RESEARCH NOTE

Identification of Two Secreted in xylem 6 (*SIX6*) Genes in *Fusarium oxysporum* f. sp. *fragariae* of Korea Isolates

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ABSTRACT

Strawberry Fusarium wilt, caused by *Fusarium oxysporum* f. sp. *fragariae* (FOF), poses a significant threat to strawberry production. FOF secretes effector proteins called "secreted in xylem" (SIX) into the plant's xylem, disrupting the defense responses in the plant. In this study the proteins SIX6a and SIX6b in FOF races 1 and 2, showing race-specific expression patterns, were identified. These genes encode effector proteins with a conserved signal peptide that is typical of fungal effectors. Differential expression in susceptible strawberries suggests that FOF races regulate these effectors differently, thereby contributing to variations in pathogenicity. Understanding these effectors is crucial for the development of race-specific resistance strategies to manage Fusarium wilt in strawberries.

Keywords: Fungal effectors, *Fusarium oxysporum* f. sp. *fragariae,* Fusarium wilt, *SIX* gene, Strawberry

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Strawberry (*Fragaria* × *ananassa*), an octoploid plant, is one of the most widely cultivated fruits worldwide. However, strawberry plants are susceptible to various pathogens, which leads to significant production losses [1]. One such pathogen is *Fusarium oxysporum* (FO), which causes Fusarium wilt. This pathogen has a broad host range among important crops, but different strains are highly host specific and are grouped into formae speciales (f. sp.) [2]. The strain responsible for Fusarium wilt in strawberries is *Fusarium oxysporum* f. sp. *fragariae* (FOF) [3]. FOF invades the roots, causes discoloration, multiplies in the xylem, disrupts nutrient movement, and ultimately kills the plant [4,5]. The pathogen produces chlamydospores that survive in unfavorable conditions, spread through the stolons, and infect first-generation daughter plants, making Fusarium wilt the most serious strawberry disease [5,6].

During infection, *F. oxysporum* secretes effectors into the host cells, thus facilitating colonization by modifying the immune response [7]. A group of small, cysteine-rich secreted proteins, known as "secreted in xylem" (SIX), have been identified in infected plants [8–10]. These proteins, present in various *Fusarium* species, serve as markers to distinguish different formae speciales (f. sp.) and races based on their presence

or sequence variations. For example, *F. oxysporum* f. sp. *lycopersici* (FOL) race 1 lacks the *SIX4* gene, and differences in *SIX3* distinguish races 2 and 3 [11]. In *F. oxysporum* f. sp. *cubense* (FOC), the *SIX8* gene is used for race identification. Research on *Fusarium* species is ongoing, with the aim of understanding their genetic diversity, pathogenicity, and fungicide response, with the ultimate goal of breeding plants resistant to *Fusarium* diseases [5,7,12–15].

Recent studies on *FOF* have identified two distinct symptoms, suggesting the presence of wilt- and yellows-causing isolates [10]. *SIX6* is found in yellows-causing isolates, whereas the wilt-causing isolates contain *SIX1a, SIX1b, SIX1c*, and *SIX13* [7,16–18]. The *SIX6* gene is present in *FOF* race 1 but absent in race 2 isolates [19]. Accordingly, we investigated the presence of the *SIX6* gene in five Korean *FOF* isolates, Fo160609, Fo080701 (race 1), Fo160403, Fo160618, and SK1 (race 2), which were obtained from the Agricultural Research & Extension Center in Nonsan (Table 1). Genomic DNA was extracted from five isolates, and sequences were analyzed to determine the presence of *SIX6*.

Table 1. *Fusarium oxysporum* f. sp. *fragariae* isolates obtained from strawberry fields in this study

Isolate	Cultivar	Source	Year	Location
Fo160403	Sulhyang	Crown	2016	Nonsan, CN
SK1	Sulhyang	Crown	2016	Hongseong, CN
Fo160618	Sulhyang	Crown	2016	Hongseong, CN
Fo080701	Redpearl	Crown	2008	Ganghwa, GG
Fo160609	Sulhyang	Crown	2016	Nonsan, CN

CN: Chungcheongnam-do (South Chungcheong Province, Korea); GG: Gyeonggi-do (Gyeonggi Province, Korea).

Five *FOF* isolates were cultured [13], and mycelia were harvested for genomic DNA extraction. Mycelia were filtered through miracloth and collected in a 50-mL Falcon tube using a sterilized spatula. The samples were either used immediately for DNA extraction or frozen in liquid nitrogen and stored at −80℃. Genomic DNA was extracted with minor modifications to the protocol [20]. Mycelia were ground in liquid nitrogen, resuspended in extraction buffer (50 mM Tris-HCl, 50 mM EDTA, 3% SDS, and 1% 2-mercaptoethanol), and treated with RNase. After vortexing and incubating at 65℃ for 1 h, chloroform was added, followed by centrifugation. The upper aqueous phase was transferred and a 24:1 mixture of chloroform and isoamyl alcohol was added, followed by centrifugation. The DNA was precipitated with 3 M NaOAc and isopropanol, washed with ethanol, and dried. The DNA pellet was resuspended in Tris-EDTA (SmartGene, Daejeon, Korea) buffer and stored at −20℃.

