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# Putative Effector Genes Distinguish Two Pathogenicity Groups of *Fusarium oxysporum* f. sp. *spinaciae*

Alexander M. Batson,<sup>1,†</sup> Like Fokkens,<sup>2</sup> Martijn Rep,<sup>2</sup> and Lindsey J. du Toit<sup>1</sup>

<sup>1</sup> Washington State University Northwestern Washington Research and Extension Center Mount Vernon, Mount Vernon, WA 98273, U.S.A.

<sup>2</sup> Molecular Plant Pathology, Swammerdam Institute for Life Sciences, University of Amsterdam, Netherlands

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**Fusarium wilt of spinach, caused by *Fusarium oxysporum* f. sp. *spinaciae*, is an important disease during warm conditions in production regions with acid soils, yet little is known about what confers pathogenicity to spinach in *F. oxysporum* f. sp. *spinaciae* genetically. To identify candidate fungal genes that contribute to spinach Fusarium wilt, each of 69 geographically diverse *F. oxysporum* isolates was tested for pathogenicity on each of three spinach inbreds. Thirty-nine isolates identified as *F. oxysporum* f. sp. *spinaciae* caused quantitative differences in disease severity among the inbreds that revealed two distinct pathogenicity groups of *F. oxysporum* f. sp. *spinaciae*. Putative effector gene profiles, predicted from whole-genome sequences generated for nine *F. oxysporum* f. sp. *spinaciae* isolates and five nonpathogenic, spinach-associated *F. oxysporum* (NPS) isolates, distinguished the *F. oxysporum* f. sp. *spinaciae* isolates from the NPS isolates, and separated the *F. oxysporum* f. sp. *spinaciae* isolates into two groups. Five of the putative effector genes appeared to be unique to *F. oxysporum* f. sp. *spinaciae*, as they were not found in 222 other publicly available genome assemblies of *F. oxysporum*, implicating potential involvement of these genes in pathogenicity to spinach. In addition, two combinations of the 14 known *Secreted in Xylem* (*SIX*) genes that have been affiliated with host pathogenicity in other formae speciales of *F. oxysporum* were identified in genome assemblies of the nine *F. oxysporum* f. sp. *spinaciae* isolates, either *SIX8* and *SIX9* or *SIX4*, *SIX8*, and *SIX14*. Characterization of these putative effector genes should aid in understanding mechanisms of pathogenicity in *F. oxysporum* f. sp. *spinaciae*, developing**

**molecular tools for rapid detection and quantification of *F. oxysporum* f. sp. *spinaciae*, and breeding for resistance to Fusarium wilt in spinach.**

*Keywords:* fungal effectors, Fusarium wilt, *Spinacia oleracea*

The demand for spinach (*Spinacia oleracea* L.) in the United States has increased dramatically since the mid-1980s, when prepackaged and triple-rinsed baby leaf spinach became readily accessible to consumers (United States Department of Agriculture Economic Research Service 2007). This market demand for baby leaf spinach that is harvested 20 to 45 days after seeding crops at populations of 6 to 9 million seeds per hectare, necessitated an increase in production of high-quality spinach seed. High-quality spinach seed can be produced only in a few locations around the globe that have the long summer day length necessary to trigger spinach plants to bolt (convert from vegetative to reproductive growth), mild temperatures for this heat-sensitive species, and low humidity and rainfall during seed maturation for optimal seed quality and seed health (Metzger and Zeevaert 1985). The maritime Pacific Northwest (PNW) region of western Oregon and western Washington is the only region in the United States that meets these stringent climatic requirements to produce commercial spinach seed (Foss and Jones 2005; Organic Seed Alliance 2007). As a result, production of spinach seed in the maritime PNW accounts for up to 20% of the total global supply of spinach seed (Foss and Jones 2005).

Despite the optimal climatic conditions for spinach seed production in the maritime PNW, the acidic soils in this region are highly conducive to spinach Fusarium wilt, caused by *Fusarium oxysporum* f. sp. *spinaciae* (Foss and Jones 2005; Gatch and du Toit 2015). In these soils, *F. oxysporum* f. sp. *spinaciae* readily persists in excess of 10 years in the absence of spinach crops, which necessitates spinach seed producers use extensive crop rotations 10 to 15+ years between spinach seed crops to avoid significant losses to the pathogen (du Toit and Ocamb 2019). In some fields, spinach seed crop rotations >15 years still have been insufficient to avoid losses of up to 100% (Gatch 2013). Historically, growers in the maritime PNW have avoided Fusarium wilt by planting spinach seed crops on land that had not previously been planted with spinach; however, land without a history of spinach or spinach seed production in this region is rare after about 100 years of spinach seed production (Gatch 2013). For fields with some risk of spinach Fusarium wilt, seed growers have used multiple practices to minimize losses to the disease, but all the economically viable management practices suppress the disease only partially or

The sequence reads of the 14 isolates of *F. oxysporum* were deposited in the sequence read archive (SRA) under the BioProject numbers PRJNA540981 and PRJNA595149. The corresponding assemblies were deposited in the NCBI GenBank repository.

<sup>†</sup>Corresponding author: A. M. Batson; alex.batson@wsu.edu

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transiently or both (du Toit et al. 2004, 2006, 2014; Gatch and du Toit 2015, 2017). Spinach cultivars with partial resistance to Fusarium wilt are available, but most are highly susceptible to the disease (Laguna 2000; O'Brien and Winters 1977). Additionally, spinach seed growers typically have little or no choice of the proprietary inbred lines they are contracted to plant for hybrid seed crops. Therefore, additional management strategies are needed to reduce losses to Fusarium wilt and improve spinach seed yields in the United States.

*F. oxysporum* is a diverse species complex comprised of isolates that are nonpathogenic saprobes, biocontrol agents (Alabouvette et al. 2009), endophytes (Gordon and Martyn 1997), human pathogens (Nucci and Anaissie 2002), and plant pathogens. Plant-pathogenic isolates of *F. oxysporum* are grouped into  $\geq 106$  host-specific formae speciales (Edel-Hermann and Lecomte 2019), each of which cause disease on a single host or, in some cases, a narrow range of closely related hosts (Armstrong and Armstrong 1976; Cafri et al. 2005; MacDonald and Leach 1976; Naiki and Kano 1977; Zhou and Everts 2007). Efforts to discriminate among *F. oxysporum* ff. spp. and to differentiate isolates of formae speciales from nonpathogenic *F. oxysporum* isolates on the basis of morphological characteristics have fallen short, as these isolates typically are indistinguishable (Leslie and Summerell 2006). Furthermore, many *F. oxysporum* ff. spp. are polyphyletic and cannot be differentiated based on multilocus DNA sequence haplotypes or DNA sequence variation among orthologous genes and noncoding loci (Baayen et al. 2000; Laurence et al. 2014; Lombard et al. 2019; Maryani et al. 2019; O'Donnell et al. 2009). Thus, time-consuming and labor-intensive pathogenicity tests have remained the standard for identification of isolates of many *F. oxysporum* ff. spp. (Leslie and Summerell 2006).

Advances in next generation sequencing and genomic studies of various *F. oxysporum* ff. spp. have improved our conceptual understanding of genetic similarities among formae speciales (Armitage et al. 2018; Ma et al. 2010; van Dam et al. 2016, 2017). Whole-genome sequencing of several closely related *Fusarium* spp. revealed that *F. oxysporum* f. sp. *lycopersici*, the causal agent of Fusarium wilt of tomato, has a genome of 59.9 Mb, while *F. graminearum*, *F. solani*, and *F. verticillioides* have genomes of 36.2, 54.0, and 41.7 Mb, respectively (Coleman et al. 2009; Ma et al. 2010). The larger genome of *F. oxysporum* f. sp. *lycopersici* is due, primarily, to the presence of four lineage-specific chromosomes that are distinct from 11 core chromosomes (Ma et al. 2010). The core genome includes chromosomes that are syntenic among *F. graminearum*, *F. solani*, and *F. verticillioides*. In contrast, accessory or lineage-specific chromosomes are typically highly repetitive, transposon-rich, and encode genes associated with pathogenicity and host-specificity (Ma et al. 2010; Schmidt et al. 2013; van Dam et al. 2017; Williams et al. 2016). Furthermore, the transfer of one lineage-specific chromosome, chromosome 14, from *F. oxysporum* f. sp. *lycopersici* to a nonpathogenic *F. oxysporum* isolate converted that recipient isolate to a pathogen of tomato (Ma et al. 2010). This phenomenon of horizontal chromosome transfer conferring host-specificity has also been demonstrated between *F. oxysporum* f. sp. *radicis-cucumerinum* and a non-pathogen of cucurbits (Li et al. 2020; van Dam et al. 2017).

Accessory chromosomes of *F. oxysporum* f. sp. *lycopersici* contain a group of 14 genes known as *Secreted in Xylem* (*SIX*) genes, which are expressed as proteins that are secreted by *F. oxysporum* f. sp. *lycopersici* into the xylem of colonized tomato plants (Rep 2005; Rep et al. 2004; Schmidt et al. 2013). These proteins are relatively small (<300 amino acids) and rich in cysteine residues (Schmidt et al. 2013). Deletion of *SIX1*

(Rep 2005), *SIX3* (Houterman et al. 2009), *SIX5* (Ma et al. 2015), and *SIX6* (Gawehns et al. 2014) from isolates of *F. oxysporum* f. sp. *lycopersici* reduced virulence of the isolates to tomato, demonstrating the role of these genes in pathogenicity of the fungus. Furthermore, the three known races of *F. oxysporum* f. sp. *lycopersici* are differentiated by *SIX1*, *SIX3*, and *SIX4*, each of which is an avirulence gene (also referred to as *AVR3*, *AVR2*, and *AVR1*, respectively) (Houterman et al. 2008, 2009; Rep et al. 2004). *SIX1*, *SIX3*, and *SIX4* encode proteins that are detected by resistance proteins in certain tomato cultivars, encoded by immunity (*I*) genes. The *I* genes that correspond to *SIX1*, *SIX3*, and *SIX4* in *F. oxysporum* f. sp. *lycopersici* were discovered among wild *Solanum* relatives of tomato (Gerdemann and Finley 1951; McGrath et al. 1987) and have been introgressed into commercial breeding lines of tomato to confer resistance to the three races of *F. oxysporum* f. sp. *lycopersici* (Catanzariti et al. 2015; Simons et al. 1998).

Subsequent to the discovery and characterization of the *SIX* genes in *F. oxysporum* f. sp. *lycopersici*, many other formae speciales of *F. oxysporum* have been demonstrated to have homologs of *SIX* genes, e.g., ff. spp. *canariensis*, *cepae*, *ciceris*, *conglutinans*, *cubense*, *fragariae*, *lini*, *melonis*, *niveum*, *palmarum*, *physali*, *pisi*, *radicis-cucumerinum*, *raphani*, *vasinfectum*, and *zingiberri* (Czislowski et al. 2018; Laurence et al. 2015; Lievens et al. 2009; Meldrum et al. 2012; Ponukumati et al. 2019; Simbaqueba et al. 2018; Taylor et al. 2016; Thatcher et al. 2012; van Dam et al. 2016; Williams et al. 2016). Lievens et al. (2009) first demonstrated that the profile of *SIX* genes could potentially be used to differentiate among isolates of different *F. oxysporum* ff. spp. as well as to distinguish isolates of formae speciales from nonpathogenic isolates. Furthermore, Taylor et al. (2016) demonstrated that *SIX3*, *SIX5*, *SIX7*, *SIX9*, *SIX10*, *SIX12*, and *SIX14* were all present in highly virulent isolates of *F. oxysporum* f. sp. *cepae*, the causal agent of Fusarium basal rot of onion, while isolates with diminished virulence to onion had fewer of these *SIX* genes. The profiles of *SIX* genes present in *F. oxysporum* f. sp. *cepae* isolates differentiated the pathogenic isolates from nonpathogenic isolates of *F. oxysporum* associated with onion plants and differentiated isolates of this forma specialis from those of eight other formae speciales evaluated in that study. Similarly, Czislowski et al. (2018) demonstrated that *SIX* gene profiles in isolates of the causal agent of Fusarium wilt of banana, *F. oxysporum* f. sp. *cubense*, were associated strongly with different races and vegetative compatibility groups (VCG) of the pathogen. The *SIX* genes also have been demonstrated to play a role in virulence of *F. oxysporum* f. sp. *cubense* to banana (Widinugraheni et al. 2018) and for the causal agent of Fusarium wilt of *Brassica* spp., *F. oxysporum* f. sp. *conglutinans* (Thatcher et al. 2012).

