont-1 strain genome was 50.99 Mb in size, encoding for 14 845 predicted genes. Comparison between the *F. virguliforme* and FSSC 11 genomes revealed 13 068 putative orthologous genes between these two FSSC species (88% and 83% of their genes, respectively); many of these orthologous regions were in syntenic regions between the two. When these regions were anchored to the chromosomal karyotype of the FSSC 11 isolate, most were placed on the larger chromosomes, but there were small syntenic regions that aligned with the dispensable chromosomes of FSSC 11 strain 77-13-4 (chromosomes 14, 15 and 17), suggesting that there could be genetic elements in the *F. virguliforme* genome that could be supernumerary. As with the supernumerary chromosomes of the FSSC 11 isolate, an estimated 18% of the genome sequence of *F. virguliforme* was composed of repeated elements, a characteristic of supernumerary chromosomes. In addition, genomic comparison with the genomes of *F. graminearum* PH-1, *F. oxysporum* f. sp. *lycopersici* 4287, *F. verticillioides* and FSSC 11 77-13-4 identified 1332 genes that were unique to *F. virguliforme* and not present in the other four genomes.

ADHESION AND PENETRATION

The infection process of hypocotyls for several phylogenetic species within the FSSC has been described on their respective host plants (Bywater, 1959; Christou and Snyder, 1962; Samac and Leong, 1989; Stahl *et al*., 1994). Isolates of FSSC 10, FSSC 11 and *F. phaseoli* were able to penetrate into the hypocotyl, either directly through the epidermis (FSSC 10) or primarily through stomates (FSSC 11 and *F. phaseoli*). Microscopic examination and quantification of the fungal sterol ergosterol *in planta* allowed the spread of a FSSC 11 infection to be followed. After invasion of the hypocotyl, hyphae of FSSC 11 spread to the upper portion of the tap root and the epicotyl, eventually colonizing the xylem and causing wilting to the above-ground portions of the host plant (Stahl *et al*., 1994).

Members of the FSSC are known to cause root rot on several plants, and root exudates can influence the germination and growth of fungi within the rhizosphere. Chlamydospores of FSSC 11 will germinate within 24 h when placed 7 mm away from a germinating seed (Short and Lacy, 1974); presumably, the carbohydrates and amino acids present in the root exudates are partially responsible for the stimulation of spore germination. One of the major root exudates from pea roots is homoserine (Van Egeraat, 1975), and the *PDA1*-CD chromosome in FSSC 11 harbours a five-gene locus that confers the ability for homoserine utilization (*HUT*) as a sole carbon and nitrogen source (Rodriguez-Carres *et al*., 2008; White, 2008). Mutation in the *HUT* cluster renders FSSC 11 less competitive in the pea rhizosphere when compared with the isogenic isolate carrying the *HUT* cluster, demonstrating that the *HUT* cluster is a rhizosphere competence trait (Rodriguez-Carres *et al*., 2008; White, 2008). In addition to carbohydrates and amino acids, flavonoids are capable of inducing spore germination in FSSC 11 and *F. phaseoli* (Ruan *et al*., 1995). These root exudates include flavones, isoflavones and pterocarpans that are synthesized as part of a defence mechanism. Stimulation of spore germination by these compounds involves the cyclic adenosine monophosphate (cAMP) pathway and is dependent on protein kinase A and regulated through cAMP phosphodiesterase (Bagga and Straney, 2000; Ruan *et al*., 1995).

The attachment of plant-pathogenic fungal spores to a susceptible host is a common initial step for the establishment of disease. A number of mechanisms have been identified that are involved in

