# Identification of pathogenicity chromosome and avirulence effector in *Fusarium oxysporum* f. sp. *cepae*

# *Fusarium oxysporum* f. sp. *cepae* における病原性染色体及び非病原力エフェクターの同定

A THESIS SUBMITTED TO THE UNITED GRADUATE SCHOOL OF AGRICULTURAL SCIENCES TOTTORI UNIVERSITY IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTORAL OF PHILOSOPHY

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2024

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# CHAPTER 1

# GENERAL INTRODUCTION

## 1.1 BACKGROUND AND OBJECTIVES

### 1.1.1 Onion (*Allium cepa* L.)

Onion (*Allium cepa* L.) is a globally important vegetable, with the global production of dry onions having reached 104.5 million tons in 2020 and production quantity of onion have been yearly increasing (Figure 1). The main countries of onion production are India and China, accounting for 50 % of onion production in the world. In Japan, onion is widely cultivated with more than 1 million tons per year, and was accredited as a "Designated vegetable" by the Ministry of Agriculture, Forestry and Fisheries of Japan, which indicates onion is a highly important vegetable not only in Japan but also world (FAOSTAT, 2022). In addition, it is known that onions abundantly contain flavonoids, including flavonols and anthocyanins (Griffiths et al., 2002). Therefore, it is believed that onion is a helpful resource to effect against cancer, heart disease, aging, etc. As above, onion is a globally important commercial crop, however, onion has recently been threatened by a variety of plant pathogens, including *Fusarium* species, which cause Fusarium basal rot disease (FBR) and lead to substantial production losses (Haapalainen et al., 2022; Sasaki et al., 2015b; Southwood et al., 2015; Taylor et al., 2016). Moreover, no onion cultivar has shown full resistance toward FBR so far. Hence, the development of onion cultivar that has a strong resistance phenotype toward FBR is globally desirable.

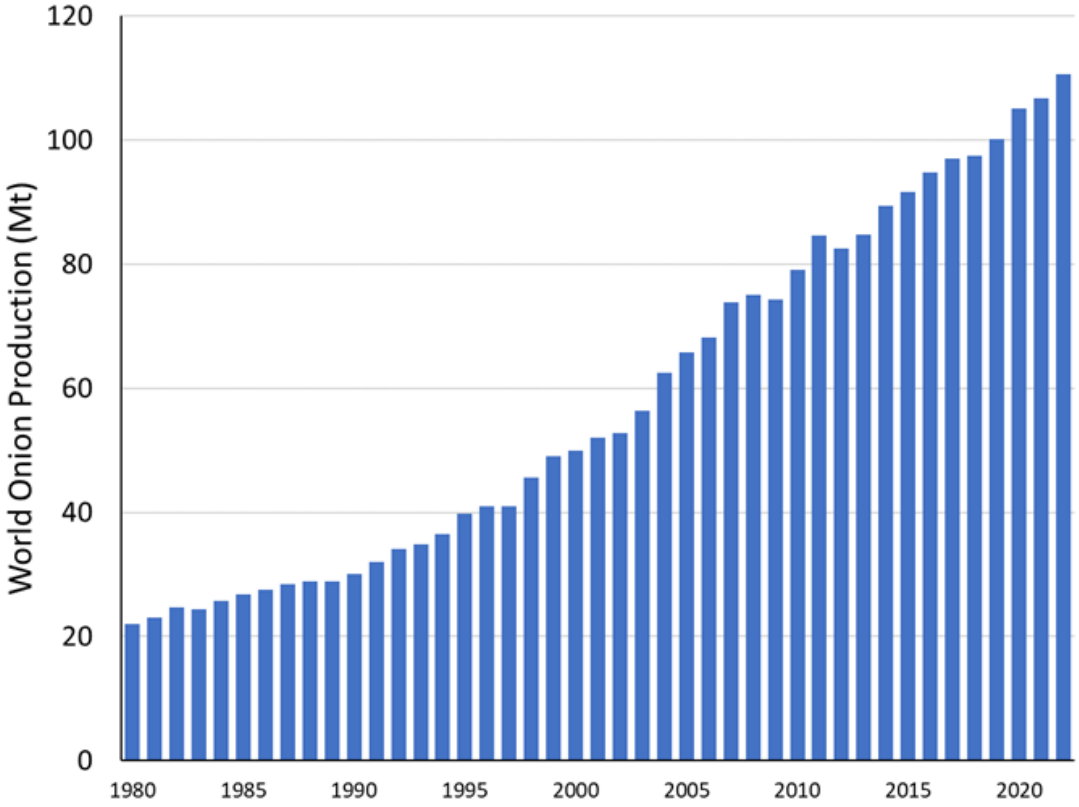


**Figure 1.** The transition of world dry onion production from 1980 to 2022.

### 1.1.2 Shallot (*A. cepa* L. Aggregatum group)

Shallot (*A. cepa* L. Aggregatum group) is an annual herbaceous plant belonging to the family Amaryllidaceae, which is widely used as a condiment in Southeast Asian countries. The shallot extract contains an alkaloid, flavonoid, triterpenoid, steroid, tannin, saponin, and phenolic. Further, Flavonoid, saponin, essential oil, allicin, and quercetin has a function of antimicrobe effects (Ahmadi et al., 2018; Anggarani et al., 2021; Tagoe et al., 2011; Teshima et al., 2013; Yin et al., 2003) and shallot is highly resistant to plant pathogens, including *Fusarium oxysporum*, causative agent of FBR (Herlina et al., 2019; Vu et al., 2012). Notably, shallots are genomically compatible and can be crossbred with onion plants (Abdelrahman et al., 2015). Therefore, shallots are considered a prospective resource for disease-resistance breeding in onions (Astley et al., 1982).

### 1.1.3 *Fusarium oxysporum*

The *F. oxysporum* species complex is a cosmopolitan, soil-borne, plant pathogenic fungus. In particular, the most important biological characteristic of *F. oxysporum* is its wide host range and host specificity. At present, it has been reported that *F. oxysporum* has a broad host range of more than 120 species and has recently been recognized as the fifth most important plant fungal pathogen (Dean et al., 2012; Leslie and Summerell, 2006). Based on its host specificity, *F. oxysporum* is subdivided into different "*formae speciales*" (Laurence et al., 2012; Michielse and Rep, 2009), among which *F. oxysporum* f. sp. *cepae* (*Foc*) has been identified as the causal agent of FBR upon onion and shallot and Fusarium wilt disease (FW) upon Welsh onion (*A. fistulosum* L.), also known as Japanese bunching onion (Chand et al., 2016; Sasaki et al., 2015b; Schwartz and Mohan, 2007). The battle between *F. oxysporum* and plants is open

underground when the potential host is transplanted into the infested field with *F. oxysporum*. Afterward, *F. oxysporum* attacks its host at any point in its developmental stage, including seed, seedling, mature plant, which causes damping-off, yellowing, wilting, and death upon its host. Troublesomely, the *F. oxysporum* can survive in plant debris or soil with another form called "chlamydospore" for several years even in the absence of host plants (Nelson et al., 1981).

#### 1.1.3.1 Current status of taxonomy in *Fusarium oxysporum* species complex

To conduct the identification of *Fusarium* species, the genetic similarity of the gene locus, including internal transcribed spacer (ITS) region, mitochondrial small subunit (mtSSU), RNA Polymerase 1 and 2 (*rpb1* and *rpb2*), and translation elongation factor 1-alpha (*tef1*) is generally investigated (Maryani et al., 2019; O'Donnell et al., 1998; Sasaki et al., 2015b; Southwood et al., 2012). However, it has been also reported that identification of *Fusarium* species with single locus is insufficient to exactly distinguish between the *Fusarium* species (O'Donnell and Cigelnik, 1997). Indeed, the epitype of the *F. oxysporum* is newly established based on the multi-locus based phylogenetic analysis to correct the current taxonomic chaos in *F. oxysporum* (Lombard et al., 2019). Therefore, phylogenetic analysis with multi-locus sequences have been currently applied for reliable identification and avoidance of incorrect identification in *F. oxysporum* (Achari et al., 2020; Maryani et al., 2019; Medeiros et al., 2021). Given the situation of the aforementioned taxonomic chaos and the global negative impact of *Fusarium* species upon commercial crop production, reliable identification of *Fusarium* species with multi-locus sequences is increasingly crucial.

### 1.1.4 Impact of FBR

FBR has been reported to be caused by *Fusarium* species, resulting in a significant loss of yield and quality of onion (Dauda et al., 2018; Gunaratna et al., 2019; Ravi et al., 2014; Le et al., 2021). Furthermore, occurrence of FBR upon onion have been globally reported (Bektas and Kusek. 2019; Galván et al., 2008; Haapalainen et al., 2016; Muthukumar et al., 2022; Sasaki et al., 2015a; Smith 2009; Southwood et al., 2012; Swift 2001; Taylor et al., 2016). As previously reported, FBR was observed at any point in its developmental stage, including seed, seedling, bulb in the field, and bulb during storage (Abawi and Lorbeer 1971; Cramer 2000; Gupta and Gupta., 2013; Kalman et al., 2020). The onion got infected by *F. oxysporum* f. sp. *cepae* (*Foc*), the causal agent of FBR, shows curling and yellowing upon the lower leaf in the above ground, moreover, cause the browning of the basal part of onion and rot symptoms in the root system of onion, which eventually lead to plant death (Figure 2). Therefore, the development of disease management for FBR using management from various viewpoints is considered to be urgently needed.


**Figure 2.** symptom of FBR.

### 1.1.5 Management of FBR using resistant cultivar

The use of resistant cultivars is an effective and powerful measurement for disease control within various pathosystems between plants and pathogen. In the *Allium* species, closely related species, including *A. fistulosum*, *A. roylei* and *A. galanthum* has exhibited resistant phenotype toward FBR. Hence, those *Allium* species are exploited for disease resistance breeding toward FBR (Galván et al., 2008). It has been reported that certain onion cultivars exhibit resistant phenotype toward FBR (Gutierrez et al., 2006; Gutierrez and Cramer 2005; Lopez and Cramer 2002; Taylor et al., 2013). However, the application of resistant cultivar of onion has several hurdle since the resistant phenotype of onion is dependent on the development stage and *Fusarium* spp. responsible for FBR, moreover, the resistance mechanism of onion toward FBR remains unknown (Caligiore Gei et al., 2014). Indeed, none of onion cultivar that has complete resistance phenotype toward FBR has been developed so far. Therefore, the onion cultivar showing a complete resistance phenotype is globally desirable.

### 1.1.6 Effector

Plants have been evolved to protect themselves from pathogens attack to survive in nature. The interaction between plants and pathogens begin the perception of conserved pathogen-associated molecular pattern (PAMPs) by cell-surface localized pattern recognition receptors (PRRs), which cause the pattern-triggered immunity (PTI) in the plant side to restrict pathogen invasion (Ausubel 2005; Jones and Dangl 2006). To counter the PTI response upon plants, pathogens secrete the small molecule protein called an “effector” to manipulate the host cellular process. The effector promotes colonization in their host and determines host specificity, which is known as effector-triggered

susceptibility (ETS) (Bi et al., 2021; Chen et al., 2021; Qian et al., 2022; Qiu et al., 2023). In the *F*. *oxysporum*, Secreted In Xylem (SIX) effector protein was found in xylem sap of a tomato infected with *F*. *oxysporum* f. sp. *lycopersici* (*Fol*) (Rep et al., 2002). Hitherto, 14 SIX proteins have been reported in *Fol* (Rep et al., 2004; Rep et al., 2005; Houterman et al., 2007; Ma et al., 2010). Among the SIX proteins found in *Fol*, SIX1, SIX3, SIX5, SIX6 are involved with pathogenicity within the *Fol*-tomato pathosystem (Gawehns et al., 2014; Houterman et al., 2009; Ma et al., 2015; Rep et al., 2005). Notably, the *SIX* genes are partially conserved within the *Fusarium* species (Haapalainen et al., 2023; Jangir et al., 2021; van Dam and Rep 2017; Watanabe et al., 2023). In the *Foc*, the causal agent of FBR, *SIX3*, *5*, *7*, *9*, *10*, *12*, and *14* genes has been found in their genome. Moreover, correlation between the *SIX* genes and pathogenicity toward onion were suggested (Haapalainen et al., 2022; Sasaki et al., 2015b).

### 1.1.7 Avirulence effector

Effectors are protein secreted by pathogen to exert the pathogenicity toward its host and facilitate host colonization. However, plants have been evolutionally acquired the resistant (R) protein, recognizing the effector to stop the infection development of pathogens with the hypersensitive response (HR), which response is also called effector triggered immunity (ETI) (Cui et al., 2015; Jubic et al., 2019; Tsuda et al., 2009; Yuan et al., 2021). In the case of the effector protein that causes ETI response upon plants, the effector is designated as an “Avirulence effector”. As research on the relationship between plants and pathogens have been gradually developing, many avirulence effector have been identified in a wide variety of pathogens (Catanzariti et al., 2007; De Wit 1997; Rouxel and Balesdent 2010; Valent and Khang 2010). The genetic concept of *F*.

*oxysporum* is well characterized in the interaction between tomato plants and *Fol*. Reportedly, 14 SIX proteins have been identified in *Fol*-infecting tomato xylem sap (Houterman et al., 2007; Schmidt et al., 2013), of which SIX1(AVR3), SIX3 (AVR2)-SIX5, and SIX4 (AVR1) are avirulence effectors that activate the immunity of tomato plants, mediated by *I-3* (SRLK-type), *I-2* (CC-type), and *I* genes, respectively (Catanzariti et al., 2015; Houterman et al., 2009; Ma et al., 2015; Rep et al., 2004; Takken and Rep 2010). These resistance genes have been introduced into commercial tomato cultivars for stable and effective production (Huang and Lindhout 1997). However, *Fol* adopts several strategies to evade the tomato immune system. The *Fol* race 2 strains completely lost the *SIX4* (*AVR1*) gene to avoid the *I* gene-derived immunity (Lievens et al., 2009), whereas the *Fol* race 3 strains had a single amino acid substitution in its SIX3 (AVR2) sequence to escape *I-2*-derived immunity (Houterman et al., 2009). Further, it is known that some of the avirulence effectors were recognized by R protein of the host plant with a physical bind to trigger the innate resistance response (Cesari et al., 2013; Deslandes et al., 2003; Dodds et al., 2006). Therefore, the avirulence effector is important information on the acceleration of disease resistance breeding. Furthermore, engineered R protein that recognizes the various avirulence effector has been developed to create more durable crop toward broad spectrum plant pathogen (Cesari et al., 2022; Liu et al., 2021).

### 1.1.8 Pathogenicity chromosome

In nature, some organisms possess “B chromosome”, are conditionally dispensable (CD) chromosome contrast to “Autosome”, are necessary chromosome, that contains indispensable genomic information for life processes. In plant pathogenic fungi, the genome is frequently divided into core and CD regions. The core region encodes

housekeeping genes, whereas the CD region frequently harbors pathogenicity-related factors, toxin-related genes, metabolic gene clusters, and effector genes (Hatta et al., 2002; Ma et al., 2010; Witte et al., 2021). For example, the gene cluster for host-specific toxins is located on the small CD chromosome in *Alternaria alternata*, and the small CD chromosome in *Colletotrichum higginsianum* is necessary to suppress tryptophan-derived defense responses and causes diseases in *Arabidopsis thaliana* (Hatta et al., 2002; Plaumann et al., 2018). The CD chromosome that dominates pathogenicity toward the host is also called the pathogenicity chromosome in plant pathogens. In the *F. oxysporum* species complex, CD chromosomes have been reported in *Fol*, *F. oxysporum* f. sp. *conglutinans* (*Focn*), *F. oxysporum* f. sp. *radicis-cucumerinum* (*Forc*), *F. oxysporum* f. sp. *melonis*, *F. oxysporum* f. sp. *medicaginis*, *F. oxysporum* f. sp. *ciceris*, *F. oxysporum* f. sp. *pisi*, endophytic *F. oxysporum*, and human-infecting *F. oxysporum* (Ayukawa et al., 2021; Constantin et al., 2021; Li et al., 2020b; Ma et al., 2010; van Dam et al., 2017; Williams et al., 2016; Zhang et al., 2020). Moreover, CD chromosomes contain *SIX* genes required for full virulence toward their hosts. Notably, pathogenicity chromosomes are transferable from pathogenic to non-pathogenic strains (Ayukawa et al., 2021; Li et al., 2020a; Li et al., 2020b; Ma et al., 2010; van Dam et al., 2017).

## 1.2 OBJECTIVES

This study aimed to achieve below bullet point.

1. To verify the taxonomic position of *Foc* in the newly established epitype of *F. oxysporum*.

2. To clarify the pathogenicity differentiation toward onion and Welsh onion in *Foc* .

3. To identify the pathogenicity chromosome in *Foc*.

4. To identify the pathogenicity related factor in *Foc*.

# CHAPTER 2

# INSIGHT INTO PATHOGENICITY DIFFERENTIATION TOWARD ONION AND WELSH ONION IN *FUSARIUM OXYSPORUM* F. SP. *CEPAE*

## 2.1 INTRODUCTION

*Fusarium oxysporum* species complex is a cosmopolitan, soil-borne, filamentous fungus infecting over 120 economically important crops (Leslie and Summerell 2006). *F. oxysporum* species are divided into "*formae speciales*" according to host specificity and further subdivided into "race" based on cultivar-specific pathogenicity, which renders *F. oxysporum* taxonomy complicated (Edel-Hermann and Lecomte 2019). To classify the *F. oxysporum* species, the genetic similarity of the gene loci, including internal transcribed spacer (ITS) region, mitochondrial small subunit (mtSSU), RNA Polymerase 1 and 2 (*rpb1* and *rpb2*), and translation elongation factor 1-alpha (*tef1*), has been investigated (Maryani et al., 2019; O'Donnell et al., 1998; Sasaki. 2015a; Southwood et al., 2012). However, the identification of *Fusarium* species using a single locus is insufficient to distinguish between different *Fusarium* species (O'Donnell, K., and Cigelnik, E 1997). The epitype of *F. oxysporum* has been established based on multiple loci, including calmodulin (*cmdA*), *rpb2*, and *tef1* genes, using phylogenetic analysis to correct the taxonomic classification of *F. oxysporum* (Lombard et al., 2019). Moreover, phylogenetic analysis using multi-locus sequences has been used to prevent the incorrect identification of *F. oxysporum* (Achari et al., 2020; Maryani et al., 2019; Medeiros et al., 2021).

Among *F. oxysporum* strains, *F. oxysporum* f. sp. *cepae* (*Foc*) is the causative agent of

FBR and FW in onions and Welsh onions, which negatively affect the onion and Welsh onion production worldwide (Dissanayake et al., 2009a; Haapalainen et al., 2016; Haapalainen et al., 2023; Sasaki et al., 2015b; Southwood et al., 2012). Therefore, many studies have focused on the pathogenicity-related factors in *Foc* to understand the mechanisms by which *Foc* infects onion and Welsh onions. *Secreted in xylem* (*SIX*) genes promote colonization and host infection in *F. oxysporum* (An et al., 2019; Gawehns et al., 2014; Ma et al., 2010; Thatcher et al., 2012). *SIX* genes are frequently found on a unique chromosome, known as the pathogenicity chromosome, in *F. oxysporum*. Pathogenicity chromosomes are necessary for pathogenicity to determine the host range and are transferable among strains (Ayukawa et al., 2021; Li et al., 2020b; Ma et al., 2010; van Dam et al., 2017; Williams et al., 2016). *F. oxysporum* have been evolutionally acquired the virulence factor via chromosome transfer to exert host-specific pathogenicity toward individual plant species. Likewise, it has also been reported that pathogenicity differentiation of *F. oxysporum* is correlated with individual phylogenetic evolution (O'Donnell et al., 1998).

To date, *SIX2*, *3*, *5*, *7*, *9*, *10*, *12*, and *14* gene homologs have been identified in the genome of onion- and Welsh onion-infecting *Foc* strains (Armitage et al., 2018; Sakane et al., 2023). In the onion-infecting *Foc*, *SIX3*, *5*, *7*, *9*, *10*, *12*, and *14* genes are located on a single pathogenicity chromosome that is required for full pathogenicity toward onion (Chapter 3 in this study). Additionally, *SIX3*, *5*, and *9* genes are highly upregulated during *Foc* infection of onion (Armitage et al., 2018; Taylor et al., 2016). *Foc* strains showing high expression levels of the *SIX9* gene are relatively more pathogenic toward onions than those showing low expression levels of *SIX9* gene (Haapalainen et al., 2022). Furthermore, *SIX2* and *SIX9* genes are located on a 3-Mb chromosome and expressed in

Welsh onion-infecting *Foc* (Sakane et al., 2023). Pathogenicity-related factors have been extensively elucidated in *Foc* strains; however, the reasons for their host specificity toward onions and Welsh onions remain unknown.

This study aimed to examine the taxonomic position of *Foc*, the causal agent of FBR and FW in onions and Welsh onions, in the newly established epitype of *F*. *oxysporum* and clarify their host specificity for onions and Welsh onions.

## 2.2 MATERIALS AND METHODS

### 2.2.1. Fungal strain and plant material

Fungal isolates previously identified as *F. oxysporum* based on morphology and ribosomal DNA ITS region sequences, used in Chapter 2 are listed in Table 1. The onion cv. “Kitamomiji 2000” (Shippou Co., Ltd., Kagawa, Japan) and Welsh onion cv. “Fuyuhiko” (Nakahara Seed Product Co., Ltd., Fukuoka, Japan) were used in Chapter 2.

**Table 1.** *Fusarium* species isolated from the diseased onions and Welsh onion seedlings in Chapter 2.

| | | | *SIX* gene | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Isolate | Host | Origin | 2 | 3 | 5 | 7 | 9 | 10 | 12 | 14 |
| AC145 | Onion | Netherlands | − | + | + | + | + | + | + | + |
| AP117 | Onion | Australia | − | + | + | + | + | + | + | + |
| Ru-13 | Onion | Russia | − | + | + | + | + | + | + | + |
| TA | Onion | Japan | − | + | + | + | + | + | + | + |
| AF67 | Welsh onion | Japan | − | − | − | − | − | − | − | − |
| AF74 | Welsh onion | Japan | + | − | − | − | − | − | − | − |
| AF97 | Welsh onion | Japan | − | − | − | − | − | − | − | − |
| AF113 | Welsh onion | Japan | − | − | − | − | + | − | − | − |
| AF17 | Welsh onion | Japan | + | − | − | − | + | − | − | − |
| AF22 | Welsh onion | Japan | + | − | − | − | + | − | − | − |
| AF90 | Welsh onion | Japan | + | − | − | − | + | − | − | − |
| AF94 | Welsh onion | Japan | + | − | − | − | + | − | − | − |

### 2.2.2. DNA isolation, polymerase chain reaction (PCR) and sequencing

Total genomic DNA was extracted from each fungal isolate cultured in the potato dextrose broth (PDB) medium (Becton, Dickinson and Company, Franklin lakes, USA) for five days at 25 °C, and the fungal mycelia were harvested by filtering the broth through a sterile filter paper (Advantec, Tokyo, Japan). Fungal DNA was extracted from the harvested mycelia using the Dr. GenTLE (from yeast) High Recovery Kit (TAKARA, Osaka, Japan).

For PCR and sequencing analysis, 20 μL reaction mixture containing 10 μL of Quick Taq HS Dye Mix (Toyobo, Osaka, Japan), 0.2 μM of designated primer, and 20 ng of genomic DNA was used. Thermal cycling conditions consisted of 2 min at 94 °C, followed by 30 cycles of 30 s at 94 °C, 30 s at 55–68 °C, and 1 min at 68 °C. PCR products were separated via electrophoresis on a 1.2% (w/v) agarose gel (NIPPON GENE, Tokyo, Japan), stained with ethidium bromide (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), and visualized under UV light. For sequencing analysis, the PCR amplicon was purified via ethanol precipitation and labeled with the BigDye Terminator v3.1 Cycle sequencing Kit (Applied Biosystems, Foster City, CA, USA). The labeled DNA amplicons were sequenced using the ABI 3500xL genetic analyzer (Applied Biosystems). The primers used in Chapter 2 are listed in Table 2. The sequences of calmodulin (*cmdA*), RNA polymerase II second-largest subunit (*rpb2*), and translation elongation factor 1 (*tef1*) of each strain used in this study have been deposited into the DNA Data Bank of Japan.

**Table 2.** Primer used in chapter 2.

| Primer name | Sequence (5'-3') | purpose | Reference |
|---|---|---|---|
| CAL-228F | GAGTTCAAGGAGGCCTTCTCCC | Sequencing for *cmdA* | Carbone and Kohn (1999) |
| CAL2Rd | TGRTCNGCCTCDCGGATCATCTC | Sequencing for *cmdA* | Groenewald et al. (2013) |
| EF-1 | ATGGGTAAGGARGACAAGAC | Sequencing for *tef1* | O'Donnell et al. (1998) |
| EF-2 | GGARGTACCAGTSATCATGTT | Sequencing for *tef1* | O'Donnell et al. (1998) |
| 5f2 | GGGGWGAYCAGAAGAAGGC | Sequencing for *rpb2* | Reeb et al. (2004) |
| 7cr | CCCATRGCTTGYTTRCCCAT | Sequencing for *rpb2* | Liu et al. (1999) |
| SIX2-F | TCCTCAACGGCGCATTAGTC | Detection of *SIX2* | Chapter 2 |
| SIX2-R | GCAAGATGACTGTGAACCCAC | Detection of *SIX2* | Chapter 2 |
| SIX3-C-F | CATGTCCATGACATGGGTTTGC | Detection of *SIX3* | Sasaki. (2015a) |
| SIX3-C-R | GATCTCGTAGTTGGCGATGGC | Detection of *SIX3* | Sasaki. (2015a) |
| SIX5-C-F | GCGCTTCGAGTACATCTCTG | Detection of *SIX5* | Sasaki. (2015a) |
| SIX5-C-R | CTAGGATGCATCACAATAGA | Detection of *SIX5* | Sasaki. (2015a) |
| SIX7-Q-F | CTTTTCCATTTCGCCCTGTTT | Detection of *SIX7* | Chapter 3 |
| SIX7-Q-R | TGGTGTTGAGCCGGTGCTA | Detection of *SIX7* | Chapter 3 |
| SIX9-F | TCAGCAGTTGCGGCAATGAC | Detection of *SIX9* | Chapter 2 |
| SIX9-R | CACAGTGCATTGTCCCATCTC | Detection of *SIX9* | Chapter 2 |
| SIX10-Q-F | TTTGCTCAAGGCTTCAACCA | Detection of *SIX10* | Chapter 3 |
| SIX10-Q-R | CGCGGTCCCGTAGATTAGTC | Detection of *SIX10* | Chapter 3 |
| SIX12-Q-F | GTTCTCGCCCAAAGCCATT | Detection of *SIX12* | Chapter 3 |
| SIX12-Q-R | TCACCTCCGGCTCGTAGTT | Detection of *SIX12* | Chapter 3 |
| SIX14-Q-F | CTTGGATGTCGTATGCCGGA | Detection of *SIX14* | Chapter 3 |
| SIX14-Q-R | GCCTTCCTTACCTTGGACCC | Detection of *SIX14* | Chapter 3 |
| FoTEF-Q2-F | CATCGGCCACGTCGACTCT | Detection of *TEF* | van der Does et al. (2008) |
| FoTEF-Q2-R | AGAACCCAGGCGTACTTGAA | Detection of *TEF* | van der Does et al. (2008) |

### 2.2.3. Phylogenetic analysis

Multi-locus based phylogenetic tree was constructed using Maximum Likelihood (ML) method. The sequence of the individual loci, including *cmdA*, *rpb2*, and *tef1* were aligned using Clustal 2.1 (Thompson et al., 1994). A phylogenetic tree was constructed using MEGA 11 (Tamura et al., 2021).

### 2.2.4. Onion bulb inoculation test

Each strain was grown on the potato dextrose agar (PDA) medium (SHIOTANI M.S, Hyogo, Japan) and incubated in a growth chamber at 25 °C for five days. Onion bulbs were surface-sterilized with 0.05% NaOCl for 3 min. Then, the central basal part of the sterilized onion bulbs was hollowed out with a 5-mm cork borer. The edge of the colony was also hollowed out with a 5-mm cork borer and embedded in the hollowed basal part of the sterilized onion bulbs. A planar PDA medium plug was embedded in the basal tissue of hollowed-out onions as a control. The inoculated onion bulb was placed inside a plastic bag with a wet paper towel and incubated at 25 °C in a temperature-controlled room. After three weeks, symptoms in the inoculated onion bulbs were observed. Symptomatic areas, including mycelia and brown discoloration regions, were manually captured and estimated from the photographs using the ImageJ software (Figure 3) (Schneider et al., 2012). All tests were conducted with at least three samples per iteration. All experiments were repeated twice.

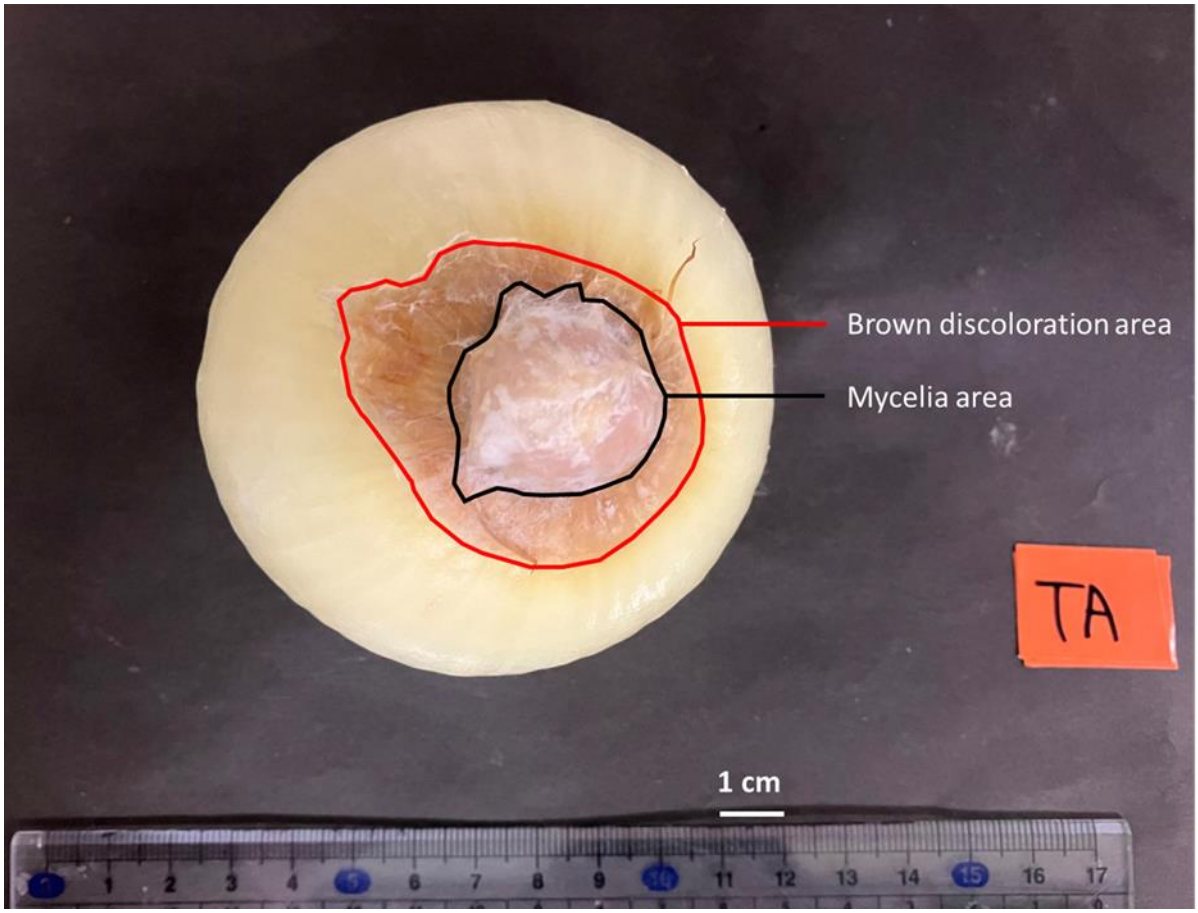


**Figure 3.** Evaluation of symptom area on onion inoculated with *Foc*. Encircled area with black or red line indicates symptom area, including mycelia and brown discoloration area, respectively. The white line indicates 1 cm scale in this photo.

