Genetic and Pathogenic Variability of *Fusarium oxysporum* f. sp. *cepae* Isolated from Onion and Welsh Onion in Japan

Kazunori Sasaki, Katsuya Nakahara, Shuhei Tanaka, Masayoshi Shigyo, and Shin-ichi Ito

First author: The United Graduate School of Agricultural Sciences, Tottori University, 4-101 Koyama-Minami, Tottori, 680-8553, Japan; and second, third, fourth, and fifth authors: Department of Biological and Environmental Sciences, Faculty of Agriculture, Yamaguchi University, Yamaguchi, 753-8515, Japan.

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ABSTRACT

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Fusarium oxysporum f. sp. *cepae* causes Fusarium basal rot in onion (common onion) and Fusarium wilt in Welsh onion. Although these diseases have been detected in various areas in Japan, knowledge about the genetic and pathogenic variability of *F. oxysporum* f. sp. *cepae* is very limited. In this study, *F. oxysporum* f. sp. *cepae* was isolated from onion and Welsh onion grown in 12 locations in Japan, and a total of 55 *F. oxysporum* f. sp. *cepae* isolates (27 from onion and 28 from Welsh onion) were characterized based on their rDNA intergenic spacer (IGS) and

translation elongation factor-1 α (EF-1 α) nucleotide sequences, vegetative compatibility groups (VCGs), and the presence of the *SIX* (secreted in xylem) homologs. Phylogenetic analysis of IGS sequences showed that these isolates were grouped into eight clades (A to H), and 20 onion isolates belonging to clade H were monophyletic and assigned to the same VCG. All the IGS-clade H isolates possessed homologs of *SIX3*, *SIX5*, and *SIX7*. The *SIX3* homolog was located on a 4 Mb-sized chromosome in the IGS-clade H isolates. Pathogenicity tests using onion seedlings showed that all the isolates with high virulence were in the IGS-clade H. These results suggest that *F. oxysporum* f. sp. *cepae* isolates belonging to the IGS-clade H are genetically and pathogenically different from those belonging to the other IGS clades.

The common onion (*Allium cepa* L.) is one of the most important vegetables grown worldwide, with a production of 82 mt (1.1 mt in Japan) in 2012 (9). Welsh onion (*A. fistulosum* L.), also known as Japanese bunching onion, is one of the most commonly cultivated vegetables in East Asia, especially in Japan and China (14,44). Young seedlings as well as adult Welsh onions are high-value farm products used as ingredients for hors d'oeuvres, soup, and sushi in Japan. Welsh onion is therefore cultivated continuously throughout the year to cater to the market demand in Japan (7). Both onion and Welsh onion are popular vegetables in Japan; their areas of cultivation are 24,900 and 23,000 ha, ranking sixth and seventh in vegetable acreage in 2012, respectively (9). Both onion and Welsh onion are attacked by many fungal pathogens, including *Fusarium oxysporum* in Japan (7,19,35).

Fusarium basal rot of onion, caused by *F. oxysporum* f. sp. *cepae* W. C. Snyder & H. N. Hansen, is an important soilborne disease that occurs widely in onion-growing areas and during the storage of onion bulbs (31). *F. oxysporum* f. sp. *cepae* also causes Fusarium wilt in Welsh onion (7,18,35). Fusarium basal rot and wilt have become major diseases causing considerable economic damage to both onion and Welsh onion production in recent years in Japan (7). Although the reasons for the increase in the occurrence of both diseases have not been determined, long-term continuous cropping and environmental changes, including higher temperatures and heavier summer rainfall in recent years in Japan, may be influencing the increase.