In comparison with other *F. oxysporum* ff. spp., the genetics behind host specificity and pathogenicity in *F. oxysporum* f. sp. *spinaciae* have not been studied extensively. Fiely et al. (1995) demonstrated three VCGs present among *F. oxysporum* f. sp. *spinaciae* isolates collected from a variety of geographic locations. Subsequently, *F. oxysporum* f. sp. *spinaciae* was found to be polyphyletic with respect to two loci, the *translation elongation factor 1- $\alpha$*  (*TEF1- $\alpha$* ) gene and the intergenic spacer (IGS) region of ribosomal DNA (rDNA). Based on these two regions, representative isolates of *F. oxysporum* f. sp. *spinaciae* in VCGs 0330 and 0332 grouped together, while an isolate belonging to VCG 0331 was placed in a separate clade (O'Donnell et al. 2009). A few studies have included *F. oxysporum* f. sp. *spinaciae* isolates in screens for *SIX* genes, but *F. oxysporum* f. sp. *spinaciae* isolates were not the main focus of those investigations. For example, Lievens et al. (2009) included three *F. oxysporum* f. sp. *spinaciae* isolates in a genotypic screen for *SIX1* to *SIX7* of 255 isolates representing 16

formae speciales and 15 nonpathogenic *F. oxysporum* isolates. None of these *SIX* genes was detected in the three *F. oxysporum* f. sp. *spinaciae* isolates. At that time, only *SIX1* to *SIX7* had been identified. Later, Covey et al. (2014) screened isolates of *F. oxysporum* ff. spp. *betae* and *spinaciae* for *SIX1*, *SIX6*, and other loci. Neither *SIX1* nor *SIX6* was detected in the three *F. oxysporum* f. sp. *spinaciae* isolates evaluated.

Analysis of the genomic locations of *SIX1* to *SIX7* on lineage-specific chromosome 14 of *F. oxysporum* f. sp. *lycopersici* revealed that these effector genes were associated with transposable elements, i.e., a miniature inverted-repeat transposable element (MITE) in the 5' promoter region called a miniature Impala (mimp) (Schmidt et al. 2013). The association of mimps with the promoter region of each of the *SIX* genes facilitated the discovery of *SIX9* to *SIX14* (Schmidt et al. 2013), and, subsequently, mimps were used as markers to discover other putative effector genes in other *F. oxysporum* ff. spp. (Armitage et al. 2018; Schmidt et al. 2016; van Dam et al. 2016). The presence or absence of *SIX* genes and mimp-predicted effector genes has been used to differentiate some *F. oxysporum* ff. spp. and even to differentiate among races of specific formae speciales (Czislowski et al. 2018; Lievens et al. 2009; Taylor et al. 2016; van Dam et al. 2016), which holds promise for the potential development of molecular tools that can discriminate isolates of different formae speciales. van Dam et al. (2016) demonstrated that *F. oxysporum* ff. spp. *cucumerinum*, *niveum*, *melonis*, *radicis-cucumerinum*, and *lycopersici* were differentiated by the presence or absence of candidate effector genes. Isolates of *F. oxysporum* ff. spp. that caused wilt of Cucurbitaceae genera and species (i.e., ff. spp. *cucumerinum*, *melonis*, *niveum*, and *radicis-cucumerinum*) had similar predicted effector gene profiles, suggesting that host specificity is determined by an isolate's repertoire of effector genes.

Identifying effector genes associated with *F. oxysporum* f. sp. *spinaciae* may provide insights into the molecular interactions between *F. oxysporum* f. sp. *spinaciae* and spinach. In addition, identifying genes that play a putative role in pathogenicity will enable development of molecular tools to differentiate isolates of the pathogen rapidly and aid spinach breeders in developing cultivars with resistance to Fusarium wilt. The goal of this research was to determine the potential genetic basis of pathogenicity of the causal agent of spinach Fusarium wilt, *F. oxysporum* f. sp. *spinaciae*, through identifying putative effector genes in this pathogen.

## RESULTS

### Distinct patterns of virulence differentiate two pathogenicity groups of *F. oxysporum* f. sp. *spinaciae*.

To identify *F. oxysporum* isolates pathogenic on spinach, pathogenicity trials were conducted with 69 isolates collected from multiple locations, plant hosts, substrates, and years (Supplementary Table S1). Fifty of the *F. oxysporum* isolates had been associated with wilting spinach plants, soil in which spinach crops were grown, or spinach seed. Each of the remaining 19 *F. oxysporum* isolates were derived from other host substrates and were confirmed by the respective supplier as pathogenic isolates of *F. oxysporum* ff. spp. *betae* (beet), *cepaе* (onion), *ciceris* (chickpea), and *pisi* (pea) (Supplementary Table S1). Each isolate was tested for pathogenicity on each of three proprietary spinach inbred lines (coded A, B, and C) that have been described as highly susceptible, moderately susceptible, or partially resistant to spinach Fusarium wilt, respectively (Gatch and du Toit 2015). Therefore, the wilt response was predicted to be quantitative. Inbred A was expected to display the most severe symptoms of wilt, followed

by B, and then C. Although three inbreds were tested in these trials, inbreds A and C represented what were considered highly susceptible and partially resistant inbreds to Fusarium wilt, so only the wilt severity of inbreds A and C are presented in Figure 1, as these two inbreds showed the greatest differentiation among fungal isolates. The number of treatment combinations of spinach inbred lines and isolates was prohibitively large to assess in a single trial, so isolates were assessed for pathogenicity over six trials.

Thirty-six of the 69 isolates caused symptoms of Fusarium wilt on at least one of the three spinach inbred lines evaluated (Fig. 1) that were significantly greater than that of the control plants treated with water ( $P < 0.05$ ). None of the isolates tested in trials 5 and 6 caused spinach plants to wilt, so the results are not shown. Isolate Fus254, the positive control isolate, caused the most severe wilt observed on inbred A in each trial. The negative control isolate Fus187 did not cause wilt in any of the trials. In trials 1 to 4, the severity of wilt differed among fungal isolates ( $P < 0.0001$  for the analysis of variance [ANOVA] main effect of isolates) and among inbred lines ( $P < 0.0001$ ). In addition, the interaction of inbred lines and fungal isolates was significant in each trial ( $P \leq 0.0295$ ). Two distinct patterns of virulence were observed among the *F. oxysporum* f. sp. *spinaciae* isolates (Fig. 1). The 17 (45%) isolates that induced the most severe wilt symptoms on inbred A were placed in pathogenicity group 1 ( $P < 0.05$ ) and 19 (50%) isolates that either induced similarly severe wilt symptoms on all three inbred lines ( $P \geq 0.05$ ) or the most severe symptoms on inbred C ( $P < 0.05$ ) were assigned to pathogenicity group 2 (Fig. 1; Supplementary Table S1). Two isolates, Fus172 and Fus287, induced such mild symptoms of wilt that they could not be placed in either group. Of the 30 *Fusarium* isolates tested that did not induce wilt symptoms on the spinach inbreds, 11 were collected originally from wilting spinach plants, spinach seed, or soil in which spinach had been grown and were identified as NPS isolates (Supplementary Table S1). The remaining 19 *Fusarium* isolates belonged to other *F. oxysporum* ff. spp. and did not cause wilt symptoms on the spinach inbreds.

Seven *F. oxysporum* f. sp. *spinaciae* isolates and five NPS isolates were tested again on spinach inbreds A and C in preparation for selecting isolates for whole-genome sequencing. The *F. oxysporum* f. sp. *spinaciae* isolates selected based on diversity of geographic origin, year of isolation, and the severity of wilt induced on the three inbred spinach lines included Fus057, Fus059, Fus254, and Fus322, representing pathogenicity group 1, and Fus167 and Fus173, representing pathogenicity group 2. Although Fus001 did not induce wilting of spinach plants in the initial pathogenicity tests, this isolate had been demonstrated previously to be a pathogen of spinach (Okubara et al. 2013), so the isolate was included in this additional testing. Five NPS isolates (Fus017, Fus187, Fus191, Fus250, and Fus259) were selected based on the lack of virulence to spinach in the initial pathogenicity trials as well as diversity in the years in which the isolates were collected. Pathogenicity tests with these 12 isolates were performed twice on both inbreds A and C. Similar to results of the initial pathogenicity trials (Fig. 1), a differential interaction between spinach inbreds and fungal isolates was observed (Table 1; Supplementary Figs. S1 and S2), with the isolates distinguished into the same two pathogenicity groups based on quantitative differences in wilt severity on inbreds A and C (Table 1). Isolates identified as nonpathogenic on spinach (NPS isolates Fus017, Fus187, Fus191, Fus250, and Fus259) in the initial pathogenicity tests did not induce either of the spinach inbreds to wilt (Table 1; Supplementary Fig. S2). Fus001 caused a greater severity of wilt on inbred C than A in trial 1 but caused a similar severity of wilt on the two spinach inbreds in trial 2,

similar to the other group 2 isolates Fus167 and Fus173 (Table 1). Thus, Fus001 was assigned to pathogenicity group 2 (Table 1; Supplementary Figs. S1 and S2).

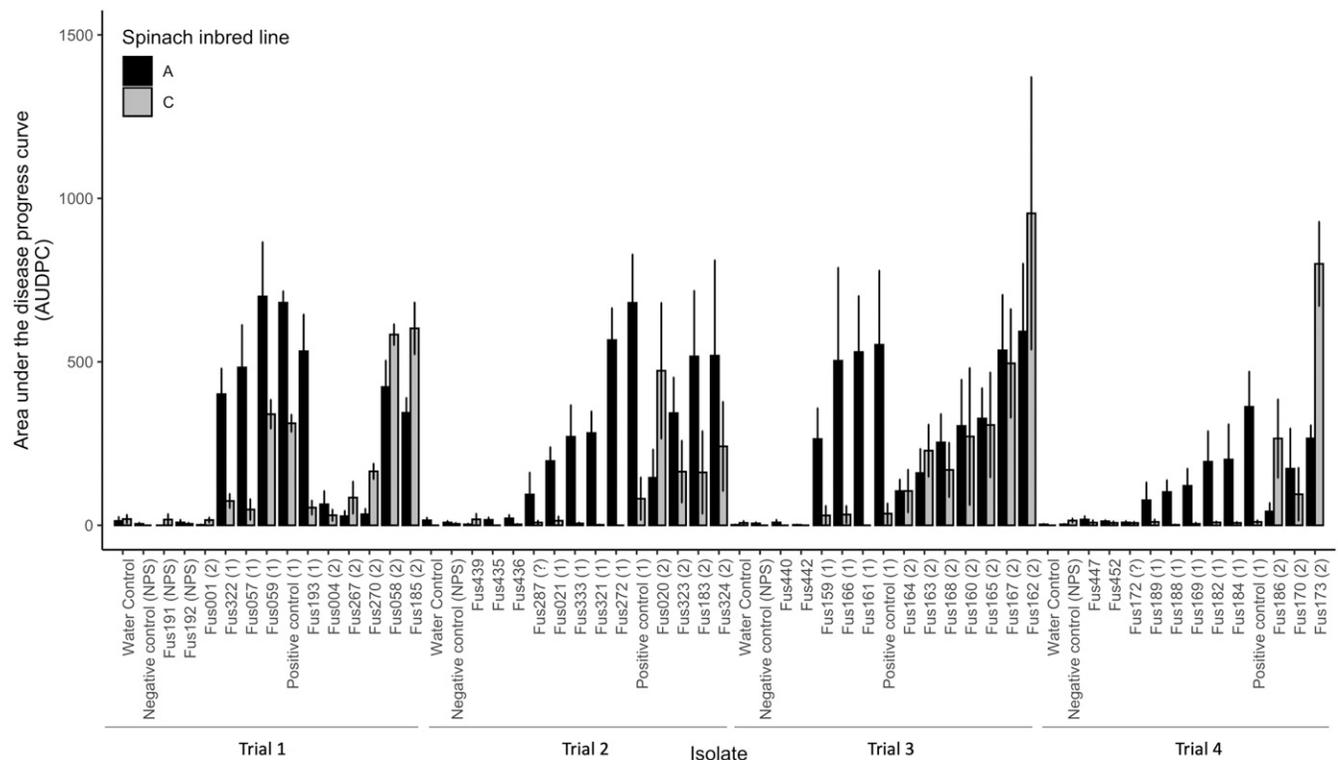
### *F. oxysporum* f. sp. *spinaciae* isolates are monophyletic based on *TEF1-α*.