### 2.2.5. Welsh onion seedling inoculation test

Fungal isolates were cultured in the PDB medium for seven days in a growth chamber at 25 °C, with shaking at 120 rpm, and subsequently filtered through three layers of sterilized gauze to collect the spores for inoculation. The spores were rinsed once with sterile water. The number of spores in the suspension was determined using a hemocytometer (Erma Inc., Saitama, Japan) and adjusted to a concentration of $1 \times 10^4$ spores/mL. Welsh onion seeds were surface-disinfected with 0.05% NaOCl for 10 min and rinsed with water for 10 min. The disinfected seeds were inoculated with a spore suspension ($1 \times 10^4$ spores/mL) of each fungal strain or sterilized water (as a control) for 1 h. After treatment, the seven inoculated Welsh onion seeds were sown into plastic pots containing a 4:1 sand:compost mixture and incubated in a growth chamber at 25 °C under a 16 h light/8 h dark photoperiod. Finally, the lengths of Welsh onion seedlings were measured 21 days post-inoculation, and the average length of the leaves was calculated.

### 2.2.6. RNA extraction and reverse-transcriptase polymerase chain reaction (RT-PCR)

For RT-PCR, RNA was extracted from the Welsh onion roots and onion bulbs inoculated with each strain at 7- or 21-d post-inoculation using Sepasol-RNA I Super G (Nacalai Tesque, Inc., Kyoto, Japan). Then, the total RNA containing genomic DNA was treated with DNase I (TAKARA) to isolate the genomic DNA. PrimeScript RT-PCR Kit (TAKARA) was used to synthesize cDNA, following the manufacturer's instructions. For RT-PCR of *SIX* genes, 20 μL reaction mixture containing 10 μL of Quick Taq HS Dye Mix (Toyobo), 0.2 μM of designated primer, and 1 μL of cDNA was used. Thermal cycling conditions consisted of 2 min at 94 °C, followed by 30 cycles of 30 s at 94 °C, 30

s at 55 °C, and 1 min at 68 °C. PCR products were separated via electrophoresis on a 1.2% (w/v) agarose gel (NIPPON GENE), stained with ethidium bromide (FUJIFILM Wako Pure Chemical Corporation), and visualized under UV light.

### 2.2.7. Statistical analysis

Experimental data were represented as the standard error of the mean. Statistically significant differences were determined using One-way ANOVA post hoc Tukey HSD test.

## 2.3 RESULTS

### 2.3.1. Verification of taxonomic position of *Foc* strains in the newly established epitype of *F. oxysporum*

To verify the taxonomic position of *Fusarium* species strains (previously described as *Foc*) in the newly established epitype of *F. oxysporum*, phylogenetic analysis of the combined sequences of *cmdA*, *rpb2*, and *tef1* genes of each strain was conducted. Based on the phylogenetic analysis results, eight isolates (AC145, AP117, Ru-13, TA, AF67, AF74, AF97, and AF113) were grouped into a clade of *F. nirenbergiae*, whereas four isolates (AF17, AF22, AF90, and AF94) were grouped into a clade of *Fusarium* sp. (Figure 4).

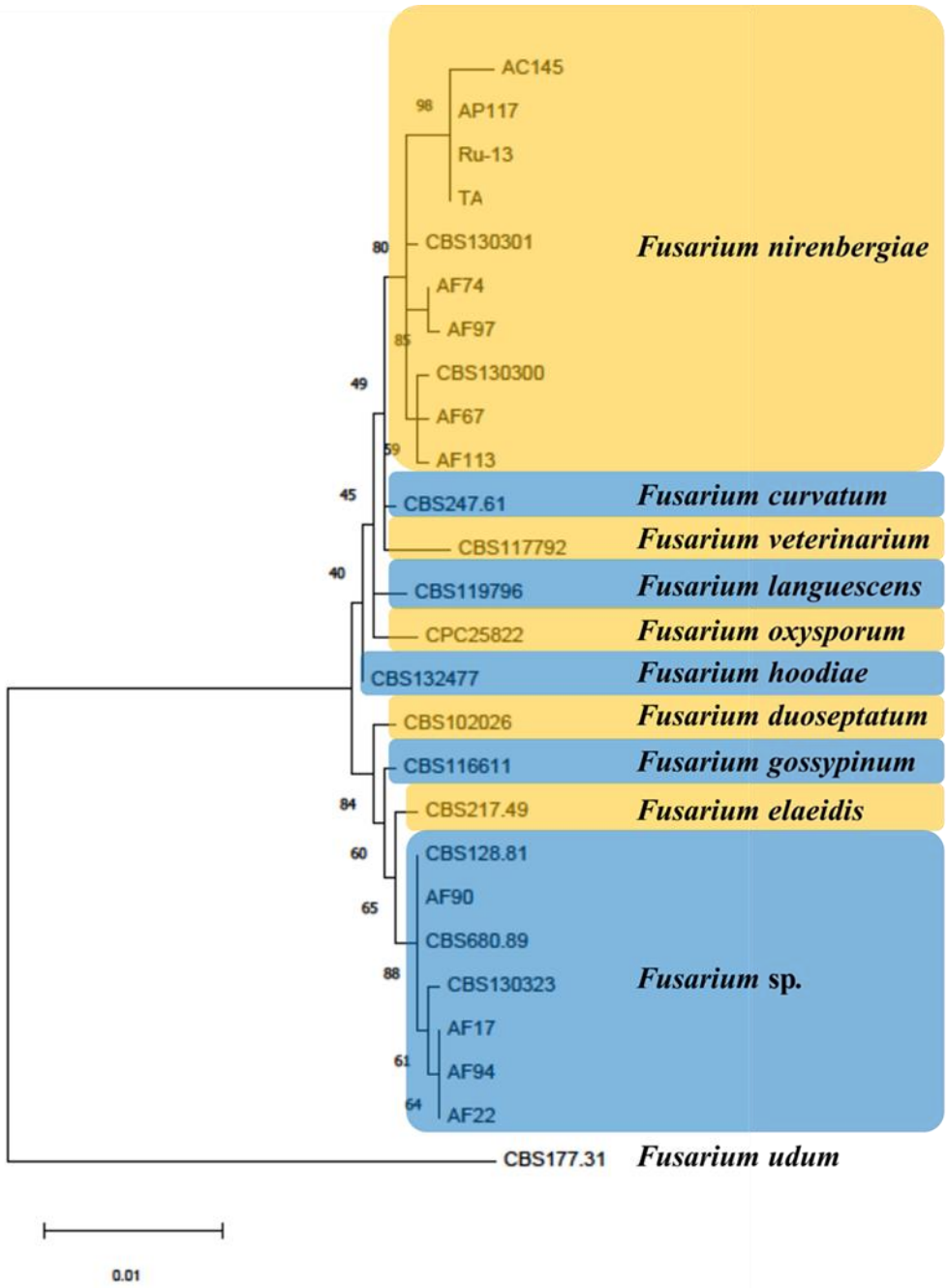


**Figure 4.** Maximum Likelihood (ML) phyloglam inferred from the sequence from the sequence of the *cmdA*, *rpb2*, and *tef1* of isolates from strains used in Chapter 2. The numbers on branches shows the percentages of congruent clusters in 1,000 bootstrap trials.

### 2.3.2. Variation of *SIX* gene possession and gene expression profile

Pathogenicity is associated with the presence of *SIX* genes in *F. oxysporum*. Here, the presence of *SIX* genes using PCR was verified to assess the diversity of *SIX* genes in the strains isolated from onions and Welsh onion seedlings. PCR analysis revealed that the strains (AC145, AP117, Ru-13, and TA) isolated from onions commonly harbored *SIX3*, *5*, *7*, *9*, *10*, *12*, and *14* genes. In contrast, the strains isolated from Welsh onion seedlings (AF17, AF22, AF90, and AF94) commonly harbored the *SIX2* and *SIX9* genes. Notably, AF74 possessed only the *SIX2* gene, and AF113 possessed only the *SIX9* gene. Moreover, AF67 and AF97 had no *SIX* genes in their genome (Table 1).

*SIX3*, *5*, and *9* gene expression levels are highly upregulated in onion-infecting *Fusarium* strains, whereas *SIX2* and *SIX9* genes are expressed in Welsh onion-infecting *Fusarium* strains, suggesting those *SIX* genes were involved with pathogenicity of the strains toward its respective hosts (Armitage et al., 2018; Sakane et al., 2023). Here, the *SIX3*, *5*, and *9* gene expression in the *Fusarium* strains isolated from onions and *SIX2* and *SIX9* gene expression in the *Fusarium* strains isolated from Welsh onion seedlings during infection were investigated. Gene expression analysis revealed that the *Fusarium* strains isolated from onions expressed *SIX3*, *5*, and *9* not only during onion infection but also during Welsh onion infection. In contrast, *Fusarium* strains isolated from Welsh onion seedlings only expressed *SIX9* during both onion and Welsh onion infections. Notably, gene expression of *SIX2* was dependent on the microbial strain and host. (Figure 5).

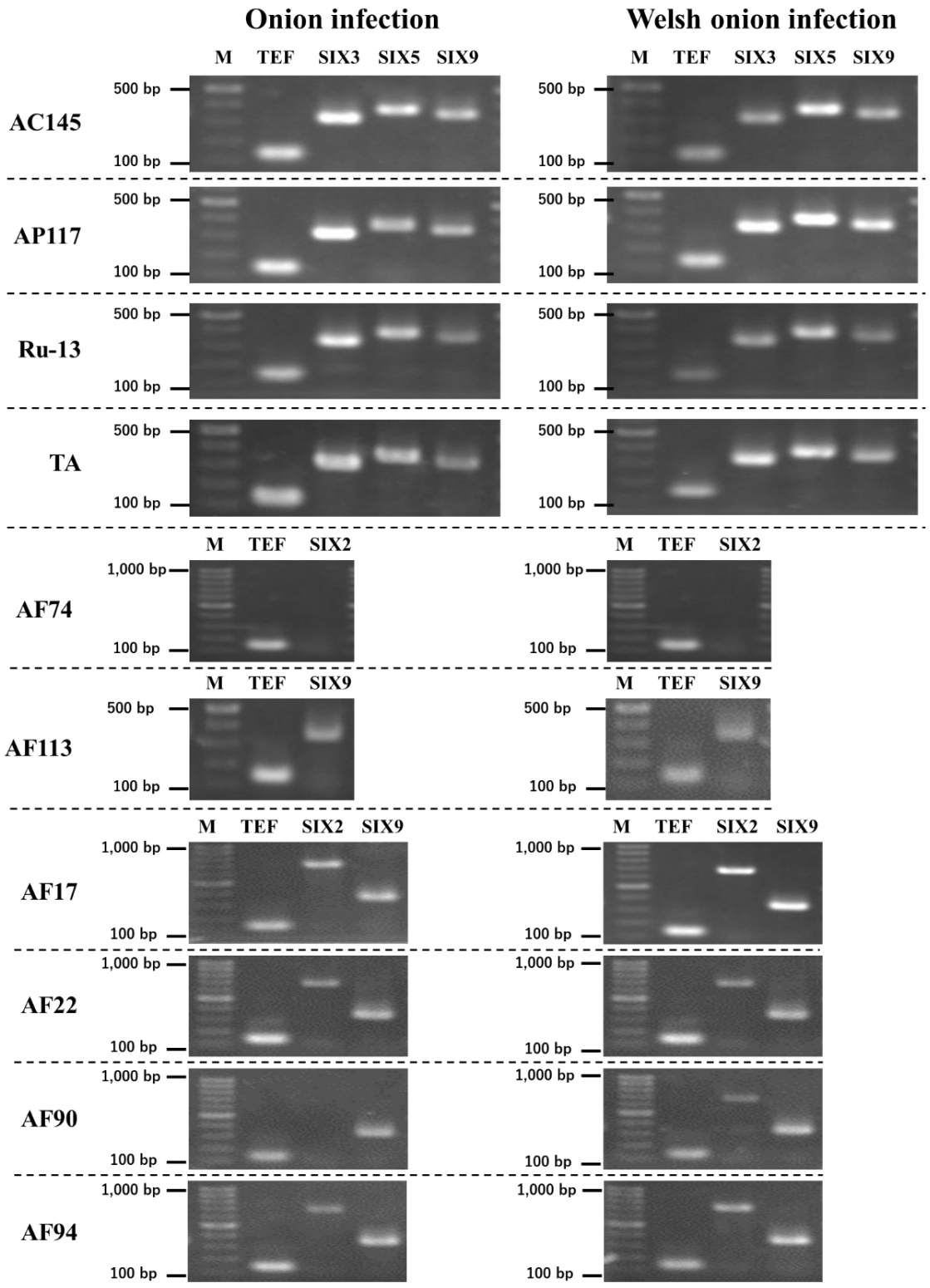


**Figure 5.** Gene expression of *SIX2*, *SIX3*, *SIX5*, and *SIX9* during infection of onion and Welsh onion. *TEF* gene is used as positive control.

### 2.3.3. Inoculation tests toward onion bulbs and Welsh onion seedlings

The strains used in Chapter 2 were isolated from onions and Welsh onion seedlings. Hence, the host specificity of the strains for onion and Welsh onions was investigated. To determine the pathogenicity toward onions, inoculation tests were performed on onion bulbs. Notably, all strains (AC145, AP117, Ru-13, and TA) isolated from onions caused severe disease symptoms in the onion bulbs, whereas the strains (AF17, AF22, AF67, AF74, AF90, AF94, AF97, and AF113) isolated from Welsh onion seedlings caused significantly fewer disease symptoms in the onion bulbs than those isolated from onions (Figure 6). Moreover, the symptoms in bulb onions caused by the strains (AF17, AF22, AF67, AF74, AF90, AF94, AF97, and AF113) were comparable to those observed in the control, with no significant difference. The inoculation test of Welsh onion seedlings revealed that all isolates, except AF97, caused significant inhibition of Welsh onion growth (Figure 7).

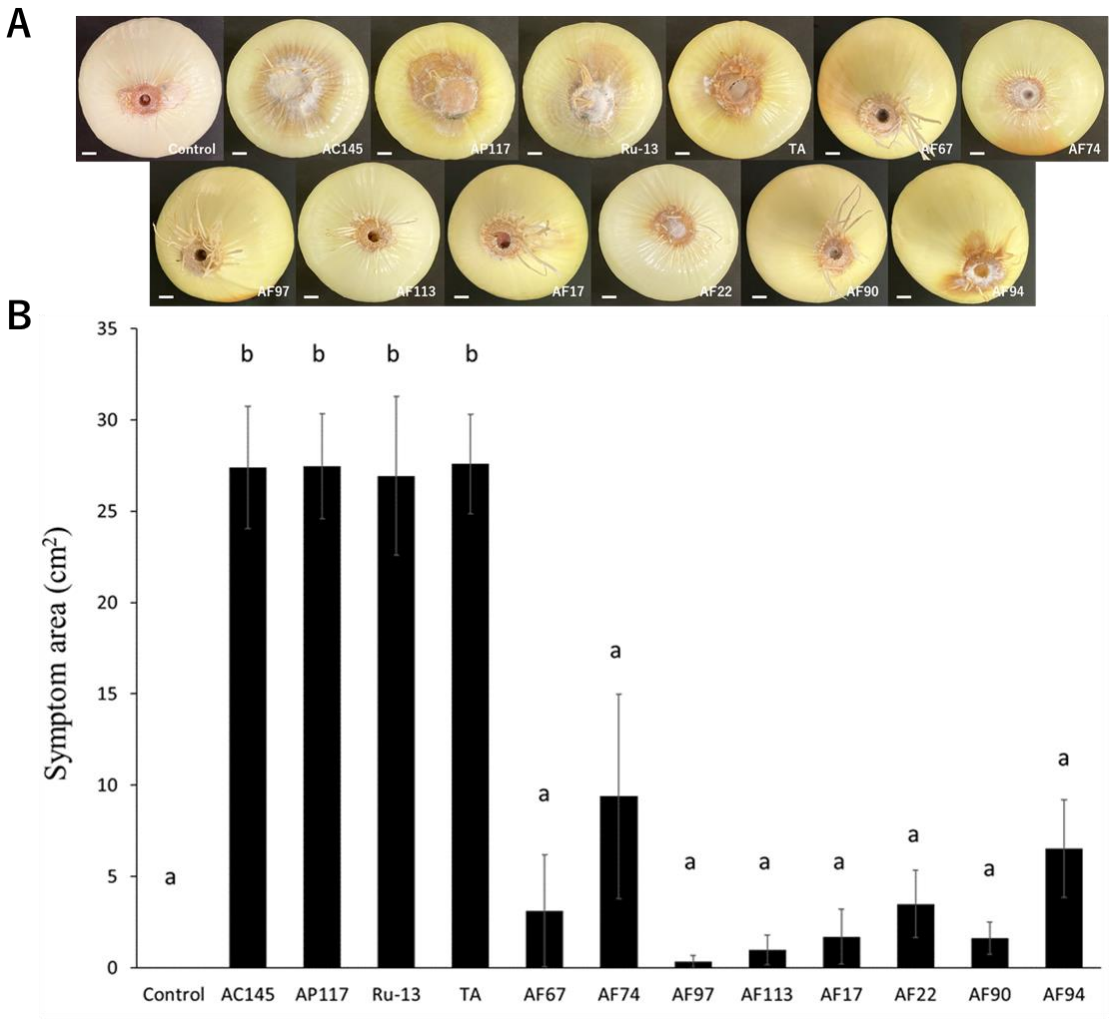


**Figure 6.** Onion bulb inoculation test. (A) Representative photograph of onion bulb inoculation test with *Fusarium* species isolates used in Chapter 2. The white bar indicates the 1 cm scale bar. (B) Evaluation of symptom area in inoculated onion bulbs. The results of two independent experiments were combined. The statistically significant difference at $p < 0.05$ was analyzed by One-way ANOVA post hoc Tukey HSD test. All data were presented as the standard error of the mean (n = 6).

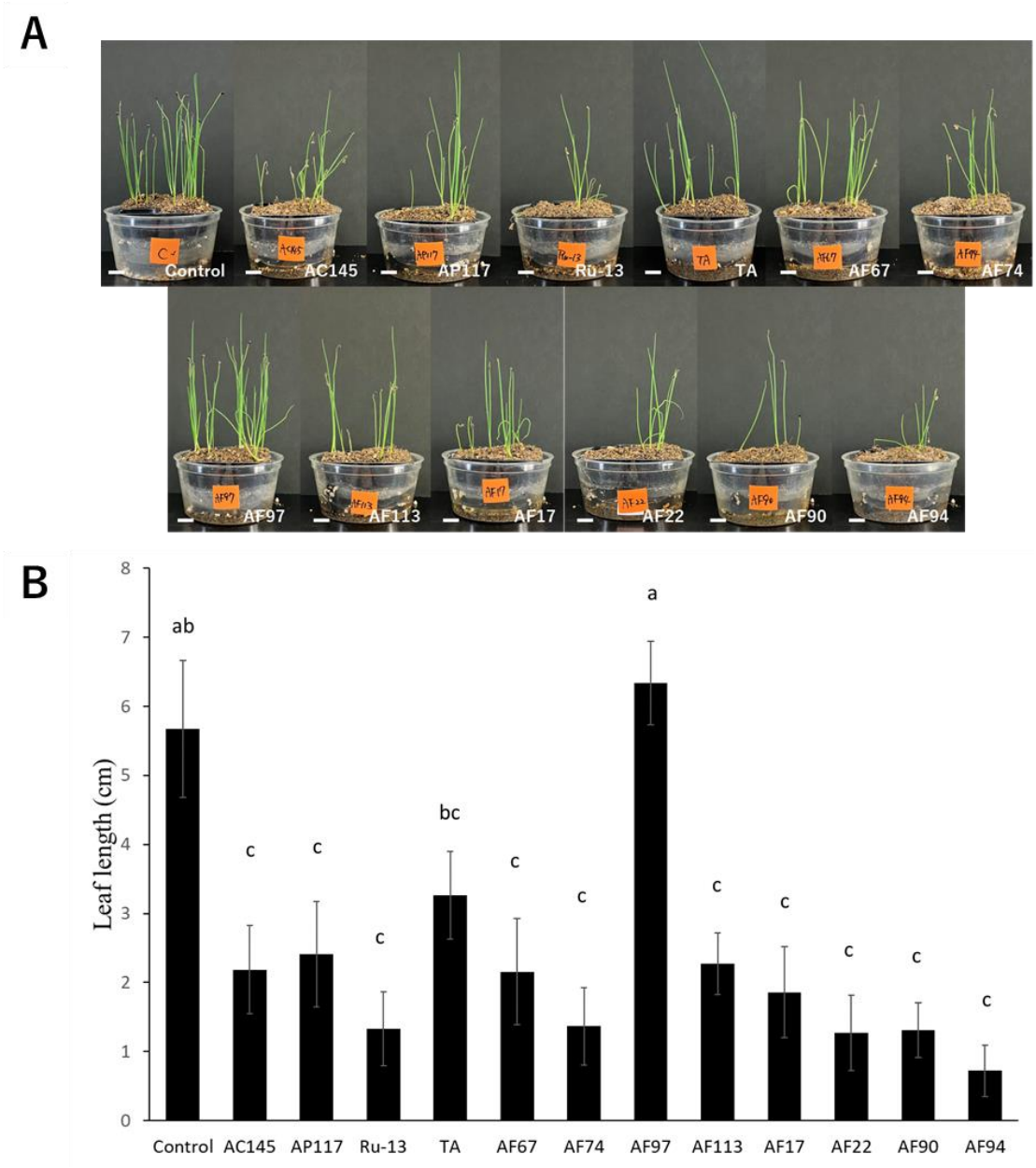


**Figure 7.** Welsh onion seedling inoculation test. (A)Representative photograph of Welsh onion seedling inoculation test with *Fusarium* species isolates used in Chapter 2. The white bar indicates the 1 cm scale bar. (B) Evaluation of average leaf length of Welsh onion seedling. The results of two independent experiments were combined. The statistically significant difference at $p < 0.05$ was analyzed by One-way ANOVA post hoc Tukey HSD test. All data were presented as the standard error of the mean (n = 49).

## 2.4 DISCUSSION

Plants are cultivated globally to meet the human food demand. However, cultivated plants are at a risk of infection by pathogenic fungi, bacteria, and viruses (Dean et al., 2012; Mansfield et al., 2012; Scholthof et al., 2011). Furthermore, due to the global climate change caused by global warming, plants and plant pathogenic organisms are shifting to more ideal places than harsh environments to continue their life cycle (Chakraborty 2013). Therefore, precise identification of plant pathogenic organisms and understanding of their host specificity are important for sustainable crop production and disease management.

In Chapter 2, phylogenetic analysis based on multi-locus sequencing of *cmdA*, *rpb2*, and *tef1* sequences, *SIX* gene analysis, and inoculation tests were performed to explore the taxonomic position of *Foc* in the newly established epitype of *F. oxysporum* and assess the host specificity of *Foc* causing the FBR and FW in onions and Welsh onions.

Based on the phylogenetic analysis of the combined sequences of *cmdA*, *rpb2*, and *tef1*, the isolates used in Chapter 2 were divided into two clades, *F. nirenbergiae,* and *Fusarium* sp., in the newly established epitype of *F. oxysporum*. Notably, *F. oxysporum* strains belonging to the same *formae speciales* were divided into different clades in the newly established epitype of *F. oxysporum*, suggesting that pathogenicity-related factors determining the host range are transferred via horizontal gene or chromosome transfer (Lombard et al., 2019). This implies the transfer of pathogenicity-related factors among the *Fusarium* strains isolated from onions and Welsh onion seedlings.

To promote host infection, plant pathogenic organisms secrete effector proteins into the host tissue. In the *Fusarium* species, SIX proteins, known as effector, secretes into xylem tissue have been broadly found (Haapalainen et al., 2022; Rep et al., 2004; Watanabe et

al., 2023). Therefore, *SIX* gene possession analysis was conducted on the strains isolated from onions and Welsh onion seedlings. As a result of the *SIX* gene possession analysis using PCR, the strains (AC145, AP117, Ru-13, and TA) isolated from onions commonly possessed *SIX3*, *5*, *7*, *9*, *10*, *12*, and *14*, whereas the strains isolated from Welsh onion seedlings harbored both *SIX2* and *SIX9* (AF17, AF22, AF90, and AF94), *SIX2* (AF74), *SIX9* (AF113), or no *SIX* genes (AF67 and AF97). Notably, according to the results of the inoculation test for Welsh onion seedlings, all strains except AF97 were pathogenic to Welsh onions. This indicates that the AF97 strain is opportunistic toward Welsh onions. Among the strains used in Chapter 2, AF17, 22, 90, and 94, which have *SIX2* and *SIX9* genes, caused relatively uniform severe disease in Welsh onion seedlings. Furthermore, the *SIX2* and *SIX9* genes are located on a single 3-Mb chromosome in the Welsh onion-infecting *Fusarium* strain (Sakane et al., 2023). These results imply that the 3-Mb sized chromosome containing *SIX2* and *SIX9* genes was evolutionarily acquired from other strains or constructed the 3-Mb sized chromosome themselves, finally leading to pathogenicity toward Welsh onion. However, obvious relationship between pathogenicity toward Welsh onion and the *SIX* gene was not observed in Chapter 2 because the AF67 strains were pathogenic toward Welsh onion but did not harbor any *SIX* genes, and the gene expression of *SIX2* was dependent on the strain and host. Thus, further investigation of various *Fusarium* strains isolated from diseased Welsh onion seedlings would be helpful in understanding pathogenicity differentiation and the relationship between pathogenicity toward Welsh onion and *SIX* genes.

Inoculation tests toward onion bulbs and Welsh onion seedlings revealed that only the strains (AC145, AP117, Ru-13, and TA) isolated from onions exerted aggressive pathogenicity toward onion bulbs, whereas all isolates, except for AF97, caused wilt

symptoms toward Welsh onion seedlings, implying that onion-infecting *Fusarium* strains evolutionarily acquired the *SIX3*, *5*, *7*, *9*, *10*, *12*, and *14* genes for enhanced onion pathogenicity. Previous studies (Sasaki et al., 2015b; Taylor et al., 2016) reported that *Fusarium* species strains containing *SIX3* and *SIX5* show relatively higher pathogenicity toward onions than those not containing these *SIX* genes, which is consistent with these results. All *SIX* genes (*SIX3*, *5*, *7*, *9*, *10*, *12*, and *14*) in the onion-infecting Japanese TA strain are located on a single pathogenicity chromosome (Chapter 3 in this study). Therefore, *SIX* genes (*SIX3*, *5*, *7*, *9*, *10*, *12*, and *14*) possessed by strains isolated from onions were evolutionarily acquired via horizontal chromosome transfer to enhance the pathogenicity toward onions. Notably, the strains isolated from onions in Chapter 2 were collected from Japan, Australia, the Netherlands, and Russia. Moreover, the strains (AC145, AP117, Ru-13, and TA) exhibited a common *SIX* gene profile and caused severe disease in onion bulbs, implying the global distribution of the clonal pathogenic *Fusarium* strains. Indeed, the Japanese TA strain used in Chapter 2 exhibited high genome similarity to the pathogenic British *Foc*_FUS2 strain (Chapter 3 in this study).

Here, inoculation tests toward onion bulbs and Welsh onion seedlings revealed that the strains harboring *SIX9* genes were aggressively pathogenic in at least either onion and Welsh onion, indicating the importance of *SIX9* in pathogenicity. Gene expression of *SIX9* is upregulated during infection and associated with the degree of pathogenicity toward onions (Duan et al., 2020; Haapalainen et al., 2022; Taylor et al., 2016). Here, *SIX9* gene was expressed during infection of both onions and Welsh onions, suggesting its roles in the pathogenicity toward onions and Welsh onions. Moreover, *SIX9* gene might involve with a common invasion function in various plant species rather than host-specific pathogenicity. Indeed, in the *F. oxysporum* spp., *F. oxysporum* f. sp *cubense*, *lycopersici*,

*narcissi*, *niveum*, *palmarum*, *pisi*, *radicis-cucumerinum*, *raphani*, *sesame*, *spinaciae*, *Arabidpsis*-infecting *F. oxysporum*, and non-pathogenic *F. oxysporum* possess the *SIX9* gene (Batson et al., 2021; Chu et al., 2023; Constantin et al., 2021; Duan et al., 2020; Hudson et al., 2021; Jenkins et al., 2021; Ponukumati et al., 2019; Taylor et al., 2016; Taylor et al., 2019b; Thatcher et al., 2012; van Dam et al., 2017). Recently, a gene knockout approach was used to reveal the functions of *SIX* genes. Therefore, further research on the *SIX9* gene using gene knockout approach is necessary to clarify the specific roles of the *SIX9* gene in pathogenicity.

In summary, the strains previously identified as *Foc* were classified into *F. nirenbergiae* and *Fusarium* sp., but not *F. oxysporum*, in the newly established epitype of *F. oxysporum* via phylogenetic analysis of the combined sequences of *cmdA*, *rpb2*, and *tef1*. Furthermore, to verify the host specificity of the strains isolated from onions and Welsh onion seedlings, inoculation tests were performed on onions and Welsh onions as potential hosts. The results revealed the diverse host specificity of the *Fusarium* strains isolated from onions and Welsh onion seedlings. The inoculation tests also provided insights into the evolutionary pathogenicity differentiation of the *Fusarium* strains causing the FBR and FW. A non-pathogenic *Fusarium* strain evolved to become pathogenic in Welsh onions via the vertical or horizontal acquisition of pathogenicity-related factors. Subsequently, the *Fusarium* strains likely evolved pathogenicity toward onions via the acquisition of pathogenicity chromosomes containing *SIX* genes. As only 12 strains were investigated in Chapter 2, more strains should be analyzed in future studies to verify the above hypothesis. Nevertheless, this study would provide more certain proof or insight into pathogenicity differentiation in the causative agent of FBR and FW in *Allium* species.

# CHAPTER 3

# IDENTIFICATION OF PATHOGENICITY CHROMOSOME IN *FUSARIUM OXYSPORUM* F. SP. *CEPAE*

## 3.1 INTRODUCTION

Onion (*Allium cepa* L.) is a globally important vegetable, with the production of dry onions having reached 104.5 million tons in 2020 (FAOSTAT, 2022). However, onions are threatened by plant pathogenic fungi, including *Fusarium oxysporum*, which lead to substantial production losses (Dissanayake et al., 2009b; Kodama. 1983; Takakuwa et al., 1977).

The *F*. *oxysporum* species complex is a ubiquitous, soil-borne, plant pathogenic fungus with a broad host range of more than 120 species and has recently been recognized as the fifth most important plant fungal pathogen (Dean et al., 2012; Leslie and Summerell, 2006). Based on its host specificity, *F*. *oxysporum* is subdivided into different "*formae speciales*" (Laurence et al., 2012; Michielse and Rep, 2009), among which *F*. *oxysporum* f. sp. *cepae* (*Foc*) has been identified as the causal agent of FBR and FW in *Allium* species, including onions (Chand et al., 2016; Sasaki et al., 2015b; Schwartz and Mohan, 2008). Plant pathogens secrete small proteins called effectors, which promote colonization in their host and determine host specificity in plant pathogens (Ayukawa et al., 2021; Houterman et al., 2009; Thatcher et al., 2012). Several effector-like proteins have been identified in Welsh onion and onion-infecting *Foc*. Among these, Cep28 has been shown to play a role in the pathogenicity of Welsh onions and fungal growth, exhibiting a trade-off relationship (Sakane et al., 2023). However, the functions of most of the identified

effector candidates in onion-infecting *Foc* have not yet been experimentally investigated.