this process in different phytopathogenic fungi (Tucker and Talbot, 2001); the molecular process of conidial adhesion has best been described for FSSC 10. Attachment of macroconidia to the unwounded surface of a susceptible host is a virulence factor for this FSSC species (Jones and Epstein, 1990), and is an active process that requires respiration and protein synthesis (Jones and Epstein, 1989). The production of spore tip mucilage is induced by treatment of the spores with zucchini fruit extract (a plant host of FSSC 10), conferring adherence at either or both apical tips of the macroconidium within 10 min after treatment (Kwon and Epstein, 1993). A 90-kDa glycoprotein was associated with adhesion of the spore tip mucilage and, when bound by concanavalin A or an antibody against the 90-kDa glycoprotein, adhesion of the macroconidium was abrogated (Kwon and Epstein, 1993, 1997). In addition, adherence was reduced in the presence of snowdrop lectin, a compound that specifically binds α-mannose, and two transglutaminase inhibitors, suggesting that the 90-kDa glycoprotein is cross-linked to the spore tip mucilage (Kwon and Epstein, 1997). The production of spore tip mucilage has been implicated in adhesion in another member of the FSSC, *F. phaseoli*, and could be a common attachment mechanism in fungi (Schuerger and Mitchell, 1993). In addition to the glycoprotein, random mutagenesis and classical genetics have revealed that FSSC 10 utilizes another attachment mechanism encoded at a genetic locus termed *Att1* (Epstein *et al*., 1994). Studies with an Att1$^-$ isolate indicated that macroconidium attachment was dependent on the concentration of zucchini extract applied to the conidia and the temperature (Epstein *et al*., 1994).

After spore attachment, the easiest route to invade the host plant is via the stomates. The role of direct penetration of plant tissue in disease development is uncertain. Passive barriers, such as cutin, a waxy polymer component of the cuticle, must be overcome to infect the plant. Variability in the role of cutinase in pathogenesis has been reported for plant-pathogenic fungi. These enzymes have been studied extensively in FSSC 11, where isolates may have several cutinase genes (Rogers *et al*., 1994; Stahl and Schäfer, 1992). Expression of cutinase is induced in the presence of cutin monomers (Podila *et al*., 1988; Woloshuk and Kolattukudy, 1986), and the enzyme can be detected at infection sites on pea (Shaykh *et al*., 1977). A number of regulatory elements in the promoter of cutinases control expression, in particular a palindromic sequence which binds cutinase transcription factor 1α (CTF1α), which positively regulates expression in the presence of cutin (Kämper *et al*., 1994; Li and Kolattukudy, 1997; Li *et al*., 2002). A second transcription factor, termed CTF1β, is involved in the constitutive expression of some cutinase genes in FSSC 11 (Li *et al*., 2002). The expression of a FSSC 11 cutinase gene in *Mycosphaerella* spp., a fungus unable to infect unwounded papaya fruit, conferred to the transgenic fungus the ability to infect intact tissue (Dickman *et al*., 1989). An extracellular esterase in FSSC 11 is responsible for cutinase and esterase activity (Murphy *et al*., 1996; Rogers *et al*., 1994; Stahl and Schäfer, 1992). Disruption of the gene encoding this cutinase, *cut1*, demonstrated that the enzyme is not essential for virulence on pea (Stahl and Schäfer, 1992; Stahl *et al*., 1994), or results in a significant decrease in virulence (Rogers *et al*., 1994). The orthologue of *cut1* has been studied in the closely related fungus FSSC 1 (*F. petroliphilum*; formerly *N. haematococca* MP V, *F. solani* f. sp. *cucurbitiae* 'race 2'). As with FSSC 11, *cutA* is induced in the presence of cutin from various cucurbits (Hawthorne *et al*., 2001); however, disruption of the *cutA* gene in FSSC 1 did not cause a significant reduction in virulence on intact fruit of several varieties of *Cucurbita maxima* and *C. moschata* (Crowhurst *et al*., 1997). The induction of cutinase activity in FSSC 1 is dissimilar to that observed in FSSC 11, and therefore it appears that the regulation of *cutA* in FSSC 1 is different from that of *cut1* in FSSC 11 (Hawthorne *et al*., 2001). Another cucurbit pathogen in the FSSC (FSSC 10) lacked cutinase activity, which may explain the difference in tissue specificity between these phylogenetically distinct cucurbit pathogens (Hawthorne *et al*., 2001).

In addition to cutinases, enzymes digesting pectin in the plant cell wall have been implicated in the penetration of plant barriers. A number of pectin lyases have been characterized in FSSC 11, including two that are involved in virulence on pea. The enzyme encoded by *pelA* is induced in the presence of pectin, whereas the enzyme encoded by *pelD* is only expressed *in planta*, where it is induced by asparagine and homoserine, two amino acids found at high levels in pea seedlings (Rogers *et al*., 2000; Yang *et al*., 2005). Deletion of either *pelA* or *pelD* individually did not cause a significant reduction in virulence; however, a *ΔpelA/ΔpelD* mutant was severely impaired in virulence, causing only a few mild lesions (Rogers *et al*., 2000).