Phylogenetic relationships among the *F. oxysporum* f. sp. *spinaciae* isolates were inferred based on sequence alignment of the *TEF1-α* locus from the 69 isolates included in the pathogenicity tests described above. Despite the diverse geographic origins and years in which the *F. oxysporum* f. sp. *spinaciae* isolates were collected, all 39 *F. oxysporum* f. sp. *spinaciae* isolates had identical sequences for this locus and grouped in one moderately supported clade (71% bootstrap support [Fig. 2]). Only one NPS isolate, Fus263, clustered with the *F. oxysporum* f. sp. *spinaciae* isolates. The other 10 NPS isolates were dispersed throughout the phylogenetic tree and were more closely related to isolates of other *F. oxysporum* ff. spp. than to *F. oxysporum* f. sp. *spinaciae* (Fig. 2). The isolates of *F. oxysporum* ff. spp. *ciceris* and *cepa* tested were monophyletic, whereas the isolates of *F. oxysporum* ff. spp. *lycopersici* and *pisi* were polyphyletic (Fig. 2). Some isolates did not group within the *F. oxysporum* species complex. For example, NPS isolate Fus317 was identified as a member of the *F. fujikuroi* species complex (Fig. 2). Moreover, of the nine isolates classified by the original supplier as *F. oxysporum* f. sp. *betae*, only two were identified as *F. oxysporum* based on the *TEF1-α* sequences. The other seven were identified as *F. equiseti* ( $n = 1$ ) or unknown *Fusarium* spp. ( $n = 6$ ) (Fig. 2).

### Whole-genome assemblies of *F. oxysporum* f. sp. *spinaciae* were larger and more fragmented than those of NPS isolates.

To evaluate genetic differences among *F. oxysporum* f. sp. *spinaciae* isolates in pathogenicity group 1, group 2, and NPS isolates, 14 isolates were selected for whole-genome sequencing, including all 12 isolates subjected to the additional pathogenicity tests described above. The genome of isolate Fus167 was sequenced twice, once by each of two institutions, resulting in two genome assemblies for the same isolate named Fus167vA and Fus167vB (Table 2). To obtain a higher-quality reference genome, the well-characterized isolate Fus254 (pathogenicity group 1) was sequenced with the Pacific Biosciences (PacBio) Sequel platform (Menlo Park, CA, U.S.A.), while the other 13 isolates were sequenced with Illumina technology.

The genome assemblies generated from Illumina paired-end reads of the nine *F. oxysporum* f. sp. *spinaciae* isolates and five NPS isolates ranged from 47.6 to 56.7 Mbp (Table 2) and were assembled into 387 to 4,042 contigs. Overall, the *F. oxysporum* f. sp. *spinaciae* assemblies generated with Illumina data were more fragmented (2,696 to 4,042 contigs) and larger (54.7 to 56.7 Mbp) than the genome assemblies of the NPS isolates generated with Illumina data (387 to 1,518 contigs and 47.6 to 51.4 Mbp) (Table 2). Of the Illumina-generated genome assemblies, the *F. oxysporum* f. sp. *spinaciae* group 1 assemblies were more fragmented and approximately 300 Kbp smaller, on average, than the *F. oxysporum* f. sp. *spinaciae* group 2 assemblies (Table 2). In comparison, the Fus254 (*F. oxysporum* f.



**Fig. 1.** Mean  $\pm$  standard error of the area under the disease progress curve for four replications of each of 49 *Fusarium* isolates characterized for pathogenicity on each of the two proprietary spinach inbred lines A and C. Results for four of the six pathogenicity trials are shown since none of the isolates of *F. oxysporum* ff. spp. and other *F. oxysporum* associated with spinach that were tested in trials 5 and 6 was pathogenic on spinach. Each trial included a positive control isolate (Fus254) of *F. oxysporum* f. sp. *spinaciae*, a negative control isolate of *F. oxysporum* that is not pathogenic on spinach (Fus187), and a water control treatment. Numbers or characters in parentheses following each isolate indicate the designation of a *F. oxysporum* f. sp. *spinaciae* pathogenicity group (i.e., 1 = group 1, 2 = group 2, ? = no group designation) or whether the isolate was determined to be nonpathogenic on spinach but associated originally with spinach (NPS).

**Table 1.** Analysis of variance (ANOVA) testing the effects of spinach inbred line and isolate of *Fusarium oxysporum* on the area under the disease progress curve (AUDPC)<sup>x</sup>

ANOVA factor <sup>y</sup>	AUDPC <sup>z</sup>			
	Trial 1		Trial 2	
	Inbred A	Inbred C	Inbred A	Inbred C
Fus057	1,681.25 a	242.71 f	1,684.67 a	281.75 e
Fus059	1,556.46 a	395.83 ef	1,751.46 a	699.71 cd
Fus254	848.38 bc	192.71 f	1,705.08 a	660.33 cde
Fus322	1,210.21 ab	201.46 f	548.92 de	88.96 f
Fus001	430.21 def	851.46 bc	1,165.79 b	1,066.92 b
Fus167	230.42 f	628.92 cde	1,163.75 b	1,222.96 b
Fus173	708.33 cd	1,154.79 ab	697.96 cd	953.75 bc
Fus017	4.79 ghij	7.71 hij	0.00 g	7.29 g
Fus187	1.04 j	6.25 ghij	0.00 g	14.58 g
Fus191	10.83 gh	1.04 j	0.00 g	87.50 g
Fus250	26.50 g	0.00 j	0.00 g	5.83 g
Fus259	59.38 ghi	0.00 j	2.92 g	0.00 g
Water	0.00 j	2.08 ij	0.00 g	0.00 g

<sup>x</sup> Results of ANOVA testing the effects of spinach inbred line and isolate of *F. oxysporum* on the AUDPC assessed in two trials in which spinach plants were grown in potting medium inoculated with each isolate to identify isolates of the spinach Fusarium wilt pathogen *F. oxysporum* f. sp. *spinaciae*.

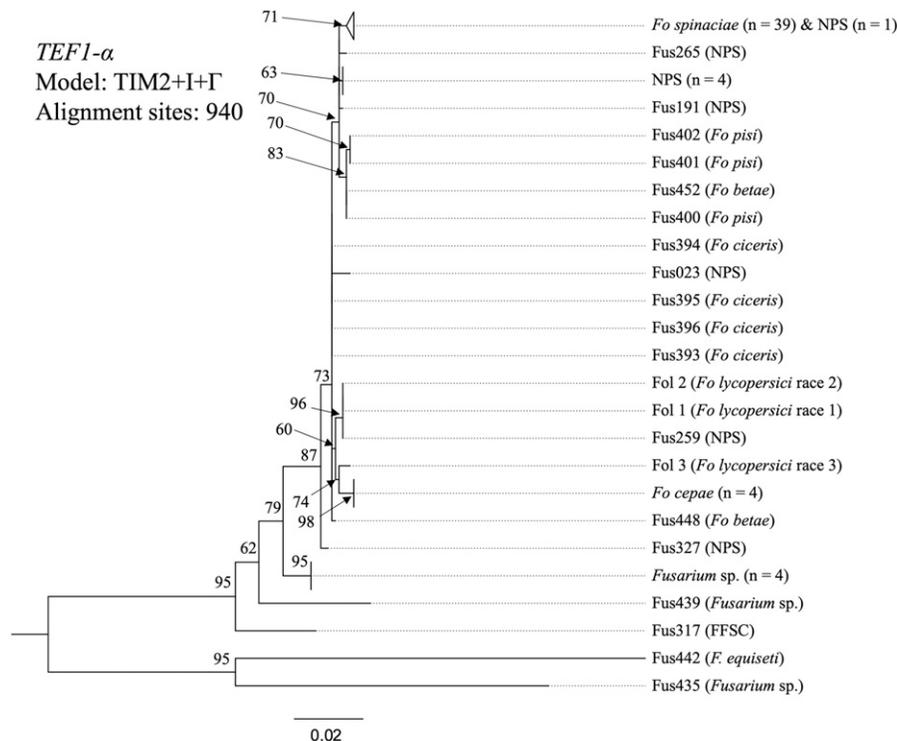
<sup>y</sup> Each value is the mean of four replications. The experiment in both trials was a randomized complete block layout of a factorial treatment design of two spinach inbred lines and 13 inoculation treatments. The main effects of spinach inbred line and inoculation treatment as well as the interaction term were significant in both trials. Raw data were either log- or rank-transformed due to heterogeneity of variances or nonnormal residuals or both. Within each trial, means with at least one letter in common are not different statistically, based on Fisher's protected least significant difference ( $P < 0.05$ ).

<sup>z</sup> AUDPC based on wilt severity ratings completed weekly from 14 to 35 days after planting.

sp. *spinaciae* group 1) PacBio assembly was 56.3 Mbp divided over 279 contigs. Each genome assembly was similar in terms of the percentage of complete single-copy orthologs identified with the BUSCO (benchmarking universal single-copy orthologs) program (Simão et al. 2015), which ranged from 98.8%, for the PacBio assembly of Fus254, to 99.2% (3,680 to 3,695 BUSCOs) (Table 2). This indicated that, although fragmented, the genome assemblies were largely complete.

### Phylogenomic analysis of *F. oxysporum* f. sp. *spinaciae* isolates distinguished the two pathogenicity groups.

To determine the relationship of the *F. oxysporum* f. sp. *spinaciae* and NPS isolates to other *F. oxysporum* isolates with publicly available genome assemblies, single-copy orthologs ( $n = 3,127$ ) identified with BUSCO (Simão et al. 2015) were used to infer gene trees for constructing a summary tree with the program ASTRAL-III (Zhang et al. 2018). The genome assemblies of the *F. oxysporum* f. sp. *spinaciae* isolates, five NPS isolates, and 13 other *F. oxysporum* isolates evaluated in this study were grouped into three lineages that were well supported by the percentage of quartet trees (>70%) and local posterior probabilities (Fig. 3). The phylogenetic relationships of genomes of the other *F. oxysporum* ff. spp. were similar to those described in other studies (Armitage et al. 2018; van Dam et al. 2016). The nine *F. oxysporum* f. sp. *spinaciae* isolates sequenced in this study occupied two closely related but distinct lineages (Fig. 3). The node supporting *F. oxysporum* f. sp. *spinaciae* group 1 isolates was present in 52.8% of the quartet trees (1,651 quartet trees), and the node supporting *F. oxysporum* f. sp. *spinaciae* group 2 isolates was present in 58.4% of the quartet trees (1,826 quartet trees) (Fig. 3). Isolates assigned



**Fig. 2.** Unrooted maximum likelihood tree inferred for translation elongation factor 1- $\alpha$  (*TEF1-α*) sequences of *Fusarium* isolates subjected to pathogenicity tests, on two spinach inbred lines, to identify isolates of *F. oxysporum* f. sp. *spinaciae*, the causal agent of spinach Fusarium wilt. The number at each node represents the percentage of trees from 1,000 bootstrap replicates with that node. The scale bar indicates the average number of substitutions per site. *Fo* = *Fusarium oxysporum*, NPS = nonpathogenic, spinach-associated *F. oxysporum* isolate, FFSC = *F. fujikuroi* species complex isolate. Values in parentheses refer to the number of isolates within a collapsed, monophyletic clade.