In plant pathogenic fungi, the genome is divided into core and conditionally dispensable (CD) regions. The core region encodes housekeeping genes, whereas the CD region frequently harbors pathogenicity-related factors, toxin-related genes, metabolic gene clusters, and effector genes (Hatta et al., 2002; Ma et al., 2010; Witte et al., 2021). For example, the gene cluster for host-specific toxins is located on the small CD chromosome in *Alternaria alternata*, and the small CD chromosome in *Colletotrichum higginsianum* is necessary to suppress tryptophan-derived defense responses and causes diseases in *Arabidopsis thaliana* (Hatta et al., 2002; Plaumann et al., 2018). The CD chromosome that dominates pathogenicity toward the host is also called the pathogenicity chromosome in plant pathogens. In addition, CD chromosomes have been reported in *F. oxysporum* f. sp. *lycopersici* (*Fol*), *F. oxysporum* f. sp. *conglutinans* (*Focn*), *F. oxysporum* f. sp. *radicis-cucumerinum* (*Forc*), *F. oxysporum* f. sp. *melonis*, *F. oxysporum* f. sp. *medicaginis*, *F. oxysporum* f. sp. *ciceris*, *F. oxysporum* f. sp. *pisi*, endophytic *F. oxysporum*, and human-infecting *F. oxysporum* (Ayukawa et al., 2021; Constantin et al., 2021; Li et al., 2020b; Ma et al., 2010; van Dam et al., 2017; Williams et al., 2016; Zhang et al., 2020). Moreover, CD chromosomes contain *SIX* (*Secreted In Xylem*) genes required for full virulence toward their hosts. Notably, pathogenic chromosomes are transferable from pathogenic to non-pathogenic strains (Ayukawa et al., 2021; Li et al., 2020a; Li et al., 2020b; Ma et al., 2010; van Dam et al., 2017). The British strain *Foc*_FUS2 harbors the *SIX3*, *5*, *7*, *9*, *10*, *12*, and *14* genes, and these *SIX* genes are located in the 3.9-Mb-sized PS region, comprising four lineage-specific (LS) contigs (contigs 10, 16, 19, and 21) (Taylor et al., 2016; Armitage et al., 2018). Sasaki et al. (2015b) reported that the *SIX3* gene is located on an approximately 4-Mb-sized chromosome in the Japanese strain

*Foc*_TA.

Chapter 3 aimed to identify the pathogenicity chromosomes in *Foc*. Twenty-one candidate effectors were identified from the genome sequence data of *Foc*_TA. Among these candidates, four were expressed during infection and were located on the 4-Mb-sized chromosome in *Foc*_TA. Subsequent assays, including a vegetative growth assay, pathogenicity test, and genome analysis, using *Foc*_TA strain with a loss of the 4-Mb-sized chromosome, demonstrated that this plays a crucial role as a pathogenicity chromosome in *Foc*_TA.

## 3.2 MATERIALS AND METHODS

### 3.2.1. Fungal strain and plant material

The fungal strain *Foc*_TA was isolated from a diseased onion bulb in Hokkaido, Japan (Sasaki et al., 2015b). The onion cv. 'Momiji3go' (Shippou Co., Ltd.) and 'Kitamomiji 2000′ (Shippou Co., Ltd.) were used in Chapter 3.

### 3.2.2. Genome analysis for effector candidate prediction

Genomic DNA of the *Foc*_TA strain was extracted using the PureLink Genomic DNA Mini Kit (Life Technologies, Foster City, CA) and sequenced using SOLiD5500. The acquired reads were initially mapped to the core genome region of FOL4287, which encompasses chromosomes 1, 2, 4, 5, 7, 8, 9, 10, 11, 12, and 13. Subsequently, the unmapped reads that did not align with FOL4287 were extracted and considered as lineage-specific reads for the *Foc*_TA strain. The collected reads were then assembled into contigs in *Foc*_TA using Genomics Workbench (CLC Bio, Aarhus, Denmark). Protein sequences were predicted from lineage-specific contigs using AUGUSTUS (https:// augustus.gobics.de/) with the settings for *Fusarium graminearum* species. Proteins < 350 aa with at least two cysteine residues predicted to be secreted using SignalP 4.1 run under default settings (Nielsen. 2017) were selected as effector candidates. Chromosomal localization of the selected effector candidates in the genome of the *Foc*_FUS2 strain was determined using a BLAST search. Briefly, to identify the location of the identified effector candidates in *Foc*_FUS2 genome, blastp analysis using NCBI BLAST with amino acid sequence of the effector candidates was conducted.

### 3.2.3. Seedling inoculation test

The onion cv. ‘Momiji3go’ was used for the seedling inoculation test. Fungal isolates were cultured in potato dextrose broth (PDB) for 7 days at 25 ◦C with continuous shaking at 120 rpm. The cultures were filtered through three sterilized layers of gauze to collect spores for inoculation and were rinsed once with sterilized water. The number of spores in the suspension was determined using a hemocytometer and adjusted to 1 $\times 10^6$ spores/mL. For the seedling inoculation test, seeds were sown in a plastic pot containing a 4:1 mixture of sand and compost and incubated for 7 days. The seedlings were uprooted, and roots were dipped into the prepared spore suspension for 1 h. The inoculated onion seedlings were then transplanted into plastic pots containing the soil mixture and incubated in a growth chamber at 25 ◦C with a 16 h light/8h dark photoperiod.

### 3.2.4. RNA extraction and reverse-transcriptase polymerase chain reaction (RT-PCR) analysis

Total RNA was extracted from the inoculated onion seedlings (9 days post-inoculation) and fungal mycelia cultured in liquid minimal medium for 5 days using Sepasol-RNA I Super G (Nacalai Tesque Inc.). The extracted RNA was converted into cDNA using a PrimeScript RT-PCR Kit (TAKARA) following the manufacturer’s instructions. cDNA (1 μL) was used as a template in a total 20 μL PCR reaction volume. PCR was performed using designated primers (Table 3).

**Table 3.** Primer used in chapter 3.

| Primer name | Sequence (5'-3') | purpose | Reference |
|---|---|---|---|
| g35-F | CCAGATTCTTTCCGTCCTTG | Detection of *g35* | Chapter 3 |
| g35-R | CTTGGTGTCAACCCACTCTG | Detection of *g35* | Chapter 3 |
| g73-F | ATGCATTAATGCTCGCACGG | Detection of *g73* | Chapter 3 |
| g73-R | AGATGCCTGCGCTTCATTGG | Detection of *g73* | Chapter 3 |
| g150-F | CGCAAGAACCTTGTTCCTCTC | Detection of *g150* | Chapter 3 |
| g150-R | GCTATGAAGCCCTTGTCTAG | Detection of *g150* | Chapter 3 |
| g172-F | CCAATGCTGCAGCACTCAAC | Detection of *g172* | Chapter 3 |
| g172-R | ATACCGTTGTTCCATCGGAC | Detection of *g172* | Chapter 3 |
| g201-F | AACCCAACATGGTTGAGGTG | Detection of *g201* | Chapter 3 |
| g201-R | GACGAGCAACCAAGATTCTG | Detection of *g201* | Chapter 3 |
| g687-F | TGTGCTTTCATTCCGGCTAG | Detection of *g687* | Chapter 3 |
| g687-R | TGTTTGGTGCTTCGTAACCG | Detection of *g687* | Chapter 3 |
| g713-F | ATCGAATGATGGATGCCAGC | Detection of *g713* | Chapter 3 |
| g713-R | ATCTGTTCCTACACAGGTCG | Detection of *g713* | Chapter 3 |
| g759-F | AGTTCACCCTTCCAACTGCG | Detection of *g759* | Chapter 3 |
| g759-R | GTCCAGCATCTCTGACACAC | Detection of *g759* | Chapter 3 |
| g761-F | CGGCTATGATCCGAGGTATC | Detection of *g761* | Chapter 3 |
| g761-R | TTGATGAGCCAGTAGGCCTC | Detection of *g761* | Chapter 3 |
| g785-F | GGAGCTCAAGCAGGTGTTTG | Detection of *g785* | Chapter 3 |
| g785-R | AAGTACCACCCATCCTCAGC | Detection of *g785* | Chapter 3 |
| g938-F | TCCAGGATCGTCTCTCGATG | Detection of *g938* | Chapter 3 |
| g938-R | TCTAGGGCACAGTGGCAATC | Detection of *g938* | Chapter 3 |
| g1064-F | TTGTCCTCAGCTCCTTCCTC | Detection of *g1064* | Chapter 3 |
| g1064-R | AGCTGCAGGGAACCTGAATG | Detection of *g1064* | Chapter 3 |
| g1064-F1 | CCAGGCGTGTATCTTCTTGAG | Disruption of *g1064* | Chapter 3 |
| g1064-F2 | gtcgtgactgggaaaaccctggcgGATTGCTTAGGAGCTGGAGTG | Disruption of *g1064* | Chapter 3 |
| g1064-F3 | tcctgtgtgaaattgttatccgctTGTGTATGTTATGTCTGCGG | Disruption of *g1064* | Chapter 3 |
| g1064-F4 | GAGAGATTTGGCCAAACACC | Disruption of *g1064* | Chapter 3 |
| g1227-F | TCATCTTGACATTCCTCGGC | Detection of *g1227* | Chapter 3 |
| g1227-R | ACGTCGTTTCGGCGGTTAC | Detection of *g1227* | Chapter 3 |
| g1351-F | TAGCCACACTCGTCTCATCG | Detection of *g1351* | Chapter 3 |
| g1351-R | ATCAAGGGCCTCATCAGCTC | Detection of *g1351* | Chapter 3 |
| g1396-F | TACGGCGCATACATGTATCC | Detection of *g1396* | Chapter 3 |
| g1396-R | ACCTACTACGACAGCTACAG | Detection of *g1396* | Chapter 3 |

| | | | |
|---|---|---|---|
| g1721-F | GTCTAGCAGCTTGTGATTCG | Detection of *g1721* | Chapter 3 |
| g1721-R | ACTGGTATGGCTGTTGGTAC | Detection of *g1721* | Chapter 3 |
| g1757-F | AGTTCTTGCTCTCGCTGGAG | Detection of *g1757* | Chapter 3 |
| g1757-R | TGCACTTGCCAAGGAACCAG | Detection of *g1757* | Chapter 3 |
| g1757-F1 | GCCTACTGTACGGTCATCCTC | Disruption of *g1757* | Chapter 3 |
| g1757-F2 | gtcgtgactgggaaaaccctggcgGACGCTGACTGATGGAAAAG | Disruption of *g1757* | Chapter 3 |
| g1757-F3 | tcctgtgtgaaattgttatccgctAGGCAGCTAGAGGCTCCCAG | Disruption of *g1757* | Chapter 3 |
| g1757-F4 | AGCTCAGAGCGATCAGGTAGG | Disruption of *g1757* | Chapter 3 |
| g1908-F | CAAGTCGACTGCTATGTTGGC | Detection of *g1908* | Chapter 3 |
| g1908-R | CCAGGGTGCTAGTATATGCG | Detection of *g1908* | Chapter 3 |
| g1908-F1 | GGTCATACAGCCCGTGTGTC | Disruption of *g1908* | Chapter 3 |
| g1908-F2 | gtcgtgactgggaaaaccctggcgCTAAGTACTTCCTATTAACT | Disruption of *g1908* | Chapter 3 |
| g1908-F3 | tcctgtgtgaaattgttatccgctGGTACGATGTCGACCCTAAG | Disruption of *g1908* | Chapter 3 |
| g1908-F4 | GCTGGTGCCTCAGGCACGAA | Disruption of *g1908* | Chapter 3 |
| g1962-F | TACTCTCCTTACCCAGGCTG | Detection of *g1962* | Chapter 3 |
| g1962-R | TTGTTCCACATCCGGTGAGC | Detection of *g1962* | Chapter 3 |
| g2174-F | AAGCACATCCAACTGCCAGG | Detection of *g2174* | Chapter 3 |
| g2174-R | ACAGTCGCGCTTTCCAAAGC | Detection of *g2174* | Chapter 3 |
| g2174-F1 | AGTATTCGCAGGTGTGCAGG | Disruption of *g2174* | Chapter 3 |
| g2174-F2 | gtcgtgactgggaaaaccctggcgGACGAAAGTGTGTGACTTGA | Disruption of *g2174* | Chapter 3 |
| g2174-F3 | tcctgtgtgaaattgttatccgctGTAATAGTTGGAGGGGGTTA | Disruption of *g2174* | Chapter 3 |
| g2174-F4 | CATAGCGATAGAACCCTTCTTCG | Disruption of *g2174* | Chapter 3 |
| g2239-F | GAAGCGCTCTGTCTTGTCG | Detection of *g2239* | Chapter 3 |
| g2239-R | AAGGGTGGTAGTAACGCCTG | Detection of *g2239* | Chapter 3 |
| SIX3-C-F | CATGTCCATGACATGGGTTTGC | Detection of *SIX3* | Sasaki. 2015a |
| SIX3-C-R | GATCTCGTAGTTGGCGATGGC | Detection of *SIX3* | Sasaki. 2015a |
| SIX3-split-F1 | AGCTACTAGCCGAGCTAAAGAC | Complementation of *SIX3* | Sasaki. 2015a |
| SIX3-split-F4 | AGCTCGATTGATGTCTAGTCGC | Complementation of *SIX3* | Sasaki. 2015a |
| SIX5-C-F | GCGCTTCGAGTACATCTCTG | Detection of *SIX5* | Sasaki. 2015a |
| SIX5-C-R | CTAGGATGCATCACAATAGA | Detection of *SIX5* | Sasaki. 2015a |
| SIX5-split-F1 | TCTTAAGGAGTGGGCGTAGAAC | Disruption and complementation of *SIX5* | Sasaki. 2015a |
| SIX5-split-F2 | gtcgtgactgggaaaaccctggcgGTGATGAAGAGTAGTAGAG | Disruption of *SIX5* | Sasaki. 2015a |
| SIX5-split-F3 | tcctgtgtgaaattgttatccgctTCTGTCATTGTGACCAGTG | Disruption of *SIX5* | Sasaki. 2015a |
| SIX5-split-F4 | ATGTCAAGAGCGCGCGAAGCTC | Disruption and complementation of *SIX5* | Sasaki. 2015a |
| SIX7-Q-F | CTTTTCCATTTCGCCCTGTTT | Detection of *SIX7* | Chapter 3 |
| SIX7-Q-R | TGGTGTTGAGCCGGTGCTA | Detection of *SIX7* | Chapter 3 |

| | | | |
|---|---|---|---|
| SIX9-Q-F | CCCAAGGAACTGCGCTAGAT | Detection of *SIX9* | Chapter 3 |
| SIX9-Q-R | CTTAACATGCTGGCCGTCAA | Detection of *SIX9* | Chapter 3 |
| SIX10-Q-F | TTTGCTCAAGGCTTCAACCA | Detection of *SIX10* | Chapter 3 |
| SIX10-Q-R | CGCGGTCCCGTAGATTAGTC | Detection of *SIX10* | Chapter 3 |
| SIX12-Q-F | GTTCTCGCCCAAAGCCATT | Detection of *SIX12* | Chapter 3 |
| SIX12-Q-R | TCACCTCCGGCTCGTAGTT | Detection of *SIX12* | Chapter 3 |
| SIX14-Q-F | CTTGGATGTCGTATGCCGGA | Detection of *SIX14* | Chapter 3 |
| SIX14-Q-R | GCCTTCCTTACCTTGGACCC | Detection of *SIX14* | Chapter 3 |
| BFJ65_g17715(SIX9)-F | TGACAAGTACAACGGACCGG | Complementation of *SIX9* | Chapter 3 |
| BFJ65_g17715(SIX9)-R | AAGCTTCCTCGAATTGGGCA | Complementation of *SIX9* | Chapter 3 |
| BFJ65_FTF1_ORF_F | GCTCCTTGGAAGCGTTCTACA | Detection of *FTF1* | Chapter 3 |
| BFJ65_FTF1_ORF_R | CCGGTAAGGCGCTTTGAGA | Detection of *FTF1* | Chapter 3 |
| BFJ65_FTF1-F1 | CCGCTCTGACGGATTCTAC | Disruption of *FTF1* | Chapter 3 |
| BFJ65_FTF1-F2 | gtcgtgactgggaaaaccctggcg CGTTCGGATCCTGCCTGATT | Disruption of *FTF1* | Chapter 3 |
| BFJ65_FTF1-F3 | tcctgtgtgaaattgttatccgct AGATGGAGGCTTGCAAGGTC | Disruption of *FTF1* | Chapter 3 |
| BFJ65_FTF1-F4 | TTAGGCGACCACATGATGCA | Disruption of *FTF1* | Chapter 3 |
| M13F | CGCCAGGGTTTTCCCAGTCACGAC | Creation of *hph* construct | Catlett et al. 2003 |
| M13R | AGCGGATAACAATTCACACAGGA | Creation of *hph* construct | Catlett et al. 2003 |
| FoTEF-Q2-F | CATCGGCCACGTCGACTCT | Detection of *TEF* | van der Does et al. 2008 |
| FoTEF-Q2-R | AGAACCCAGGCGTACTTGAA | Detection of *TEF* | van der Does et al. 2008 |

### 3.2.5. Contour-clamped homogeneous electric field (CHEF) gel electrophoresis and CHEF southern blot

Fungal protoplasts were prepared using the method described by Akamatsu et al. (1999) with slight modifications. The enzyme solution contained 10 mg/mL lysing enzyme (Sigma-Aldrich, St. Louis, MO) and 4 mg/mL yatalase (TAKARA). The protoplast concentration was adjusted to $2 \times 10^8$ cells/mL in STE buffer (1 M sorbitol, 50 mM EDTA, and 25 mM Tris-HCl, pH 7.5), and an equal amount of 1.4 % low-melting agarose gel was added. Protoplast resuspended gel solution was applied to a 50-well plug mold (Bio-Rad, Hercules, CA) and incubated for 20 min at 4 ◦C. Chromosomes were separated using the CHEF-DR II system (Bio-Rad) in 0.5 × Tris/borate/EDTA buffer at 14 ◦C for 96 h using switch times between 600 and 1,800 s at 2 V/cm. The chromosome of *Hansenula wingei* (Bio-Rad) was used as the chromosome size marker. Chromosomal localization was confirmed using digoxigenin-labeled probes and a DIG DNA Labeling and Detection Kit (Roche Diagnostics, Basel, Switzerland) according to the manufacturer's instructions.

### 3.2.6. Generation of gene knockout construct

A fusion PCR strategy was used to generate a gene knockout construct (Kuwayama et al., 2002). The 5′ and 3′ flanking regions of each gene were amplified using designated F1-F4 primer sets (Table 3). The *hph* (hygromycin B resistance) cassette was amplified from the pHRC vector using the M13F/M13R primer set. The three obtained amplicons were fused by fusion PCR using the designated F1-F4 primer set.

### 3.2.7. Fungal transformation

Fungal protoplasts were prepared as previously described (Akamatsu et al., 1999) with slight modifications. The enzyme solution contained 10 mg/mL lysing enzymes (Sigma-Aldrich) and 4 mg/mL yatalase (TAKARA). The protoplast concentration was adjusted to $1.0 \times 10^8$ cells/mL in STC buffer (1.2 M sorbitol, 50 mM CaCl2, 10 mM Tris-HCl, pH 7.4). Polyethylene glycol (PEG)-mediated transformation was performed to generate a gene knockout mutant. Briefly, 20 μg of the gene knockout construct and 920 μL of 60 % PEG solution were added to the protoplast suspension (Kawabe et al., 2004). The hygromycin B-resistant mutant was selected as a candidate for gene knockout mutant and incubated on potato dextrose agar (PDA) containing hygromycin B (100 g/mL). Fungal DNA was extracted as previously described (Saitoh et al., 2006). Gene knockout was verified by PCR using Quick Taq HS (Toyobo), following the manufacturer's instructions, with the designated primer sets. Furthermore, gene knockout was verified using southern blot analysis. In brief, the upstream or downstream region of each gene was amplified using the designated primer set, and digoxigenin was labeled as a hybridization probe. The total DNA was extracted from the mycelia of wild-type *Foc*_TA and candidate of gene knockout mutants cultured for 5 days. Thereafter, 10 μg of total DNA of the wild-type *Foc*_TA and candidates of gene knockout mutants was digested using the restriction enzyme that does not cut ORF of each gene and, after blotting, hybridized using the hybridization probe. The digoxigenin-labeled probe was detected using a CDP-StarTM detection reagent (Roche Diagnostics) according to the manufacturer's instructions.

### 3.2.8. Bulb inoculation assay

The bulb inoculation assay was conducted as previously described in section 2.2.4.

### 3.2.9. Generation of 4-Mb-sized chromosome loss strains

The chromosome loss experiment was conducted as described by VanEtten et al. (1998). Briefly, the *FocSIX5* gene was replaced with the *hph* cassette to generate the hygromycin-B-resistant-ΔSIX5 strain. The ΔSIX5 strain was incubated in PDB for 3 days at 25 ◦C, with shaking at 120 rpm. Subsequently, benomyl was added to the culture at a final concentration of 12.5 μg/mL, and the liquid medium was cultured for four more days. Cultures were filtered through three sterilized layers of gauze to collect spores, which were incubated on minimal medium (3% sucrose, 1% KNO3, 0.17% yeast nitrogen base without amino acids and ammonium sulfate, and 0.04% Triton X-100) for 3 days. Using an autoclaved cloth, the minimal medium was overlaid onto the PDA medium containing hygromycin B (100 g/mL). The PDA medium was incubated for 3 days, and hygromycin-B-sensitive strains were selected as putative 4-Mb-sized chromosome loss strains by comparing the minimal medium and PDA medium. Hygromycin B-sensitive strains were isolated from the original minimal medium.

### 3.2.10. Vegetative growth assay

Wild-type *Foc*_TA, ΔSIX5 strain, and 4-Mb-sized chromosome loss strains were cultured on PDA at 25 ◦C for 5 days. The colony edge was collected with a 5-mm cork borer and placed at the center of the PDA. The plates were then incubated at 25 ◦C for 5 days, and colony diameters were measured.

### 3.2.11. Copy number variation analysis with 4-Mb-sized chromosome loss strains

Genomic DNAs of the *Foc*_TA, ΔSIX5 strain, and 4-Mb-sized chromosome loss strains were extracted using a PureLink Genomic DNA Mini Kit (Life Technologies). The

150 bp paired end reads were acquired using Illumina NovaSeq 6000. Reads were aligned to the *Foc*_FUS2 reference genome (Armitage et al., 2018) using BWAMEM (v0.7.17-r1188) (Li and Durbin, 2009). Copy number variation analysis was performed using a CNV kit to infer and visualize copy (Talevich et al., 2016).

### 3.2.12. Statistical analysis

Experimental data are presented as the standard error of the mean. The statistical significance of the differences between the mean values was determined using one-way ANOVA post hoc Tukey HSD test.

## 3.3 RESULTS

### 3.3.1. Most of the selected effector candidates expressed during infection were overrepresented on the 4-Mb-sized chromosome in *Foc*_TA

The effector candidates in *Foc*_TA were identified based on the whole genome sequence acquired from highly virulent *Foc*_TA isolated from symptomatic diseased onions. To predict effector candidates located in the lineage-specific region of *Foc*_TA, the obtained sequence read data were mapped to the core chromosome of the FOL4287 strain (Ma et al., 2010), and lineage-specific contigs were constructed using unmapped reads. Twenty-one effector candidates were identified from the lineage-specific contigs. Of the identified effector candidates, five (*g759*, *g1064*, *g1757*, *g1908*, and *g2174*) were expressed 9 days after inoculation in onion (Table 4). The genes, except for *g759*, were located on a 4-Mbsized chromosome (Figure 8). Furthermore, *g1064*, *g1757*, *g1908*, and *g2174* were located in chromosomal regions orthologous to contigs 16 (*g1064*), 19 (*g1757* and *g2174*), and 21 (*g1908*) in the *Foc*_FUS2 reference genome (Table 4).

**Table 4.** Effector candidates in *Fusarium oxysporum* f. sp. *cepae*_TA strain.

| Effector candidate | Length(aa) | Number of cysteines | Expression *in planta*[a] | Gene location in *Foc*_Fus2 genome |
|---|---|---|---|---|
| **g35** | 200 | 2 | N | Chromosome 2 (Contig 17) |
| **g73** | 299 | 8 | N | Contig 10 |
| **g150** | 157 | 5 | N | Chromosome 12 (Contig 18) |
| **g172** | 124 | 4 | N | Chromosome 1 |
| **g201** | 227 | 8 | N | Chromosome 8 |
| **g687** | 92 | 4 | N | Contig 10 |
| **g713** | 140 | 8 | N | - |
| **g759** | 126 | 8 | Y | Chromosome 13 (contig 11) |
| **g761** | 317 | 7 | N | Contig 10 |
| **g785** | 246 | 9 | N | Contig 10 |
| **g938** | 163 | 6 | N | Chromosome 10 |
| **g1064** | 143 | 6 | Y | Contig 16 |
| **g1227** | 80 | 6 | N | Contig 16 |
| **g1351** | 231 | 2 | N | Contig 10 |
| **g1396** | 136 | 6 | N | Contig 10 |
| **g1721** | 257 | 2 | N | Chromosome 7 |
| **g1757** | 77 | 7 | Y | Contig 19 |
| **g1908** | 132 | 4 | Y | Contig 21 |
| **g1962** | 136 | 5 | N | Chromosome 2 (Contig 17) |
| **g2174** | 119 | 3 | Y | Contig 19 |
| **g2239** | 106 | 9 | N | Contig 22 |

[a] Y; Expressed in planta. N; Not expressed in planta.

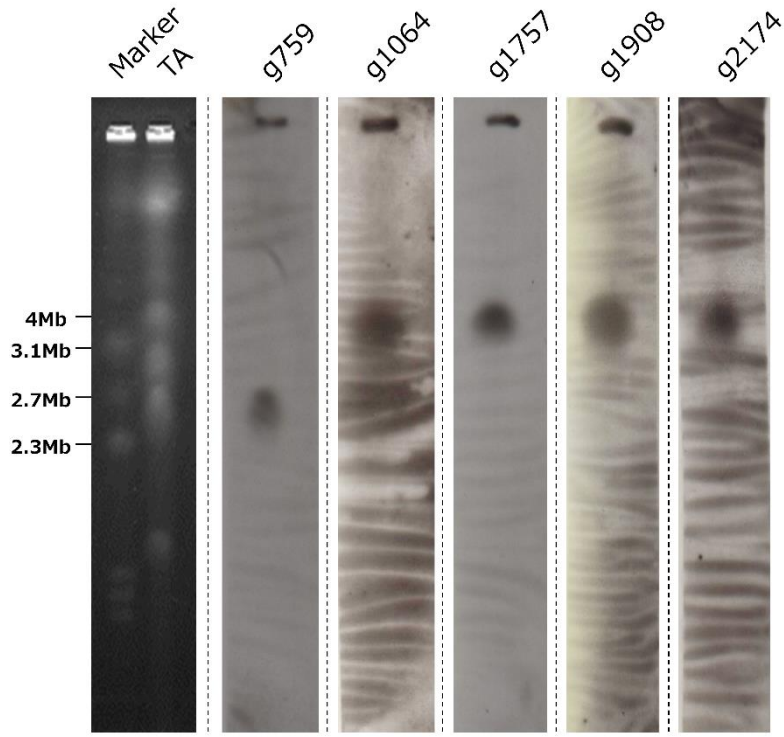


**Figure 8.** Chromosomal localization of expressed effector candidates in *Foc*_TA.

TA lane: Karyotype of the wild-type *Foc*_TA strain. g759, g1064, g1757, g1908, and g2174 lane: Chromosomal localization of each effector candidate in the *Foc*_TA strain. The black bar indicates the 4-Mb-sized chromosome and the size of the marker (*Hansenula wingei*).

### 3.3.2. Hygromycin-B-sensitive strains were impaired in their pathogenicity toward the onion

The 4-Mb-sized chromosome loss strain was generated to confirm whether the 4-Mb-sized chromosome is related to pathogenicity toward onions. *SIX5* gene was selected as a marker to isolate the putative 4-Mb-sized chromosome loss strain based on its localization on the 4-Mb-sized chromosome as revealed by chromosomal localization analysis (Figure 9). The *SIX5* gene knockout mutant (ΔSIX5) was created with a hygromycin-B-resistance gene, and a pathogenicity test was performed with wild-type *Foc*_TA and ΔSIX5 mutant. Based on the pathogenicity test, ΔSIX5 mutant was comparable to wild-type *Foc*_TA in pathogenicity toward onion (Figure 10). Nine hygromycin-B-sensitive strains (TCL1, TCL3–TCL10) were isolated as putative 4-Mb-sized chromosome loss strains, and the karyotype of the strains was investigated using CHEF gel electrophoresis. Although 4-Mb-sized chromosome loss was not observed using CHEF gel electrophoresis, several karyotype variations were observed in the TCL1 and TCL9 strains (Figure 11). The TCL1 strain lost approximately 3-Mb-sized chromosomes. In addition, a novel, ~0.9-Mb-sized chromosome appeared in the TCL9 karyotype. PCR amplification of the *g1064*, *g1757*, *g1908*, *g2174*, and *SIX3* genes located on the 4-Mb-sized chromosome showed that none of the amplicons were present in the hygromycin-B-sensitive strains (Figure 12), suggesting a loss of the 4-Mb-sized chromosome, as a whole or as a part, in these strains. Subsequently, the colony formation capabilities of the hygromycin-B-sensitive strains were analyzed, and no apparent differences in colony formation were observed (Figure 13). A bulb inoculation test was conducted to determine the pathogenic phenotype of the hygromycin B-sensitive strains. The wild-type *Foc*_TA and ΔSIX5 strains caused typical basal rot disease in onion bulbs, whereas onion bulbs inoculated with hygromycin-B-

sensitive strains (TCL, TCL3–TCL10) were asymptomatic (Figure 14).

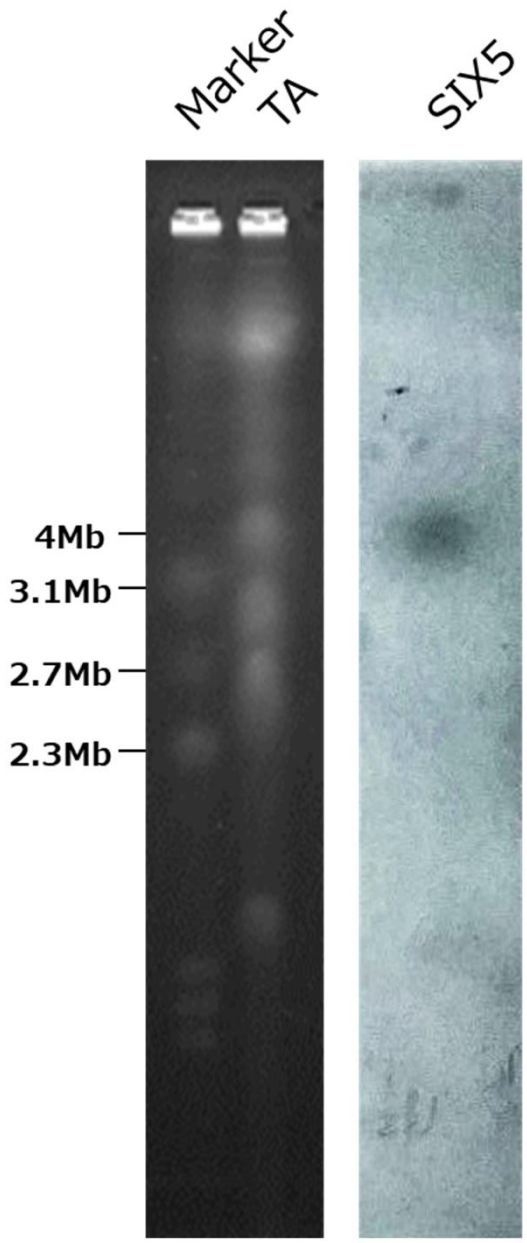


**Figure 9.** Chromosomal localization of the *SIX5* gene. Chromosomal localization of the *SIX5* gene. TA lane shows the karyotype of the *Foc*_TA strain. SIX5 lane indicates the chromosomal localization of the *SIX5* gene in the *Foc*_TA strain. The black bar indicates the 4-Mb-sized chromosome and the size of the marker (*Hansenula wingei*).