Genetic and pathogenic characterization of *F. oxysporum* f. sp. *cepae* distributed in Japan is useful for efficient management of onion and Welsh onion production. The vegetative compatibility group (VCG) approach has been demonstrated to be a valuable

method for characterizing the genetic variability of *F. oxysporum* f. sp. *cepae* (3,33,34,43). Widodo et al. (43) reported that *F. oxysporum* f. sp. *cepae* isolated from onion in Hokkaido, the northernmost island of Japan, were grouped into four VCGs and two single self-compatible isolates. Vegetative compatibility is mediated by the multiple vegetative incompatibility (*vic*) loci (21). The isolates classified in the same VCG were closely related genetically because they form stable heterokaryons only if each *vic* loci of the isolate is identical. Another method used in the genetic characterization within a forma specialis (f. sp.) of *F. oxysporum* involves analyzing the nucleotide sequences of the ribosomal DNA intergenic spacer (IGS) region (25); this method has also been applied in investigating genetic variability in *F. oxysporum* f. sp. *cepae* (3,6,33). Dissanayake et al. (6) isolated *F. oxysporum* f. sp. *cepae* from wilted Welsh onion in Japan and reported that these isolates share polyphyletic origins as well as a relationship with the phylogenetic position of the isolates based on IGS sequences and their pathogenicity to Welsh onion cultivars. However, no information is available about the genetic and pathogenic variability of *F. oxysporum* f. sp. *cepae* isolates from onion in Japan. In addition, there have been very few studies on the genetic and pathogenic variability among the *F. oxysporum* f. sp. *cepae* isolates from onion and Welsh onion.

Unlike *F. oxysporum* f. sp. *cepae*, the genetic and pathogenic variability in *F. oxysporum* f. sp. *lycopersici*, the causative agent of Fusarium wilt in tomato, have been studied in great detail. Recent studies revealed that *F. oxysporum* f. sp. *lycopersici* strains secrete effector proteins into xylem, referred to as the secreted in xylem (*SIX*) proteins. So far, 14 *SIX* genes (*SIX1* to *SIX14*) have been identified in *F. oxysporum* f. sp. *lycopersici* (12,23,24,27,28,39,40). Although *SIX* genes were initially believed to be unique to *F. oxysporum* f. sp. *lycopersici*, some of them have been detected in other forma specialis as well (4,11,16,23,37). Thus far, however, the *SIX3* and *SIX5* genes have been detected only in the genome of *F. oxysporum* f. sp. *lycopersici*. These results led us to hypothesize

Corresponding author: S. Ito; E-mail address: shinsan@yamaguchi-u.ac.jp

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that *F. oxysporum* f. sp. *cepae* may have *SIX* gene homologs, which may be associated with its pathogenicity.

In this study, the genetic and pathogenic variability in the *F. oxysporum* f. sp. *cepae* isolates from onion and Welsh onion grown in Japan were analyzed with respect to their VCG and IGS sequences. The *F. oxysporum* f. sp. *cepae* isolates were also examined for the presence of *SIX* genes in their genomes.

MATERIALS AND METHODS

Fungal isolates. Onion and Welsh onion plants with typical symptoms of Fusarium basal rot and Fusarium wilt, respectively, were collected from 12 locations (Hokkaido, Fukushima, Saitama, Shizuoka, Aichi, Mie, Kyoto, Hyogo, Tokushima, Kochi, Saga, and Kagoshima) in Japan. Basal plates of diseased plants were surface-sterilized in 80% ethanol for 30 s, which was followed by suspending them in 0.5% NaOCl for 30 s, and then rinsing in sterile distilled water. Basal plates (3 to 5 mm in diameter) were placed onto *Fusarium* selective medium (20) and incubated for 1 week at 25°C in the dark. The pure cultures of the isolates were obtained using a single-spore culture technique (22).

DNA extraction and identification of *Fusarium* spp. based on their ITS sequences. For DNA extraction, the fungal isolates were cultured in potato dextrose broth (PDB) for 3 days at 25°C in the dark, and the fungal mycelia were harvested by filtering the broth through sterile filter paper (Advantec, Tokyo, Japan). DNA was extracted from the mycelia using a Dr. GenTLE (from Yeast) (Takara Bio, Shiga, Japan), according to the manufacturer's instructions.