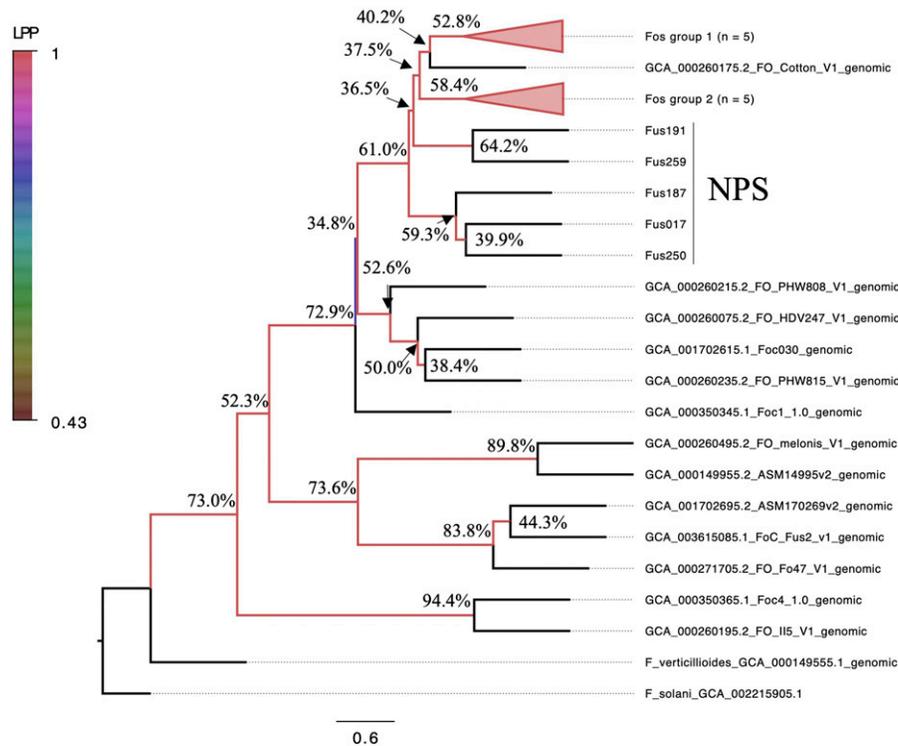
to *F. oxysporum* f. sp. *spinaciae* group 1 shared a more recent common ancestor with a representative isolate of *F. oxysporum* f. sp. *vasinfectum*, a pathogen of cotton, than with isolates of *F. oxysporum* f. sp. *spinaciae* group 2, with that ancestor represented by 40.2% of the quartet trees (1,257 quartet trees).

Additionally, the five NPS isolates were more closely related to each other at these loci than to any of the *F. oxysporum* f. sp. *spinaciae* isolates evaluated. Two isolates of *F. oxysporum* f. sp. *cubense* tropical race 4 included in this tree, i.e., isolates Foc4 and Fo\_II5, were described recently as a new species,

**Table 2.** Assembly statistics of 15 *Fusarium oxysporum* genomes for identification of putative effectors that define the spinach *Fusarium* wilt pathogen *F. oxysporum* f. sp. *spinaciae*

Assembly <sup>u</sup>	Designation <sup>v</sup>	No. of contigs	Total length (Mbp)	Largest contig (Kbp)	N50 (Kbp) <sup>w</sup>	L50 <sup>x</sup>	Complete BUSCOs (%) <sup>y</sup>
Fus254 (JAALGI000000000)	Group 1	279	56.3	1,713.9	625.5	28	98.8
Fus057 (JAALGR000000000)	Group 1	4,042	56.2	690.3	94.4	140	99.1
Fus059 (JAALGQ000000000)	Group 1	4,007	56.1	1,028.0	94.4	142	99.0
Fus166 (JABCQY000000000)	Group 1	2,993	54.7	1,680.6	137.5	77	99.1
Fus322 (JAALGJ000000000)	Group 1	4,003	56.1	601.9	92.8	149	99.1
Fus001 (JAALGT000000000)	Group 2	3,098	56.2	1,383.9	156.6	85	99.2
Fus165 (JABCQZ000000000)	Group 2	2,841	55.7	1,638.4	281.2	44	99.1
Fus167vA (JAALGP000000000) <sup>z</sup>	Group 2	3,748	56.7	759.4	141.7	105	99.0
Fus167vB (JABCQX000000000) <sup>z</sup>	Group 2	2,696	55.6	2,715.7	316.7	43	99.0
Fus173 (JAALGO000000000)	Group 2	3,021	56.2	974.3	146.0	89	99.0
Fus017 (JAALGS000000000)	NPS	1,518	51.4	1,168.8	255.0	56	98.9
Fus187 (JAALGN000000000)	NPS	998	49.2	1,307.7	239.4	57	99.0
Fus191 (JAALGM000000000)	NPS	387	47.6	1,263.7	329.9	42	99.1
Fus250 (JAALGL000000000)	NPS	1,139	50.1	1,132.4	277.9	49	99.1
Fus259 (JAALGK000000000)	NPS	821	49.3	1,115.9	308.4	45	99.1

- <sup>u</sup> Name and GenBank accession number of the genome assembly. The PacBio Sequel sequencing platform was used to generate read data for Fus254; the Illumina HiSeq platform was used for all the others.
- <sup>v</sup> Designated as either *Fusarium oxysporum* f. sp. *spinaciae* pathogenicity group 1 or group 2 or as NPS for *F. oxysporum* isolates that were not pathogenic on spinach although originally obtained from a spinach plant, spinach seed, or soil in which spinach was grown. Pathogenicity groups are isolates that induced differential severity of wilt symptoms on two spinach inbred lines.
- <sup>w</sup> N50 = the median contig length in a genome assembly, i.e., the length of the shortest contig among the minimum number of contigs in an assembly whose summed length is  $\geq 50\%$  of the length of the genome assembly.
- <sup>x</sup> L50 = the minimum number of contigs whose summed length is  $\geq 50\%$  of the length of the genome assembly.
- <sup>y</sup> Percentage of single copy orthologs in a genome as assessed with the BUSCO program (Simão et al. 2015).
- <sup>z</sup> The genome assemblies for Fus167vA and Fus167vB were generated from the same isolate, Fus167.



**Fig. 3.** Phylogenetic tree demonstrating the relationship among 28 *Fusarium oxysporum* isolates, one *F. verticillioides* isolate, and one *F. solani* isolate. The tree was inferred from 3,127 single-copy ortholog-gene trees present in each *Fusarium* genome assembly, using the program ASTRAL-III. Fos = *F. oxysporum* f. sp. *spinaciae* (pathogenicity group), NPS = *F. oxysporum* isolate that was not a pathogen on spinach but was obtained originally from a spinach plant, spinach seed, or soil in which spinach was grown. The two *F. oxysporum* f. sp. *spinaciae* pathogenicity groups were based on isolates that induced differential severity of wilt on two spinach inbred lines. Colored branches indicate node support based on local posterior probabilities (LPPs), while the percentages adjacent to the nodes indicate the quartet support. The scale bar indicates coalescence units. The final tree was rooted to *F. solani*.

*F. odoratissimum* (Lombard et al. 2019; Maryani et al. 2019), and represented a clade distinct from the other *F. oxysporum* isolates with 94.4% quartet support and 100% bootstrap support on the concatenated tree (Fig. 3; Supplementary Fig. S1).

There were minor topological discrepancies between the ASTRAL-inferred tree and the tree inferred from a concatenated alignment of 3,127 BUSCOs (Fig. 3; Supplementary Fig. S3). In both trees, the five NPS isolates belonged to one of two clades; however, the relationship of the NPS clades to *F. oxysporum* f. sp. *spinaciae* isolates differed slightly between the trees (Fig. 3; Supplementary Fig. S3). In both trees, the nodes that supported the different placements had relatively low support, either 36.5% of quartet tree support in the ASTRAL-inferred tree or 56% bootstrap support in the concatenated phylogeny (Supplementary Fig. S3). In addition, the relationship between *F. oxysporum* f. sp. *cucumerinum* isolate Foc030 (GCA\_001702615.1) and *F. oxysporum* isolates HDV247 (GCA\_000260075.2) and PHW815 (GCA\_000260235.2) differed between the two trees (Fig. 3; Supplementary Fig. S3). However, in both trees, the relationship among *F. oxysporum* f. sp. *spinaciae* group 1 isolates, *F. oxysporum* f. sp. *spinaciae* group 2 isolates, a *F. oxysporum* f. sp. *vasinfectum* isolate (GCA\_000260175.2), and other recognized clades of *F. oxysporum* isolates was congruent.

### Homology searches identify putative effectors in *F. oxysporum* f. sp. *spinaciae*.

The 14 known SIX gene homologs in *F. oxysporum* f. sp. *lycopersici* were queried against each of the *F. oxysporum* f. sp. *spinaciae* and NPS genome assemblies with the command line tool BLASTN. Two combinations of SIX genes were identified in the 10 *F. oxysporum* f. sp. *spinaciae* genome assemblies, *SIX8* and *SIX9* or *SIX4*, *SIX8*, and *SIX14* (Table 3). The genome assemblies of all five *F. oxysporum* f. sp. *spinaciae* group 1 isolates (Fus057, Fus059, Fus166, Fus254, and Fus322) had homologs of *SIX4*, *SIX8*, and *SIX14* whereas the five genome assemblies of the four isolates of *F. oxysporum* f. sp. *spinaciae* group 2 (Fus001, Fus165, Fus167vA, Fus167vB, and Fus173) had *SIX8* and *SIX9* (Table 3). Only one of the five NPS isolates, Fus187, had a complete copy of *SIX14*. No other SIX genes were detected in the five NPS genome assemblies (Table 3). The *SIX4*, *SIX8*, and *SIX14* DNA sequences of *F. oxysporum* f. sp. *spinaciae* group 1 isolates were 85, 87, and 92% identical, respectively, to the equivalent SIX homologs in *F. oxysporum* f. sp. *lycopersici*. A 217-bp insertion was detected in the *SIX9* homolog of *F. oxysporum* f. sp. *spinaciae* group 2 isolates which, when translated in-frame, resulted in a premature stop codon in the hypothetical protein (data not shown). Two copies of *SIX8* were identified in the genome assemblies of all *F. oxysporum* f. sp. *spinaciae* group 2 isolates, with approximately 80% DNA sequence identity to each other. Interestingly, one of the copies of *SIX8* in the genome assemblies of *F. oxysporum* f. sp. *spinaciae* group 2 isolates was identical to that of the *SIX8* homolog found in the genome assemblies of the five *F. oxysporum* f. sp. *spinaciae* group 1 isolates, while the other copy of *SIX8* in the *F. oxysporum* f. sp. *spinaciae* group 2 isolates had 91% DNA sequence identity to that of *F. oxysporum* f. sp. *lycopersici*.

Three copies of *SIX14* were identified in the PacBio assembly of Fus254 (Table 3). Two of the three copies were identical. The third copy had a single insertion of a cytosine residue at base pair 64, which, when translated, would result in a premature stop codon (data not shown). A complete copy of *SIX14* could not be extracted from the genome assemblies of *F. oxysporum* f. sp. *spinaciae* group 1 isolates Fus057, Fus059, and Fus322, as the gene was only assembled partially for those assemblies, i.e., the first 63 base pairs of the gene were present

at the end of a contig. The gene was assembled improperly at the same base pair where a cytosine insertion was observed in one copy of *SIX14* in the PacBio assembly of Fus254.