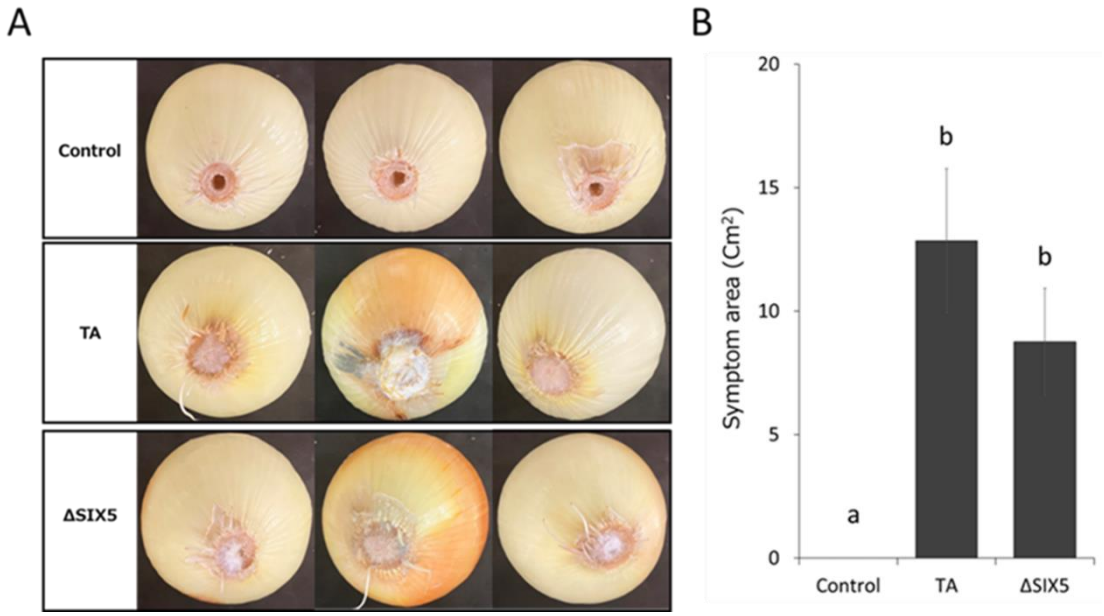


**Figure 10**. Pathogenicity test with *SIX5* gene knockout mutant. (A) Representative photograph of onion inoculated by wild-type *Foc*_TA and *SIX5* gene knockout mutant (ΔSIX5). (B) Evaluation of symptom area of onion inoculated by wild-type *Foc*_TA and *SIX5* gene knockout mutant (ΔSIX5). Statistical analyses were performed with One-way ANOVA post hoc Tukey HSD test. All data were presented as the standard error of the mean. Different letters indicate significant differences ($P<0.05$).

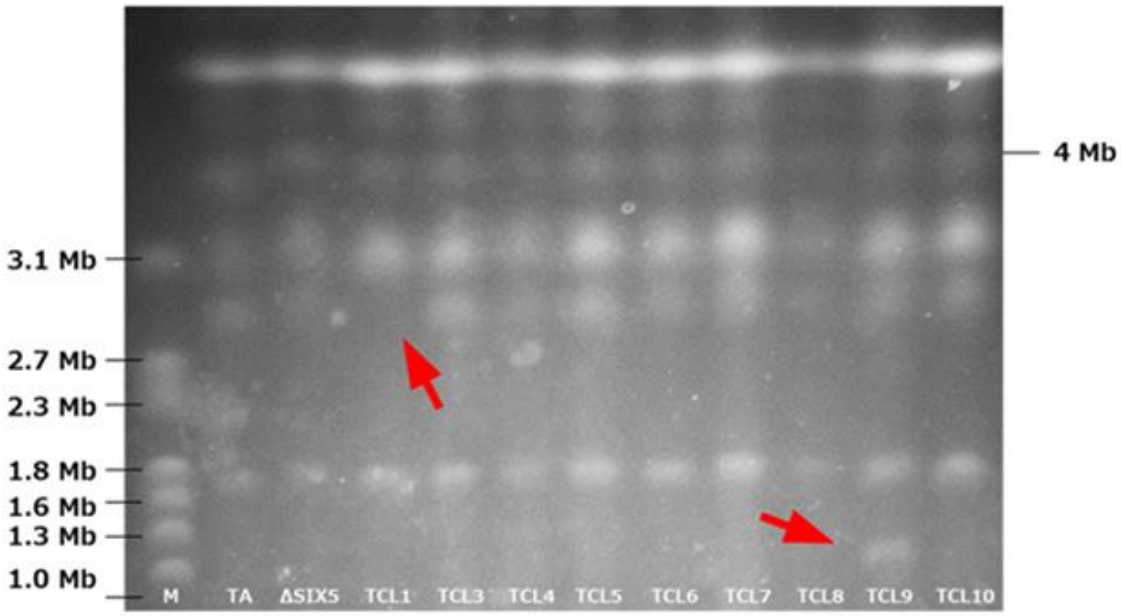


**Figure 11.** Investigation of karyotype in 4-Mb-sized chromosome loss strains. Karyotype investigation for the hygromycin-B-sensitive strains. The red arrow indicates the karyotype differentiation between strains.

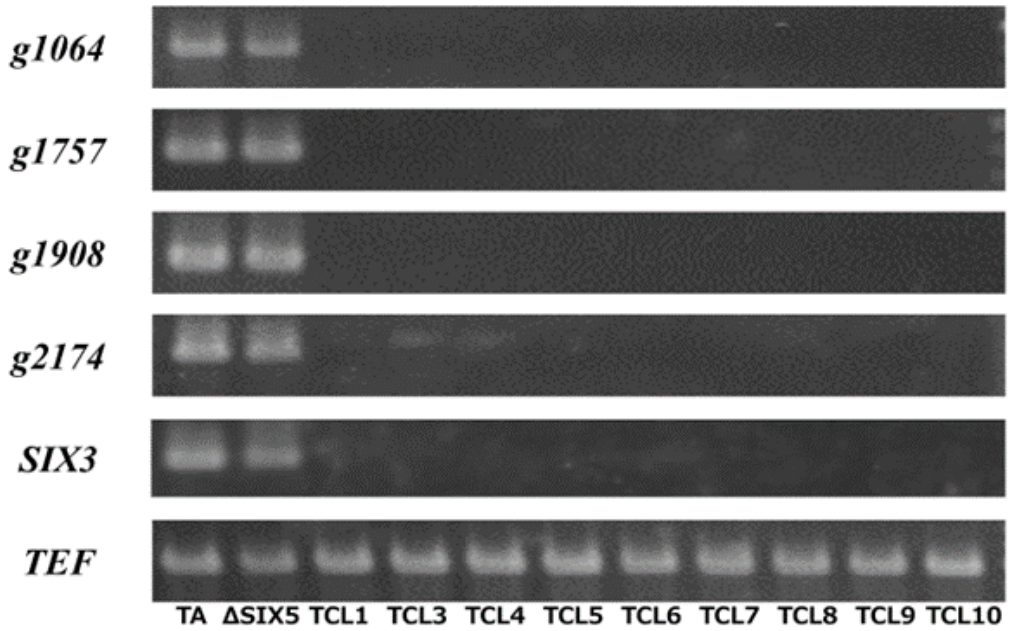


**Figure 12.** Investigation of *g1064*, *g1757*, *g1908*, *g2174*, *SIX3* gene possession, in 4-Mb-sized chromosome loss strains. The *TEF* gene was used as a positive control.

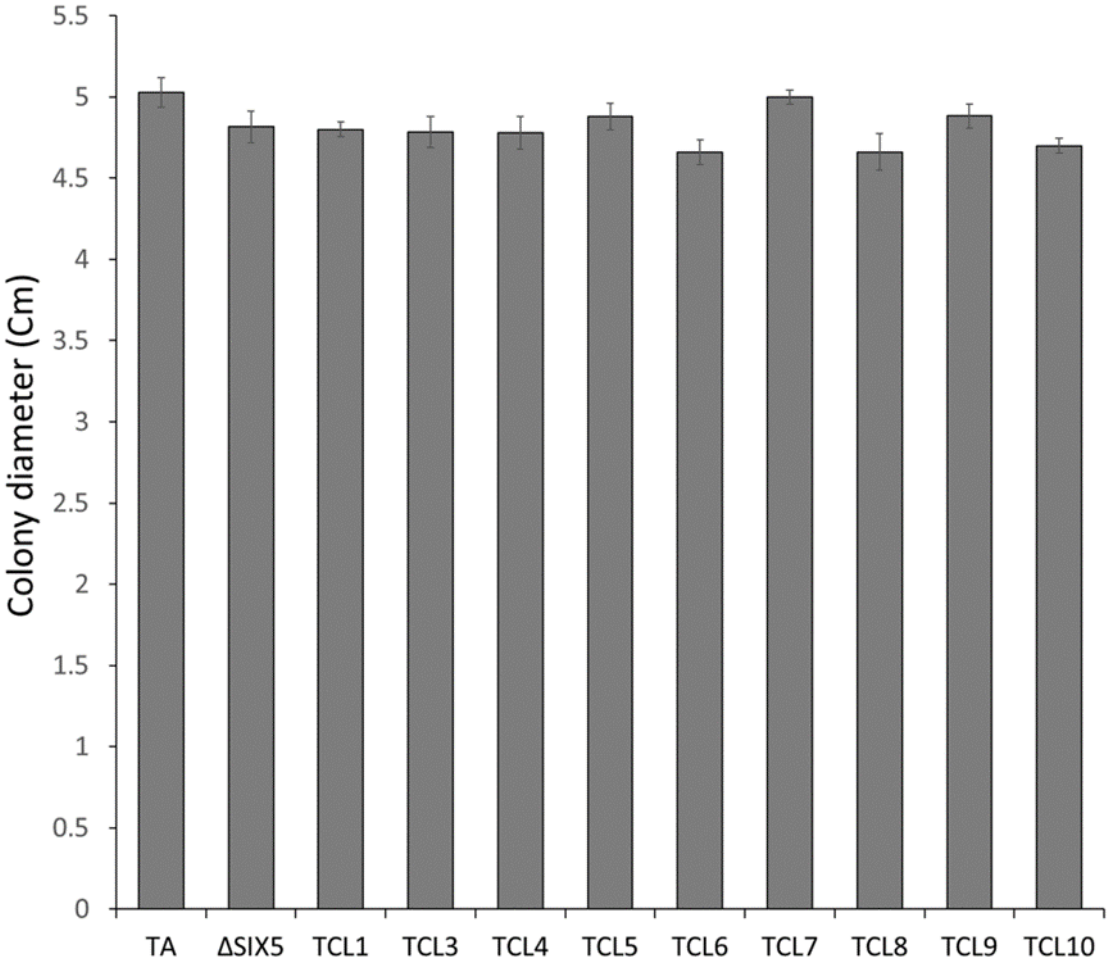


**Figure 13.** Investigation of colony formation capability in 4-Mb-sized chromosome loss strains. Statistical analyses were performed with one-way ANOVA post hoc Tukey HSD test. All data were presented as the standard error of the mean. No significant difference was observed among those strains.

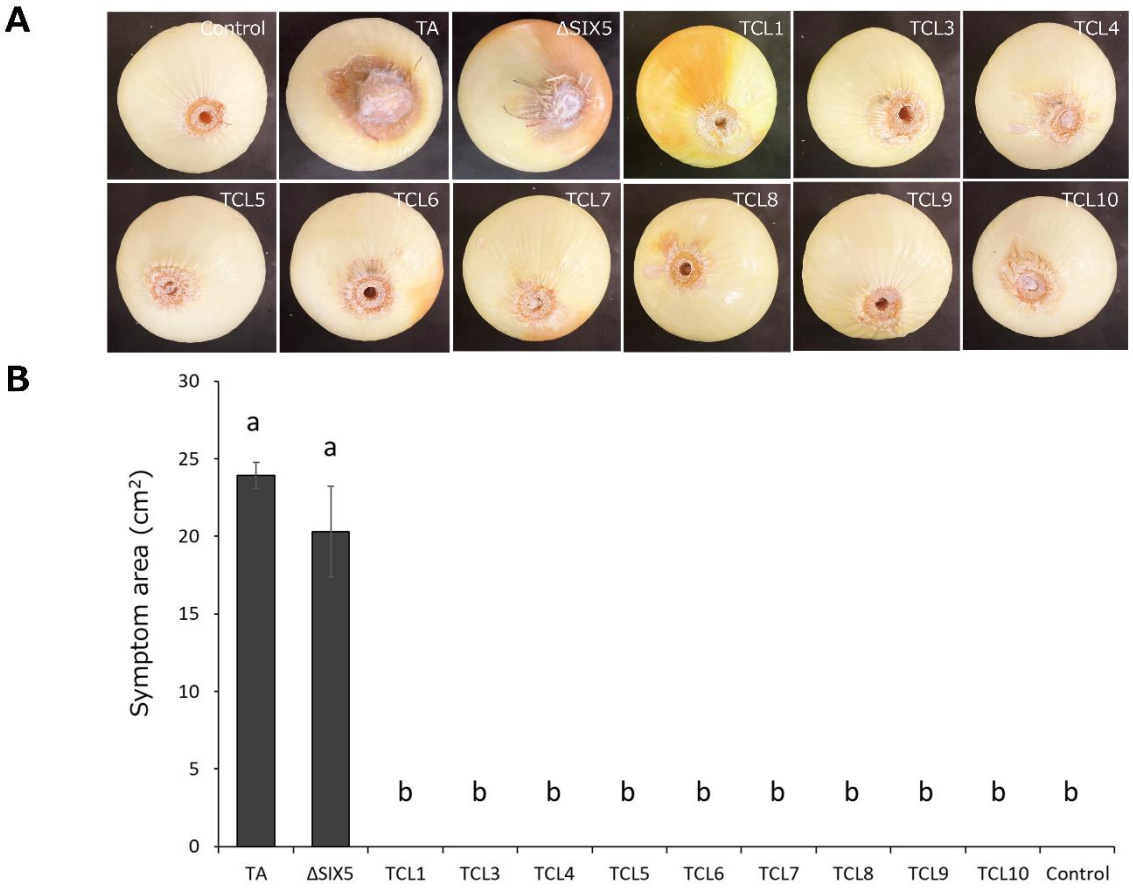


**Figure 14.** Confirmation of the pathogenicity in the hygromycin-B-sensitive strains. (A) Representative photograph of bulb inoculation assay with hygromycin-B sensitive strains. (B) Evaluation of symptom area in inoculated bulb onion. Statistical analyses were performed with one-way ANOVA post hoc Tukey HSD test. All data were presented as the standard error of the mean. Different letters indicate significant differences ($P < 0.01$).

### 3.3.3. Copy number variation analysis revealed that a 4-Mb-sized chromosome is present as a pathogenicity chromosome in *Foc*_TA

Based on the pathogenicity test, it was found that a potential relationship between the 4-Mb-sized chromosome and pathogenicity. Moreover, several karyotype patterns were observed in the hygromycin-B-sensitive strains (TCL1 and TCL9). Therefore, the genome of the wild-type *Foc*_TA, ΔSIX5, TCL1, TCL6, and TCL9 strains were analyzed to clarify the relationship between karyotype patterns and pathogenicity. Genome analysis of the *Foc*_TA strain revealed that the reads acquired from the *Foc*_TA strain mapped to the *Foc*_FUS2 reference genome at a rate of 99.48 %, suggesting that the genome construction of *Foc*_TA strain was almost the same as that of the *Foc*_FUS2 strain. Copy number variation analysis of the *Foc*_FUS2 reference genome was performed to verify karyotype patterns in TCL1, TCL6, and TCL9. Notably, the TCL1 and TCL6 strains lost chromosomal regions orthologous to contigs 10, 16, 19, and 21 in *Foc*_FUS2, which were proposed to be the PS regions in *Foc*_FUS2. TCL1 additionally lost chromosomal regions orthologous to contigs 13, 15, and 18, totaling 3 Mb in the *Foc*_FUS2 genome. Likewise, TCL9 lost chromosomal regions that were orthologous to contigs 16, 19, and 21 and part of contig 10 (approximately 1.4 Mb) (Figure 15). Moreover, PCR analysis of *SIX* genes (*SIX5*, *7*, *9*, *10*, *12*, and *14*) located in the PS region showed that the TCL1, TCL6, and TCL9 strains lost the *SIX5*, *7*, *9*, *10*, *12*, and *14* genes (Figure 16). Finally, to confirm whether chromosomal regions orthologous to contigs 10, 16, 19, and 21 comprised a single 4-Mb-sized chromosome in *Foc*_TA, chromosome localization analysis of *g1396*, located in the chromosomal region orthologous to contig 10 was conducted; the results revealed that *g1396* was also located on the 4-Mb-sized chromosome (Figure 17). Given the genome similarity between *Foc*_TA and *Foc*_FUS2 strains, the 4-Mb-sized

chromosome in *Foc*_TA was comprised of chromosomal regions that are orthologous to contigs 10, 16, 19, and 21 in *Foc*_FUS2 and functions as a pathogenicity chromosome. Furthermore, based on the pathogenicity test and copy number variation analysis, it was determined that a 3.1-Mb region is essential for full pathogenicity toward onions.

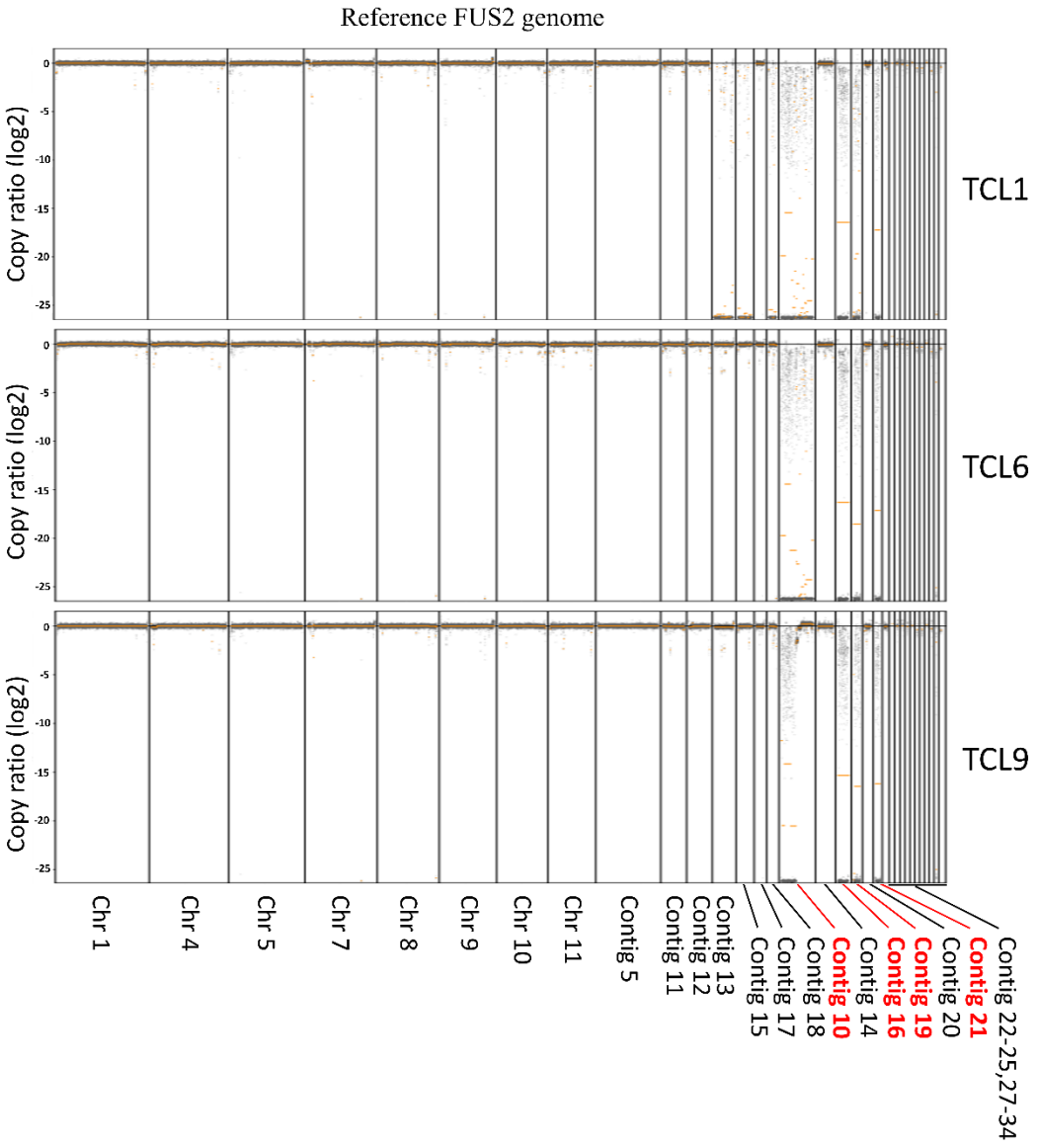


**Figure 15.** Copy number variation analysis for the hygromycin-B-sensitive strain TCL1, TCL6, and TCL9. Red lines and characters indicate genomic regions that are commonly lost in the TCL1, TCL6, and TCL9 strain genomes compared with those in the *Foc*_FUS2 reference genome.

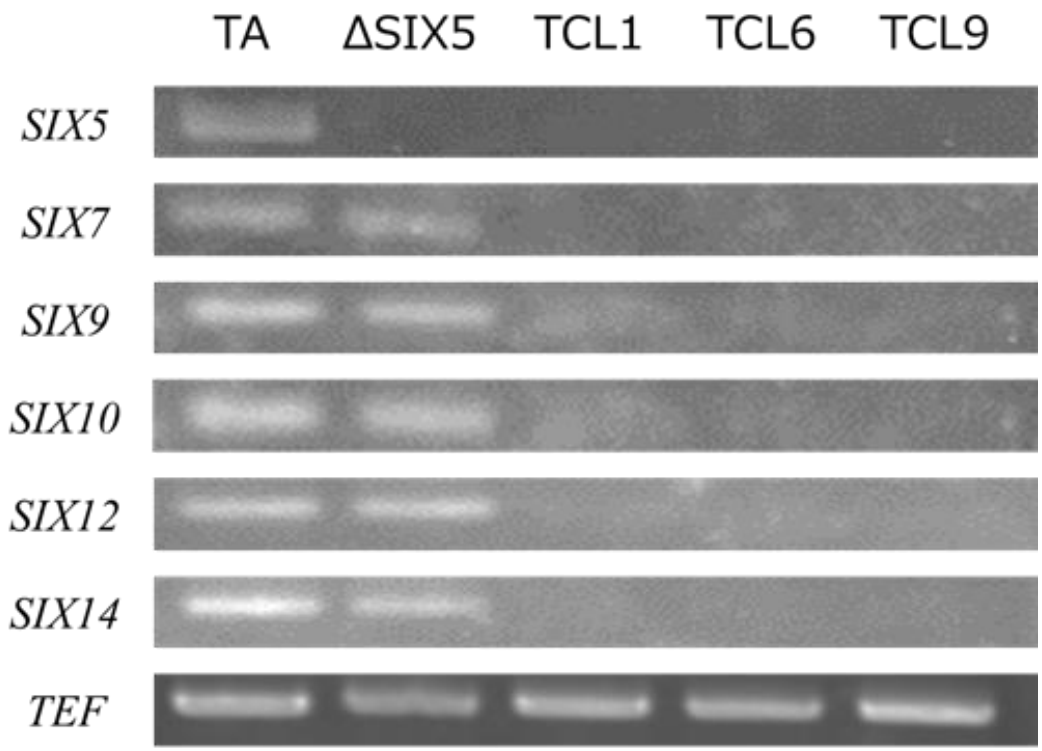


**Figure 16.** Polymerase chain reaction (PCR) diagnosis of *SIX5*, *7*, *9*, *10*, *12*, and *14* genes. Each *SIX* gene was amplified with the designated F-R primer pair via PCR using DNA of TA, ΔSIX5, TCL1, TCL6, and TCL9 strains. The *TEF* gene was used as a positive control.

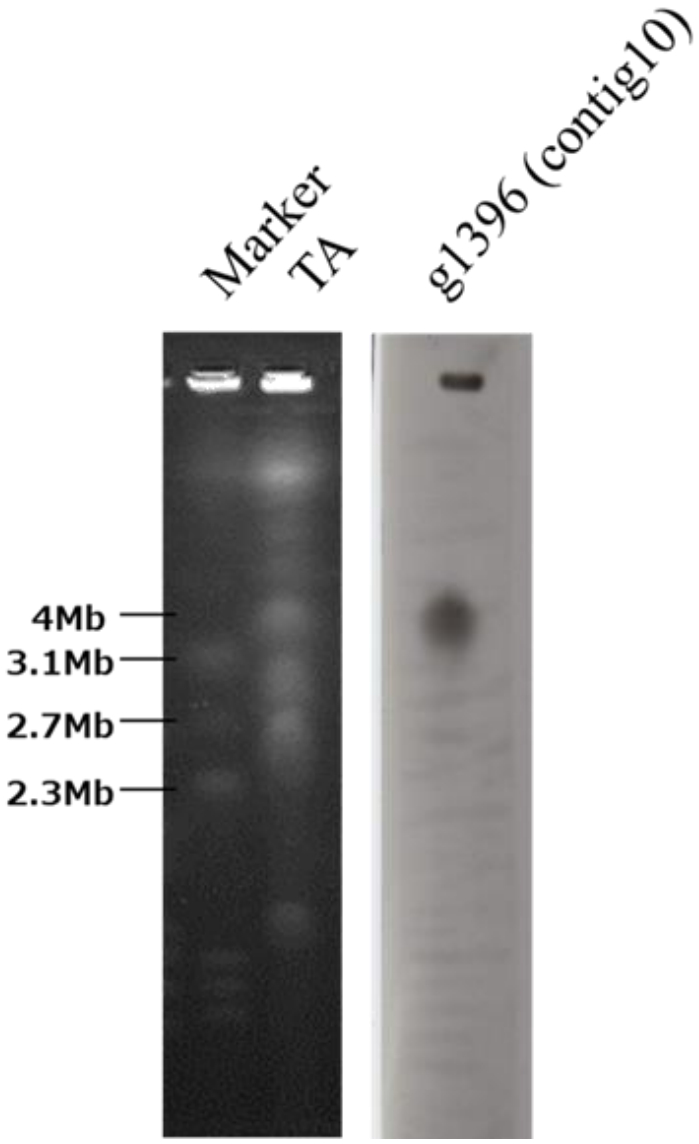


**Figure 17.** Chromosomal localization of *g1396*, located on contig10. Left panel (TA) shows karyotype of *Foc*_TA strain by CHEF gel electrophoresis. Right panel (*g1396*) shows result of chromosomal localization of *g1396* gene in *Foc*_TA strain by CHEF southern blot. The black bar indicates the 4-Mb-sized chromosome and size of marker (*Hansenula wingei*).

### 3.3.4. Selected effector candidates (*g1064*, *g1757*, *g1908*, and *g2174*) were not involved with pathogenicity toward onion

To identify the novel effectors involved with pathogenicity in *Foc*_TA, selected effector candidates (*g1064*, *g1757*, *g1908*, and *g2174*), located on the 4-Mb-sized chromosome were individually disrupted (Figure 18 and 19). Subsequently, onion bulbs were inoculated with each gene knockout mutant. However, all gene knockout mutants caused severe basal rot disease in the onion bulb, with no significant difference compared to the *Foc*_TA strain (Figure 20).

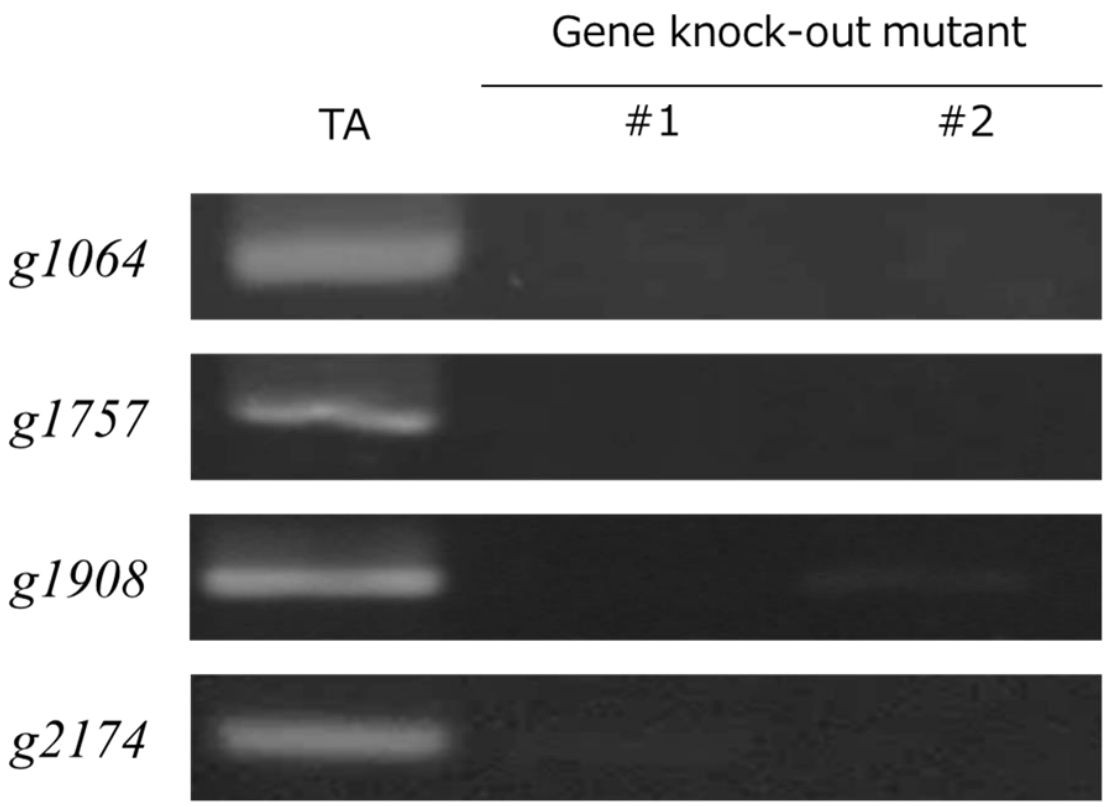


**Figure 18.** Polymerase chain reaction (PCR) diagnosis of *g1064*, g*1757*, *g1908*, and *g2174* gene knockout mutant. Each gene was amplified with the designated F-R primer pair via PCR using the DNA of *Foc*_TA strain and two independent gene knockout mutants (#1 and #2).

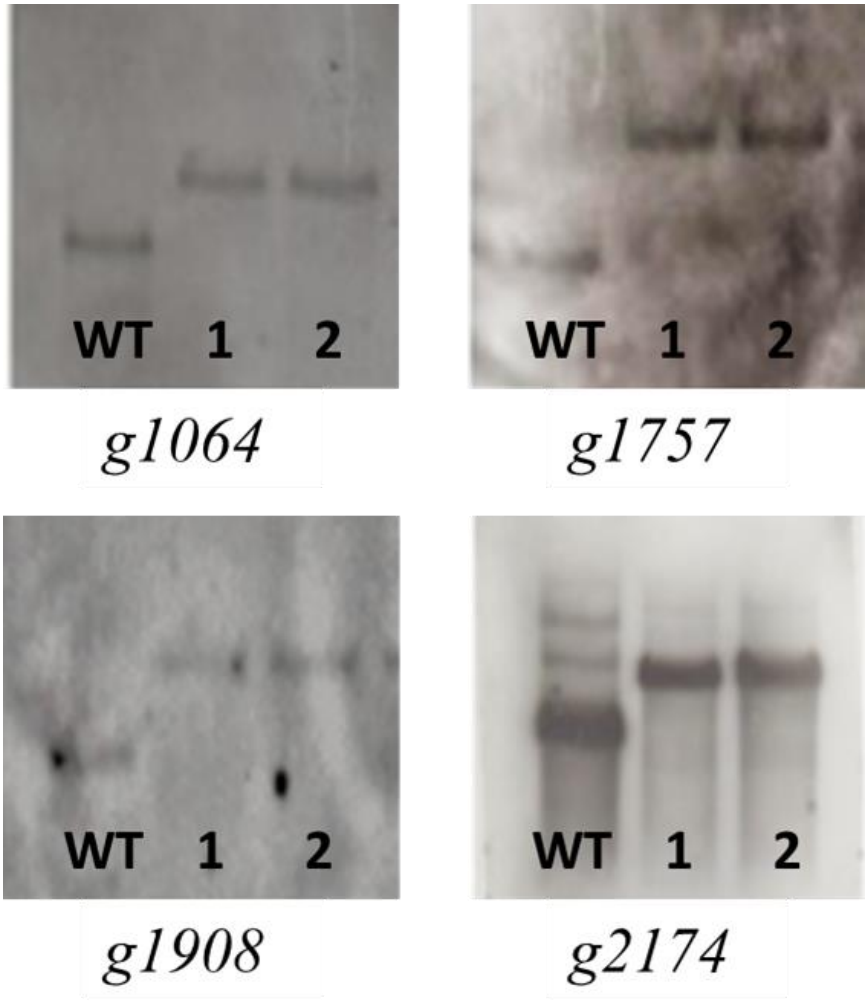


**Figure 19.** Confirmation of *g1064*, g*1757*, *g1908*, and *g2174* gene disruption by southern blotting analysis. WT lane: southern blotting result of wild-type *Foc*_TA, each number lane indicate the result of candidate of gene knockout mutants.