The fungal species were identified on the basis of morphology (22) and their ribosomal DNA internal transcribed spacer (ITS) region sequences. Polymerase chain reaction (PCR) amplification of the ITS regions was performed using the primers ITS1 and ITS4 (42). The PCR consisted of 20 ng of genomic DNA, 2 µl of 10× buffer (Takara Bio), 1.6 µl of 2.5 mM each dNTP, 0.2 µM of each primer, and 0.2 U of *Ex-Taq* polymerase (Takara Bio) in a total reaction mixture of 20 µl. An initial denaturation step of 94°C for 2 min was followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 40 s, with

a final extension step at 72°C for 5 min. PCR products were purified by ethanol precipitation and labeled using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). The DNA fragments were sequenced using an ABI PRISM 3100 genetic analyzer (Applied Biosystems).

Phylogenetic analysis. The partial rDNA-IGS region was amplified using primers PNFo and PN22 (8). The translation elongation factor-1α (EF-1α) sequence was amplified using primers EF1 and EF2 (26). PCR conditions were the same as described for the ITS region, except that the extension time for the IGS region was 1.5 min. Sequencing was performed using primers PNFo, PN22, and IGS2 (10) for IGS, and EF1 and EF2 for EF-1α. The reaction conditions were identical to those described for the ITS region. Nucleotide sequences of the partial rDNA-IGS and EF-1α were aligned using ClustalW (38). Phylogenetic trees were constructed by the neighbor-joining method (29) based on Kimura's two-parameter model (17) using MEGA v.4.0 (36). The statistical reliability of each node was assessed using 1,000 bootstrap iterations. *F. verticillioides* (accession no. AY249379) was used as an outgroup in the IGS-based phylogenetic analysis. *Fusarium* spp. isolates NRRL28387 (accession no. AF246832) and NRRL25184 (accession no. AF008514) were used as outgroups in the EF-1α-based phylogenetic analysis.

Vegetative compatibility test and inter-retrotransposon sequence-characterized amplified regions markers. Vegetative compatibility tests were performed according to the method of Correll (5). Nitrate nonutilizing mutants were generated on minimal medium containing 4% KClO₃, and were classified as *nit1*, *nit3*, and *NitM* based on the differences in their utilization patterns of the nitrogen source in the medium. Heterokaryon formation was tested by pairing *nit1* and *NitM* mutants of the isolates.

Multiplex PCR was performed using inter-retrotransposon sequence-characterized amplified regions (IR-SCAR) markers, i.e., the primer sets of HTH-1F/2R (244 bp) and Hansec-1F/2R (392 bp) (32) in a 20-µl reaction system containing 10 µl of Quick Taq HS DyeMix (Toyobo, Shiga, Japan), 0.2 µM of both the primer pairs, and 20 ng of genomic DNA. An initial denaturation step for 2 min at 94°C was followed by 35 cycles of denaturation for 30 s at 94°C, annealing for 30 s at 60°C, and extension for 30 s at 68°C. Amplified

TABLE 1. Primers used in this study^a

Primer name	Sequence (5'–3')	Target	Reference
ITS1	TCCGTAGGTGAACCTGCGG	ITS	(42)
ITS4	TCCTCCGCTTATTGATATGC	ITS	(42)
PNFo	CCCGCCTGGCTGCGTCCGACTC	IGS	(8)
PN22	CAAGCATATGACTACTGGC	IGS	(8)
IGS2	GCCGGATTGTGCTCCCTTCT	IGS	(10)
EF1	ATGGGTAAAGGARGACAAGAC	EF-1α	(26)
EF2	GGARGTACCAGTSATCATGTT	EF-1α	(26)
Hansec-1F	TTATTGCCCTCATCGAAAG	IR-SCAR (392 bp)	(32)
Hansec-2R	ACCAGCATGCAGCAACAGTC	IR-SCAR (392 bp)	(32)
HTH-1F	CATCGGAAGTGACATGGTTG	IR-SCAR (244 bp)	(32)
HTH-2R	AGGCTTTTCCAGCATTTGAA	IR-SCAR (244 bp)	(32)
P12-F1	CCCCGAATTGAGGTGAAG	<i>SIX1</i>	(28)
P12-R1	AATAGAGCCTGCAAAAGCATG	<i>SIX1</i>	(28)
SIX2-F2	CAACGCCGTTTGAATAAGCA	<i>SIX2</i>	(39)
SIX2-R2	TCTATCCGCTTTCTTCTCTC	<i>SIX2</i>	(39)
SIX3-F1	CCAGCCAGAAGGCCAGTTT	<i>SIX3</i>	(39)
SIX3-R2	GGCAATTAACCACTCTGCC	<i>SIX3</i>	(39)
SIX4-F1	TCAGGCTTCACTTAGCATAC	<i>SIX4</i>	(23)
SIX4-R1	GCCGACCGAAAAACCCTAA	<i>SIX4</i>	(23)
SIX5-F1	ACACGCTCTACTACTTCA	<i>SIX5</i>	(23)
SIX5-R1	GAAAACCTCAACGCGGCAAA	<i>SIX5</i>	(23)
SIX6-F1	CTCTCTGAACCATCAACTT	<i>SIX6</i>	(23)
SIX6-R1	CAAGACCAGGTGTAGGCATT	<i>SIX6</i>	(23)
SIX7-F1	CATCTTTTCGCCGACTTGGT	<i>SIX7</i>	(23)
SIX7-R1	CTTAGCACCCCTTGAGTAACT	<i>SIX7</i>	(23)