The pathogenicity of the *FOF* race 1 and race 2 isolates was assessed by inoculating susceptible strawberry plants with spore suspensions (1×10^6 spores/mL) and planting them in autoclaved sand in a growth chamber. Roots from five plants were harvested at 0, 2, 4, 6, 8, and 10 days after inoculation (dai) and flash-frozen in liquid nitrogen. RNA was extracted using Trizol® reagent, and first-strand cDNA was synthesized. Real-time reverse-transcription polymerase chain reaction (RT-PCR) was performed to quantify *SIX* gene expression, which was analyzed on a Roche LightCycler 480 using SYBR Green I Master Mix (Bio-Rad CFX96TM Real-time System platform, Berkeley, USA). The *SIX6* gene was

originally identified in strawberry (*Fragaria* × *ananassa*) plants following *FOF* infection [17,18]. *SIX6* acts as an avirulence factor (*AvrFW1*) for *FOF* race 1 by specifically interacting with a resistance gene located at the *FW1* locus in strawberry cultivars [19]. Using the *AvrFW1* sequence provided by Dillar-Ermita et al. [19], we successfully cloned *SIX6*. We then analyzed nine candidate *SIX6* genes from five *FOF* race 1 and race 2 isolates from Korea using *FOF*-specific primers (*FOFra*) and primers specific for *SIX6a* and *SIX6b*. The results showed the presence of 600- and 700-bp bands for races 1 and 2, respectively (Fig. 1A).

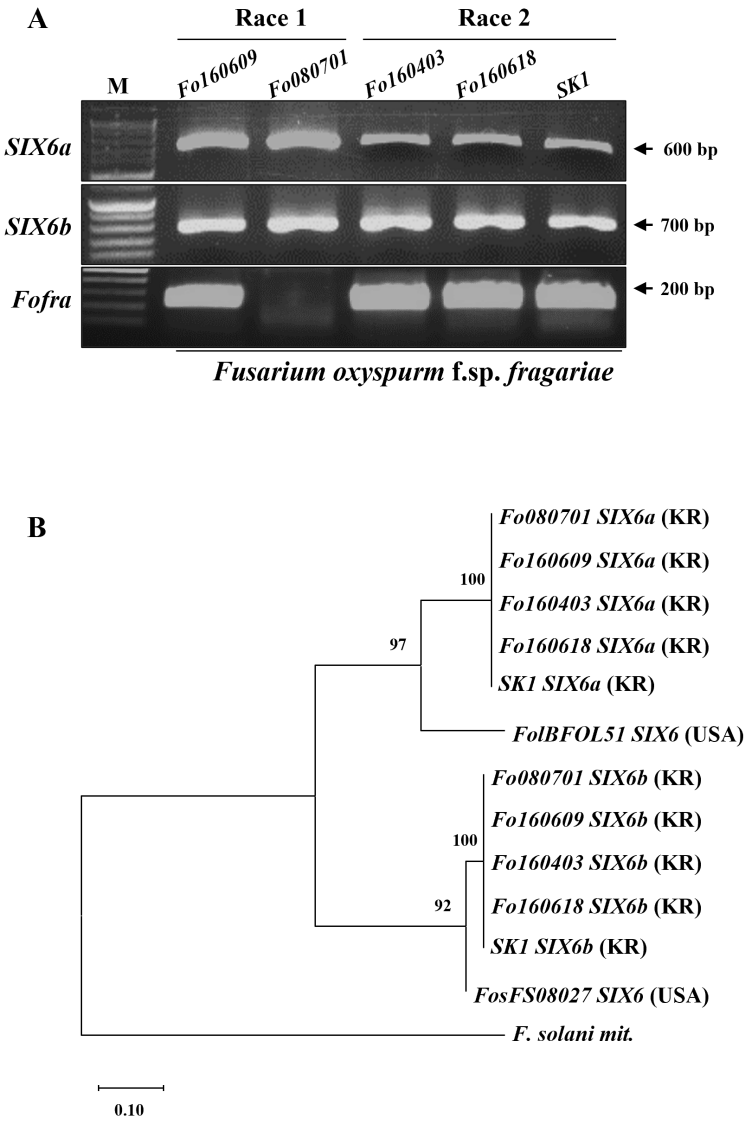

Fig. 1. Identification of the SIX6a and SIX6b gene from 5 different *Fusarium oxysporum* f. sp. *fragariae* (FOF) race 1 and race 2 isolates [13]. (A) Multiplex polymerase chain reaction (PCR) with FOF-specific primers(*FOFra*), a specific primer of *SIX6a* and *SIX6b* gene. The 600 bp and 700 bp bands were detected in *FOF* race 1 and race 2. (B) Maximum likelihood tree for 5 *FOF* isolates from diseased Korea strawberry, based on an alignment of *SIX6a* and *SIX6b* sequences. Numbers represent bootstrap percentage values from 1,000 replicates. Scale bar indicates 0.10 substitutions per site. The tree is rooted through three *F. solani* isolates (outgroup).