### Putative effector gene profiles differentiate *F. oxysporum* f. sp. *spinaciae* and NPS isolates.

A pipeline that identifies mimp-associated effector genes (van Dam et al. 2016) was used to predict putative effector genes in the *F. oxysporum* f. sp. *spinaciae* and NPS genome assemblies. In total, 52 putative effector genes were predicted, and the effector gene profiles of the *F. oxysporum* f. sp. *spinaciae* isolates and NPS isolates differentiated the isolates into three distinct groups, i.e., *F. oxysporum* f. sp. *spinaciae* group 1 isolates, *F. oxysporum* f. sp. *spinaciae* group 2 isolates, and NPS isolates (Fig. 4). Twenty-two of the mimp-associated genes were predicted among all the *F. oxysporum* f. sp. *spinaciae* isolates as well as the NPS isolates, suggesting that these genes are likely associated with the core genome of *F. oxysporum* isolates associated with spinach. Twelve predicted genes were specific to the 10 *F. oxysporum* f. sp. *spinaciae* genome assemblies representing nine *F. oxysporum* f. sp. *spinaciae* isolates (Fig. 4). An additional five effector genes were specific to the assemblies of all five *F. oxysporum* f. sp. *spinaciae* group 2 isolates (Fig. 4). Homologs of *SIX4*, *SIX8*, *SIX9*, and *SIX14* were detected using this pipeline (Fig. 4). However, a *SIX14*-like homolog was identified in all of the genome assemblies of the *F. oxysporum* f. sp. *spinaciae* group 2 isolates, the NPS isolate Fus187, and two *F. oxysporum* f. sp. *spinaciae* group 1 isolates (Fus166 and Fus254), even though this gene was not detected in these same genome assemblies (Table 3).

**Table 3.** Presence or absence profile of homologs of the 14 known *Secreted in Xylem (SIX)* genes from *Fusarium oxysporum* f. sp. *lycopersici* identified in the genome assemblies of 10 *F. oxysporum* f. sp. *spinaciae* isolates and five *F. oxysporum* isolates that were not pathogenic on spinach but were associated originally with spinach

Isolate	Designation <sup>x</sup>	<i>Secreted in Xylem (SIX) genes<sup>y</sup></i>				
		<i>SIX4</i>	<i>SIX8a</i>	<i>SIX8b</i>	<i>SIX9</i>	<i>SIX14</i>
Fus057	Group 1	+ (1)	–	+ (1)	–	+ (1)
Fus059	Group 1	+ (1)	–	+ (1)	–	+ (1)
Fus166	Group 1	+ (1)	–	+ (1)	–	+ (1)
Fus254	Group 1	+ (1)	–	+ (1)	–	+ (3)
Fus322	Group 1	+ (1)	–	+ (1)	–	+ (1)
Fus001	Group 2	–	+ (1)	+ (1)	+ (1)	–
Fus165	Group 2	–	+ (1)	+ (1)	+ (1)	–
Fus167vA <sup>z</sup>	Group 2	–	+ (1)	+ (1)	+ (1)	–
Fus167vB <sup>z</sup>	Group 2	–	+ (1)	+ (1)	+ (1)	–
Fus173	Group 2	–	+ (1)	+ (1)	+ (1)	–
Fus017	NPS	–	–	–	–	–
Fus187	NPS	–	–	–	–	+ (1)
Fus191	NPS	–	–	–	–	–
Fus250	NPS	–	–	–	–	–
Fus259	NPS	–	–	–	–	–

<sup>x</sup> Isolates were identified as *Fusarium oxysporum* f. sp. *spinaciae* pathogenicity groups 1 or 2 or NPS. Pathogenicity groups are based on *F. oxysporum* f. sp. *spinaciae* isolates that induced differential severity of wilt on two spinach inbred lines. NPS means the *F. oxysporum* isolate was not pathogenic on spinach although it was obtained originally from a spinach plant, spinach seed, or soil in which spinach was grown.

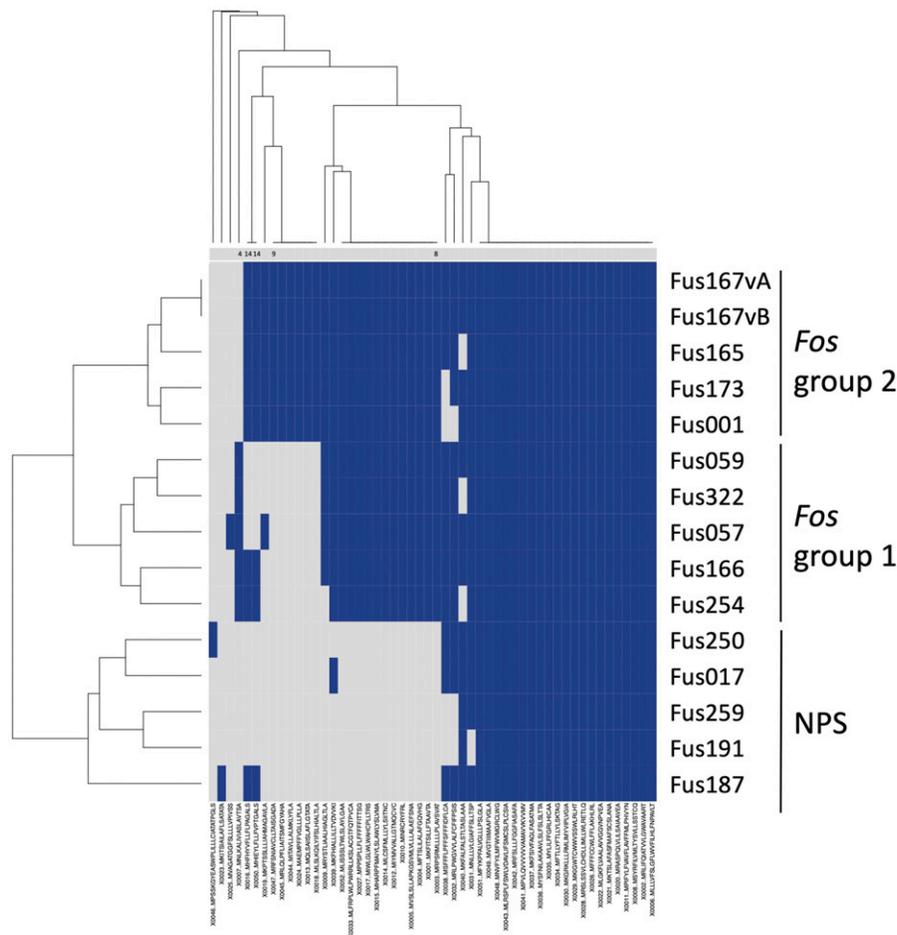
<sup>y</sup> The presence of a SIX gene homolog is indicated by + and the absence of a SIX gene homolog is indicated by –. Numbers in parentheses represent the copy number of the SIX gene homolog identified by a BLASTN search with default parameters or by mapping Illumina reads with Bowtie 2 (Langmead and Salzberg 2012). *SIX1* to *SIX3*, *SIX5* to *SIX7*, and *SIX10* to *SIX13* were not detected in any of the *F. oxysporum* f. sp. *spinaciae* or NPS genome assemblies.

<sup>z</sup> Genome assemblies Fus167vA and Fus167vB were generated from the same isolate, Fus167.

When submitted to a BLASTX search against the non-redundant protein sequence database, 30 (58%) of the mimp-associated genes coded for hypothetical proteins reported in the genomes of other *F. oxysporum* isolates or *Fusarium* spp., or the predicted genes did not resemble any BLASTX annotated genes. The remaining 22 genes coded for predicted peptides that were homologous to various secretory carbohydrate-degrading enzymes, kinases, nucleotide-binding transcription factors, and the *SIX* genes. When the 52 effector genes predicted were queried against other publicly available *F. oxysporum* genome assemblies ( $n = 222$ ), four were found exclusively in the *F. oxysporum* f. sp. *spinaciae* isolates of both pathogenicity groups (MLFRPLWLPWRRLLKSLACGTFQTPVCA, MIYMVVALLGTMQCVC, MLCSFMLLLYLSIITNC, and MLISSSLTWLSLAYLGAA) and one was found only in the genomes of *F. oxysporum* f. sp. *spinaciae* group 2 isolates (MVGTHIIMAAFVGLA).

MLFRPLWLPWRRLLKSLACGTFQTPVCA was the only *F. oxysporum* f. sp. *spinaciae* effector that was 100% identical for all nine *F. oxysporum* f. sp. *spinaciae* isolates. When the DNA and amino acid sequences of the putative effector gene MIYMVVALLGTMQCVC were compared, two haplotypes

were found. Eight isolates (Fus001, Fus057, Fus059, Fus165, Fus166, Fus173, Fus254, and Fus322) had identical DNA and amino acid sequences at this locus, while this homolog in the two genome assemblies for isolate Fus167 differed from that of the other isolates at four DNA base pairs or two amino acids. Similarly, two haplotypes of MLCSFMLLLYLSIITNC were found in the assemblies of all the *F. oxysporum* f. sp. *spinaciae* isolates. Eight isolates (Fus001, Fus057, Fus059, Fus165, Fus166, Fus173, Fus254, and Fus322) had identical DNA and amino acid sequences at this locus, which encode a valid peptide. However, the MLCSFMLLLYLSIITNC homolog identified in both assemblies of isolate Fus167 lacked 29 bp in the DNA alignment, which introduced a premature stop codon in the predicted translation. These three putative effector genes had no similarity to proteins in the InterPro database. The fourth gene found in the *F. oxysporum* f. sp. *spinaciae* genome assemblies, MLISSSLTWLSLAYLGAA, was not assembled completely for Fus057, Fus059, and Fus322 in *F. oxysporum* f. sp. *spinaciae* group 1. However, the partial DNA sequences of this effector in these assemblies were 100% identical to that of the other two *F. oxysporum* f. sp. *spinaciae* group 1 isolates, Fus166 and Fus254. Two copies of MLISSSLTWLSLAYLGAA



**Fig. 4.** Presence or absence plot of putative effector genes found in *Fusarium oxysporum* f. sp. *spinaciae* (*Fos*) isolates in comparison with nonpathogenic isolates of *F. oxysporum* associated with spinach (NPS isolates), divided into two pathogenicity groups of isolates that induced differential symptoms of wilt on spinach inbred lines and a group of nonpathogenic, spinach-associated *F. oxysporum* isolates (NPS). Putative effector genes were identified using the pipeline developed by van Dam et al. (2016). Open reading frames (ORFs) were identified within 2,000 base pairs downstream of a miniature impala transposable element that had characteristics of effector genes. Individual ORFs were each selected as a putative effector gene if the putative peptide had a predicted signal peptide (assessed with SignalP 4.0) and the number of amino acids was between 25 and 600. Solid blue boxes indicate the presence of an ORF in a genome assembly, while shaded gray boxes indicate the absence of an ORF. The genome assemblies (rows) are clustered hierarchically by putative effector profile (left y axis), and the putative effector genes (columns) are clustered by distribution within each assembly (top x axis). Numbers at the top of columns represent the putative effector genes homologous to one of the 14 known *Secreted in Xylem* (*SIX*) genes (e.g., 4 represents *SIX4*, 8 represents *SIX8*). The genome assemblies Fus167vA and Fus167vB were generated from the same isolate, Fus167.

were found in isolates Fus059 and Fus254, which differed at two base-pair positions and resulted in two synonymous amino acid substitutions in the predicted peptide. The predicted protein encoded by MLISSSLTWLSLAYLGAA had similarity to proteins with extracellular carbohydrate (mannan) hydrolase activity. The putative effector gene MVGTIIIMAAFVGLA was only detected in the assemblies of *F. oxysporum* f. sp. *spinaciae* group 2 genome assemblies (Fig. 4). This putative effector gene and the predicted peptide were 100% identical for the *F. oxysporum* f. sp. *spinaciae* group 2 isolates. The predicted protein had gene ontology terms implicating kinase activity, proton-exporting ATPase activity, plasma membrane localization, and positive regulation of the target of rapamycin complex 1 (TORC1) signaling.