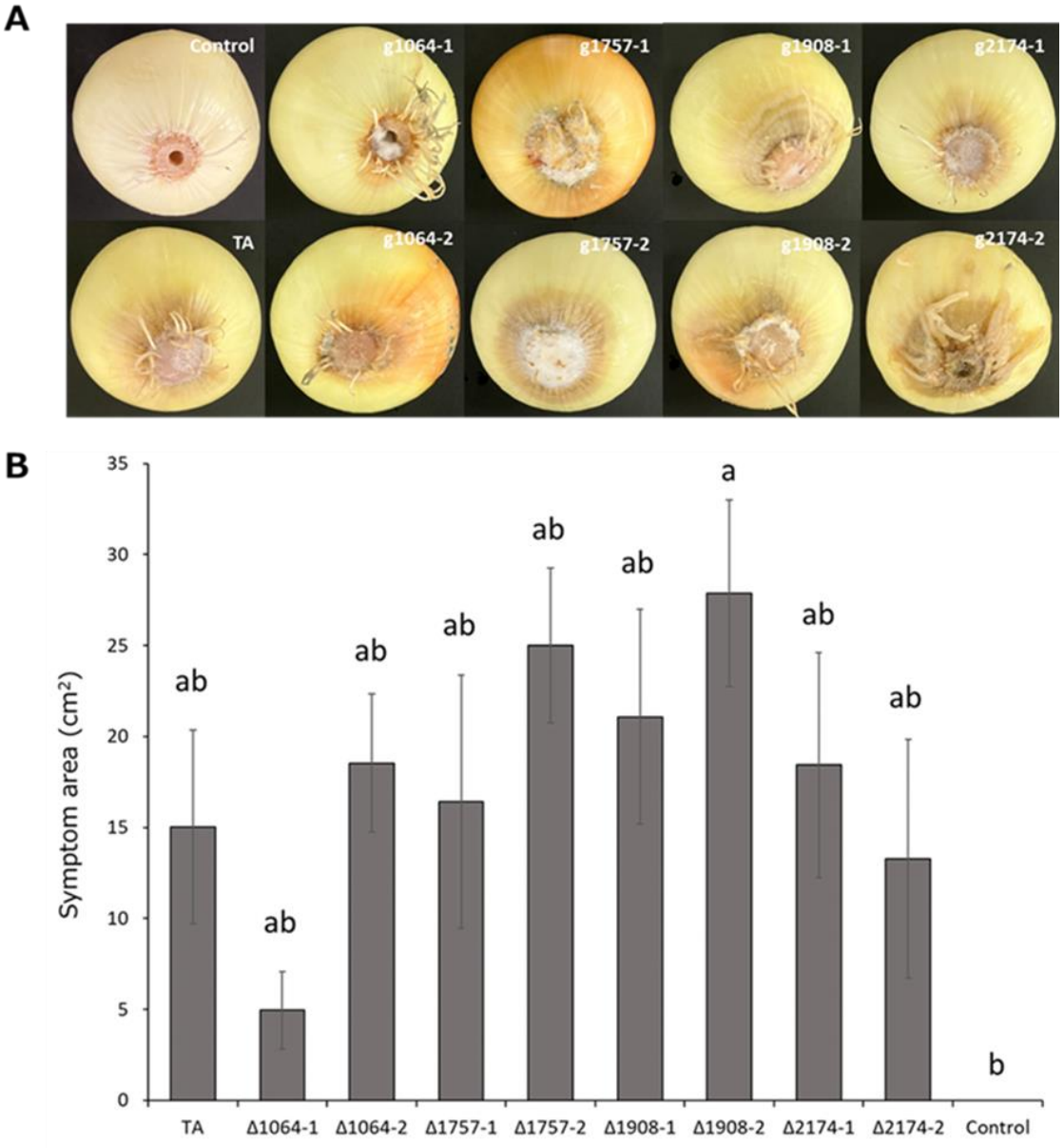


**Figure 20.** Bulb inoculation test with *g1064*, *g1757*, *g1908*, and *g2174* gene knockout mutant. (A) Representative photograph of bulb inoculation assay with *g1064*, *g1757*, *g1908*, and *g2174* gene knockout mutant. (B) Evaluation of symptom area in inoculated bulb onion. Statistical analyses were performed with One-way ANOVA post hoc Tukey HSD test. All data were presented as the standard error of the mean. Different letters indicate significant differences ($P<0.05$).

### 3.3.5. Gene complementation with *SIX3*, *SIX5*, and *SIX9* gene did not rescue pathogenicity in the non-pathogenic TCL6 strain

Using the gene knockout approach, none of the novel effector in *Foc*_TA were identified. Therefore, *SIX3*, *5*, and *9* genes, which are highly expressed during infection and located in the PS region (Armitage et al., 2018), were complemented into the TCL6 strain (Figure 21). Gene expression of *SIX3*, *5*, and *9* in the each TCL6 complemented mutant were confirmed by RT-PCR (Figure 22). All the gene complementation mutants were inoculated into onion bulbs. However, none of the complementation mutants exhibited pathogenicity on the inoculated onion, as exerted by the TCL6 strain (Figure 23).

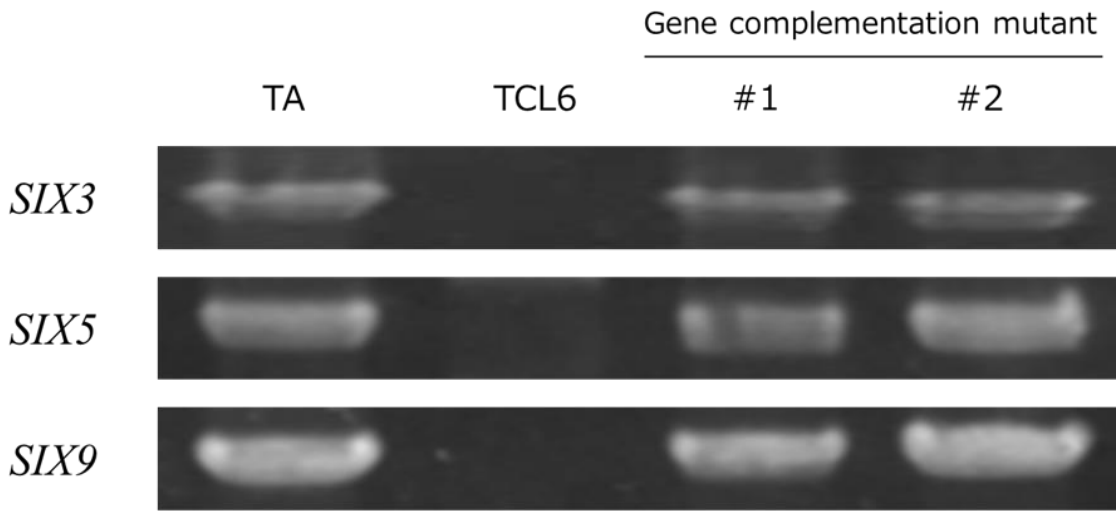


**Figure 21.** Polymerase chain reaction (PCR) diagnosis of *SIX3*, *SIX5*, and *SIX9* gene complementation mutant. Each gene was amplified with the designated F1-F4 or F-R primer pair via PCR using DNA of *Foc*_TA, TCL6 strains, and two independent gene complementation mutants (#1 and #2).

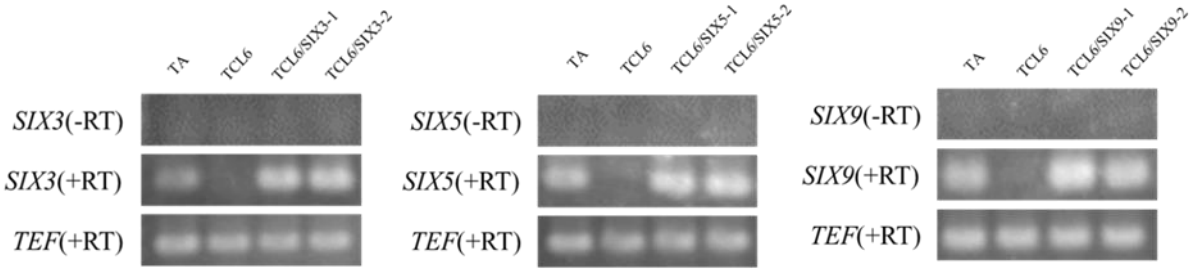


**Figure 22.** Gene expression of *SIX3*, *SIX5*, and *SIX9* in TCL6 mutant complemented with *SIX3*, *SIX5*, and *SIX9* gene on minimal medium. Extracted RNA sample was used as negative control (–RT), while reverse transcribed RNA (+RT) and its *TEF* gene were used as positive controls.

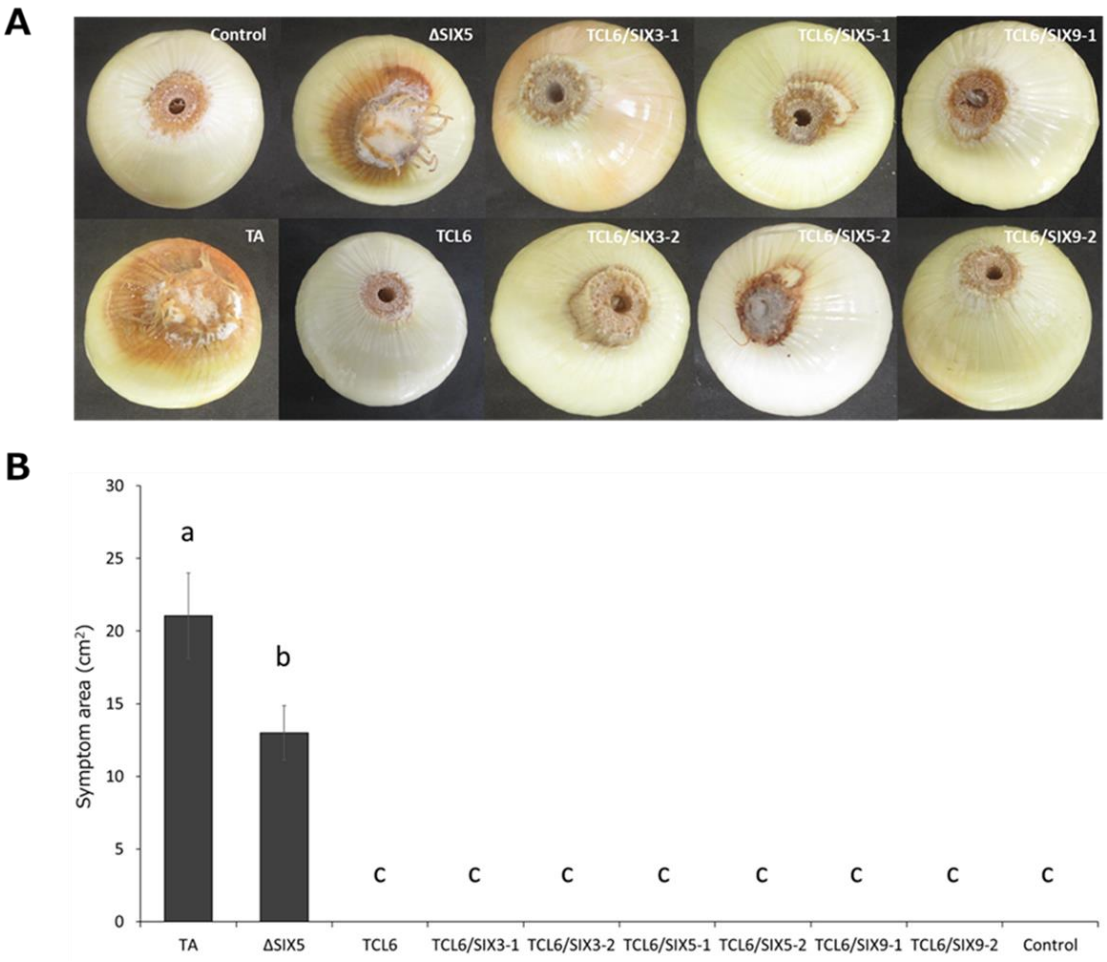


**Figure 23.** Bulb inoculation test with TCL6 strain complemented with *SIX3*, *SIX5*, and *SIX9* gene. (A) Representative photograph of bulb inoculation assay with TCL6 strain complemented with *SIX3*, *5*, and *9* gene. (B) Evaluation of symptom area in inoculated bulb onion. Statistical analyses were performed with One-way ANOVA post hoc Tukey HSD test. All data were presented as the standard error of the mean. Different letters indicate significant differences ($P<0.01$).

### 3.3.6. Gene disruption of *FTF1* (*Fusarium transcription factor 1*) was compromised their pathogenicity toward onion

In plant pathogenic fungi, it has been reported that some transcription factors are involved with pathogenicity toward its host. In the *F. oxysporum* f. sp. *phaseoli*, *FTF1* (*Fusarium transcription factor 1*) was suggested to correlate with the pathogenicity toward common bean (Niño-Sánchez et al., 2016). Notably, two copies of *FTF1* homologs (*BFJ65_g18276* and *BFJ65_g 18330*) were found in *Foc*_TA genome and located on the 4-Mb-sized chromosome. In *Foc*_TA, the *FTF1* homologs (*FocFTF1*) was expressed during infection (Figure 24), thus, both two copies of *FocFTF1* (*BFJ65_g18276* and *BFJ65_g 18330*) were disrupted with consensus knockout construct amplified using BFJ65_FTF1_F1-F4 primer set to investigate whether the *FocFTF1* is involved with pathogenicity toward onion (Figure 25 and Figure 26). Interestingly, gene knockout mutants of both *FTF1* homologs (*BFJ65_g18276* and *BFJ65_g 18330*) caused significantly fewer disease symptoms in the onion bulbs when compared to wild-type *Foc*_TA (Figure 27).

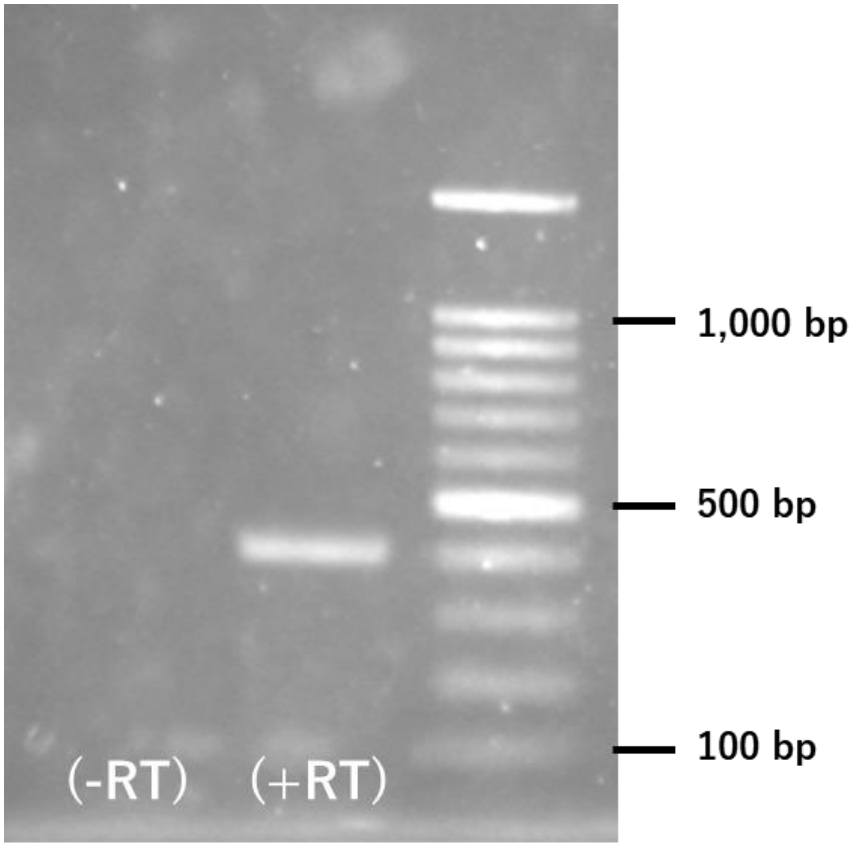


**Figure 24.** Gene expression of *FocFTF1* during infection. Extracted RNA sample was used as negative control (–RT), while reverse transcribed RNA (+RT).

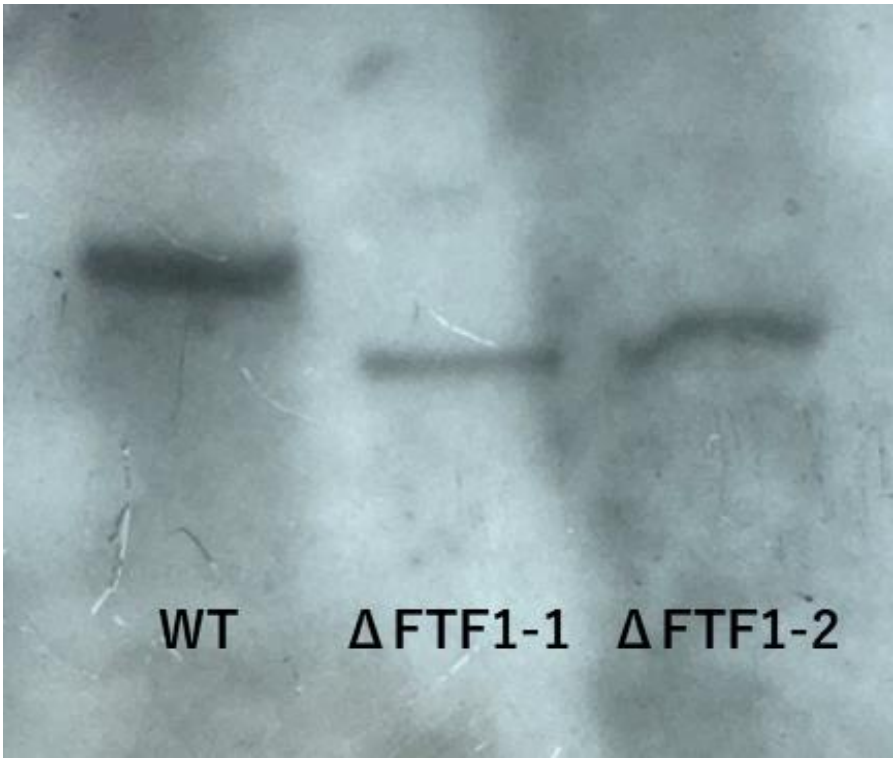


**Figure 25.** Confirmation of *FocFTF1* gene disruption by southern blotting analysis. WT lane: southern blotting result of wild-type *Foc*_TA, ΔFTF1-1 and ΔFTF1-2 indicate the result of candidate of *FocFTF1* gene knockout mutants.

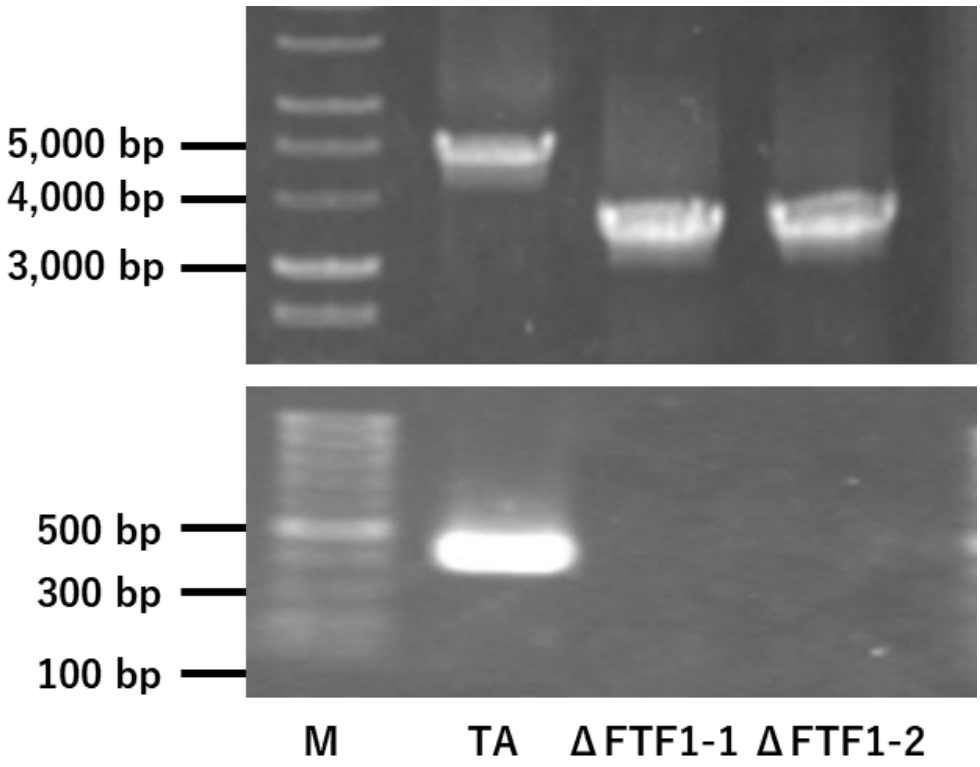


**Figure 26.** Polymerase chain reaction (PCR) diagnosis of *FocFTF1* gene knockout mutant. The *FocFTF1* gene was amplified with the designated primer pair via PCR using the DNA of *Foc*_TA strain and two independent gene knockout mutants (ΔFTF1-1 and ΔFTF1-2). M indicates that marker lane. Top and bottom panel indicates the result of PCR diagnosis using BFJ65_FTF1_F1-F4, and BFJ65_FTF1_ORF_F-R primer sets, respectively.

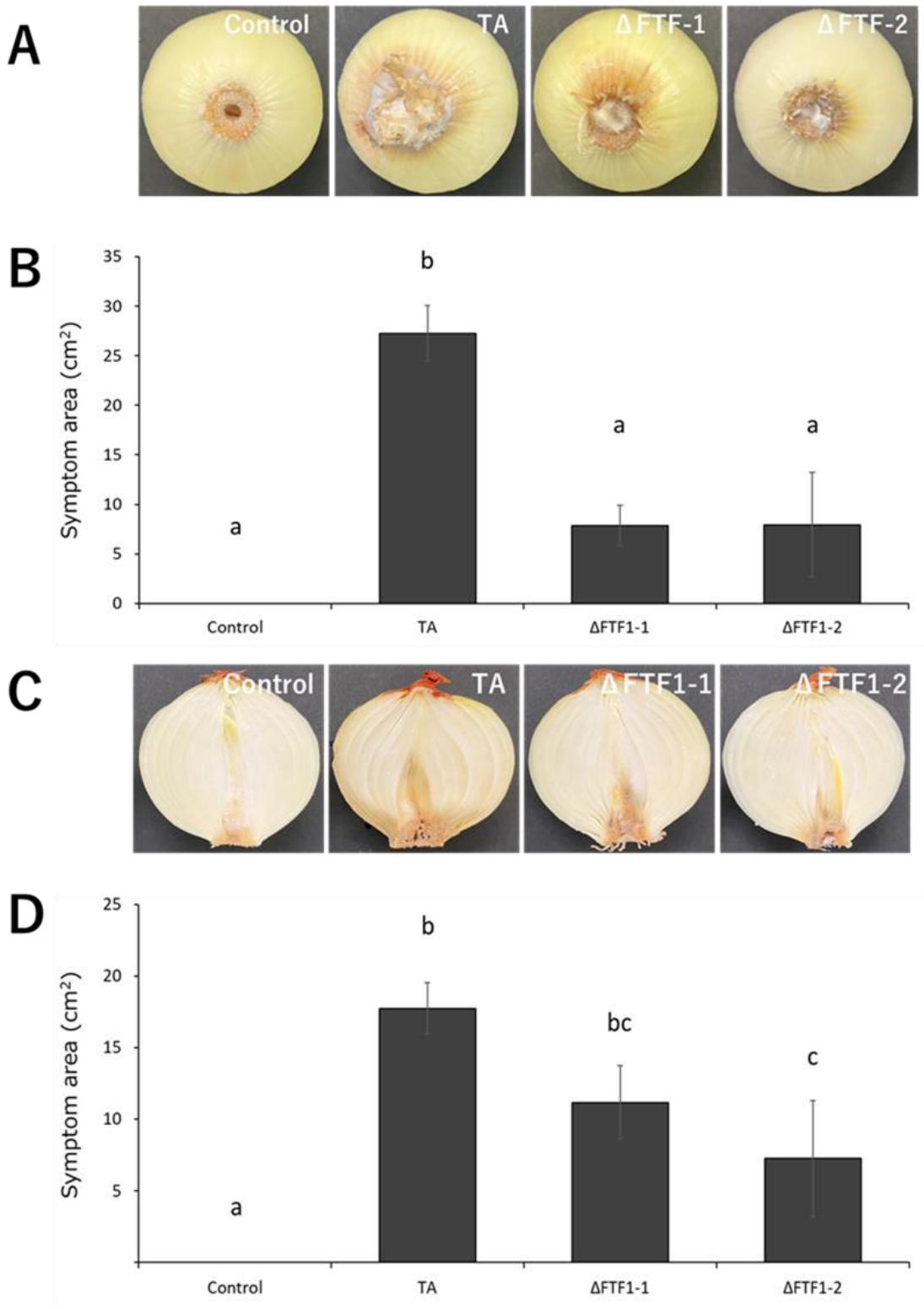


**Figure 27.** Bulb inoculation test with *FocFTF1* gene knockout mutants.

(A) Representative outside photograph of bulb inoculation assay with *FocFTF1* gene

knockout mutants. (B) Evaluation of outside symptom area in inoculated bulb onion. Statistical analyses were performed with One-way ANOVA post hoc Tukey HSD test. All data were presented as the standard error of the mean. Different letters indicate significant differences ($P<0.05$). (C) Representative inside photograph of bulb inoculation assay with *FocFTF1* gene knockout mutants. (D) Evaluation of inside symptom area in inoculated bulb onion. Statistical analyses were performed with One-way ANOVA post hoc Tukey HSD test. All data were presented as the standard error of the mean. Different letters indicate significant differences ($P<0.05$).

## 3.4 DISCUSSION

Pathogenicity and CD chromosomes are common among plant pathogenic fungi. The presence of pathogenic CD chromosomes in *F. oxysporum* species complex has been reported (Ayukawa et al., 2021; Constantin et al., 2021; Li et al., 2020b; Ma et al., 2010; van Dam et al., 2017; Williams et al., 2016; Zhang et al., 2020). In addition, the pathogenicity chromosomes harboring the *SIX* genes are necessary for pathogenicity toward their host and are transferable between strains (Ayukawa et al., 2021; Ma et al., 2010; van Dam et al., 2017). In the British strain *Foc*_FUS2, the PS region was approximately 3.9 Mb, comprising four contigs (contigs 10, 16, 19, and 21) containing all *SIX* genes (*SIX3*, *5*, *7*, *9*, *10*, *12*, and *14*) (Armitage et al., 2018). This study clarified that the identified effector candidates (*g1064*, *g1757*, *g1908*, and *g2174*) mapped to chromosomal regions orthologous to contigs 16, 19, and 21 in the *Foc*_FUS2 reference genome were located on the 4-Mb-sized chromosome in *Foc*_TA strain. Moreover, the 4-Mb-sized chromosome loss strains (TCL1, TCL6, and TCL9) were impaired in their pathogenicity toward onions; however, with no change in their colony formation capability. These results strongly suggest that the PS region comprises a single 4-Mb-sized chromosome, and the 4-Mb-sized chromosome acts as the pathogenicity chromosome in *Foc*. This is the first report of the presence of a pathogenicity chromosome in *Foc*. In Chapter 3, karyotype variations were observed in the hygromycin-B-sensitive strains. TCL1 additionally lost a 3-Mb-sized chromosome, comprising chromosomal regions that are orthologous to contigs 13, 15, and 18. However, no effect on colony formation capability was detected in any of the hygromycin-B-sensitive strains, indicating that the 4-Mb and 3-Mb-sized chromosomes in the hygromycin-B-sensitive strains were not necessary for colony formation capability in *Foc*_TA. Moreover, TCL9

lost part of the 4-Mb-sized chromosome (3.1 Mb) and its pathogenicity toward onions, suggesting that the 3.1-Mb region within the 4-Mb-sized chromosome is required for full pathogenicity to onions. Nevertheless, the entire pathogenic chromosome 14 in *Fol* is not necessary to exert full virulence in tomato plants (Li et al., 2020a; Vlaardingerbroek et al., 2016). Furthermore, it was investigated whether this 4-Mb-sized chromosome is transferable between strains; however, a chromosome transfer event was not observed (data not shown). Flow cytometry and cell sorting techniques may help observe this phenomenon. Nevertheless, the chances of the 4- Mb-sized chromosome being non-transferable cannot be excluded because the 4-Mb-sized chromosome is relatively larger than the known transferable chromosomes *Fol* (~2 Mb), *Fom* (~2 Mb), and *Forc* (~2 Mb) in *F. oxysporum*, *C. gloeosporioides* (~2 Mb), and *A. alternata* (~1 Mb) (Akagi et al., 2009; He et al., 1998; Li et al., 2020b; Ma et al., 2010; van Dam et al., 2017).

Plant pathogens use effectors to modulate immunity. These effectors are often preferentially located on specific chromosomes and plasmids (Mehrabi et al., 2011; Mecsas and Strauss, 1996). In Chapter 3, the 4-Mb-sized chromosome was identified as pathogenicity chromosome in *Foc* toward onion infection, clearly indicating that the 4-Mb-sized chromosome contains pathogenicity-related factors. Thus, next challenge will be to identify the pathogenicity-related factors located on the 4-Mb-sized chromosome. The 4-Mb-sized chromosome contains *SIX3*, *SIX5*, and *SIX9* genes, and the expression levels of *SIX3*, *SIX5*, and *SIX9* located in the PS region of *Foc*_FUS2 are drastically upregulated during infection (Armitage et al., 2018). Moreover, Haapalainen et al. (2022) reported a relationship between the expression level of the *SIX9* gene and aggressive pathogenicity toward onions. Therefore, it is likely that *SIX* genes located on the 4-Mb-sized chromosome are associated with pathogenicity. Of note, although *FocSIX5* gene

knockout mutant caused basal rot disease in onion bulbs, the strength of pathogenicity seemed slightly compromised. This suggests that SIX5 plays a role in pathogenicity toward onion. Previous reports indicated that SIX5 in *Fol* enhances the spread of effectors to adjacent cells, thereby facilitating infection (Blekemolen et al., 2022; Cao et al., 2018). Notably, the SIX5 homolog is conserved among genera of plant pathogens other than *F. oxysporum*, implying that the function of SIX5 is conserved over significant evolutionary distances. This suggests that its function might be widely conserved and involves the manipulation of the fundamental defense systems of various plant species (Talbi et al., 2023). Certain effectors are broadly conserved among plant pathogens and are core effectors that overcome the ancestral defense systems of plants (Hemetsberger et al., 2015; Irieda et al., 2019). Given that SIX5 in *Foc* may play a role in pathogenicity toward onion, additional experiments are required to clarify its exact function. Moreover, gene knockout (of *g1064*, *g1757*, *g1908*, and *g2174*) and complementation (of *SIX3*, *SIX5*, and *SIX9* into TCL6 strain) mutants were generated to identify the novel effector gene required for pathogenicity toward onion. Unfortunately, none of the genes applied for gene knockout and complementation mutants changed their pathogenicity toward onion. These results suggest that the function of selected effector candidates might be redundant and the pipeline of effector candidate selection should be considered using the advanced tools since effector prediction tools, including EffectorP and Phyre2 were recently developed to effectively identify effector of fungi and oomycete (Kelley et al., 2015; Sperschneider and Dodds 2022). Furthermore, none of the 4-Mb chromosome loss TCL6 strain complemented with *SIX3*, *SIX5*, and *SIX9* genes, highly upregulated during infection recovered their pathogenicity toward onion, suggesting that other pivotal factor required for pathogenicity toward onion is located on the 4-Mb-sized chromosome.