TOLERANCE TO PLANT DEFENCE MECHANISMS

As a part of the defence reaction to pathogens, plants release small molecular weight antimicrobial compounds. These compounds can be either preformed and compartmentalized in an inactive state inside the plant cell, termed phytoanticipins, or synthesized *de novo* in response to the pathogen, termed phytoalexins (VanEtten *et al*., 1994). Regardless of the time of synthesis, a fungal pathogen must be able to tolerate these antimicrobial compounds in order to establish an infection on the host plant and, eventually, to develop disease. Pathogens primarily possess three mechanisms by which they accomplish this feat: (i) enzymatic degradation or modification of the compound to a less toxic molecule; (ii) efflux of the antimicrobial prohibiting an intracellular concentration that has a physiological effect; and (iii) modification of the target site of the compound (Coleman and Mylonakis, 2009; Morrissey and Osbourn, 1998; VanEtten *et al*., 2001). Of these, degradation/modification and efflux have been described in members of the FSSC.

Tolerance mechanisms of FSSC 11 against the phytoalexin (+)pisatin produced by garden pea have been studied extensively and represent one of the best examples in plant–pathogen interactions. On challenge with FSSC 11, pea plants begin to synthesize pisatin, which inhibits the pathogen. FSSC 11 isolates that are highly pathogenic on pea carry a gene encoding a cytochrome P450, termed pisatin demethylase (PDA; VanEtten *et al*., 1980). PDA catalyses the demethylation of pisatin at the C3 position, forming the much less toxic (+)-6a-hydroxymaackiain. Currently, nine alleles of PDA have been identified from FSSC 11 isolates by classical genetics (Funnell *et al*., 2002; Kistler and VanEtten, 1984; Mackintosh *et al*., 1989; Miao *et al*., 1991a, b); however, only two of these, *PDA1* and *PDA4*, are linked to high virulence on pea (Maloney and VanEtten, 1994; VanEtten *et al*., 1980). Deletion of *PDA1* in FSSC 11 strains yielded transformants that were reduced in virulence on garden pea and more sensitive to pisatin (Wasmann and VanEtten, 1996). Alternatively, expression of *PDA1* in an FSSC 11 isolate that was previously *PDA*$^{-}$ increased virulence on pea (Ciuffetti and VanEtten, 1996). Expression of *PDA1* is induced *in planta* and on treatment with pisatin (Liu *et al*., 2003; Ruan *et al*., 1995; Straney and VanEtten, 1994). A pisatin-responsive activating factor is hypothesized to bind to a 35-bp region −514 to −480 bp relative to the start site of *PDA1* activating transcription (Straney and VanEtten, 1994). Expression of *PDA1* is repressed *in vitro* under high nutrient conditions, such as glucose and amino acids (Khan and Straney, 1999; Straney and VanEtten, 1994), and is independent of cAMP signalling (Ruan *et al*., 1995). This nutritional repression is mediated by a region in the *PDA1* promoter that resides −287 to −429 bp relative to the start codon (Khan and Straney, 1999).

PDA resides on a small supernumerary chromosome that is not required for the fungus to grow under laboratory conditions, and has been called the *PDA1*-CD chromosome (Miao *et al*., 1991a). The FSSC 11 77-13-4 isolate that has lost the 1.6-Mb *PDA1*-CD chromosome is non-pathogenic on pea. However, when the isolate carries a disrupted *PDA1* gene, it retains some pathogenicity, and therefore other virulence factors for pea must reside on the same CD chromosome as *PDA1* (Wasmann and VanEtten, 1996). Subsequent studies have found that *PDA1* indeed resides within an ~25-kb cluster of genes that are involved in pea pathogenicity (PEP; Fig. 2). Three genes (*PEP1*, *PEP2* and *PEP5*) have been shown to contribute to virulence on pea when expressed in a non-pathogenic isolate of FSSC 11 (Han *et al*., 2001). *PEP1* may encode two proteins, 330 and 320 amino acids in length, that are in different reading frames, and *PEP2* encodes a protein of 233 amino acids in length. *PEP5* is the only *PEP* gene with a postulated function based on amino acid sequence, as PEP5 encodes an efflux pump of the major facilitator superfamily, although the substrate of PEP5 is unknown. As with *PDA1*, *PEP1*, *PEP2* and *PEP5* were expressed *in planta* and after treatment with pisatin (Liu *et al*., 2003). A correlation between the degree of virulence on pea and the *PEP* genes has been observed, where FSSC 11 isolates that are highly virulent carry all the *PEP* genes and, in many instances, multiple copies (Temporini and VanEtten, 2002).