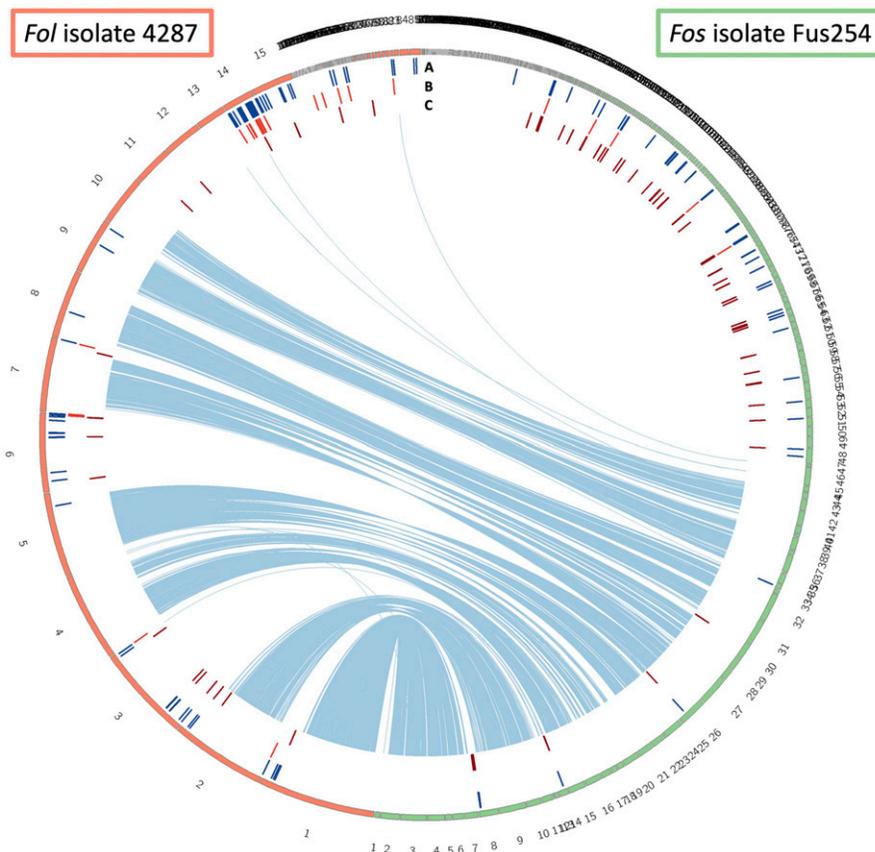
#### Comparison of the genomes of *F. oxysporum* f. sp. *lycopersici* isolate 4287 and *F. oxysporum* f. sp. *spinaciae* isolate Fus254.

A comparison of the genomes of *F. oxysporum* f. sp. *spinaciae* isolate Fus254 and *F. oxysporum* f. sp. *lycopersici* isolate 4287 revealed the presence or absence of core and lineage-specific regions in both genomes (Fig. 5). Chromosomes 3, 6, 11, 12, 13, 14, and 15 in *F. oxysporum* f. sp. *lycopersici* isolate 4287 shared few or none of the single-copy BUSCOs common among Sordariomycetes with the genome assembly of *F. oxysporum* f. sp. *spinaciae* isolate Fus254 (Fig. 5). As demonstrated

in previous studies (Ma et al. 2010; Schmidt et al. 2013), chromosome 14, a pathogenicity chromosome in *F. oxysporum* f. sp. *lycopersici*, harbors *SIX1* to *SIX3* and *SIX5* to *SIX14* (Fig. 5). As expected, this chromosome was also enriched with mimps (85 single-stranded mimp motifs). The genome assembly of Fus254 was highly partitioned. All of the *SIX* genes and nearly all the *F. oxysporum* f. sp. *spinaciae*-predicted effector genes identified in the genome assemblies of Fus254 and this race 2 isolate of *F. oxysporum* f. sp. *lycopersici* were present in genomic regions separate from the BUSCOs (Fig. 5).

## DISCUSSION

The main objective of this study was to identify the putative genetic basis of pathogenicity of *F. oxysporum* f. sp. *spinaciae*. In this study, for the first time, putative effector genes were predicted from whole-genome assemblies of *F. oxysporum* f. sp. *spinaciae* isolates, which lead to the discovery of five candidate effector genes uniquely associated with *F. oxysporum* f. sp. *spinaciae*. The presence or absence profile of the effector genes identified through a homology search or by *ab initio* prediction downstream of mimps facilitated the differentiation of *F. oxysporum* f. sp. *spinaciae* isolates from *F. oxysporum* isolates that were not pathogens of spinach. Although the function of these *F. oxysporum* f. sp. *spinaciae*-specific putative effector genes remains unknown, their identification provides an



**Fig. 5.** Organization and comparison of genome assemblies of *Fusarium oxysporum* f. sp. *lycopersici* (*Fol*) isolate 4287 (GenBank accession GCA\_000149955.2) (red) and *F. oxysporum* f. sp. *spinaciae* (*Fos*) isolate Fus254 (green). Boxes and numbers at the outer edge of the circle represent chromosomes (*F. oxysporum* f. sp. *lycopersici*) or contigs (*F. oxysporum* f. sp. *spinaciae*) and chromosome or contig number, respectively. **A**, Miniature impala motifs are highlighted in dark blue; **B**, the location of *Secreted in Xylem* genes are highlighted in light red; and **C**, the location of putative effector genes identified with the pipeline used in this study are noted in maroon. The putative effectors (**C**) were searched for in the assemblies of *F. oxysporum* f. sp. *spinaciae* isolate Fus254 and *F. oxysporum* f. sp. *lycopersici* isolate 4287. Light blue lines that connect contigs of *F. oxysporum* f. sp. *lycopersici* and *F. oxysporum* f. sp. *spinaciae* each represent the position of one single-copy, orthologous gene out of 3,533 total genes identified with the BUSCO program (Simão et al. 2015) and the ortholog dataset for Sordariomycete orthologs (dataset *odb9*).

invaluable foundation for determining the genetic basis of pathogenicity and host specificity of the causal agent of spinach Fusarium wilt. In addition to identification of putative effector genes, the *F. oxysporum* f. sp. *spinaciae* isolates evaluated in this study were differentiated into two distinct pathogenicity groups based on quantitative differences in wilt severity between two spinach inbreds. Isolates of the two *F. oxysporum* f. sp. *spinaciae* groups could be differentiated from each other based on group-specific profiles of candidate effector genes, which may contribute to the differences in wilt severity that *F. oxysporum* f. sp. *spinaciae* group 1 and group 2 isolates induced on the two spinach inbreds.

Of the *Fusarium* isolates confirmed as *F. oxysporum* f. sp. *spinaciae* by inoculation of spinach inbred lines, all but two exhibited differential virulence on two of the three spinach inbreds evaluated. This discriminated the *F. oxysporum* f. sp. *spinaciae* isolates into two pathogenicity groups. Although quantitative differences in virulence among *F. oxysporum* f. sp. *spinaciae* isolates have been demonstrated (Fiely et al. 1995) as well as quantitative differences in susceptibility of spinach cultivars to *F. oxysporum* f. sp. *spinaciae* (Laguna 2000; O'Brien and Winters 1977), the distinct differential susceptibility of spinach cultivars to isolates of *F. oxysporum* f. sp. *spinaciae* observed in this study has not been described previously. Interestingly, two races of *F. oxysporum* f. sp. *spinaciae* were proposed by Armstrong and Armstrong (1976); however, those races were based on the observation that some *F. oxysporum* f. sp. *spinaciae* isolates induced wilt on spinach and beet, *Beta vulgaris* subsp. *vulgaris*, which they called race 1 isolates, whereas other isolates only induced wilt on beet, which they called race 2 isolates. This race designation proposed by Armstrong and Armstrong (1976) was not associated with differential severity of wilt caused by the isolates on spinach cultivars but on host specificity to different genera of plants. In this study, *F. oxysporum* f. sp. *spinaciae* isolates were assigned to two pathogenicity groups rather than races, since pathogenic races defined for other *F. oxysporum* ff. spp. have been based on differences in susceptibility of cultivars within a host species (Edel-Hermann and Lecomte 2019; Gordon and Martyn 1997; Kistler 1997). In this study, the *F. oxysporum* f. sp. *spinaciae* isolates evaluated induced wilt on all three spinach inbred lines tested, but the severity of wilt differed quantitatively between two inbred lines, with isolates of *F. oxysporum* f. sp. *spinaciae* group 1 causing more severe wilt on spinach inbred A than inbred C, whereas isolates of *F. oxysporum* f. sp. *spinaciae* group 2 caused more severe wilt on inbred C than inbred A. Naming the two *F. oxysporum* f. sp. *spinaciae* groups as races could be misinterpreted to convey solely qualitative differences in susceptibility of spinach lines to isolates of the two pathogenicity groups.

Quantitative differences in virulence detected among isolates of *F. oxysporum* f. sp. *spinaciae* within the same pathogenicity group, and quantitative differences between isolates from the two pathogenicity groups identified in this study may reflect genetic loci that confer different degrees of virulence on specific spinach cultivars. For example, *F. oxysporum* f. sp. *spinaciae* isolate Fus267 induced more severe wilt on spinach inbred lines B and C than on inbred A, and Fus058 induced even more severe wilt on inbred C than did Fus267. Generating whole-genome sequences of *F. oxysporum* f. sp. *spinaciae* isolates from the same pathogenicity group that demonstrated different levels of virulence on spinach (such as Fus267 and Fus058) could be valuable for identifying effector genes or genomic regions that influence pathogenicity quantitatively. Based on prior studies (Gatch and du Toit 2015), two of the three inbred spinach inbred lines used in this study to identify *F. oxysporum* f. sp. *spinaciae* isolates coincidentally

differentiated a large majority of the *F. oxysporum* f. sp. *spinaciae* isolates into the two pathogenicity groups. Additional patterns of virulence among *F. oxysporum* f. sp. *spinaciae* isolates might potentially be identified by testing *F. oxysporum* f. sp. *spinaciae* isolates for pathogenicity on more diverse spinach germplasm (Batson et al. 2020; Gyawali et al. 2019).

Although Fusarium wilt can be a devastating disease of spinach and is the most important limitation for spinach seed production in the United States (Foss and Jones 2005), little is known about the genetic basis of pathogenicity of *F. oxysporum* f. sp. *spinaciae*. Similar to other host-specific *F. oxysporum* isolates that have been demonstrated to have unique profiles of effector genes, this study demonstrated a distinct association of isolates of the two *F. oxysporum* f. sp. *spinaciae* pathogenicity groups with candidate effector gene profiles identified by whole-genome sequencing of *F. oxysporum* f. sp. *spinaciae* isolates and NPS isolates. In addition, the presence or absence profiles of these candidate effector genes differentiated the *F. oxysporum* f. sp. *spinaciae* isolates into the same two groups defined by the pathogenicity tests on two spinach inbreds. Four of the 52 candidate effector genes were unique to the *F. oxysporum* f. sp. *spinaciae* isolates compared with all other publicly available *F. oxysporum* genome assemblies, and one putative effector gene was unique to isolates of *F. oxysporum* f. sp. *spinaciae* pathogenicity group 2.