^a Internal transcribed spacer (ITS); intergenic spacer (IGS); elongation factor-1α (EF-1α); and inter-retrotransposon sequence-characterized amplified regions (IR-SCAR).

products were separated by electrophoresis on a 2% agarose gel, stained with ethidium bromide, and then visualized under UV light.

Amplification of *SIX* homologs. Fungal DNAs were examined for the presence of the seven *SIX* genes (*SIX1* to *SIX7*) by performing PCR using the specific primer pairs listed in Table 1. PCR amplification was carried out in a reaction mixture of 20 µl containing 10 µl of Quick Taq HS DyeMix (Toyobo, Shiga, Japan), 0.2 µM of each primer, and 20 ng of genomic DNA. Thermal cycling conditions consisted of 2 min at 94°C, followed by 35 cycles of 30 s at 94°C, 30 s at 50°C, and 45 s at 68°C. Amplified products were separated by electrophoresis on a 2% agarose gel, stained with ethidium bromide, and then visualized under UV light. The

genomic DNA of *F. oxysporum* f. sp. *lycopersici* (race 1) was used as the positive control.

Chromosomal Southern blot analysis. Fungal protoplasts were prepared using the methods of Akamatsu et al. (1), with slight modifications: the enzyme solution contained lysing enzymes at 10 mg/ml (Sigma-Aldrich, Tokyo, Japan) and Yatalase at 4 mg/ml (Takara Bio). Chromosomes were separated by performing contour-clamped homogeneous electric field (CHEF) gel electrophoresis in a 1.0% Certified Megabase Agarose gel (Bio-Rad, Hercules, CA) by using the CHEF-DR II system (Bio-Rad). Electrophoresis was performed in 0.5× TBE buffer for 260 h at 4°C, using switch times between 1,200 and 4,800 s at 1.5V/cm.

Fig. 1. Phylogenetic tree generated using neighbor-joining methods based on intergenic spacer region sequences of *Fusarium oxysporum* isolated from onion and Welsh onion. The distances were determined by Kimura's two-parameter method. The numbers beside branches represent the percentages of congruent clusters in 1,000 bootstrap trials, with values greater than 60%. Scale bar indicates 1% sequence dissimilarity.

Chromosomes of *Saccharomyces cerevisiae* (Bio-Rad) and *Schizosaccharomyces pombe* (Bio-Rad) were used as molecular size markers. *F. oxysporum* f. sp. *lycopersici* (race 2) was used as the positive control. Chromosome-sized DNA separated by electrophoresis was transferred to a Hybond-N+ membrane (GE Healthcare, Buckinghamshire, England) using 20× SSC for 24 h. Chromosome localization of the *SIX3* gene, Hansec, and HTH fragments was determined by performing membrane hybridization using digoxigenin (DIG)-labeled *SIX3*, Hansec, and HTH probes that were prepared using a DIG DNA Labeling and Detection Kit (Roche Diagnostics, Mannheim, Germany), according to the manufacturer's instructions.