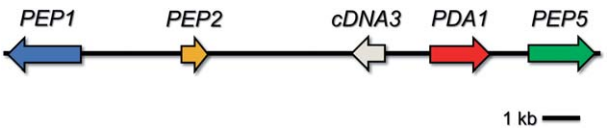

Fig. 2 The *PDA1* and *PEP* clusters in *Fusarium solani* species complex (FSSC) 11. The *PEP* cluster is ~20 kb in size and resides on chromosome 14, a conditionally dispensable chromosome. *PDA1* encodes a cytochrome P450 and *PEP5* encodes a putative major facilitator superfamily (MFS) transporter. The functions of *PEP1*, *PEP2* and *cDNA3* are unknown. PDA, pisatin demethylase; PEP, pea pathogenicity.

In addition to isolates of FSSC 11, functional *PDA* genes have been identified in isolates that are currently placed within FSSC 8 (*Fusarium neocosmosporiellum*; formerly *Neocosmospora vasinfecta*; O'Donnell *et al*., 2013) and other closely related fusaria (i.e. *Neocosmospora bonniensis* and *Neocosmospora tenuicristata*; Temporini and VanEtten, 2004; Milani *et al*., 2012). In addition to *PDA*, homologues of *PEP1*, *PEP2* and *PEP5* are present in *Ne. bonniensis* in a cluster as in FSSC 11 (Temporini and VanEtten, 2004). Although the *Fusarium* sp. originally described as *Ne. bonniensis* was not known to be a pea pathogen, when inoculated on pea roots, the fungus was able to cause necrosis to the root system and stunting (Temporini and VanEtten, 2004). The discontinuous phylogenetic distribution of *PDA* and *PEP* genes within the FSSC supports the potential evolutionary origin of these genes via horizontal gene transfer, as *PDA* genes are not found in other FSSC species that are closely related to FSSC 11 (Temporini and VanEtten, 2004). As observed with the dispensable chromosomes in the sequenced FSSC 11 77-13-4 isolate (Coleman *et al*., 2009), a G + C and codon bias exists between the *PDA* and *PEP* genes when compared with genes that have a conserved 'housekeeping' function (Liu *et al*., 2003; Milani *et al*., 2012).

In addition to PDA, members of FSSC 11 have a second tolerance mechanism to pisatin. In the absence of enzymatic detoxification, the fungus is able to overcome the inhibitory effects through a 'non-degradative' mechanism that is induced by pisatin and other similarly structured compounds (Denny and VanEtten, 1983a,b; Denny *et al*., 1987). The ABC transporter NhABC1, which is responsible for the efflux of pisatin, belongs to the pleotropic drug resistance (PDR or ABCG) family of efflux pumps (Coleman *et al*., 2011). *ΔNhABC1* mutants were slightly reduced in sensitivity to pisatin; however, there was a significant reduction in virulence on pea. Double mutants of *NhABC1* and *PDA1* were more sensitive to pisatin than either single mutant. Virulence assays with the *ΔNhABC1*/*ΔPDA1* mutants showed that they were significantly reduced in virulence when compared with either single mutant. Although the FSSC 11 isolate used in this study was not a pathogen of potato, NhABC1 also conferred tolerance to the

potato phytoalexin rishitin. Phylogenetic analysis of *NhABC1* revealed that it is a member of a *Fusarium*-specific clade of ABC transporters that include other transporters involved in virulence on plants (Fleissner *et al*., 2002; Gardiner *et al*., 2013; Skov *et al*., 2004).