The *SIX6* gene has been identified in race 1 yellows-fragariae isolates [19]. To determine whether *SIX6* was present in Korean isolates [13], we designed primers for *SIX6* and performed PCR on two race 1 isolates and three race 2 isolates. We identified two homologous genes, *SIX6a* and *SIX6b*, in race 1 and race 2 isolates (Fig. 1A). Both genes exhibited 100% identical nucleotide sequences with no variations. Basic Local Alignment Search Tool (BLAST) analysis also revealed that *SIX6* gene sequences in Korean isolates matched those of yellows-fragariae isolates from other countries. The sequencing results confirmed that *SIX6* contains a conserved signal peptide at the N-terminus.

The phylogenetic tree was constructed using MEGA11 with a bootstrap value of 1,000, employing the maximum likelihood method and the Kimura 2-parameter model [21]. The tree was based on the nucleotide sequences of *SIX6a* and *SIX6b* from five Korean isolates and two American isolates. The *FOF SIX6a* and *SIX6b* genes exhibited 52.8% nucleotide sequence identity. *SIX6a* homologs from the five Korean *FOF* isolates demonstrated 75.0% sequence identity with the closest homolog derived from the *FOL* isolate *BFOL-51*. In contrast, *SIX6b* homologs from the same five Korean isolates [13] showed significantly higher sequence identity (98.1%) with the closest homolog found in *F. oxysporum* f. sp. *sesami* isolate FS08027 (Fig 1B).

To determine whether *SIX6a* and *SIX6b* are also expressed during the infection stage, we inoculated in *FOF* race 1 and race 2 isolates that inoculated into the cultivar 'Sulhyang' at a concentration of 1×10^6 spores/mL, and the disease severity was investigated [13]. Both the strains showed yellowing and wilting symptoms in the infected plants. Race 2 exhibited more severe wilting symptoms than did race 1 (Fig. 2A). Second, RNA was collected from *FOF* race 1-and race 2-infected strawberry plants at 2, 4, 6, 8, and 10 dai. The expression of *SIX6a* and *SIX6b* was monitored using RT-PCR, and *SIX6a* and *SIX6b* transcripts were detected at selected time points in the infected plants. Interesting results were obtained from the inoculation experiments with *FOF* race 1 and race 2 isolates. In the *FOF* race 1 isolate, the expression of both *SIX6a* and *SIX6b* began to increase at 2 dai, with the highest expression levels observed at 6 dai. Furthermore, there were no significant differences in the expression levels of these two genes. In contrast, the *FOF* race 2 isolate exhibited distinct expression patterns: the expression of *SIX6a* increased on day 2 but then declined, and *SIX6b* showed no detectable expression initially but exhibited high expression levels on day 10 (Fig. 2B and 2C).