Pathogenicity test using root-dip method. Fungal isolates were cultured in PDB for 3 days at 25°C, and the cultures were filtered through three layers of sterile gauze cloth to remove the mycelia. The spore concentrations were estimated using a hemocytometer and adjusted to 1 × 10⁶ conidia/ml. Onion cultivars Higuma (Takii Seed, Kyoto, Japan) and Kitamomiji 2000 (Shippo Seed, Kagawa, Japan) were used as the host plants for fungi since both

cultivars are extensively cultivated in Japan. Ten-day-old onion seedlings were inoculated by immersing the roots for 1 h at 25°C in a spore suspension of the isolates; control plant roots were immersed in sterile water. Seedlings were then replanted in plastic pots containing an autoclaved 4:1 mixture of sand and Takii seedling soil (Takii Seed) and maintained in a growth chamber at 25°C, with a photoperiod of 16 h light/8 h dark. The percentages of the wilted plants were determined at 3 weeks postinoculation. Each fungal isolate was inoculated in 10 seedlings and in duplicate. The experiments were replicated twice. The isolates were divided into three virulence classes based on the mean percentage of wilted plants in the two onion cultivars: high virulence (>70%), moderate virulence (30 to 70%), and low virulence (<30%).

RESULTS

Identification of fungal isolates from onion and Welsh onion. A total of 382 *Fusarium* isolates were obtained: 248 isolates

Fig. 2. Phylogenetic tree generated using neighbor-joining method based on translation elongation factor sequences of *Fusarium oxysporum* isolated from onion and Welsh onion. The distances were determined by Kimura's two-parameter method. The numbers beside branches represent the percentages of congruent clusters in 1,000 bootstrap trials, with values greater than 60%. Scale bar indicates 0.5% sequence dissimilarity.

from onion and 134 isolates from Welsh onion. The fungal species were identified on the basis of morphology and their ribosomal DNA ITS region sequences. The majority of isolates from onion (165 isolates) and Welsh onion (86 isolates) were identified as *F. oxysporum*. The second major *Fusarium* sp. isolated from both types of plant was *F. solani*: 69 isolates from onion and 31 from

Welsh onion. *F. proliferatum* was also isolated: one isolate from onion and nine isolates from Welsh onion.

Phylogenetic analysis. A total of 55 *F. oxysporum* isolates, including 27 isolates from onion and 28 from Welsh onion, which together represented a broad geographical range and had been stored at 4°C until use, were analyzed phylogenetically based on

TABLE 2. Characteristics (pathogenicity, vegetative compatibility groups [VCGs], and *SIX* genes) of *Fusarium oxysporum* isolated from onion and Welsh onion in Japan