Notably, results of Chapter 3 suggested that *FocFTF1* gene is involved with pathogenicity toward onion because gene knockout mutants of *FocFTF1* were significantly compromised their pathogenicity toward onion.

Chapter 3 provides evidence that the 4-Mb-sized pathogenicity chromosome in *Foc*_TA contains *SIX3*, *5*, *7*, *9*, *10*, *12*, and *14* genes. In contrast, the *Foc*_AF22 strain, isolated from Welsh onion, has *SIX2* and *SIX9* located on the putative 3-Mb-sized pathogenicity chromosome (Sakane et al., 2023), suggesting that *Foc* has a high diversity in effector inventory, which could explain why *Foc* can infect toward a wide range of *Allium* species. Among the effector candidates in *Foc*, only *SIX9* was identified as the *SIX* gene common to both onion-infecting and Welsh onion-infecting *Foc*, suggesting that *SIX9* may be a common effector that promotes infection toward *Allium* species. A comparative analysis between the 4-Mb-sized chromosome in the *Foc*_TA strain and the 3-Mb-sized chromosome in the *Foc*_AF22 strain would provide a deeper understanding of the common and host-specific effectors required for the pathogenicity toward *Allium* species.

In Chapter 3, the results demonstrated that the 4-Mb-sized chromosome in *Foc*_TA plays a crucial role as a pathogenicity chromosome in onion infections. Hence, next challenge is to identify the specific pathogenicity-related factors that will bring a deeper understanding of the infection mechanisms employed by *Foc*.

# CHAPTER 4

# IDENTIFICATION OF AVIRULENCE EFFECTOR IN *FUSARIUM OXYSPORUM* F. SP. *CEPAE*

## 4.1 INTRODUCTION

The *Fusarium oxysporum* species complex is a ubiquitous, soil-borne, and plant-pathogenic fungus with a wide host range comprising more than 120 species. Therefore, it has recently been recognized as the fifth most important plant pathogen (Dean et al.,2012; Leslie and Summerell 2006). Based on host specificity, *F. oxysporum* species are generally distinguished as "*formae speciales*" (Laurence et al., 2012; Michielse and Rep 2009), among which *F. oxysporum* f. sp. *cepae* (*Foc*) has been identified as the causative agent of FBR in onions (*Allium cepa* L.). Globally, onion production is threatened by *Foc* (Haapalainen et al., 2022; Sasaki et al., 2015b; Southwood et al., 2015; Taylor et al., 2016). Hence, understanding plant defense systems against *Foc* and *Foc*-infective mechanisms is important for achieving sustainable onion production. Moreover, durable genetic resources are desirable for breeding disease-resistant onions.

Shallot (*A. cepa* L. Aggregatum group) is an annual herbaceous plant belonging to the family Amaryllidaceae, which is widely used as a condiment in Southeast Asian countries. It contains antimicrobial compounds (Teshima et al., 2013; Yin et al., 2003) and is highly resistant to pathogens, including *F. oxysporum* (Herlina et al., 2019; Vu et al., 2012). Shallots are genomically compatible and can be crossbred with onion plants (Abdelrahman et al., 2015). Therefore, shallots are considered a versatile breeding resource for onions (Astley et al., 1982).

Plants have evolved multilayered barrier systems to protect themselves against pathogens. Plants recognize pathogen-associated molecular patterns (PAMPs) using plant cell-surface localized pattern-recognition receptors (PRR) to induce pattern-triggered immunity (PTI). To impede the PTI response, pathogens secrete proteins with signal peptide motifs that translocate into the host tissue to manipulate the PTI response, known as effector-triggered susceptibility (ETS) (Jones and Dangl 2006; Rovenich et al., 2014). Subsequently, plants recognize pathogenic effector molecules using resistance proteins to trigger a robust immune response called effector-triggered immunity (ETI) (Yuan et al., 2021).

In *F. oxysporum*, the genetic concept of *F. oxysporum* is well characterized in *F. oxysporum* f. sp. *lycopersici* (*Fol*)-tomato pathosystem. Reportedly, *Fol* secretes the SIX (secreted in the xylem) effector protein into the xylem to facilitate colonization during infection. To date, 14 SIX proteins have been identified in *Fol*-infecting tomato xylem sap (Houterman et al., 2007; Schmidt et al., 2013), of which SIX1(AVR3), SIX3 (AVR2), SIX5, and SIX6 are required for the virulence of tomato plants. Conversely, SIX1(AVR3), SIX3 (AVR2)-SIX5, and SIX4 (AVR1) are avirulence effectors that activate the immunity of tomato plants, mediated by *I-3* (SRLKtype), *I-2* (CC-type), and *I* genes, respectively (Catanzariti et al., 2015; Gawehns et al., 2014; Houterman et al., 2009; Ma et al., 2015; Rep et al., 2004; Takken et al., 2010). These resistance genes have been introduced into commercial tomato cultivars for stable and effective production (Huang and Lindhout 1997). However, *Fol* adopts several strategies to evade the tomato immune system. The *Fol* race 2 strain completely lost the *SIX4* (*AVR1*) gene to avoid the *I* gene-derived immunity (Lievens et al., 2009), whereas the *Fol* race 3 strain had a single amino acid substitution in its SIX3 (AVR2) sequence to escape *I-2*-derived immunity (Houterman et

al., 2009). In the *Foc* genome, the sequences of some *SIX* genes (*SIX3*, *SIX5*, *SIX7*, *SIX9*, *SIX10*, *SIX12*, and *SIX14*) are conserved (Taylor et al., 2016; Armitage et al., 2018). Among these *SIX* genes, *SIX5* (*FocSIX5*) is the most highly upregulated during *Foc* infection in onions (Armitage et al., 2018); however, its virulence in *Foc* has not yet been elucidated. In Chapter 4, *FocSIX5* gene-modified mutants of *Foc* were generated and was conducted pathogenicity tests on both *Foc*-susceptible onions and *Foc*-resistant shallots using *FocSIX5* gene-modified mutants to investigate the function of FocSIX5.

## 4.2 MATERIALS AND METHODS

### 4.2.1. Plant material and fungal strain

Shallots (*A*. *cepa* L. Aggregatum group) cv. Chiang Mai (SAMD00027216) (Abdelrahman et al., 2015) and onion cv. “Kitamomiji 2000” (Shippou Co., Ltd.) and “Tarzan” (Shippou Co., Ltd.) were used for Chapter 4. The *Foc*_TA isolate from the fungal strain *F*. *oxysporum* f. sp. *cepae* (*Foc*) was collected from a diseased onion bulb in Hokkaido, Japan (Sasaki et al., 2015b).

### 4.2.2. Pathogenicity test toward onion and shallot plants

For the pathogenicity test on onion bulbs, the pathogenicity testing was conducted as previously described in section 2.2.4. For the pathogenicity test of shallot and onion seedlings, shallot cv. Chiang Mai (Abdelrahman et al., 2015) and onion cv. “Tarzan” were used. Fungal isolates were cultured in potato dextrose broth for seven days in a growth chamber with a temperature of 25 ◦C, with shaking at 120 rpm, and the cultures were filtered through three layers of sterilized gauze to collect spores for inoculation. The spores were then collected and rinsed once with sterile water. The number of spores in the suspension was determined using a hemocytometer and adjusted to a concentration of $1 \times 10^6$ spores/mL. Shallot bulbs and onion seeds were sown in plastic pots filled with a mixture of sand and compost at a ratio of 4:1. The pots were incubated for seven days in a temperature-controlled room with a temperature of 25 ◦C and a 16:8 light–dark cycle, after which the seedlings were uprooted and the central portion of the root was excised. The cut portion of the root was dipped into the prepared spore suspension and sterilized water (as a control) for 1 h. Subsequently, the inoculated seedlings were transplanted into plastic pots containing the same soil mixture. The pots were then placed in a temperature-

controlled room with a temperature of 25 ◦C and 16:8 light-dark cycle. The shallot disease index was scored five weeks post inoculation, as described previously (Herlina et al., 2021), with slight modifications: 0, no chlorosis; 1, necrosis on the tip of the leaf; 2, leaf curving with a pale green or yellowish color; 3, leaf curving and drying out; and 4, leaf death. The disease index was evaluated for each leaf, and an average disease index was calculated for each plant using the following equation: disease index = (4 × n [number of leaf deaths] + 3 × n [number of leaves curving and drying out] + 2 × n [number of leaves curving with a pale green or yellowish color] + 1 × n [number of necrosis on the tip of the leaf] + 0 × n [leaf number with no chlorosis])/total number of evaluated leaves. The biomass of all the shallot plants was also measured. The pathogenicity test was conducted at least twice with at least three seedlings per iteration.

### 4.2.3. Evaluation of the biomass of *Foc* from shallot plants inoculated with *Foc*

The evaluation of the biomass of *Foc* from shallot plants inoculated with *Foc* was carried out as previously described (Sasaki. 2015a). In brief, the 7300 real-time PCR system (Applied Biosystems) was used for quantitative PCR. THUNDERBIRD SYBR qPCR Mix (Toyobo) was used for the quantitative PCR reaction. In 20 µl of the reaction solution, 0.3 µM of primer (FocSIX3-F2 and FocSIX3-R2) and 1 µl of template DNA extracted from shallot plants inoculated with *Foc* at five weeks after inoculation were added, and after treatment at 95°C for 1 minute, 40 cycles of 95°C for 15 seconds and 60°C for 1 minute were performed. The melting curve temperature of the PCR product was determined by further increasing the temperature from 60°C to 95°C. For the standard curve, a plasmid containing the *FocSIX3* gene fragment linearized by restriction enzyme *Pst*I was adjusted to $10^1$-$10^8$ copies/µl and used as a template.

### 4.2.4. RNA extraction and quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR)

To conduct the quantitative reverse-transcription polymerase chain reaction (qRT-PCR), RNA was extracted from onion and shallot roots inoculated with *Foc*_TA at 3, 7, and 14 days post inoculation (dpi). Total RNA was extracted from three independent onion and shallot root samples using Sepasol-RNA I Super G (Nacalai Tesque Inc.). For reverse transcription, 500 ng of total RNA was used in a 10 µL reaction volume with the ReverTra Ace qPCR RT Master Mix with gDNA Remover (Toyobo), following the manufacturer's instructions. The resulting cDNA was diluted (1:1), and 1 µL of the diluted cDNA was used as a template in a 20 µL total volume of THUNDERBIRD SYBR qPCR Mix (Toyobo). The relative amounts of *FocSIX5* gene transcripts were calculated and normalized to that of the *TEF* gene. Real-time quantitative PCR was performed using a 7300 system (Applied Biosystems).

### 4.2.5. Sequence alignment and prediction of signal peptide

Amino acid sequences were aligned using the Clustal 2.1 (Thompson et al., 1994). Signal peptides were predicted using SignalP-5.0 (Almagro Armenteros et al., 2019). Sequence data of SIX5 were obtained from the NCBI database: (Fol4287 (XP_018257286), FUS2 (ALQ80804), Fus062 (QMX85381), Fus125 (QMX85382), Fus127 (QMX85383), Fus129 (QMX85384), A21 (ALQ80805), Fox129 (UVW62045), and AP117 (LC731005)).

### 4.2.6. Generation of gene knockout and complementation constructs

Generation of gene knockout construct was conducted as previously described in section

3.2.6. For a generation of gene complementation construct, the construct, including upstream and downstream, and open reading frame (ORF) of *FocSIX5* was amplified with SIX5-split-F1/F4 primer sets. A geneticin-resistance gene cassette was amplified from the pII99 plasmid (Namiki et al., 2001).

**Table 5.** Primer used in chapter 4.

| Primer name | Sequence (5'-3') | purpose | Reference |
|---|---|---|---|
| FocSIX3-F2 | GGTTATGCTAGAACATTCTCCC | Quantification of *Foc* biomass | Sasaki. (2015a) |
| FocSIX3-R2 | CAGTTTGTATGCCTCCAATGGAG | Quantification of *Foc* biomass | Sasaki (2015a) |
| SIX5-C-F | GCGCTTCGAGTACATCTCTG | Detection of *FocSIX5* | Chapter 4 |
| SIX5-C-R | CTAGGATGCATCACAATAGA | Detection of *FocSIX5* | Chapter 4 |
| SIX5-Q-F | TGCCACCACTCAGCTTCAGA | Quantification of *FocSIX5* | Chapter 4 |
| SIX5-Q-R | TGAAATGTGGACCAAGTGCTCTA | Quantification of *FocSIX5* | Chapter 4 |
| SIX5-split-F1 | GGGATAGGTAAGCAAGCAGCTTG | Disruption and complementation of *FocSIX5* | Chapter 4 |
| SIX5-split-F2 | GTCGTGACTGGGAAAACCCTG | | |
| | GCGGTGATGAAGAGTAGTAGAG | Disruption of *FocSIX5* | Chapter 4 |
| SIX5-split-F3 | TCCTGTGTGAAATTGTTATCCG | | |
| | CTTCTGTCATTGTGACCAGTG | Disruption and Verification of knockout of *FocSIX5* | Chapter 4 |
| SIX5-split-F4 | ATGTCAAGAGCGCGCGAAGCTC | Disruption, complementation, and Verification of gene knockout of *FocSIX5* | Chapter 4 |
| FoTEF-Q2-F | CATCGGCCACGTCGACTCT | Quantification of *TEF* | van der Does et al. (2008) |
| FoTEF-Q2-R | AGAACCCAGGCGTACTTGAA | Quantification of *TEF* | van der Does et al. (2008) |
| M13F | CGCCAGGGTTTTCCCAGTCACGAC | Creation of *hph* construct | Catlett et al. (2003) |
| M13R | AGCGGATAACAATTCACACAGGA | Creation of *hph* construct | Catlett et al. (2003) |

### 4.2.7. Protoplast preparation

Fungal protoplasts were prepared as previously described in section 3.2.7.

### 4.2.8. Fungal transformation

Polyethylene glycol (PEG)-mediated fungal transformation was performed as previously described in section 3.2.7. The DNA of the candidate of the *FocSIX5* gene knockout mutant was extracted using a simple extraction method described previously (Saitoh et al., 2006). *FocSIX5* gene knockout was verified via PCR using Quick Taq HS (Toyobo), following the manufacturer's instructions, with the SIX5-C-F/SIX5-C-R and SIX5-split-F1/SIX5-split-F4 primer sets. Furthermore, *FocSIX5* gene knockout was verified using southern blot analysis. In brief, the downstream region of the *FocSIX5* gene was amplified using the SIX5-split-F3/SIX5-split-F4 primer set, and digoxigenin was labeled as a hybridization probe. The total DNA was extracted from the mycelia of wild-type *Foc*_TA and candidate of *FocSIX5* gene knockout mutants cultured for 5 days. Thereafter, 10 µg of total DNA of the wild-type *Foc*_TA and candidate of *FocSIX5* gene knockout mutants was digested using the *Eco*RV restriction enzyme and, after blotting, hybridized using the hybridization probe. The digoxigenin-labeled probe was detected using a CDP-StarTM detection reagent (Roche Diagnostics) according to the manufacturer's instructions. Finally, two *FocSIX5* gene knockout mutants (ΔSIX5-1 and ΔSIX5-2) were generated and used for further investigation. To obtain the *FocSIX5* gene complementation mutants, 10 µg of the complementation construct and 10 µg of the geneticin-resistance cassette were co-transformed into fungal protoplasts. Geneticin-resistance mutants were selected as candidate of *FocSIX5* gene complementation mutant and incubated on PDA-containing G418 (100 µg/mL). DNA of the candidate of *FocSIX5*

gene complementation mutant was extracted (Saitoh et al., 2006), and *FocSIX5* gene complementation was verified through PCR using the SIX5-C-F/SIX5-C-R and SIX5-split-F1/SIX5-split-F4 primer sets. Consequently, two *FocSIX5* gene complementation mutants (ΔSIX5-1 + SIX5 [Δ-1 + SIX5] and ΔSIX5-2 + SIX5 [Δ-2 + SIX5]) and two *FocSIX5* gene complementation mutants with *FocSIX5* gene variant carrying the G200A SNP (ΔSIX5-2 + SIX5R67K-1 [Δ-2 + SIX5R67K-1] and ΔSIX5-2 + SIX5R67K-2 [Δ-2 + SIX5R67K-2]) were generated and used for further investigation.

### 4.2.9. Vegetative growth assays

Wild-type *Foc*_TA and the *FocSIX5* gene knockout and gene complementation mutants were cultured on PDA in a growth chamber with a temperature of 25 ◦C for five days. The colony edge was collected using a 5 mm cork bore, and the mycelia plug was placed in the center of PDA plates and incubated in a growth chamber with a temperature of 25 ◦C for five days. The colony diameters were measured. All the tests were conducted with at least three samples per iteration. All experiments were repeated at least twice.

### 4.2.10. Statistical analysis

The experimental data are presented as the mean and standard error. The statistical significance of the differences between the mean values was determined using the Student's *t*-test or one-way analysis of variance with post hoc ANOVA and post hoc Tukey HSD test

## 4.3 RESULTS

### 4.3.1. Confirmation of pathogenicity of *Foc*_TA toward onion and shallot plants

Inoculation tests were performed to confirm the pathogenicity of *Foc*_TA on onion and shallot plants. These results showed that *Foc*_TA caused severe basal rot disease in onion bulbs. However, shallot seedlings inoculated with *Foc*_TA exhibited only slight necrosis of the leaf tip (Figure 28).

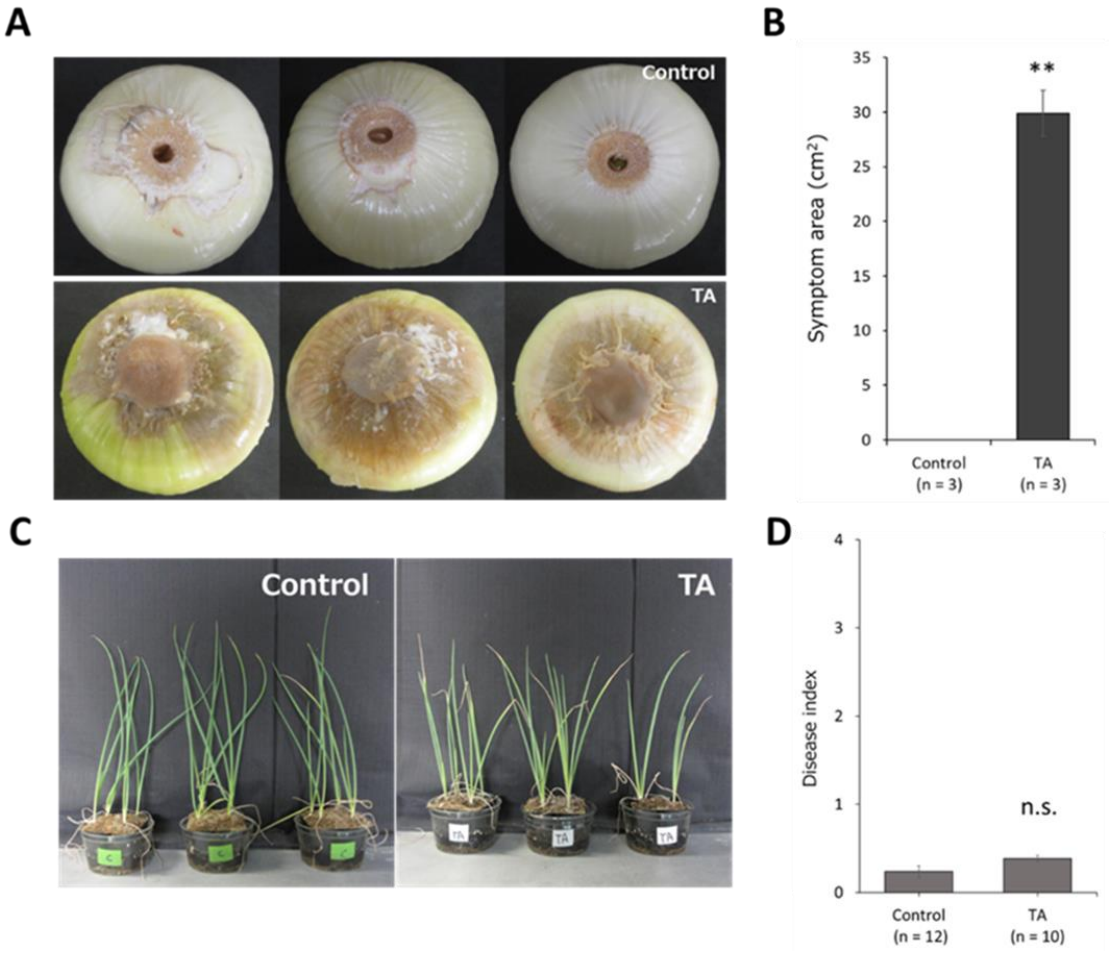


**Figure 28.** Results of the pathogenicity test of *Foc*_TA toward onion and shallot plants. (A) Symptoms of non-inoculated and *Foc*_TA-inoculated onions. (B) Symptom area on inoculated onion bulb. n represents sample size. Asterisks indicate a significant difference (** $p < 0.01$) compared to the control using a Student's *t*-test. (C) Symptoms of non-inoculated and *Foc*_TA-inoculated shallots. (D) Average disease index of shallot plants inoculated with wild-type *Foc*_TA five weeks after inoculation. n represents the sample size. n.s. denotes "not significant" compared to control using a Student's *t*-test. All data are presented as mean and standard error.

### 4.3.2. Expression of *FocSIX5* gene in onion and shallot plants during infection

*FocSIX5* gene is drastically upregulated during onion infection (Armitage et al., 2018). Therefore, qRT-PCR was performed to examine the expression of *FocSIX5* gene in *Foc*_TA during onion and shallot infections. qRT-PCR showed that the *FocSIX5* gene was expressed in shallot and onion roots inoculated with *Foc*_TA (Figure 29).

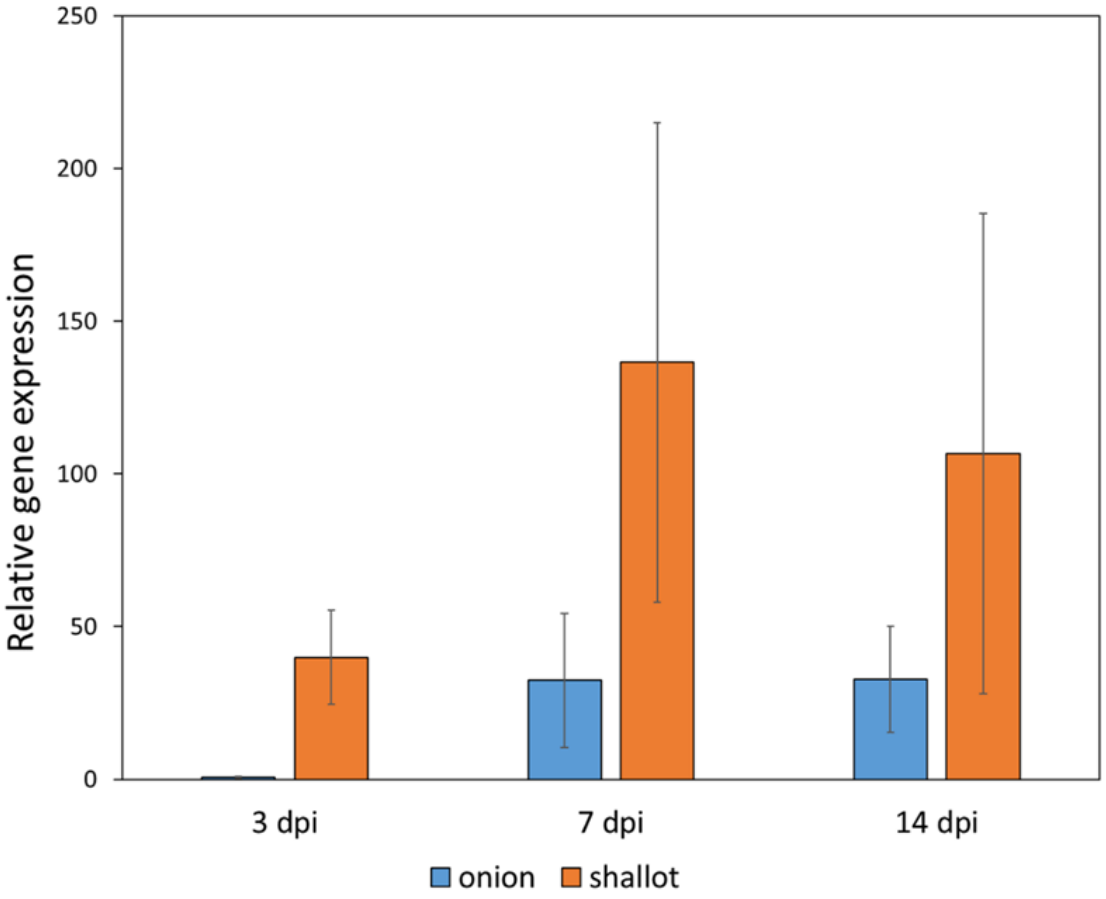


**Figure 29.** Relative gene expression of *FocSIX5* in *Foc*_TA during infection in onion and shallot. mRNA expression levels of *FocSIX5* genes at 3, 7, and 14 days post-inoculation (dpi) were determined using qRT-PCR. Relative amounts of transcripts of the *FocSIX5* gene were calculated and normalized to that of the *TEF* gene. Data are presented as mean and standard error (n = 3).

### 4.3.3. Characterization of FocSIX5

The amino acid sequences of FocSIX5 and SIX5 in FOL 4287 (FolSIX5, Accession no. XP_018257286) were compared. FocSIX5 was predicted to be a secretory peptide harboring seven cysteine residues, encoding 122 amino acids with 13.4 KDa (Accession of. LC730887). According to the BLAST analysis, FocSIX5 was 74% similar to FolSIX5, and the signal peptide of FocSIX5/FolSIX5 was predicted to be cleaved at the alanine residue. The cysteine residues were conserved between FocSIX5 and FolSIX5 (Figure 30)

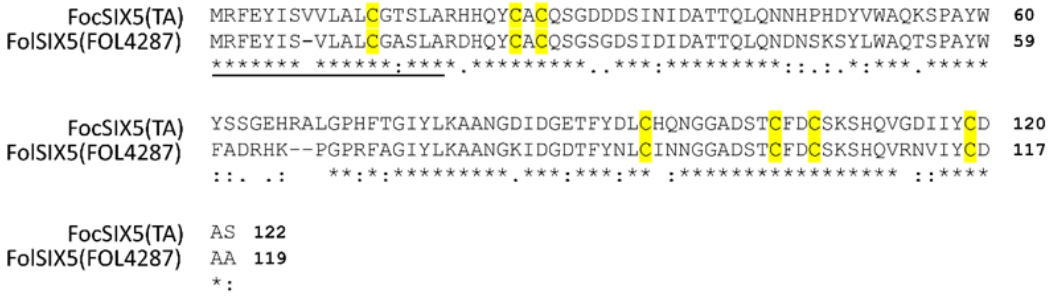


**Figure 30.** Alignment of the amino acid sequences of FocSIX5 and FolSIX5. The underline in the sequence alignment shows the signal peptides predicted using SignalP5.0. The yellow boxes indicate cysteine residues in the amino acid sequences. The asterisk (*) indicate identical amino acids.

### 4.3.4. Generation of a *FocSIX5* gene-modified mutant

To clarify the involvement of FocSIX5 in pathogenicity, *FocSIX5* gene knockout mutants were generated via marker-exchange homolog recombination with a hygromycin B resistance gene (*hph*) cassette (Figure 31). *FocSIX5* gene knockout mutants were complemented by reintroducing the *FocSIX5* construct and a geneticin-resistance cassette. Subsequently, gene modification was verified using a polymerase chain reaction (PCR) using the designated primer set, southern blot analysis, and RT-PCR analysis (Figures 32, Figure 33, and Figure 34).

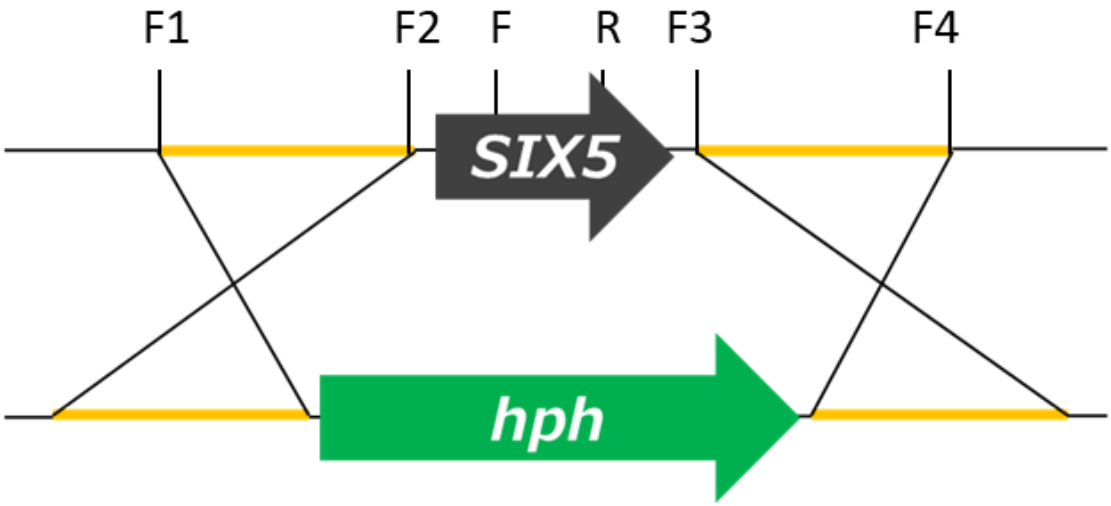


**Figure 31.** Schematic of the marker-exchange homologous recombination between *FocSIX5* gene and the hygromycin B resistant (*hph*) cassette. Yellow bars indicate identical upstream/downstream sequences for homologous recombination. The vertical solid lines illustrate each primer sites.