The 14 known *SIX* genes discovered in *F. oxysporum* f. sp. *lycopersici* and used to profile other *F. oxysporum* ff. spp. (Czislowski et al. 2018; Lievens et al. 2009; Ponukumati et al. 2019; Taylor et al. 2016) were investigated in this study for the potential to profile *F. oxysporum* f. sp. *spinaciae* isolates. The results demonstrated, for the first time, that homologs of *SIX4*, *SIX8*, *SIX9*, or *SIX14*, singly or in combination, are present in *F. oxysporum* f. sp. *spinaciae* genomes. Furthermore, isolates of the two pathogenicity groups of *F. oxysporum* f. sp. *spinaciae* were differentiated by the presence or absence profile of the *SIX* genes; *SIX8* and *SIX9* were detected in all five *F. oxysporum* f. sp. *spinaciae* group 2 isolates sequenced, while *SIX4*, *SIX8*, and *SIX14* were detected in the assemblies of all five *F. oxysporum* f. sp. *spinaciae* group 1 isolates sequenced. Interestingly, only one of the *SIX* genes, *SIX14*, also was found in the genome assemblies of one of five NPS isolates sequenced (Fus187). Other isolates of *F. oxysporum* not known to be pathogenic on any plant species have been demonstrated to have *SIX* genes (Deltour et al. 2018; Taylor et al. 2016; Tesdall et al. 2017; Rocha et al. 2016). Some isolates have even been shown to possess a profile of *SIX* genes that matched that of the tomato pathogen *F. oxysporum* f. sp. *lycopersici* (Jelinski et al. 2017). These observations suggest that the presence of specific *SIX* genes alone may not be a sufficient predictor of virulence in *F. oxysporum* ff. spp., i.e., other genetic factors may influence virulence on specific hosts, such as the presence of other effector genes or the regulation of transcription of the *SIX* genes.

The presence or absence of the putative effectors identified in this study distinguished *F. oxysporum* f. sp. *spinaciae* and NPS isolates. Furthermore, the profile of candidate, mimp-predicted effector genes clearly distinguished isolates of *F. oxysporum* f. sp. *spinaciae* group 1 from isolates of *F. oxysporum* f. sp. *spinaciae* group 2. This corroborated previous studies that found unique combinations of effector genes associated with certain *F. oxysporum* ff. spp. (Lievens et al. 2009; Taylor et al. 2016; Williams et al. 2016; van Dam et al. 2016). However, the roles of these putative effector genes as well as the *SIX* genes in host-specificity of the *F. oxysporum* f. sp. *spinaciae* isolates to spinach are unknown. Some of the predicted functions of these genes are related to carbohydrate degradation, which may aid in breaking down plant cell-wall components, nutrient acquisition, or overcoming host defenses (de Jonge et al. 2010;

Tonukari et al. 2000; van Esse et al. 2008). However, a majority of the mimp-predicted genes encoded hypothetical proteins or proteins without an inferred function, which may encode novel peptides involved in spinach-*F. oxysporum* f. sp. *spinaciae* interactions. In addition, four of the predicted effector genes were unique to both *F. oxysporum* f. sp. *spinaciae* groups compared with other *F. oxysporum* genome assemblies, and a fifth effector gene was present only in *F. oxysporum* f. sp. *spinaciae* group 2 isolates. Further studies are warranted for elucidating the functions of these putative effector genes, by determining whether these genes are expressed during infection of spinach and are required for virulence to spinach.

In this study, representative isolates of *F. oxysporum* f. sp. *spinaciae* group 1 occupied a phylogenetic lineage that was closely related to but distinct from isolates of *F. oxysporum* f. sp. *spinaciae* group 2. This is congruent with findings from O'Donnell et al. (2009), who separated three *F. oxysporum* f. sp. *spinaciae* isolates into two lineages. The three *F. oxysporum* f. sp. *spinaciae* isolates evaluated by O'Donnell et al. (2009) had been characterized for pathogenicity to spinach by Fiely et al. (1995), who found that these isolates belonged to three different VCGs and isolates of the two lineages differed in the severity of wilt they induced on the spinach cultivar Grandstand. Interestingly, *F. oxysporum* f. sp. *spinaciae* group 1 isolates were more closely related to an isolate of *F. oxysporum* f. sp. *vasinfectum* than to *F. oxysporum* f. sp. *spinaciae* group 2 isolates. This supports the notion that phylogenetic lineages of isolates of some formae speciales may be poor predictors of host-specificity, especially given that entire chromosomes that confer pathogenicity can potentially be transferred between isolates (Ma et al. 2010; Shahi et al. 2016; van Dam et al. 2017).

Molecular assays for rapid identification of isolates of specific *F. oxysporum* ff. spp. have been developed based on *SIX* genes (Carvalho et al. 2019) as well as other putative effector genes identified through comparative genomics (van Dam et al. 2018). The unique DNA sequences of the *F. oxysporum* f. sp. *spinaciae* homologs of the *SIX* genes and the *F. oxysporum* f. sp. *spinaciae*-specific effector genes identified in this study indicate that these loci may be promising markers for development of one or more molecular diagnostic assays for *F. oxysporum* f. sp. *spinaciae*. An assay that enables rapid and cost-effective identification and, potentially, quantification of *F. oxysporum* f. sp. *spinaciae* in culture or in situ is needed in place of time- and resource-intensive pathogenicity tests. Although a real-time PCR assay was developed for detection of *F. oxysporum* f. sp. *spinaciae* based on a single nucleotide polymorphism in the IGS region of rDNA (Okubara et al. 2013), that test failed to discriminate isolates of *F. oxysporum* f. sp. *spinaciae* from a majority of NPS isolates tested (70% of NPS isolates cross-reacted with the assay) as well as isolates of a few other formae speciales. In the absence of a highly specific, sensitive, and quantitative molecular assay for *F. oxysporum* f. sp. *spinaciae*, a soil bioassay has been used annually in the maritime PNW region of the United States since 2010 to quantify the risk of spinach Fusarium wilt in spinach seed growers' fields. Spinach seed of inbred lines that differ in susceptibility to Fusarium wilt are planted into soil sampled from each field, and symptoms of Fusarium wilt are rated over the course of seven weeks to quantify the risk of Fusarium wilt (Gatch and du Toit 2015). Although the soil bioassay is a highly effective, direct, and quantitative test for spinach Fusarium wilt risk that has been used effectively as a risk management tool for evaluating almost 500 fields over 10 years, the assay is labor-intensive, not easily scalable, and requires a minimum of two months to yield results. This emphasizes the need to develop a molecular assay that provides growers with a rapid and cost-effective response. Such an assay could be designed based on

the putative effector genes identified in this study that differentiated isolates of *F. oxysporum* f. sp. *spinaciae* from NPS isolates. Furthermore, the assay could be designed based on the effector genes that differentiate *F. oxysporum* f. sp. *spinaciae* isolates of the two pathogenicity groups to reflect potential differences in risk based on genetic background of the spinach lines to be planted in a field (Batson et al. 2020). However, an advantage of the soil bioassay over a DNA-based molecular assay is that accurate quantification of the risk of a disease like Fusarium wilt is not determined solely by the number of propagules of the pathogen. Numerous other factors, such as soil pH, competitive soil microflora, and soil micronutrient concentrations, can have a significant influence on disease pressure (Gatch and du Toit 2015; Malvick et al. 1994; Oyarzun et al. 1994) but would not be accounted for using a DNA-based assay.

## MATERIALS AND METHODS

### Pathogenicity trials.

Sixty-nine *Fusarium oxysporum* isolates were tested for pathogenicity on each of three proprietary spinach inbred lines, A, B, and C. The isolates were tested in groups over a set of six trials due to space constraints. The number of unique *F. oxysporum* isolates tested in each trial ranged from 12 to 14. The isolates tested in trials 5 and 6 were all expected to be nonpathogenic to spinach, based on information provided by the supplier of these isolates, except for the positive control isolate of *F. oxysporum* f. sp. *spinaciae* included in each trial, Fus254. For this reason and to accommodate space constraints, only inbred A was used in trial 6. Each pathogenicity test was conducted as a randomized complete block design with four replicate blocks of the factorial treatment combinations. In each trial, spinach plants were inoculated with *F. oxysporum* f. sp. *spinaciae* isolate Fus254 as a positive control treatment. A spinach-associated *F. oxysporum* isolate that was not pathogenic on spinach but which originated from spinach (NPS isolate Fus187) served as one negative control treatment, and water was used as a second negative control treatment in each trial.

Spinach seeds of each inbred were planted in RediEarth propagation mix (SunGro Horticulture, Agawam, MA, U.S.A.) in 6-cell packs (TLC Polyform, Inc., Salem, OR, U.S.A.). The seedlings were maintained in a greenhouse or a growth chamber, depending on the time of year, with a 9-h day, 15-h night diurnal cycle, to prevent bolting, at 22°C by day and 18°C by night. *F. oxysporum* isolates were cultured at room temperature (23 ± 1°C) under ambient light on half-strength potato dextrose agar (PDA) (Difco Laboratories, Sparks, MD, U.S.A.) amended with 100 ppm of chloramphenicol. A microconidial suspension of each isolate was prepared by inoculating 250 ml of Kerr's broth (Kerr 1963) with three 1-mm<sup>3</sup> colonized agar plugs taken from the leading edge of a colony. The inoculated broth was incubated at room temperature under ambient light on a gyratory shaker at 125 rpm for 7 days. Each microconidial suspension was then filtered through cheesecloth, and the spore concentration was quantified with a hemocytometer. For each of the six trials, spinach plants were inoculated either by drenching the root plugs of 12-day-old spinach plants or by planting seed into propagation mix inoculated with the spore suspension. For the latter, the microconidial suspension was sprayed onto the propagation mix in a Gustafson batch seed treater (Gustafson LLC., Shakopee, MN, U.S.A.), and was tumbled for 5 min to mix the propagation medium and inoculum thoroughly. The inoculated propagation medium was then distributed into the 6-cell packs, and the seeds were sown. In all six trials, the concentration of microconidia was 1.25 ×

10<sup>4</sup> spores per milliliter of propagation mix. Twelve days after spinach seeds had been sown, the temperature in the greenhouse or growth chamber was increased to 28°C by day and 24°C by night, to promote transpirational demand and, thereby, development of *Fusarium* wilt symptoms (Naiki and Morita 1983). The spinach plants were rated for severity of wilt weekly, using an ordinal scale of 0 (no wilt) to 5 (dead plant). Ratings were converted to a *Fusarium* wilt severity index (Gatch and du Toit 2015). The area under the disease progress curve (AUDPC) was calculated based on this index, using the trapezoidal method (Sparks et al. 2008).

The repeat pathogenicity trials for the isolates selected for whole-genome sequencing were performed on spinach inbred lines A and C by inoculating the propagation mix, as described previously, to achieve 3.75 × 10<sup>4</sup> microconidia per milliliter of propagation mix. The trial was conducted in a growth chamber (Percival, Perry, IA, U.S.A.) for 39 days (trial 1) and was then repeated in a greenhouse (trial 2) for 35 days, as the daylength had shortened enough in the autumn to prevent bolting of the spinach plants. In addition to scoring wilt severity, the above-ground dry biomass of the spinach plants in each replicated plot (6-pack) for each treatment combination was determined after drying the plants at 65°C for 72 h.

#### Data analyses for the pathogenicity trials.

Data for the pathogenicity trials were summarized and visualized with the R-package suite Tidyverse (Wickham et al. 2019). Statistical analyses were performed in SAS version 9.4 (SAS Institute, Cary, NC, U.S.A.) with PROC MIXED, and PROC CORR was used to calculate Pearson's correlation coefficients for weekly disease ratings, the AUDPC, and dry biomass per plant. Log, square root, or arcsine square root transformations were used to transform response data when one or both parametric assumptions of normally distributed residuals or homogeneous variances were not met. If the transformed response data did not meet parametric assumptions for ANOVA, the response variable was rank-transformed and statistical analyses performed on the ranked data. Mean separation tests were calculated with Fisher's protected least significant difference at *P* < 0.05, and means were grouped with pdmix800 (Saxton 1998).