Isolate	Host	Regional origin	Year	Percentage of wilted plants ^a		VCG ^b	IR-SCAR marker ^c	<i>SIX</i> genes ^d	Accession number ^e	
				Higuma	Kitanomiji				IGS	EF-1 α
AC12	Onion	Aichi	2010	5	15	HIS	–	–	AB936592	AB938050
AC13	Onion	Aichi	2010	0	0	SMV16	–	–	AB936593	AB938051
AC15	Onion	Aichi	2010	5	5	SMV17	–	–	AB936594	AB938052
AC22	Onion	Hokkaido	2011	45	20	G4	+	+	AB936595	AB938053
AC26	Onion	Hokkaido	2011	45	45	G4	+	+	AB936596	AB938054
AC49	Onion	Hokkaido	2011	35	70	G4	+	+	AB936597	AB938055
AC76	Onion	Hokkaido	2011	15	0	G5	–	–	AB936598	AB938056
AC85	Onion	Hokkaido	2011	60	70	G4	+	+	AB936599	AB938057
AC89	Onion	Hokkaido	2011	65	80	G4	+	+	AB936600	AB938058
AC109	Onion	Hokkaido	2011	25	70	G4	+	+	AB936601	AB938059
AC117	Onion	Hokkaido	2011	55	65	G4	+	+	AB936602	AB938060
AC123	Onion	Hokkaido	2011	55	85	G4	+	+	AB936603	AB938061
AC130	Onion	Hokkaido	2011	80	85	G4	+	+	AB936604	AB938062
AC140	Onion	Hokkaido	2011	85	65	G4	+	+	AB936605	AB938063
AC150	Onion	Hokkaido	2011	100	95	G4	+	+	AB936606	AB938064
AC158	Onion	Hokkaido	2011	50	65	G4	+	+	AB936607	AB938065
AC164	Onion	Hokkaido	2011	55	55	G4	+	+	AB936608	AB938066
AC173	Onion	Hokkaido	2011	35	35	G4	+	+	AB936609	AB938067
AC184	Onion	Hokkaido	2011	40	30	G4	+	+	AB936610	AB938068
AC205	Onion	Hokkaido	2011	100	90	G4	+	+	AB936611	AB938069
AC214	Onion	Hokkaido	2011	20	2	n.g.	–	–	AB936612	AB938070
AC217	Onion	Hokkaido	2011	25	30	G5	–	–	AB936613	AB938071
AC222	Onion	Hokkaido	2011	10	25	SMV18	–	–	AB936614	AB938072
AC241	Onion	Hokkaido	2011	45	55	G4	+	+	AB936615	AB938073
AC245	Onion	Saga	2012	n.t.	n.t.	G4	+	+	AB936616	AB938074
AC248	Onion	Saga	2012	n.t.	n.t.	G4	+	+	AB936617	AB938075
TA	Onion	Hokkaido	–	95	100	G4	+	+	AB936618	AB938076
AF1	Welsh onion	Saitama	2006	45	15	n.g.	–	–	AB936564	AB938022
AF15	Welsh onion	Kyoto	2006	10	15	SMV1	–	–	AB936565	AB938023
AF17	Welsh onion	Kagoshima	2006	25	30	SMV2	–	–	AB936566	AB938024
AF22	Welsh onion	Kagoshima	2006	55	55	SMV3	–	–	AB936567	AB938025
AF31	Welsh onion	Hyogo	2006	20	20	G1	–	–	AB936568	AB938026
AF52	Welsh onion	Tokushima	2007	20	35	SMV4	–	–	AB936569	AB938027
AF60	Welsh onion	Kochi	2007	35	25	SMV5	–	–	AB936570	AB938028
AF66	Welsh onion	Kyoto	2008	5	5	SMV6	–	–	AB936571	AB938029
AF67	Welsh onion	Kyoto	2008	0	25	SMV7	–	–	AB936572	AB938030
AF72	Welsh onion	Kyoto	2008	10	10	SMV8	–	–	AB936573	AB938031
AF74	Welsh onion	Fukushima	2009	0	0	G2	–	–	AB936574	AB938032
AF75	Welsh onion	Fukushima	2009	5	5	G2	–	–	AB936575	AB938033
AF77	Welsh onion	Fukushima	2009	30	35	HIS	–	–	AB936576	AB938034
AF88	Welsh onion	Shizuoka	2010	90	35	SMV9	–	–	AB936577	AB938035
AF89	Welsh onion	Shizuoka	2010	15	45	G1	–	–	AB936578	AB938036
AF90	Welsh onion	Shizuoka	2010	40	60	SMV10	–	–	AB936579	AB938037
AF91	Welsh onion	Shizuoka	2010	35	95	HIS	–	–	AB936580	AB938038
AF93	Welsh onion	Shizuoka	2010	25	15	G1	–	–	AB936581	AB938039
AF94	Welsh onion	Shizuoka	2010	40	90	SMV11	–	–	AB936582	AB938040
AF95	Welsh onion	Shizuoka	2010	40	35	SMV12	–	–	AB936583	AB938041
AF96	Welsh onion	Tokushima	2010	15	25	SMV13	–	–	AB936584	AB938042
AF97	Welsh onion	Tokushima	2010	25	0	SMV14	–	–	AB936585	AB938043
AF98	Welsh onion	Tokushima	2010	5	0	G3	–	–	AB936586	AB938044
AF101	Welsh onion	Tokushima	2010	15	30	G3	–	–	AB936587	AB938045
AF103	Welsh onion	Mie	2010	5	25	n.g.	–	–	AB936588	AB938046
AF113	Welsh onion	Saitama	2010	0	15	SMV15	–	–	AB936589	AB938047
AF124	Welsh onion	Hokkaido	2011	n.t.	n.t.	n.g.	–	–	AB936590	AB938048
AF125	Welsh onion	Hokkaido	2011	n.t.	n.t.	n.g.	–	–	AB936591	AB938049

^a For determining the percentage of wilted plants, two onion cultivars ('Higuma' and 'Kitanomiji 2000') were inoculated with *F. oxysporum* by the root-dip method. Ten seedlings were used in each experiment in duplicate. n.t. indicates not tested.