The phytoalexins (–)maackiain and (–)medicarpin are produced by chickpea (*Cicer arietinum* L.) in response to pathogens. Some FSSC 11 isolates are highly virulent on chickpea and are tolerant to phytoalexins; conversely, isolates more sensitive to maackiain are less virulent on this host plant (Lucy *et al*., 1988). Isolates of FSSC 11 were able to perform three metabolic modifications on maackiain and medicarpin via monooxygenases and were able to detoxify the phytoalexins to the less inhibitory (–)-6a-hydroxypterocarpan, the 1a-hydroxydienone and the isoflavanone derivatives (Denny and VanEtten, 1981, 1982; Lucy *et al*., 1988). Three maackiain detoxification genes (*MAK*1–*MAK*3) have been identified by genetic studies (Covert *et al*., 1996; Miao and VanEtten, 1992). All FSSC 11 isolates that are capable of metabolizing maackiain are also able to modify medicarpin to analogous byproducts, suggesting that the enzymes encoded by the *MAK* genes are responsible for this modification (Denny and VanEtten, 1982). When *MAK1* was hybridized to genomic DNA of isolates carrying the other *MAK* genes, no cross-hybridization was observed, supporting divergence among the *MAK* genes in FSSC 11 (Covert *et al*., 1996). MAK1 encodes a flavin adenine dinucleotide (FAD)-containing monooxygenase of 460 amino acids in length (Covert *et al*., 1996), which is responsible for the detoxification of maackiain to the less toxic compound 1a-hydroxymaackiain (Covert *et al*., 1996; Miao and VanEtten, 1992). Despite the divergence between the *MAK1* and *MAK2* genes, MAK2 is also responsible for converting maackiain to 1a-hydroxymaackiain, whereas MAK3 converts the phytoalexin to 6a-hydroxymaackiain (Miao and VanEtten, 1992). Although all three *MAK* genes encode enzymes capable of metabolizing maackiain, only *MAK1* and *MAK2* correlate with high virulence on chickpea (Miao and VanEtten, 1992). When *MAK1* was disrupted in a highly virulent isolate on chickpea, virulence was reduced. Conversely, when the gene was placed into a *mak*$^{-}$ isolate, there was an increase in virulence on chickpea (Enkerli *et al*., 1998). When *MAK1*-disrupted mutants were placed on medium containing maackiain, growth was reduced by ~20% (Enkerli *et al*., 1998). Genetic studies indicated that *MAK1* was linked with *PDA6-1* and resided on the same dispensable chromosome (Covert *et al*., 1996; Miao and VanEtten, 1992). An isogenic isolate that had lost the 1.6-Mb chromosome was reduced in virulence to a similar degree as the disrupted *mak*$^{-}$ mutant, demonstrating that there were no other virulence factors for chickpea on the *MAK1*/*PDA6-1* dispensable chromosome (Enkerli *et al*., 1998).

A correlation between tolerance to the bean (*Phaseolus vulgaris*) phytoalexin kievitone and virulence has been observed for *F. phaseoli* (formerly *F. solani* f. sp. *phaseoli*), in which isolates that produce extracellular kievitone hydratase are highly virulent on bean and, conversely, are less virulent when kievitone hydratase activity is abolished (Smith *et al*., 1982, 1984). Kievitone hydratase is an acidic glycoprotein that catalyses a hydration reaction on the dimethylallyl moiety, forming the less toxic compound kievitone hydrate (Cleveland and Smith, 1983; Zhang and Smith, 1983). The gene encoding kievitone hydratase, *khs*, generates a preprotein of 350 amino acids in length, in which the N-terminal 19 amino acids are hydrolysed during maturation and secretion (Li *et al*., 1995). Pulse field gel electrophoresis has revealed that *khs* resides on chromosomes usually ranging in size from approximately 2 to 3.5 Mb in *F. phaseoli* and *F. cuneirostrum* (reported as *F. phaseoli*), whereas it is absent in almost all of the other FSSC isolates included in the analysis (Suga *et al*., 2002), suggesting that the chromosomes in *F. phaseoli* and *F. cuneirostrum* that harbour *khs* may be supernumerary. It should be noted that, in addition to kievitone, *P. vulgaris* produces other phytoalexins, including phaseollidin, phaseollin and phaseollin isoflavan. Phaseollidin is hydroxylated in a similar manner to kievitone by an extracellular enzyme, termed phaseollidin hydratase, forming phaseollidin hydrate (Turbek *et al*., 1992). Biochemical analysis of *F. phaseoli* indicates that phaseollin is detoxified to the less toxic compound 1a-hydroxyphaseollone by a monooxygenase (Kistler and VanEtten, 1981).