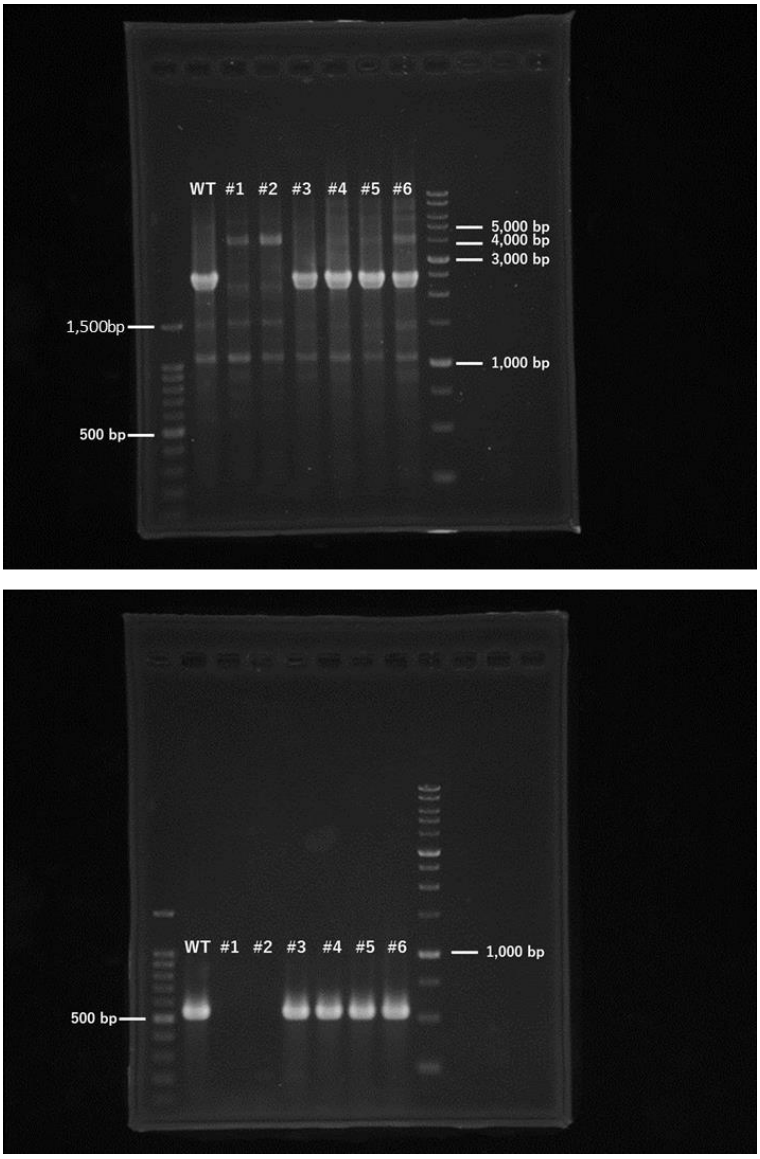


**Figure 32.** Confirmation of transformation by polymerase chain reaction (PCR) in mutants with modifications in the *FocSIX5* gene. The upper and lower panels show PCR amplification of upstream and downstream region, including *FocSIX5* gene with a SIX5-split-F1-SIX5-split-F4 primer set (upper panel) and *FocSIX5* gene coding region with the SIX5-C-F- SIX5-C-R primer set (lower panel). WT: *Foc*_TA. #1: ΔSIX5-1. #2: ΔSIX5-

2. #3: ΔSIX5-1+SIX5. #4: ΔSIX5-2+SIX5 #5: ΔSIX5-2 + $SIX5^{R67K}$-1. #6: ΔSIX5-2 + $SIX5^{R67K}$-2.

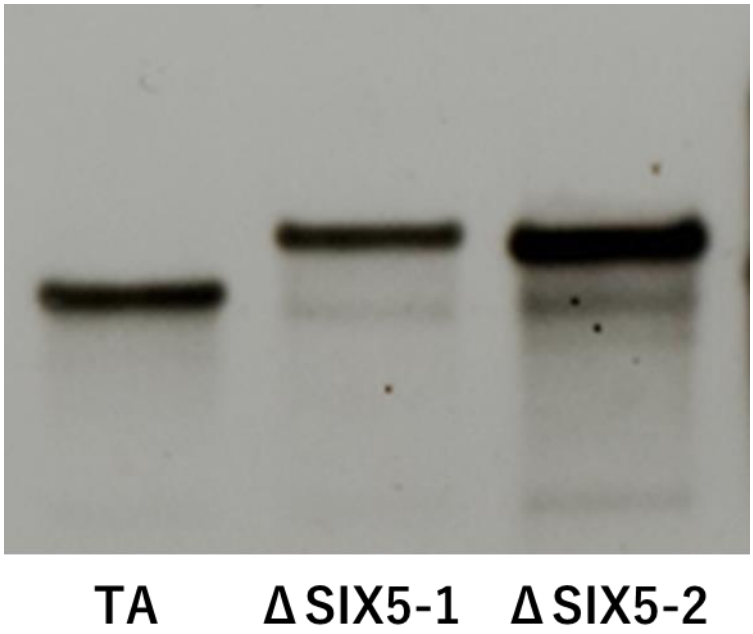


**Figure 33.** Confirmation of *FocSIX5* gene disruption by southern blotting analysis.

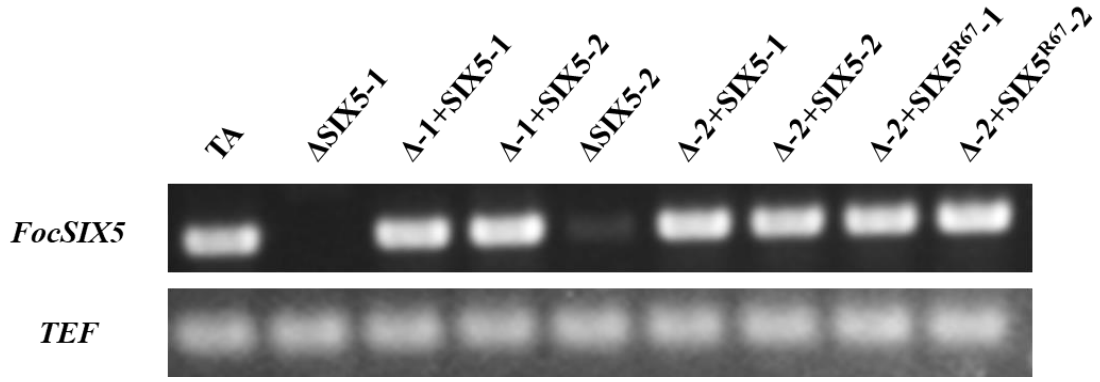


**Figure 34.** Gene expression of *FocSIX5* in *Foc*_TA during infection in shallot. The *TEF* gene was used as a positive control.

### 4.3.5. Mycelial growth of *FocSIX5* gene-modified mutant

To investigate the effects of *FocSIX5* gene modification on phenotypic traits, the fungal development in wild-type *Foc*_TA, *FocSIX5* gene knockout, and *FocSIX5* gene complementation mutants was evaluated. No marked differences were observed in mycelial growth among the wild-type *Foc*_TA, *FocSIX5* gene knockout, or *FocSIX5* gene complementation mutants (Figure 35).

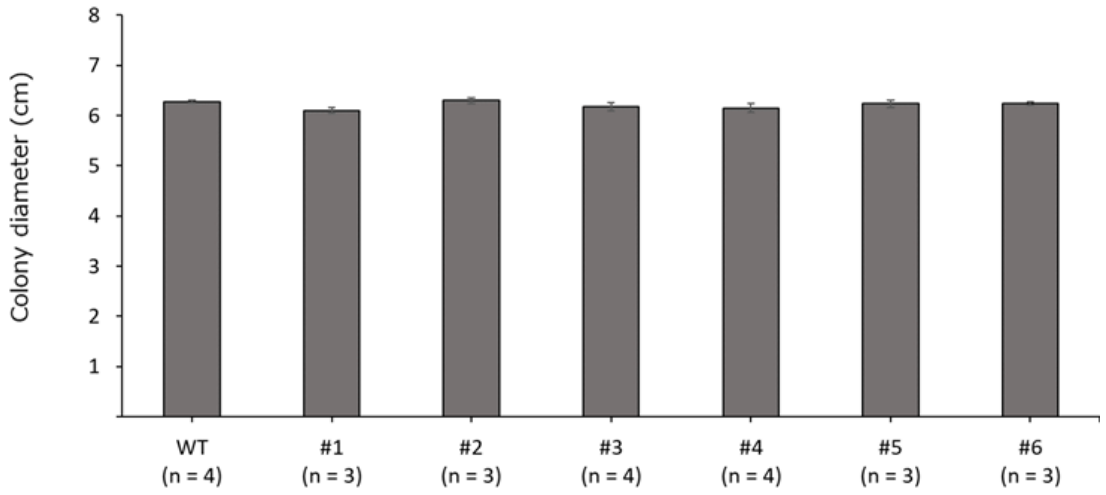


**Figure 35.** Confirmation of colony formation capability in *FocSIX5* gene-modified mutants. The statistically significant difference at $p < 0.05$ were analyzed by one-way ANOVA post hoc Tukey HSD test. n represents sample size. All data were presented as mean and standard error. WT: Foc_TA. #1: ΔSIX5-1. #2: ΔSIX5-2. #3: ΔSIX5-1+SIX5. #4: ΔSIX5-2+SIX5 #5: ΔSIX5-2 + $SIX5^{R67K}$-1. #6: ΔSIX5-2 + $SIX5^{R67K}$-2.

### 4.3.6. Effect of the *FocSIX5* gene modification on pathogenicity toward onion and shallot

*FolSIX5* acts as both a virulence and an avirulence gene in the *Fol*-tomato pathosystem (Ma et al., 2015). Thus, onion bulbs were inoculated with *FocSIX5* gene knockout (ΔSIX5-1 and ΔSIX5-2) and *FocSIX5* gene complementation mutants (ΔSIX5-1 + SIX5 [Δ-1 + SIX5] and ΔSIX5-2 + SIX5 [Δ-2 + SIX5]) to investigate whether *FocSIX5* gene is related to pathogenicity toward onion. *FocSIX5* gene knockout mutants did not remarkably compromise virulence but slightly decreased the symptom area on the onion bulb compared to the wild-type *Foc*_TA and *FocSIX5* gene complementation mutants (Figure 36). Pathogenicity tests for shallots were performed using wild-type *Foc*_TA, *FocSIX5* gene knockout, and *FocSIX5* gene complementation mutants. The shallot plants used in Chapter 4 exhibited a highly *Foc*-resistant phenotype; therefore, shallot plants inoculated with wild-type *Foc*_TA did not exhibit severe wilting symptoms. However, shallot plants inoculated with the *FocSIX5* gene knockout mutant showed more severe wilting than those inoculated with the wild-type *Foc*_TA. Furthermore, the biomass of *Foc* in the inoculated shallot with *FocSIX5* gene knockout mutant were significantly higher when compared to the inoculated shallot with *Foc*_TA (Figure 37 and Figure 38). In addition, the biomass of shallot plants inoculated with *FocSIX5* gene knockout mutant was consistently lower than that of shallot plants inoculated with the wild-type *Foc*_TA and *FocSIX5* gene knockout mutants (Figure 39). Moreover, no severe wilt symptoms were observed in shallot plants inoculated with the *FocSIX5* gene complementation mutants, demonstrating that FocSIX5 acts as an intact avirulence effector in shallots (Figure 40).

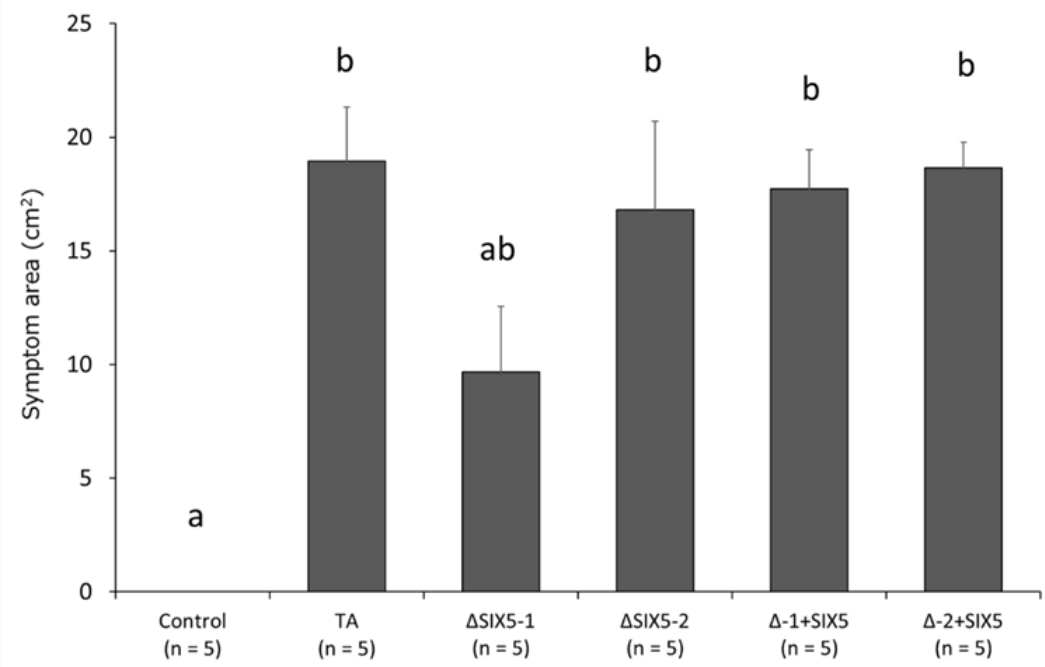


**Figure 36.** Results of the pathogenicity test toward onion bulb with *FocSIX5* gene knockout and gene complementation mutant. Symptom area on onion bulb inoculated with wild-type *Foc*_TA, *FocSIX5* gene knockout (ΔSIX5-1 and ΔSIX5-2), and *FocSIX5* gene complementation mutants (ΔSIX5-1 + SIX5 [Δ-1 + SIX5] and ΔSIX5-2 + SIX5 [Δ-2 + SIX5]) four weeks after inoculation. The statistically significant difference at $p < 0.05$ were analyzed by one-way ANOVA post-hoc Tukey HSD test. n represents sample size. All data are presented as mean and standard error.

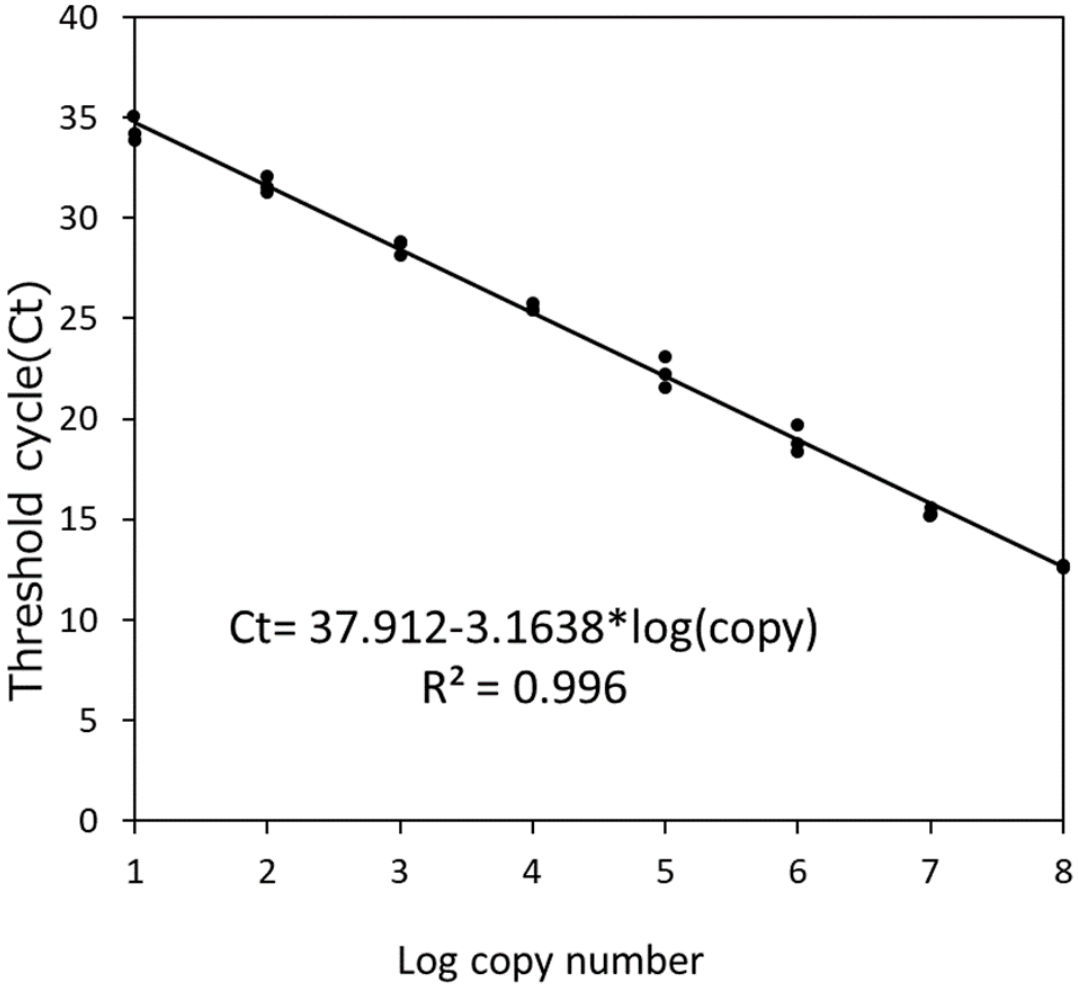


**Figure 37**. Standard regression line of serial dilution (from $10^1$ to $10^8$) with plasmid DNA containing single copy *FocSIX3* fragment. Threshold cycle were plotted with the log of plasmid standard curves.

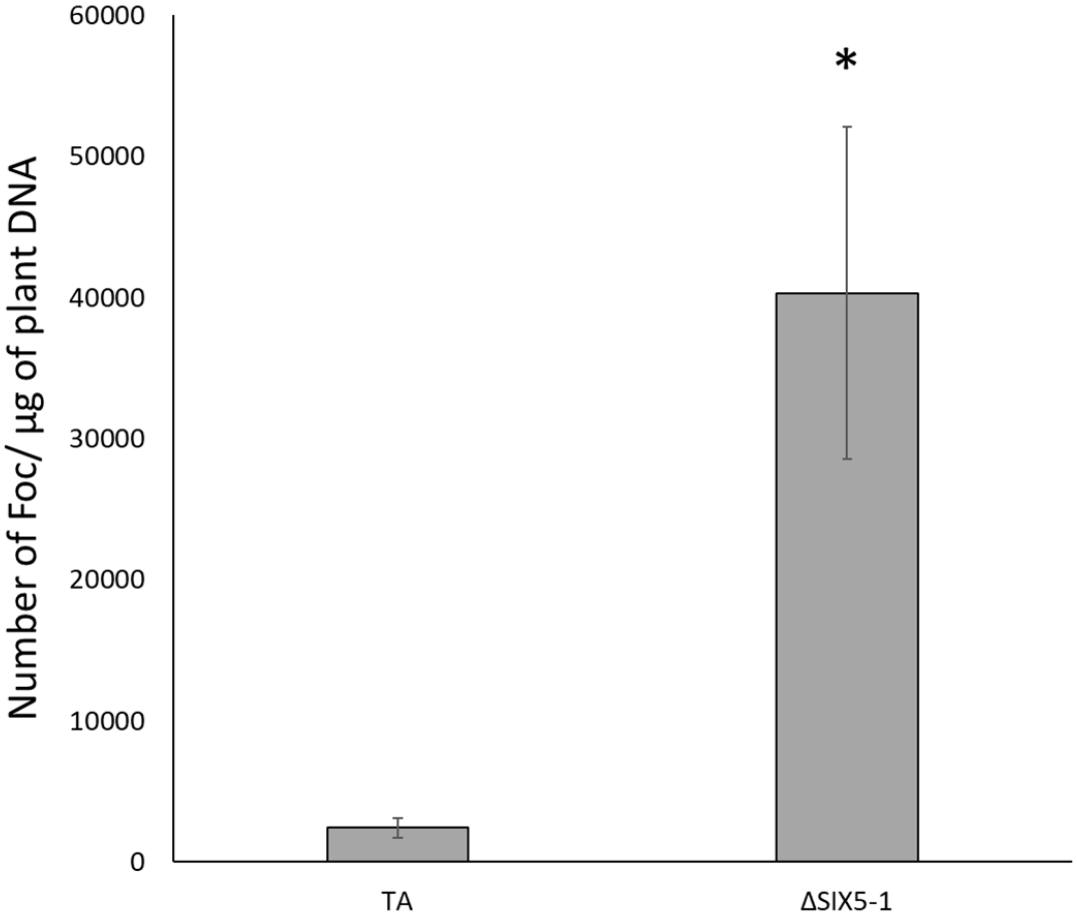


**Figure 38**. Quantification of *Foc* detected from the shallot plant inoculated with *Foc*_TA and *FocSIX5* gene knockout mutants ( Δ SIX5-1). The total DNA was extracted from three independent shallot plants inoculated with *Foc*_TA and *FocSIX5* gene knockout mutant ( Δ SIX5-1). Asterisks indicate a significant difference (* $p < 0.05$) compared to the *Foc*_TA using a Student's *t*-test. All data are presented as mean and standard error.

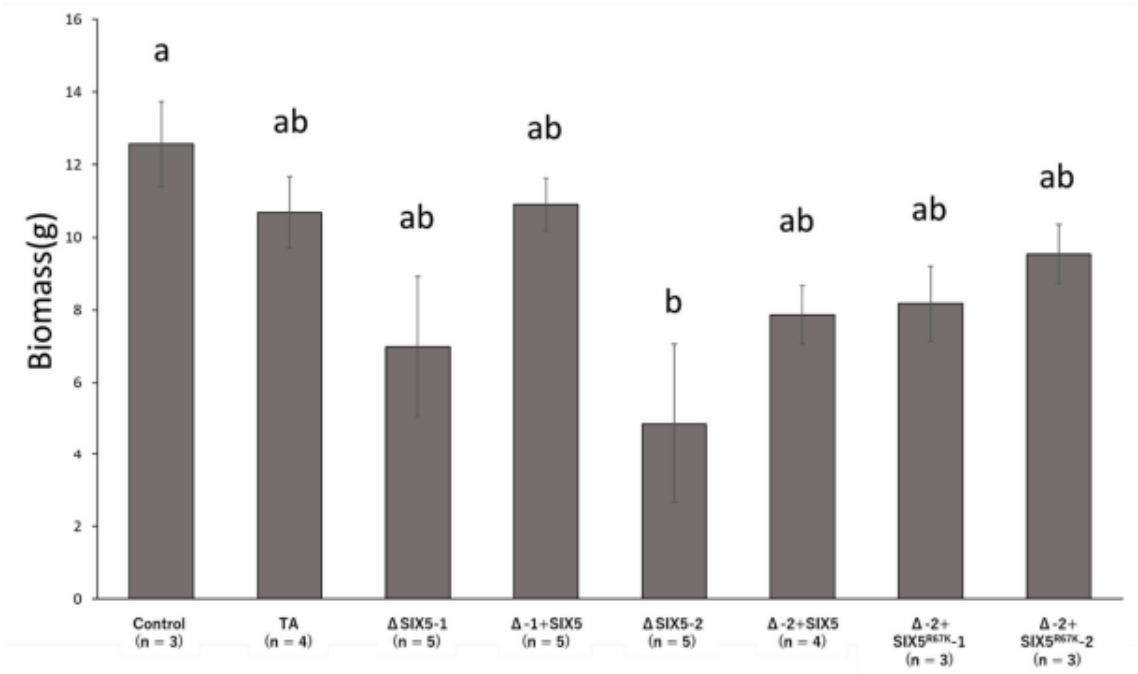


**Figure 39**. Biomass of shallot plant inoculated with wild-type *Foc*_TA, *FocSIX5* gene knockout, and complementation mutants. Average biomass of shallot plants inoculated with wild-type *Foc*_TA, *FocSIX5* gene knockout (ΔSIX5-1 and ΔSIX5-2) and gene complementation mutant (ΔSIX5-1 + SIX5 [Δ-1 + SIX5], ΔSIX5-2 + SIX5 [Δ-2 + SIX5], ΔSIX5-2 + $SIX5^{R67K}$-1 [Δ-2+ $SIX5^{R67K}$-1], and ΔSIX5-2+ $SIX5^{R67K}$-2 [Δ-2+ $SIX5^{R67K}$-2]) with five weeks after inoculation. The statistically significant difference at $p < 0.05$ was analyzed by one-way ANOVA post hoc Tukey HSD test. n represents sample size. Data are presented as mean and standard error.

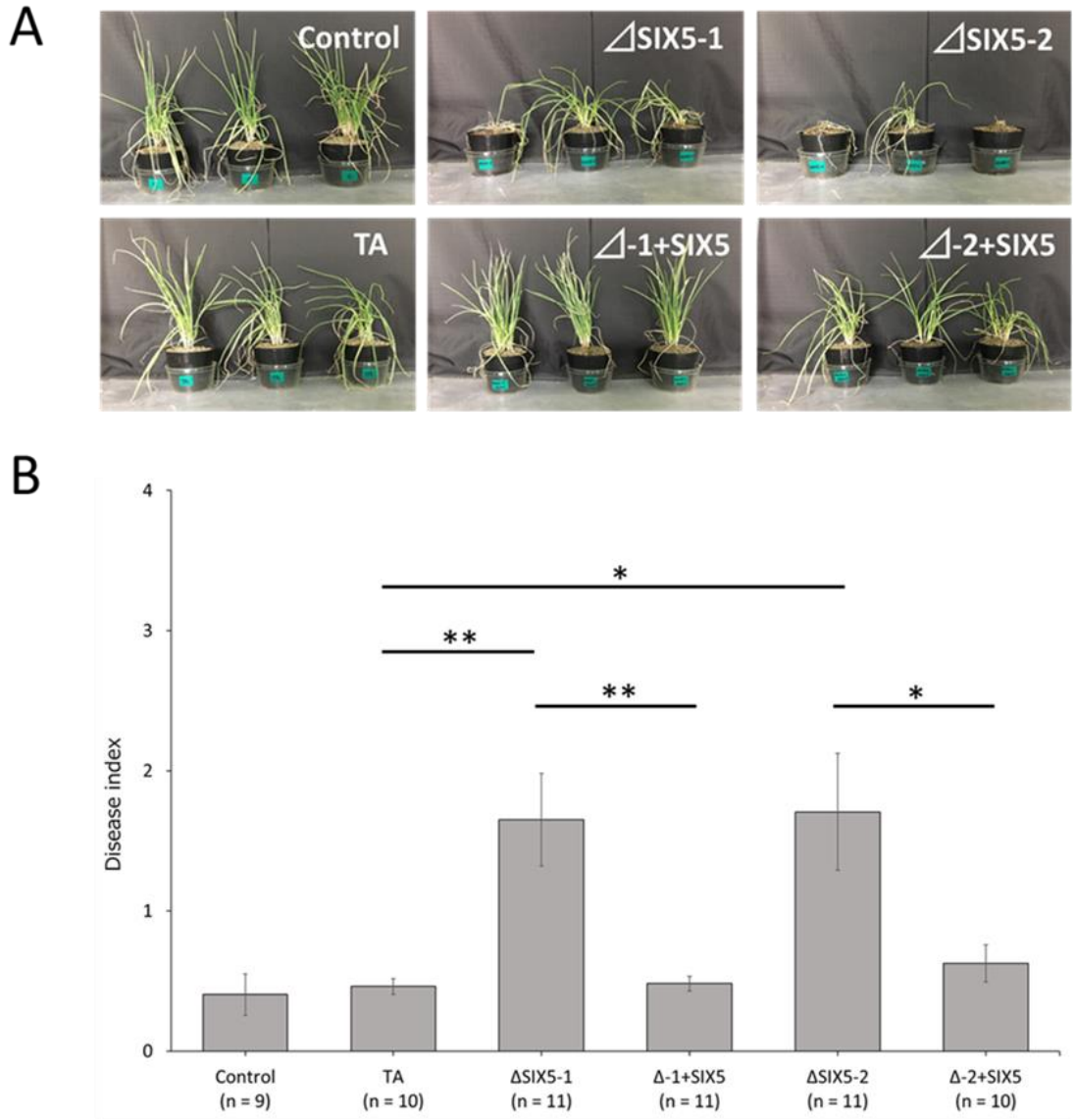


**Figure 40.** Results of the pathogenicity test toward shallot plants with *FocSIX5* gene knockout and gene complementation mutant. (A) Photographs of representative shallot plants inoculated with wild-type *Foc*_TA, *FocSIX5* gene knockout (ΔSIX5-1 and ΔSIX5-2), and *FocSIX5* gene complementation mutants (ΔSIX5-1 + SIX5 [Δ-1 + SIX5] and ΔSIX5-2 + SIX5 [Δ-2 + SIX5]) five weeks after inoculation. (B) Average disease index of shallot plants inoculated with wild-type *Foc*_TA, *FocSIX5* gene knockout (ΔSIX5-1 and ΔSIX5-2), and *FocSIX5* gene complementation mutants (ΔSIX5-1 + SIX5 [Δ-1 +

SIX5] and ΔSIX5-2 + SIX5 [Δ-2 + SIX5]) five weeks after inoculation. Results of at least two experiments were combined. Asterisks indicate a significant difference (** $p < 0.01$, * $p < 0.05$) evaluated using a Student's *t*-test. n represents sample size. Data are presented as mean and standard error.

### 4.3.7. Effect of G200A mutation on the pathogenicity toward shallot

To evade plant immunity, pathogens mutate the nucleotide sequences of their avirulence effectors, resulting in nonsynonymous substitutions. Therefore, BLAST investigation was conducted to investigate whether there were sequence variations in FocSIX5 among different isolates. Notably, a single-nucleotide polymorphism (G200A) was detected in the *FocSIX5* nucleotide sequence of the *Foc*_A21 strain isolated from the United Kingdom (Taylor et al., 2016) and the Fox129 strain isolated from Finland (Haapalainen et al., 2022), leading to a nonsynonymous substitution (R67K) (Figure 41A). Additionally, the same nonsynonymous substitution (R67K) was found in the Australian AP117 strain as in *Foc* collection (Accession No. LC731005). Thus, it was speculated that this nonsynonymous substitution may be a strategy used by *Foc* to avoid recognition by the host. To test this hypothesis, gene complementation mutants with the *FocSIX5* gene construct, including the G200A SNP (ΔSIX5-2 + $SIX5^{R67K}$-1 [Δ-2 + $SIX5^{R67K}$-1] and ΔSIX5-2 + $SIX5^{R67K}$-2 [Δ-2 + $SIX5^{R67K}$-2]) were generated (Figure 32), and performed pathogenicity tests on shallots. Contrary to the hypothesis, shallot plants inoculated with mutants expressing SIX5 protein variants carrying the R67K substitution exhibited a highly resistant phenotype, suggesting that host plant immunity was not related to a single amino acid mutation (R67K) in FocSIX5 (Figure 41B and Figure41C).