^b HSI; heterokaryon self-incompatible. n.g.; generated either *nit1* or NitM mutant. SMV; single member of VCG.

^c + indicates that two polymerase chain reaction (PCR) products (392 and 244 bp) were obtained using inter-retrotransposon sequence-characterized amplified regions (IR-SCAR) marker primers. +* indicates that only 392-bp product was obtained. – indicates that no PCR products were obtained.

^d + indicates that PCR products were obtained with specific primers of *SIX3*, *SIX5*, and *SIX7*. – indicates that no PCR products were obtained.

^e GenBank/DBJ accession number of partial rDNA intergenic spacer (IGS) and elongation factor-1 α (EF-1 α) sequences determined in this study.

their IGS and EF-1 α nucleotide sequences. A phylogenetic tree based on the IGS sequences revealed that the 55 isolates were divided into eight clades, A to H (Fig. 1). No clade was related to its geographic origin. Most of the isolates from onions grown in Hokkaido and Saga were in clade H; the IGS sequences of the isolates of clade H shared 100% nucleotide identity. A phylogenetic tree based on EF-1 α branched into two clades (Fig. 2). All the isolates belonging to clade H of the IGS-based phylogenetic tree were grouped into a subclade (B1), with 100% nucleotide identity with the EF-1 α -based phylogenetic tree; while the other isolates belonging to clades other than H in the IGS-based phylogenetic tree had no relationship with the clades in the EF-1 α -based phylogenetic tree. In both phylogenetic trees, a high degree of genetic variability was observed among the Welsh onion isolates.

Vegetative compatibility and IR-SCAR markers.

Hypochlorite-resistant mutants of each isolate were designated as *nit1*, *nit3*, or NitM based on the differences in their utilization patterns of the nitrogen source in the medium. Out of 55 isolates, five (AF1, AF103, AF124, AF125, and AC214) could not be used in the pairing test because they generated neither *nit1* nor NitM mutants. Three isolates (AF77, AF91, and AC12) showed heterokaryon self-incompatible (HSI) phenotypes. Five VCGs and 18 single members of VCG (SMV) were obtained from the 47 isolates tested (Table 2). All the isolates belonging to clade H of the IGS-based phylogenetic tree formed heterokaryons with each of the isolates in the VCG group 4 (G4).

IR-SCAR markers have been reported to identify VCG0421 and VCG0425 of *F. oxysporum* f. sp. *cepa*e (32); the IR-SCAR marker generated two bands (392 bp with Hansec primers and 244 bp with HTH primers) from *F. oxysporum* f. sp. *cepa*e VCG0421 and VCG0425. In our experiments, PCR amplification using IR-SCAR marker primers produced bands (392 and 244 bp) that were detected only in the isolates of clade H of the IGS-based phylogenetic tree. Among those, AC140, AC150, AC205, and TA generated only a 392-bp band. No amplified products were detected in the isolates other than the H-clade isolates (Table 2).

Presence of *SIX* homologs. All the isolates used in the phylogenetic analysis (55 isolates) were also examined for the presence of the seven *SIX* genes by performing PCR. The *SIX3*, *SIX5*, and *SIX7* amplicons were generated from the DNA of all the isolates of the IGS-clade H. In contrast, no *SIX* amplicons were detected in the isolates other than the H-clade isolates (Table 2).