EFFECTORS, SECRETED PROTEINS AND HOST-SPECIFIC VIRULENCE FACTORS

Although the tolerance mechanisms to phytoalexins represent the best-studied host-specific virulence factors in members of the FSSC, only a few effectors have been identified in these fungi. An extracellular DNase has been identified in *F. phaseoli* and FSSC 11 which is capable of eliciting a defence response in pea (Hadwiger, 2008; Hadwiger *et al*., 1995). Produced by the macroconidia, the single-strand nicking enzyme, termed Fsph DNase, enters the pea cell and eventually the nucleus (Gerhold *et al*., 1993), where it induces the expression of several pathogenesis-related (PR) proteins, including defensin DRR230 and the phytoalexin pisatin (Hadwiger, 2008; Hadwiger *et al*., 1995).

There were 746 small secreted proteins identified by SignalP in the sequenced genome of FSSC 11, which represents 4.75% of the entire annotated proteome (Coleman *et al*., 2009). FSSC 11 isolates are virulent on carrot and tomato in addition to pea. Genes conferring pathogenicity on carrot and ripe tomato are on chromosomes other than the *PDA1*-CD chromosome (Funnell and VanEtten, 2002). A number of effectors have been identified in *F. oxysporum* f. sp. *lycopersici* that are involved in virulence on tomato (van der Does *et al*., 2008; Rep *et al*., 2004), and multiple homologues of these proteins exist in the FSSC 11 genome.

Analysis of the *F. virguliforme* genome using SignalP identified 1155 putative secreted proteins (Srivastava *et al*., 2014). A host-selective toxin has been identified in *F. virguliforme*, a causative agent of SDS. This small secreted protein, termed FvTox1, is responsible for causing the foliar symptoms observed in plants infected with the fungus, i.e. chlorosis and necrosis, ultimately leading to the loss of leaves and pods of the infected plant (Brar *et al*., 2011). A single gene, *fvtox1*, is expressed in infected root tissue that encodes a pro-peptide. On secretion into culture medium, the N-terminal 32 amino acids are cleaved and the protein is predicted to contain a sequence for translocation through the endoplasmic reticulum and a putative chloroplast transit peptide sequence (Brar *et al*., 2011). Targeted mutation of *fvtox1* resulted in strains that caused foliar disease symptoms and chlorophyll losses that were over two-fold less than in soybean plants infected with the wild-type isolate (Pudake *et al*., 2013). A homologue of *fvtox1* is present in the genome of the sequenced FSSC 11 isolate (Nh Protein ID 122357 located on chromosome 2), encoding a protein that is 91% identical to FvTox1 (Brar *et al*., 2011). Proteomic studies of xylem sap from *F. virguliforme*-infected soybean plants uncovered five fungal proteins that were present in the xylem (Abeysekara and Bhattacharyya, 2014). All five proteins possessed an N-terminal secretion signal amino acid sequence and were all less than 30 kDa in size. The most frequently identified and abundant *F. virguliforme* protein found in the soybean xylem sap encoded a protein with a high degree of similarity to cerato-platanin, a known phytotoxin that was originally identified in *Ceratocystis fimbriata* f. sp. *platani*. Although FvTox1 was not identified in the xylem sap, it appears that multiple protein toxins are secreted into the xylem during *F. virguliforme* infection of soybean (Abeysekara and Bhattacharyya, 2014).

SECONDARY METABOLITES

As with all fusaria, members of the FSSC are capable of producing an abundant array of secondary metabolites. Unlike species belonging to the *Fusarium sambucinum* and *fujikuroi* species complexes, members of the FSSC are not known to produce trichothecene and fumonisin mycotoxins. Therefore, the biosynthetic pathways for these secondary metabolites are postulated to have evolved after the divergence of the FSSC from other fusaria (O'Donnell *et al*., 2013).

There were 12 polyketide synthase (PKS), 12 non-ribosomal peptide synthase (NRPS) and one PKS–NRPS hybrid encoding genes in the genome of FSSC 11 (Coleman *et al*., 2009). Homologues of eight *NRPS* genes are present in three other *Fusarium* genomes (*F. graminearum* PH-1, *F. verticillioides* 7600 and *F. oxysporum* 4287). Based on similarity, it can be predicted that three of these NRPS-containing biosynthetic clusters are responsible for the production of the siderophores ferricrocin, fusarinine and malonichrome or other similarly structured secondary metabolites (Hansen *et al*., 2012; O'Donnell *et al*., 2013). Of the PKS and NRPS genes in the FSSC 11 genome, seven PKS and four NRPS genes were unique to FSSC isolate 77-13-4 (Hansen *et al*., 2012). Comparison of the *F. virguliforme* Mont-1 genome with the four *Fusarium* genomes identified a single PKS (*Fv14626*) that was unique to *F. virguliforme* (Srivastava *et al*., 2014).