#### DNA extraction and molecular characterization of *Fusarium* isolates.

The *Fusarium* isolates listed in Supplementary Table S1 were each grown on half-strength PDA amended with 100 ppm of chloramphenicol, from which two 3-mm<sup>3</sup> colonized agar plugs were taken from the leading edge of the colony and placed in 75 ml of potato dextrose broth in a 125 ml Erlenmeyer flask. Each inoculated flask was placed on an orbital shaking-platform operated at 125 rpm at room temperature for 7 days under ambient light. The resulting mycelium was vacuum-filtered and frozen at -80°C for 24 h. DNA was extracted from the mycelium with the FastDNA kit (MP Biomedicals, Santa Ana, CA, U.S.A.). The concentration of DNA was assessed on a Qubit fluorometer (Invitrogen, Carlsbad, CA, U.S.A.).

The *Fusarium* isolates were each identified to species, based on morphology and the DNA sequence of a partial region of the *TEF1-α* gene. In brief, *TEF1-α* was amplified with primers 526F (GTC GTY GTY ATY GGH CAY GT) (S. Rehner *personal communication*) and 1567R (ACH GTR CCR ATA CCA CCR ATC TT) (Rehner and Buckley 2005) and the following PCR protocol: 1 cycle of 2 min at 94°C; 9 cycles of 30 s at 94°C, 30 s at 66°C, and 1°C lower for each subsequent cycle, and 1.5 min at 72°C; 28 cycles of 30 s at 94°C, 30 s at 56°C, and 1.5 min at 72°C; followed by 1 cycle for 10 min at 72°C. The PCR products were visualized on a 1.5% agarose gel (1× Tris-

borate-EDTA buffer) using GelRed (Biotium, Fremont, CA, U.S.A.). The DNA fragments were purified with ExoSAP-IT (Applied Biosystems, Foster City, CA, U.S.A.) and were quantified with a Qubit fluorometer. Purified DNA fragments were sequenced bidirectionally at Elim Biopharmaceuticals (Hayward, CA, U.S.A.). The consensus sequences for all *TEF1-α* DNA fragments were deposited in GenBank (Supplementary Table S1). A maximum likelihood tree was estimated for the *TEF1-α* sequences by manual alignment of the DNA sequences. The best-fit model for each alignment was selected by Bayesian information criterion (BIC) with ModelTest-NG (Darriba et al. 2020), and a maximum-likelihood tree was inferred using the best-fit evolutionary model with RAXML-NG (Kozlov et al. 2019) and 1,000 bootstrap replicates to estimate node support.

#### Genome sequencing.

DNA of each of *F. oxysporum* f. sp. *spinaciae* isolates Fus001, Fus057, Fus059, Fus167, Fus173, Fus254, and Fus322 and NPS isolates Fus017, Fus187, Fus191, Fus250, and Fus259 was extracted from approximately 100 mg of frozen mycelium, using the Purelink plant total DNA purification kit (Invitrogen), according to manufacturer protocol. Genomic DNA concentration and integrity were determined with a Qubit fluorometer and gel electrophoresis, respectively. Library preparation and sequencing were completed by Molecular Research DNA Lab (Shallowater, TX, U.S.A.). Eleven isolates (Fus001, Fus017, Fus057, Fus059, Fus167, Fus173, Fus187, Fus191, Fus250, Fus259, and Fus322) were sequenced with the Illumina HiSeq platform (2 × 250 bp, approximately 10 million reads per paired end). The DNA samples were prepared with the KAPA HyperPlus kits (Roche, Basel, Switzerland), following manufacturer protocol. In addition, genomic DNA isolated from *F. oxysporum* f. sp. *spinaciae* isolate Fus254 was sequenced using the PacBio Sequel platform (Pacific Biosciences). Fus254 had been characterized extensively in prior pathogenicity trials. Isolate Fus254 was sequenced with a long-read platform to generate a draft genome assembly of one *F. oxysporum* f. sp. *spinaciae* isolate with greater contiguity than the Illumina-generated assemblies. The library for Fus254 was prepared using SMRTbell template prep kit (Pacific Biosciences), following manufacturer protocol. The DNA of Fus254 was sheared to a size of approximately 7.2 Kbp, using the Covaris G-tube (Covaris Inc., Woburn, MA, U.S.A.), and the library pool was sequenced on two SMRT Cells, using a 10-h movie time, on the PacBio Sequel (Pacific Biosciences).

In addition to the 12 isolates tested three times for pathogenicity on spinach, as described above, three *F. oxysporum* f. sp. *spinaciae* isolates from the United States Department of Agriculture Agricultural Research Service (USDA ARS) culture collection at the Northern Regional Research Laboratory (NRRL) were used for whole-genome sequencing completed at the University of Amsterdam in Amsterdam, Netherlands: Fus165 = NRRL26874, Fus166 = NRRL26875, and Fus167 = NRRL26876. DNA was extracted from freeze-dried mycelium harvested from 5-day-old NO<sub>3</sub>-broth (0.17% yeast nitrogen base, 3% sucrose, 100 mM KNO<sub>3</sub>) cultures. The mycelium from each isolate was ground in a mortar with liquid nitrogen. Approximately 25 mg of ground mycelium was added to a prechilled tube containing three metal beads, and 800 μl of sodium dodecyl sulfate (SDS) extraction buffer (100 mM Tris, pH 8.0, 50 mM EDTA, 1 M NaCl, 3% SDS) was added. The samples were shaken for 2 min at max speed in a Tissue Lyser (Qiagen, Hilden, Germany) and were incubated at 65°C for 30 min. Then, 800 μl of phenol/chloroform/isoamylalcohol (25:24:1, buffer saturated phenol) was added to the lysed tissue, the sample was mixed by inversion, and was centrifuged at

maximum speed at 4°C for 15 min. DNA was precipitated from the aqueous phase with a 0.1 volume of 5 M NaCl and two volumes of 96% ethanol. The resulting DNA was purified with the Purelink plant total DNA purification kit (Invitrogen). Libraries were prepared for the three isolates with a the TruSeq Nano DNA low throughput library prep kit (Illumina, San Diego, CA, U.S.A.), following manufacturer protocol, and were sequenced with the Illumina HiSeq X Ten platform (2 × 150 bp) by the Hartwig Medical Foundation (Amsterdam).

### Raw read data and genome assembly.

The sequence reads of the 14 isolates of *F. oxysporum* were deposited in the sequence read archive (SRA) under the Bio-Project numbers PRJNA540981 and PRJNA595149. The corresponding assemblies were deposited in the NCBI GenBank repository (Table 2). Illumina reads were quality-trimmed using Trimmomatic version 0.39 (Bolger et al. 2014) with a quality threshold of 20, and the Illumina adapters were removed. PhiX phage contamination was found in the Illumina reads generated for 11 of the *F. oxysporum* isolates (in 0.01 to 0.07% of the total reads for those isolates). Reads of PhiX origin were then removed by mapping the trimmed Illumina reads to the genome of Coliphage phi-X174 (NC\_001422.1) with Bowtie 2 (Langmead and Salzberg 2012). Unmapped, PhiX-free Illumina reads of the 11 isolates were then assembled with SPAdes v3.13.0 (Bankevich et al. 2012), with default kmer sizes of 21, 33, 55, 77, 99, and 127. The Illumina reads for the genomes of Fus165, Fus166, and Fus167vB were assembled with CLC-Workbench version 8.0 (Qiagen), with default parameters. De novo assembly of the PacBio reads of isolate Fus254 was accomplished using the SMRT Analysis Hierarchical Genome Assembly Process version 3.0. The expected genome size was estimated to be 50 Mbp, based on the genomes of other *F. oxysporum* ff. spp. sequenced (Ma et al. 2010; van Dam et al. 2016). General assembly statistics were calculated with QUAST (Gurevich et al. 2013). BUSCOs were identified in the newly generated assemblies as an estimate of genome completeness with the Sordariomycete (*odb9*) database (Simão et al. 2015) (Table 2).

### Phylogenomic analyses.

Phylogenetic summary trees were inferred by comparing BUSCOs identified from whole-genome sequences of the nine *F. oxysporum* f. sp. *spinaciae* isolates, five NPS isolates, 13 publicly available *F. oxysporum* genome assemblies, and genome sequences of two other *Fusarium* spp. downloaded from GenBank (Supplementary Table S2). In total, 3,127 single-copy, nonfragmented BUSCOs common among all 30 genome assemblies were used for phylogenetic analyses. Each BUSCO was aligned among the taxa using Clustal Omega (Sievers et al. 2011), the best-fit model for each alignment was selected by BIC with ModelTest-NG (Darriba et al. 2020), and a maximum-likelihood tree was inferred for each of the BUSCOs, using the best-fit evolutionary model with RAxML-NG (Kozlov et al. 2019). The parameters used for RAxML-NG included an expanded tree search (100 parsimony and 100 maximum likelihood start trees) without bootstrapping. The 3,127 maximum-likelihood trees were used in ASTRAL-III to estimate the best species tree given the gene trees (Zhang et al. 2018). The final tree produced by ASTRAL-III was rooted to *F. solani*. In addition to the ASTRAL-inferred species tree, the 3,127 aligned BUSCOs were concatenated, resulting in 6,222,831 alignment sites from which a tree was inferred, as described previously, with the exception that a single model of evolution (GTR + I + G) was used for the entire concatenated data set, and 1,000 bootstrap replicates were performed to estimate clade support (Supplementary Fig. S3). The resulting newick trees were visualized with FigTree version 1.4.4 or the web-based tree-editor phylo.io.

Unaligned and aligned FASTA files of BUSCOs and phylogenetic trees (in newick format) will be made available on request.

### Effector gene prediction.

A prediction pipeline was used to identify putative effector genes associated with mimps among the 10 *F. oxysporum* f. sp. *spinaciae* and five NPS genome assemblies generated in this study (van Dam et al. 2016). In brief, open reading frames (ORFs) were predicted within 2,000 bp downstream of each mimp-identified ORF that translated into a protein with between 25 and 600 amino acids and had a predicted signal peptide (assessed with SignalP 4.0 [Petersen et al. 2011]). These were designated as putative effectors. In addition, homologs of the 14 known *SIX* genes of *F. oxysporum* f. sp. *lycopersici* were searched for in the genome assemblies of the *F. oxysporum* f. sp. *spinaciae* and NPS isolates, using the command line tool BLASTN with the flag ‘-outfmt 6’. When fragmented *SIX* genes were found in the assemblies, trimmed Illumina reads were mapped to the 14 *SIX* genes with Bowtie 2 (Langmead and Salzberg 2012) and the consensus sequence was extracted with bcftools (Li et al. 2009). Each predicted effector gene was investigated for putative function by using BLASTX and InterProScan with the program Blast2GO version 5.2.5 (Conesa et al. 2005). To identify *F. oxysporum* f. sp. *spinaciae*-specific effector genes from the pipeline described by van Dam et al. (2016), the predicted effector genes were searched with the BLASTN command line tool with default parameters against 222 other *F. oxysporum* genome assemblies present in GenBank as of January 1, 2020.

### Genomic comparison of *F. oxysporum* f. sp. *spinaciae* and *F. oxysporum* f. sp. *lycopersici*.

The organization of the genomes of *F. oxysporum* f. sp. *lycopersici* isolate 4287 (GCA\_000149955.2) and *F. oxysporum* f. sp. *spinaciae* isolate Fus254 were compared using Circos version 0.69-8 (Krzywinski et al. 2009). Common, single-copy Sordariomycete orthologs inferred with BUSCO ( $n = 3,533$ ) were linked to demonstrate synteny, the 14 *SIX* genes and putative effector genes were located in both genome assemblies using the command line version of BLASTN with default parameters, and mimp sequences were identified using SeqKit (Shen et al. 2016) with the following command ‘seqkit locate –bed -d -p NNCAGT[GA][GA]G[GAT][TGC]GCAA[TAG]AA’ (Fig. 5).

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### AUTHOR-RECOMMENDED INTERNET RESOURCES

FigTree version 1.4.4: <https://github.com/rambaut/figtree>  
phylo.io: <https://phylo.io>

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