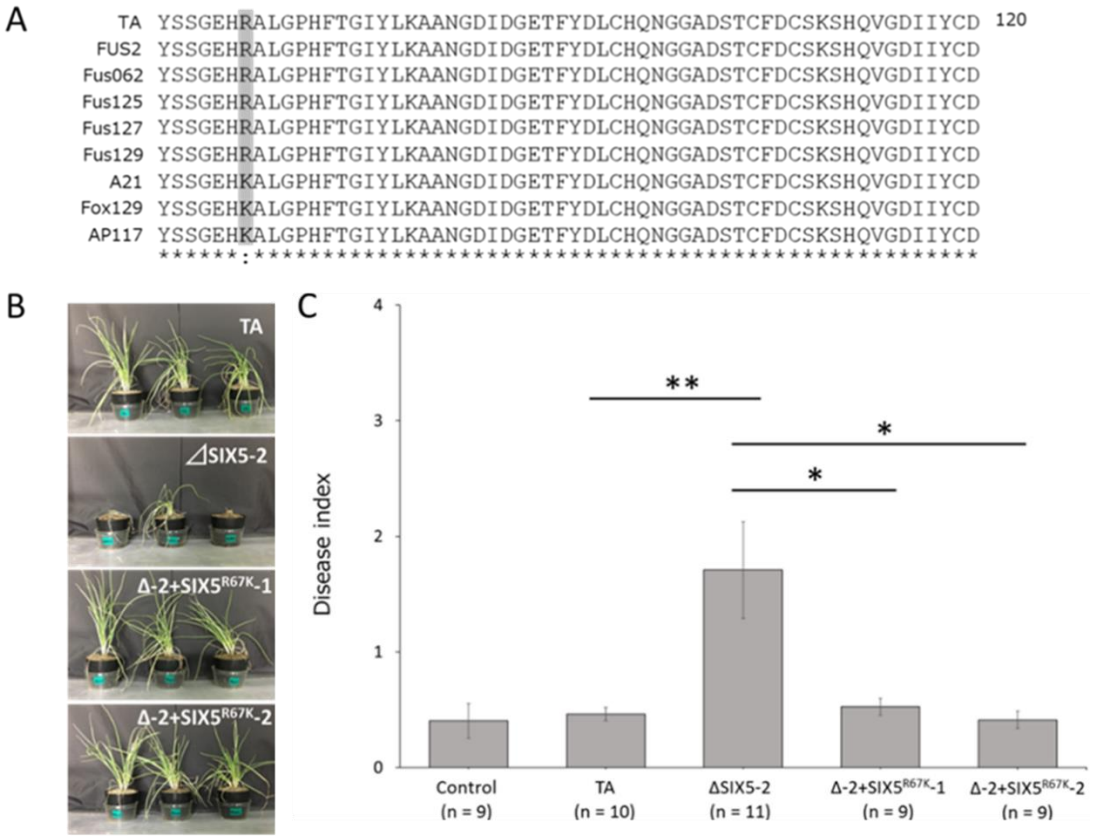


**Figure 41.** Results of the pathogenicity test after single amino acid substitution R67K in the FocSIX5 sequences. (A) Alignment of amino acid sequences of FocSIX5. The gray box shows sequence variation in FocSIX5 among *Foc* strains. (B) Photographs of representative shallot plants inoculated via *FocSIX5* gene knockout (ΔSIX5-2) and gene complementation mutant with *FocSIX5* gene variant G200A SNP (ΔSIX5-2 + SIX5$^{R67K}$-1 [Δ-2 + SIX5$^{R67K}$-1] and ΔSIX5-2 + SIX5$^{R67K}$-2 [Δ-2 + SIX5$^{R67K}$-2]) five weeks after inoculation. (C) Average disease index of shallot plants inoculated with the *FocSIX5* gene knockout mutant (ΔSIX5-2) and gene complementation mutant with *FocSIX5* gene variant carrying the G200A SNP (ΔSIX5-2 + SIX5$^{R67K}$-1 [Δ-2 + SIX5$^{R67K}$-1] and ΔSIX5-2 + SIX5$^{R67K}$-2 [Δ-2 + SIX5$^{R67K}$-2]) five weeks after inoculation. Results of at least two experiments were combined. Asterisks indicate a significant difference (**$p < 0.01$, * $p < 0.05$) evaluated using a Student's *t*-test. n represents sample size. Data are presented as

mean and standard error.

## 4.4 DISCUSSION

Plant-pathogenic fungi secrete effectors that manipulate the host immunity to induce infections. However, some effectors are recognized as avirulent by innate plant immune receptors, which cause robust plant resistance responses (Jones and Dangl 2006; Hammond-Kosack and Jones 1997; Martin et al., 2003). In *F. oxysporum*, avirulence effectors such as SIX1 (AVR3), SIX3 (AVR2), SIX5, and SIX4 (AVR1) in *Fol* and SIX6 in *F. oxysporum* f. sp. *niveum* (which infects watermelons) have been identified (Houterman et al., 2009; Ma et al., 2015; Niu et al., 2016). Among the SIX effectors in *Fol*, SIX5 is required for full virulence in susceptible tomato lines, and a SIX5 homolog is present in *Foc* (Sasaki et al., 2015b; Ma et al., 2015). Therefore, it was investigated whether SIX5 in *Foc* is related to pathogenicity in onion and shallot plant as reported for SIX5 in *Fol*-tomato pathosystem (Ma et al., 2015). In the present study, it was confirmed that *FocSIX5* in *Foc*_TA was expressed in both onion and shallot roots during the *Foc*-infection process. The *FocSIX5* gene was expressed in shallot and onion roots inoculated with *Foc*_TA, suggesting that FocSIX5 may play an important role in the pathogenicity of *Foc* in onion and shallot infections. To investigate the relationship between the *FocSIX5* gene and pathogenicity during *Foc* infection in onions and shallots, *FocSIX5* gene-modified mutants were generated. Upon conducting a pathogenicity test, onion bulbs inoculated with the wild-type *Foc*_TA strain, *FocSIX5* gene knockout mutants, or *FocSIX5* gene complementation mutants exhibited typical symptoms of FBR, and the symptom areas were not considerably different between the wild-type *Foc*_TA and *FocSIX5* gene-modified mutants. However, the symptom area in onions inoculated with *FocSIX5* gene knockout mutants was slightly decreased compared to that in wild-type *Foc*_TA or *FocSIX5* gene complementation mutants. Given that *FocSIX5* gene knockout

mutants showed the same colony formation capability as wild-type *Foc*_TA and *FocSIX5* gene complementation mutants, the *FocSIX5* gene was specifically upregulated during *Foc* infection of susceptible onion, suggesting that FocSIX5 plays a role in the colonization of host plants rather than growth in onion. Similarly, SIX5 of *Fol* is related to virulence; thus, it was speculated that *FocSIX5* gene is related to virulence in onions. Nevertheless, shallot plants inoculated with the *FocSIX5* gene knockout mutant showed more severe wilt symptoms than those inoculated with the wild-type *Foc*_TA, indicating that FocSIX5 secreted by *Foc* acts as an avirulence effector in shallots. Although the mechanism underlying the severe disease symptoms in shallot plants inoculated with the *FocSIX5* gene knockout mutants is not clear from the present study, it has been reported that host plants inoculated with avirulence geneknockout *F*. *oxysporum* mutants showed more severe wilting than those inoculated with wild-type *F*. *oxysporum* (Houterman et al., 2009; Ma et al., 2015; Niu et al., 2016). To the best knowledge, this is the first report of an avirulence effector in *Foc* toward *Allium* species.

Pathogens undergo mutations in the avirulence effector that avert host recognition and promote infection (Rouxel and Balesdent 2010; Stephens et al., 2021). The *Fol* race 3 strain has three different patterns of single amino acid substitutions in its AVR2 sequence (V41M, R45H, and R46P) to escape from *I-2*-derived immunity (Houterman et al., 2009). *FocSIX5* is widely expressed in aggressive *Foc* (Haapalainen et al., 2022; Sasaki. 2015a; Taylor et al., 2016). Therefore, it was speculated that there are variations in the FocSIX5 sequence. As expected, FocSIX5 had a single amino acid substitution, R67K, when sequence variants from different *Foc* strains were compared. To determine whether the R67K substitution affected avirulence, *FocSIX5* gene construct with an allelic variant carrying the G200A mutation was complemented. Shallot plants inoculated with mutants

expressing SIX5 protein variants carrying the R67K substitution exhibited a highly resistant phenotype, suggesting that the single amino acid substitution R67K in FocSIX5 is not sufficient to overcome shallot resistance to *Foc*. The SIX5 protein is conserved only within *Fol* and *Foc* in the *F. oxysporum* species complex with 74% identity and harbors cysteine residues at precisely the same positions. Notably, homologs of SIX5 in *Leptosphaeria maculans* (AvrLm10A, AvrLm10A_like1, and AvrLm10A_like2), the causal agent of oilseed rape stem canker were respectively shared 36% (AvrLm10A) and 52% (AvrLm10A_like1, and AvrLm10A_like2) amino acid sequence identity with FolSIX5 and acts as avirulence effector toward *Brassica nigra*, which suggests that receptor protein recognizing avirulence effector might be widely conserved in various plant species and tertiary structure of the avirulence protein could be important rather than high protein sequence identity to be recognized by receptor protein (Petit-Houdenot et al., 2019; Talbi et al., 2023; Talbi et al., 2024). As above, some avirulence effectors possessed amino acid substitution in the sequence, leading to escape the recognition of receptor protein, which suggests the substitution in the amino acid sequence could result in a change in the tertiary structure of the avirulence effector.

Interestingly, AVR2 (SIX3) and SIX5 share promoter regions in the *Fol* genome, and their encoded proteins physically interact with each other and are necessary for triggering *I-2*-derived immunity in tomato plants (Ma et al., 2015). In *Foc*, *FocSIX3* and *FocSIX5* genes are located on the same scaffold, sharing both promoter regions in the reference genome of the *Foc*_FUS2 strain (Armitage et al., 2018), and the nucleotide sequence of *FocSIX3* is 91.4% similar to that of *FolSIX3* (Sasaki 2015a). Thus, FocSIX3-FocSIX5 may physically interact with each other and play a role similar to that of AVR2-SIX5, as reported by *Fol* (Ma et al., 2015). However, shallot plants inoculated with the *FocSIX3*

gene knockout mutant did not show the same wilt symptoms as those inoculated with wild-type *Foc*_TA (data not shown), suggesting that FocSIX3 is not an avirulence effector recognized by the shallot, in contrast to the AVR2-SIX5 pair in *Fol*. Thus, it is possible that the putative immune receptor of shallots that recognizes FocSIX5 is unlikely to resemble but is partially similar to the I-2 receptor of tomatoes. The *I-2* gene encodes a nucleotide-binding and leucine-rich repeats (NB-LRR) at the N- and C-termini, respectively (Takken et al., 2010). Further studies are required to explore the NB-LRR proteins that recognize FocSIX5 in shallots. Some plant immune receptor proteins specifically interact with avirulent effectors secreted by the causative agents of the disease. For example, the immune receptor protein L6 in flax (*Linum usitatissimum*) interacts with the Avr567 avirulence protein in flax rust (*Melampsora lini*), causing a hypersensitivity reaction (Ellis et al., 2007). In addition, the resistance protein Pi-ta in rice and the avirulence effector AVR-Pi-ta in the rice blast fungus *Pyricularia oryzae* bind directly to each other to confer rice blast resistance (Jia et al., 2000). *Foc*-resistance-related loci and genes in shallots are gradually being investigated; however, the specific loci and genes involved in disease resistance remain unclear (Herlina et al., 2019; Vu et al., 2012). Thus, FocSIX5 may be a useful tool for identifying *Foc*-resistant loci or genes in shallot. In Chapter 4, only one shallot genotype exhibiting high resistance to *Foc* was used. However, other shallot genotypes resistant to *Foc* have also been reported (Sintayehu et al., 2011). Hence, comparative genome analysis of these shallot genotypes and shallot cv. Chiang Mai used in Chapter 4 could reveal *Foc*-resistant loci or genes, leading to an understanding of the resistance mechanism of shallots to *Foc* and the acquisition of a promising breeding resource for onion disease resistance. Collectively, these results indicate that the high-*Foc*-resistance shallot cv. Chiang Mai and shallot immunity-

recognizing FocSIX5 are promising breeding resources for disease resistance in onions against *Foc*.

# CHAPTER 5

# GENERAL DISCUSSION

Onion (*A. cepa* L.) is globally cultivated and commercially important crop. However, the occurrence of FBR caused by *F. oxysporum* f. sp. *cepae* (*Foc*) has been globally reported (Bektas and Kusek. 2019; Galván et al., 2008; Haapalainen et al., 2016; Muthukumar et al., 2022; Sasaki et al., 2015b; Smith 2009; Southwood et al., 2012; Swift 2001; Taylor et al., 2016). Given that mankind is facing global warming and the *Foc*, the causative agent of FBR favors the high temperature (approximately 30℃) to grow and infect onion, the occurrence of FBR is likely more spread from now on (Abawi and Lorbeer 1972; Elad and Pertot 2014; Lee and Magan 2010). Therefore, research on the interaction between *Foc* and onion is need to be increasingly carried out and gradually unveiled how *Foc* can infect onion (Sasaki et al., 2015; Taylor et al., 2019a). Moreover, taxonomic rearrangement of *F. oxysporum* was recently established with multi-locus based phylogenetic analysis as epitype of *F. oxysporum* to escape from present taxonomic chaos in *F. oxysporum*. Here, this study aimed 1) to verify the taxonomic position of *Foc* in the newly established epitype of *F. oxysporum*, 2) to clarify the pathogenicity differentiation in *Foc*, 3) to identify the pathogenicity chromosome in *Foc*, and 4) to identify the pathogenicity related factor in *Foc*.

In Chapter 2, taxonomic position of the twelve *Foc* strains isolated from diseased onion and Welsh onion seedlings were examined with the newly established epitype of *F. oxysporum* since *F. oxysporum* species complex was recently re-organized as epitype of *F. oxysporum* (Lombard et al., 2019). According to phylogenetic analysis with the

combined sequence of *cmdA*, *rpb2*, and *tef1*, the strains used in Chapter 2 were grouped into *F. nirenbergiae* and *Fusarium* sp. but not *F. oxysporum* in the newly established epitype of *F. oxysporum*, suggesting that single locus-based identification is collapsed in the newly established epitype of *F. oxysporum*. This taxonomic chaous was caused by which single locus-based classification for identification and classification system as "*formae speciales*" according to host range in *F. oxysporum* has been generally used for a long time (Armstrong 1981; Snyder and Hansen 1940). Hence, although it might take time to generally use the newly established epitype of *F. oxysporum*, avoidance of the use of single locus-based identification and "*formae speciales*" would be better to make the *F. oxysporum* taxon more stable for the future as already re-organized in *F. oxysporum* f. sp. *cubense* (Maryani et al., 2019). Moreover, the result of Chapter 2 implied pathogenicity differentiation toward onions and Welsh onions in *Fusarium* species, causative agent of FBR and FW. Regarding the insight into acquisition of pathogenicity toward Welsh onion from opportunistic to parasitic strain, further investigation is considered to be needed by comprehensive comparative of the genome between opportunistic and parasitic strains to reach out how the pathogenicity differentiation occurred in *Foc*. On the other hand, given that the strains isolated from onions commonly possessed same *SIX* genes (*SIX3*, *5*, *7*, *9*, *10*, *12*, and *14*) and exerted highly aggressive pathogenicity toward onions and Welsh onions, it is plausible that the onion-infecting strains obtained the 4-Mb-sized pathogenicity chromosome containing *SIX3*, *5*, *7*, *9*, *10*, *12*, and *14* genes to be pathogenic upon onion via horizontal chromosome transfer. However, it is not exclusive that the 4-Mb-size pathogenicity chromosome can drive the pathogenicity toward Welsh onions. Thus, research on the pathogenicity of 4-Mb-sized chromosome strain (TCL6) toward Welsh onion and comparative genome analysis will

provide a more reliable insight into pathogenicity differentiation toward onions and Welsh onions. Moreover, it was found that the *SIX9* gene was commonly possessed and expressed in onion-infecting and Welsh onion-infecting strains, suggesting that the *SIX9* gene acts as a core pathogenicity related factor toward onion and Welsh onion. Hence, investigation of *SIX9* gene function using gene knockout approach is desirable. If the *SIX9* gene is involved with pathogenicity, the *SIX9* gene could be useful tool for the detection and diagnosis of FBR and FW .

In Chapter 3, the results demonstrated that the 4-Mb-sized chromosome in *Foc* truly acts as pathogenicity chromosome required for full pathogenicity toward onion. Indeed, it has been reported that 3.9 Mb-sized region in an aggressive pathogenic *Foc*_FUS2 strain was predicted as pathogen-specific (PS) region (Armitage et al., 2018), which data strongly supports this result. Moreover, taken together with data from the results of Chapter 3 and Armitage et al. (2018), 4-Mb-sized pathogenicity chromosome contains a bunch of effector genes. Intriguingly, out of the effector genes highly expressed during infection, several effector genes shared the upstream promoter region (data not shown). Recently, some effector genes are working with the other effector in pair mode (Ma et al., 2015). Therefore, effector genes sharing the promoter region in *Foc* also functioned toward pathogenicity in corporate with the other effector. Furthermore, some transcription factors, expressed during infection were associated with pathogenicity. Of the transcription factors in *F. oxysporum*, *FTF1* is known as a regulator of *SIX1* and *SIX6* gene in *F. oxysporum* f. sp. *phaseoli* and was suggested to correlate with pathogenicity toward common bean (Niño-Sánchez et al., 2016; Niño-Sánchez et al., 2015). Notably, result of Chapter 3 suggested that *FTF1* homologs possessed by *Foc* (*FocFTF1*) contribute to virulence toward onion, implying that investigation of the gene expression regulated by

*FocFTF1* discloses novel pathogenicity-related factor in *Foc*.

In Chapter 4, the results shows that FocSIX5 acts as an avirulence effector toward shallot. Moreover, non-synonymous substitution in the amino acid sequence in FocSIX5 (R67K) was not related to evacuation of shallot immunity response, implying that resistance protein recognizing FocSIX5 would be durable disease resistance resources for onion since shallot can be crossbred with onion. Notably, it has been reported that the *SIX5* gene is widely conserved among genera of plant pathogen other than *F. oxysporum* (Talbi et al., 2023) and FolSIX5 was also identified as an avirulence effector recognized by the tomato I-2 receptor in *Fol*-tomato pathosystem (Takken and Rep 2010). Moreover, SIX5 homolog in *Leptosphaeria maculans*, the causal agent of oilseed rape stem canker also functioned as avirulence effector toward *Brassica nigra* (Petit-Houdenot et al., 2019), even though the SIX5 homolog in *L. maculans* shows relatively low protein sequence similarity when compared to that of FolSIX5. Recently, some proteins were functionally the same even though the proteins have low similarity of protein sequence if the proteins shared an important domain (Nomura et al., 2023), which this insight suggests the SIX5 homologs conserved within various plant pathogen potentially acts as avirulence effector in various pathosystem.

In conclusion, Chapter 2 clarified that previously identified *Foc* strains were categorized into *F. nirenbergiae* and *Fusarium* spp.. Moreover, the results provided insight into pathogenicity differentiation toward onion and Welsh onion in *Fusarium* species. In Chapter 3, the results indicated that 4-Mb-sized chromosome in *Foc* functioned as pathogenicity chromosome and 3.1 Mb region within 4-Mb-sized chromosome is required for full pathogenicity toward onion. This result strongly suggested that unique pathogenicity related factor is located on the 3.1 Mb region in 4-Mb-sized chromosome.

Therefore, radical investigation of the 3.1 Mb region would disclose the novel pathogenicity related factor, required for pathogenicity toward onion in *Foc*, leading to a deeper understanding the mechanism of *Foc* infection. In Chapter 4, the results demonstrated that *FocSIX5* gene acts as an avirulence effector toward shallot. To the best knowledge, this is the first report of avirulence effector in *Foc*. This study hopefully provides information on the insight of current taxonomy, pathogenicity differentiation, and pathogenicity related factor in *Foc*.

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# SUMMARY

Onion (*Allium cepa* L.) is staple crop on the tables of countries around the world and is a globally very important economic crop, with more than 100 million tons produced worldwide in 2020. However, Fusarium basal rot disease (FBR), caused by *F. oxysporum* f. sp. *cepae* (*Foc*) threatens onion production. Furthermore, the occurrence of FBR upon onion is globally spread, however, no onion variety exhibits complete resistance to the *Foc*. Therefore, breeding for disease resistance upon onion based on the pathogenicity and infection mechanism of *Foc* is an urgent issue.

This study aimed to elucidate the current taxonomic position and pathogenicity related factor in *Foc* by using multi locus-based phylogenetic, genomic sequencing techniques, and gene function analysis with the reverse genetic method.

The *F. oxysporum* species complex has been reported to infect more than 120 plant species and has been further subdivided into "*formae speciales*" based on its host range. Due to its unique classification system, the classification of the *F. oxysporum* species complex is currently chaotic. Therefore, a taxonomic reorganization of the *F. oxysporum* species complex was undertaken to resolve the chaos in the classification of the *F. oxysporum* species complex. Therefore, in Chapter 2, the taxonomic position of *Foc* was examined in the newly established epitypes of *F. oxysporum*. The results revealed that 12 strains, previously identified as *Foc* were classified as *F. nirenbergiae* and *Fusarium* sp. in the newly established epitypes of *F. oxysporum*. Notably, none of the strains were categorized as *F. oxysporum*. To determine the host specification in the strains used in Chapter 2, pathogenicity test toward onion and Welsh onion as its potential host was carried out. As a result of pathogenicity test, four strains (AC145, AP117, Ru-13, and TA), isolated from diseased onion, commonly possessed *SIX3*, *5*, *7*, *9*, *10*, *12*, and *14* genes

showed aggressive pathogenicity toward onion while all strains except for one strain (AF97) used in Chapter 2 caused significant inhibition of Welsh onion growth. The pathogenicity test also provided insight into the evolutionally pathogenicity differentiation toward onion and Welsh onion in *Fusarium* species. A non-pathogenic *Fusarium* strain that invades as endophyte evolutionally strengthens pathogenicity toward Welsh onion via vertical evolution of pathogenicity related factor or horizontal transfer of pathogenicity related factor. Subsequently, Welsh onion-infecting *Fusarium* strain evolutionally acquired pathogenicity chromosome, containing *SIX* genes to exert aggressive pathogenicity toward onion via horizontal chromosome transfer.

Plant pathogens frequently possess the "pathogenicity chromosomes", which dominate pathogenicity to the host and in which many effectors involved in pathogenicity located on the chromosome. However, it has not been reported whether pathogenicity chromosome is present in *Foc*. In Chapter 3, genome of *Foc* was analyzed and it was found that effector-like genes frequently located on a 4-Mb-sized chromosome. Therefore, nine putative 4-Mb sized chromosome loss strains (TCL1, TCL3-10) were generated by treatment of benomyl (mitotic inhibitor). The colony formation capability and pathogenicity of the 4-Mb-sized chromosome loss strains against onion were investigated, and it was found that all 4-Mb-sized chromosome loss strains impaired their pathogenicity against onion even though their colony formation capability was the same when compared to wild-type *Foc*_TA. Furthermore, genome analysis of wild-type *Foc*_TA, ΔSIX5 (the original strain lacking the 4-Mb-sized chromosome), TCL1, TCL6, and TCL9 revealed that the 4-Mb-sized chromosome functions as a pathogenicity chromosome in the *Foc*. Moreover, at least 3.1 Mb region within the 4-Mb-sized chromosome is required for pathogenicity toward onion.

Plant pathogenic microbe secretes not only “Effector”, required for pathogenicity but also "Avirulence effector" that induces resistance to its host. However, avirulence effector in *Foc* has not been elucidated so far. This is one of the reasons why the progress of disease resistance breeding in onions is significantly delayed. Because the information on the avirulence effector is important to know the resistance protein in its host. Hence, in Chapter 4, identification of avirulence effector in *Foc* was attempted. As previously reported in the pathosystem between *F*. *oxysporum* f. sp. *lycopersici* (*Fol*) and tomato, FolSIX5 acts as avirulence effector toward *I-2* tomato lineage, moreover, it has been reported that SIX5 homolog was possessed by *Foc*. Hence, it was investigated whether FocSIX5 acts as avrulence effector toward *Foc* resistance shallot that is genetically compatible and can be crossbred with onion. As a result of pathogenicity test toward *Foc* resistance shallot, *FocSIX5* gene knockout mutants caused severe disease upon *Foc* resistance shallots when compared to wild-type *Foc*_TA and *FocSIX5* gene complementation mutants. Moreover, non-synonimous substitution in FocSIX5 sequence (R67K) was not enough to overcome the shallot immunity. This result demonstrated that *FocSIX5* gene functions as an avirulence effector against *Foc* resistance shallots and shallot may be a promising disease resistance resource against FBR, and that immune receptors recognizing the FocSIX5 avirulence effector would be a molecular marker to accelerate disease resistance breeding in onion.

# JAPANESE SUMMARY

タマネギ (*Allium cepa* L.)は、世界各国の食卓に欠かせない作物であり、2020年には世界全体で 1 億 t 以上の量が生産されている世界的に非常に重要な経済作物となっている。しかしながら、土壌伝染性植物病原菌であるタマネギ乾腐病菌の感染によって引き起こされるタマネギ乾腐病が世界的に蔓延しており、タマネギ生産を脅かしているのが現状である。本病害は、原因菌が *Fusarium oxysporum* f. sp. *cepae* (*Foc*)である事が明らかになっているにも関わらず、タマネギ乾腐病菌に対する完全な抵抗性を示すタマネギ品種が存在しない。その為、タマネギ乾腐病菌の病原性及び感染メカニズムに基づくタマネギ病害抵抗性育種は喫緊の課題となっている。本研究では、国内外で分離されたタマネギ乾腐病菌の遺伝的系統解析、ゲノム解析、及び逆遺伝学的手法を用いた表現型解析を行うことでタマネギ乾腐病菌の病原性及び感染メカニズムの一端を明らかにすることを目的とした。

*F. oxysporum* 種複合体は、120 以上の植物種に感染する事が報告されており、さらに、その宿主範囲に基づき“*formae speciales*”に細分化されてきた。その特異な分類体系が原因で *F. oxysporum* 種複合体の分類はカオス状態となっている。そこで、*F. oxysporum* 種複合体の分類におけるカオス状態を解消する為、*F. oxysporum* 種複合体の分類学的再編成が行われた。その為、第 2 章では、近年構

築された *F. oxysporum* のエピタイプにおける *Foc* の分類学的位置づけを調査した。その結果、これまで *Foc* と同定されていた *Fusarium* 属菌 12 菌株は、*F. nirenbergiae* 及び *Fusarium* sp.のクレードに属する事が明らかとなった。興味深いことに、本研究で用いた全ての菌株が *F. oxysporum* には分類されなかった。さらに、本研究で用いた菌株の宿主特異性を明らかにする為に、タマネギおよびネギに対する病原性試験を行った。その結果、罹病タマネギから分離された 4 菌株（AC145 株、AP117 株、Ru-13 株および TA 株）は、共通して *SIX3*、*5*、*7*、*9*、*10*、*12*、*14* 遺伝子を保持しており、タマネギに対して強い病原性を示した。一方で、AF97 株を除くすべての菌株は、ネギの生育を阻害した。また、この病原性試験から、フザリウム属菌のタマネギおよびネギに対する病原性の進化的分化に関する仮説が得られた。仮説としては、エンドファイトとして侵入した非病原性フザリウム属菌が、病原性関連因子を垂直的もしくは水平的に獲得することで、ネギに対する病原性を示すようになった。その後、ネギに感染するフザリウム属菌が、*SIX* 遺伝子(*SIX3*、*5*、*7*、*9*、*10*、*12*、*14*)を含む病原性染色体を水平的に獲得することでタマネギに対して攻撃的な病原性を発揮する菌株となった、というものである。

植物病原菌は、宿主に対する病原性を司る、且つ病原性に関わるエフェクターが多数座乗する染色体である「病原性染色体」を保持する事が報告されている。

しかしながら、タマネギ乾腐病菌が病原性染色体を保持するかどうかはこれまでに報告されていない。その為、第 3 章ではタマネギ乾腐病菌における病原性染色体の同定を試みた。その結果、タマネギ乾腐病菌が保持するエフェクター様遺伝子の多くは 4 Mb サイズ染色体に座乗しており、タマネギ乾腐病菌においては 4 Mb サイズ染色体が病原性染色体である可能性が見出された。そこで、有糸分裂阻害剤をタマネギ乾腐病菌に処理する事で 4 Mb サイズ染色体欠損株を 9 菌株(TCL1、TCL 3-10)作出する事に成功した。作出した 4 Mb サイズ染色体欠損株のコロニー形成能およびタマネギに対する病原性を調査すると、全ての菌株におけるコロニー形成能がワイルドタイプ TA 株と同等であったにも関わらずタマネギに対する病原性が著しく低下する事が明らかとなった。さらに、ワイルドタイプ TA 株、 ΔSIX5（4 Mb 染色体欠損株の元株）株、 4 Mb 染色体欠損株 TCL1,TCL6 及び TCL9 のゲノム解析を実施したところ、タマネギ乾腐病菌においては、4 Mb サイズ染色体が病原性染色体として機能している事、4 Mb サイズ染色体の内、少なくとも 3.1 Mb の領域がタマネギに対する病原性を司る事が明らかとなった。

植物病原菌は宿主に対する病原性を司る「エフェクター」のみならず、宿主に対して抵抗性を誘導する「非病原力エフェクター」を分泌する事がわかっている。しかしながら、タマネギ乾腐病菌においては非病原力エフェクターはこれまで

に同定されておらず、この事は、タマネギ病害抵抗性育種の進展を阻んでいる一要因となっている。先行研究により、トマト萎凋病菌(*Fol*)が保持する FolSIX5 は *I-2* トマト系統に対する非病原力エフェクターとして機能する事が報告されており、*Foc* も SIX5 のホモログを保持する事が報告されている。そこで、第 4 章では *Foc* が保持する FocSIX5 の病原性及び非病原力における機能の有無を調査した。その結果、*FocSIX5* 遺伝子破壊株は、ワイルドタイプ TA 株および *FocSIX5* 遺伝子相補株と比較して、抵抗性シャロットに対して重篤な病害を引き起こした。さらに、FocSIX5 の配列における非同義的置換（R67K）は、シャロットの免疫を打破するのに十分ではないものであった。この結果は、*FocSIX5* 遺伝子が抵抗性シャロットに対する非病原力エフェクターとして機能する事、シャロットがタマネギ乾腐病菌に対する有望な病害抵抗性育種の素材と成り得る事、さらには非病原力エフェクターFocSIX5 を認識するシャロット免疫受容体がタマネギの病害抵抗性育種を促進する分子マーカーとなる事を示した。

# LIST OF PUBLICATIONS RELATED TO THIS THESIS

Sakane, K., Akiyama, M., Jogaiah, S., Ito, S., & Sasaki, K. (2024). Pathogenicity chromosome of *Fusarium oxysporum* f. sp. *cepae*. Fungal Genetics and Biology, 170, 103860. (Chapter 3)

Sakane, K., Kunimoto, M., Furumoto, K., Shigyo, M., Sasaki, K., & Ito, S. (2023). The SIX5 Protein in *Fusarium oxysporum* f. sp. *cepae* Acts as an Avirulence Effector toward Shallot (*Allium cepa* L. Aggregatum Group). Microorganisms, 11(12), 2861. (Chapter 4)

# ACKNOWLEDGEMENTS

At the beginning, I wish to express my gratitude from the bottom of my heart to Dr. Shin-ichi Ito, emeritus of plant pathology, Graduate School of Sciences and Technology for Innovation, Yamaguchi University, Japan. He willingly welcomed me to come back and gave me an appotunity to study again in his laboratory. And he always gave me constructive suggestions, advice, and guidance for my thesis. Besides, he also shared great conversation time with me and helped to cheer me up when I was down and felt anxious throughout the life of my doctorate course. The precious time with him made my motivation constantly fulfilled at any time. Hence, I'm honored to be his laboratory student. I'm sure this experience within his laboratory will be valuable and irreplaceable terms to make entire of my life fruitful.

I also wish to express my gratitude to Dr. Tadashi Takahashi, Yamaguchi University, for warmly welcoming me to his laboratory after the retirement of Dr.Shin-ich Ito and for his kind support throughout my doctorate course life. I'm not able to imagine the accomplishment of my thesis without his dedicated and thoughtful support.

Also, I would like to express my appreciation to my co-supervisor Dr. Kazunori Sasaki, Yamaguchi University, for his devoting support to making my research and paper more sophisticated. I could not finish writing my thesis within the assigned term in the doctorate course without his help.

In addition, I would like to express special thanks to my co-supervisor Dr. Makoto Ueno, Shimane University, Japan, for giving me constructive suggestions to make my thesis more advanced.

Moreover, I wish to express many thanks to my initially assigned co-supervisor Dr. Yoko Takematsu, Yamaguchi University. She gave me sharp indication to make my thesis more

developed. Besides, she also provided me with refreshment time, including special BBQ and various parties with attractive and humorous students, which belong to her laboratory.

I wish to sincerely thank Dr. Masayoshi Shigyo, Graduate School of Sciences and Technology for Innovation, Yamaguchi university, for kindly providing the samples used in this study.

Importantly, I would like to say thanks a million to my wonderful member of plant pathology laboratory. I'm sure I could not have accomplished my thesis within the assigned terms of the doctorate course without their kind and dedicated support.

Most importantly, I want to express maximum gratitude to my beloved family, Keisuke, Kazumi, Yu-ichi, and Shiori. Their encouragement and support always made my mind fulfilled and drove my motivation more active in my doctorate course activity. I sincerely feel I'm a really happy person to be member of the beloved family.