A homologue of the PKS–NRPS hybrid in FSSC 11 has been characterized in *F. verticillioides* (*FUS1*, FVEG_11086) and is responsible for the production of fusarin (Brown *et al*., 2012). FUS1 and the homologue in the FSSC 11 isolate, Nh70660, are 64% identical in amino acid sequence, and homologues of seven of the eight other genes in the *FUS* biosynthetic cluster are present in the FSSC 11 genome as a 27-kb cluster, the exception being *FUS5* (Brown *et al*., 2012; Hansen *et al*., 2012). Although genes within the *FUS* gene cluster are homologous, the arrangement of genes within the cluster is different, as well as the genes immediately flanking the clusters, indicating a difference in genomic position (Brown *et al*., 2012; Hansen *et al*., 2012). Although fusarin production has not been reported by members of the FSSC, the similarity between the *FUS* biosynthetic gene clusters of these fungi suggests that members of the FSSC may be able to produce fusarin or a similarly structured compound.

A plethora of naphthoquinones are produced by members of the FSSC. These pigmented compounds have a wide range of structural variation and biological action, including antimicrobial and phytotoxic activity (reviewed in Medentsev and Akimenko, 1998). Some of the naphthoquinones produced by the FSSC include fusarubin, javanicin, norjavanicin, solaniol, bostrycoidin, fusarubinoic acid, novarubin, marticin, nectriafurone, nectriachrysone and derivatives of these metabolites (Medentsev and Akimenko, 1998; Short *et al*., 2013). The role of naphthoquinones in the pathogenicity of FSSC on host plants is unknown. Although some of these compounds are toxic to plants and can be found in diseased tissue (Kern and Naef-Roth, 1965), genetic studies have shown that pathogenicity on pea does not correlate with naphthoquinone production (Holenstein and Défago, 1983). The purple pigment associated with the more derived clades of *Fusarium* is produced by a biosynthetic cluster containing the PKS PGL1 (Proctor *et al*., 2007), and data suggest that this pigmented compound is either fusarubin or synthesized from the compound (Brown *et al*., 2012). Although FSSC isolates are associated with a red pigment (discussed further below), a homologue of *PGL1* is present in the FSSC 11 genome. Only three of the six genes in the PGL cluster were present in FSSC 11 [*PGL1*, *NhOMT1* (*PGL2*) and *NhFDM1* (*PGL3*]; however, they were present in a syntenic block and there was a high degree of sequence similarity (>70%) between the FSSC 11 homologues and those in *F. graminearum* and *F. verticillioides* (Proctor *et al*.,

2007). Although members of the FSSC do not produce the purple pigment, they are known to produce fusarubin and multiple structural derivatives (Medentsev and Akimenko, 1998; Short *et al*., 2013).

One of the PKS genes that might be unique to the FSSC is *pksN* (Graziani *et al*., 2004b). *pksN*, Nh33672 in the sequenced genome, was found to be one of two genetic loci that are involved in the production of the red perithecial wall pigment that is characteristic of FSSC species with known sexual reproduction. Mutation of *pksN* did not cause any phenotypic alteration in mycelial growth, conidiation or ascospore production in the FSSC CBS 225.53 isolate (Graziani *et al*., 2004a,b). Although the chemical structure of the final product of pksN is unknown, the similarity of pksN to PGL1, and the known diverse array of pigmented naphthoquinones produced by members of the FSSC, allows speculation that pksN is responsible for the production of a red naphthoquinone.