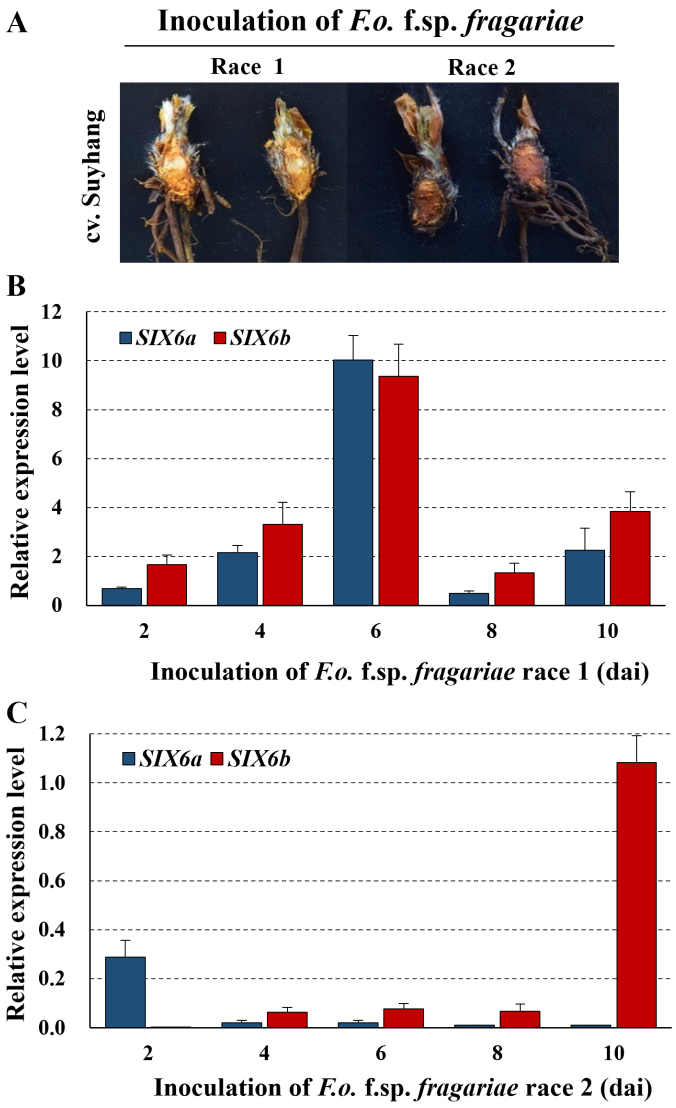

Fig. 2. Pathogenicity of *Fusarium oxysporum* f. sp. *fragariae* (*FOF*) race 1 and race 2 isolates [13] on cultivar Sulhang of strawberry using the root dip inoculation method. Photograph of symptoms were taken 30 days after inoculation (dai) (A). Expression of Secreted In Xylem (*SIX6a* and *SIX6b*) genes in strawberry roots infected with FOF race 1 (B) and race 2 (C) isolates as determined by reverse transcription qPCR of RNA. Expression was calculated relative to the β-Tubulin and the mean value displayed [Error bars represent standard error of the mean (SEM)].

The sequences of the two genes in Korean isolates showed no differences [13]. We hypothesized that the expression patterns of these genes would differ between races 1 and 2 when *FOF* was inoculated onto strawberry roots. Heavy colonization by this pathogen was observed in the vascular tissue of the susceptible cultivar 'Carmarosa' during the early stages of infection [20]. After *FOF* inoculation, samples were collected for up to 10 dai. Reverse-transcription quantitative polymerase chain reaction (RT-qPCR) results revealed that both *SIX6a* and *SIX6b* were highly expressed when race 1 was inoculated onto the susceptible cultivar. In contrast, neither of the two genes was expressed when race 2 was inoculated.

SIX1a, SIX1b, SIX1c, SIX6, and *SIX13* have been identified in *FOF* [7,16,17,18]. However, five Korean *FOF* isolates contained two homologs of *SIX6a* and *SIX6b*, whereas *FOF* isolates from California possessed only one *SIX6* gene [7,17,18]. Notably, we did not detect the presence of *SIX1a, SIX1b, SIX1c*, or *SIX13* in the Korean isolates, in contrast to *FOF* isolates from Australia and Spain, which harbored these genes [16,18]. Furthermore, race 1 isolates of *FOF* from California exclusively contained *SIX6*, whereas race 2 isolates did not [19]. In contrast, the race 1 and race 2 *FOF* isolates from Korea possessed two *SIX6* genes. This finding suggests that *FOF* strains exhibit different combinations of *SIX* genes, depending on geographic location. However, no sequence differences were observed between Korean and California isolates. The expression patterns of *SIX* genes during pathogen inoculation of susceptible strawberry roots were also similar between the Korean and California race 1 isolates [7,17,18]. Specifically, in race 1, both *SIX6a* and *SIX6b* were highly expressed during the early stages of inoculation. These results align with the observation that the vascular tissue of the root in the susceptible cultivar 'Carmarosa' is heavily colonized by the pathogen in the early stages of infection [20]. This suggests that *FOF* race 1 secretes *SIX6* to facilitate strawberry root vascular tissue colonization.

In the Korean isolates, race 2 also possessed two *SIX6* genes; however, their expression was minimal upon inoculation with susceptible strawberry roots [13]. We hypothesized that race 2 secretes other candidate effector genes responsible for the infection process in strawberry roots. Unlike the Californian isolates, where races 1 and 2 are distinguishable based on their *SIX6* gene presence, in the Korean isolates races 1 and 2 can be distinguished by assessing the expression of *SIX6a* and *SIX6b* in response to inoculation. We defined race 1 as expressing *SIX6a* and *SIX6b* for root colonization, whereas race 2 did not express these genes. This is the first report of two *SIX6* gene homologs in *FOF* isolates from Korea. These findings provide molecular evidence that this pathogen secretes different virulence factors depending on race when infecting strawberry plants. Additionally, it may be possible to identify race-specific resistance (R) genes in strawberry cultivars that exhibit resistance, and explore the molecular interactions between these R genes and the effector genes of the *FOF*.

CONFLICT OF INTERESTS

The authors declare that there are no conflicts of interest.

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