Members of the FSSC are capable of producing several other secondary metabolites. The diversity of secondary metabolites produced by members of the FSSC varies not only between species, but also within a single phylogenetic species (Short *et al*., 2013). The non-polar cyclic peptides cyclosporin A and cyclosporin C are produced by members of the FSSC, in particular isolates of FSSC 1 (*F. petroliphilum*), FSSC 2 (*F. keratoplasticum*), FSSC 3+4 (*F. falciforme*) and FSSC 5 (Short *et al*., 2013), and perhaps others (Sugiura *et al*., 1999). A unique NRPS in the genome of FSSC 11 showed 25.1% amino acid identity to SimA of the cyclosporin-producing fungus *Tolypocladium inflatum* (J. J. Coleman, unpublished data). Fusaric acid (5-butylpicolinic acid) production has been reported in the FSSC (Bacon *et al*., 1996), although a homologue of the PKS in the fusaric acid biosynthetic gene cluster in *F. verticillioides*, *FUB1*, was not present in the FSSC 11 genome. The secondary metabolite bikaverin is produced by several members of the FSSC (Chelkowski *et al*., 1992; Short *et al*., 2013), although a homologue of the PKS responsible for bikaverin synthesis (*BIK*) in *F. fujikuroi* was not found in the FSSC 11 genome (Hansen *et al*., 2012).

MEMBERS OF THE FSSC AS A MODEL SYSTEM FOR CELL BIOLOGY

Although the supernumerary chromosomes encoding host-specific virulence factors of the FSSC have been a focal point of research, members of the FSSC have been used as a system for cell biology. FSSC 11 was developed as a model system to study mitosis in fungi, in particular the formation and function of the spindle pole body and asters in anaphase (Aist, 2002). FSSC 11 was the first fungus to show that spindle pushing and aster pulling forces were both involved in mitotic spindle elongation (Aist and Bayles, 1991). Further studies have indicated that the microtubule-associated motor protein dynein is involved in the formation of mitotic asters, as mutants of cytoplasmic dynein show impaired hyphal growth, reduced astral microtubules and limited post-mitotic nuclear migration (Inoue *et al*., 1998a,b). Mutants of the other major microtubule motor protein, kinesin, resulted in morphological changes in hyphae, as well as a reduced growth rate, and subcellular abnormalities in location and size of mitochondria and the Spitzenkörper (Wu *et al*., 1998). A kinesin-related motor protein in FSSC 11, termed NhKRP1, is responsible for producing an inwardly directed force in the mitotic spindle. Mutation of *NhKRP1* significantly increased the rate of spindle elongation during anaphase A and B, as the lack of the inward force from NhKRP1 allows the astral pulling forces to elongate the spindle more rapidly (Aist, 2002; Sandrock *et al*., 1999).

Epigenetic factors have been shown to be responsible for two different morphological alterations in FSSC 11 that appear either as a typical sector ('Secteur') in the culture or as a ring surrounding the culture ('Anneau'). The factors responsible for these changes can appear spontaneously, and the frequency is dependent on cultural conditions, in particular temperature (Silar and Daboussi, 1999). Once established, transmission can occur to an undifferentiated culture at the growing edge of the mycelium through anastomoses, thereby transmitting the infectious cytoplasmic determinants, termed α and σ, for the 'Anneau' and 'Secteur' morphologies, respectively. Both determinants are under the control of two unlinked nuclear loci. Genetic studies revealed that 'Anneau' is controlled by locus *A* composed of a single gene that exists in three allelic forms: *wild-type*, *a* and *58* (Daboussi-Bareyre, 1980). The locus controlling the 'Secteur' morphology, *S* or *Ses*, is controlled by two genes, termed *SesA* and *SesB* (Graziani *et al*., 2004a). The function of the protein encoded by *SesA* (210 amino acids in length) is unknown, although homologues are only present in other filamentous fungi, whereas the *SesB* encoded protein (386 amino acids in length) has a domain that may confer esterase/lipase/thioesterase activity. The C-terminal regions of both *SesA* and *SesB* share a homology sequence that is also present in the N-terminal region of an adjacent gene, designated *het-eN* (Daskalov *et al*., 2012; Graziani *et al*., 2004a). These three proteins have characteristics of prion forming domains, and it has been proposed that the HET-eN protein induces a conformational change in sesA which then modifies sesB by interacting through the shared domain.

CONCLUSIONS

The FSSC is a diverse group of fungi capable of causing disease on a wide array of plants. As several specific virulence factors have been identified in these fungi that reside on CD supernumerary chromosomes, genomics and other next-generation technologies should greatly advance the current understanding of the interac-

tion between members of the FSSC and their respective host plants. Further knowledge of the molecular mechanisms responsible for conferring virulence to members of the FSSC could facilitate the development of alternative disease management strategies.

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