Chromosomal location of *SIX3* homolog and IR-SCAR marker amplicons. Fungal chromosomes were separated in CHEF gels, and the chromosomal locations of the *SIX* homologs were determined by performing Southern blot analysis, which revealed that the *SIX3* homolog was located on a 4 Mb-sized chromosome in the *F. oxysporum* f. sp. *cepa*e isolates of IGS-clade H (Fig. 3A). CHEF blots probed using the Hansec fragment (392 bp) also indicated the presence of the 4 Mb chromosome of the IGS-clade H isolates (AC109 and TA). However, although the HTH fragment (244 bp) hybridized to the 4 Mb chromosome of isolate AC109, it did not hybridize to any chromosome of TA; a result consistent with those of IR-SCAR PCR (Fig. 3B). These data show that the *SIX3* homologs as well as the target regions of the IR-SCAR markers were located on the same chromosome of the H-clade isolates.

Inoculation test. Fungal isolates were examined for their virulence in onion seedlings of two cultivars. Most of the onion isolates belonging to clade H of the IGS-based phylogenetic tree showed moderate or high virulence in the onion seedlings of the two onion cultivars. Among those, AC150, AC205, and TA had higher virulence, causing wilt symptoms in more than 90% of the plants tested. Onion isolates belonging to the other clades of the IGS-based phylogenetic tree showed low or no virulence in the onion seedlings (Table 2).

Most of the fungal isolates from Welsh onion showed low or moderate virulence in onion seedlings, excluding AF88, which was highly virulent in 'Higuma', and AF91 and AF95, both of which were highly virulent in 'Kitanomiji 2000' (Table 2).

DISCUSSION

To the best of our knowledge, here we revealed for the first time the phylogenetic relationships among *F. oxysporum* isolated from onion and Welsh onion in Japan. Welsh onion isolates were polyphyletic in the phylogenetic tree based on the IGS sequences, supporting previous results reported by Dissanayake et al. (6). We also found that there was no relationship between the geographical origin and each clade of the phylogenetic tree based on IGS sequences of the isolates. In contrast, most of the onion isolates were monophyletic and were confined to IGS-clade H. Seven onion isolates not belonging to IGS-clade H (AC12, AC13, AC15, AC76,

Fig. 3. Chromosomal localization of *SIX* genes and transposable elements in *Fusarium oxysporum* f. sp. *cepa*e. **A**, Karyotype profiles of *F. oxysporum* f. sp. *cepa*e by contour-clamped homogeneous electric field (CHEF) electrophoresis (left panel) and Southern blot analysis of the CHEF gel using *SIX3* as a probe (right panel). LMW: low molecular weight marker (*Saccharomyces cerevisiae*); HMW: high molecular weight marker (*Schizosaccharomyces pombe*); FOL: *F. oxysporum* f. sp. *lycopersici* (race 2). **B**, Southern blot analysis of the CHEF gel probed with 244-bp fragment (HTH), 392-bp fragment (Hansec), and *SIX3* homolog (*SIX3*).

AC214, AC217, and AC222) showed no or very low virulence in onion seedlings. It is reasonable to speculate, therefore, that these isolates may not be *F. oxysporum* f. sp. *cepae* but rather a nonpathogenic *F. oxysporum* that infects onions. Indeed, such nonpathogenic *F. oxysporum* have been isolated from symptomatic sugar beets (41). The high degree of genetic variation among Welsh onion isolates compared with onion isolates might be related to the long cultivation history of Welsh onions, which have been planted in Japan since the 8th century or earlier, while the cultivation of common onions in Japan started in 1871 using seeds imported from the United States.

In the vegetative compatibility test, heterokaryon formation was limited, even though the isolates belonged to the same clade; five types of VCGs and 18 SMVs were observed. Such VCG diversity and a high number of SMVs have also been reported in *F. oxysporum* isolated from onion in Turkey, in which 48 SMVs were detected in 75 isolates (3), and in those isolated from a common bean field in Spain in which 96 VCGs were detected in 128 isolates (2). However, the isolates belonging to the IGS-clade H formed heterokaryons only with other isolates in the same clade, suggesting that isolates contained in the clade are genetically closely related.

In the present study, we tested for VCG types of *F. oxysporum* isolated from onion and Welsh onion by using IR-SCAR marker primers that can amplify specific PCR products (392 and 244 bp) from VCG0421 and VCG0425. The IR-SCAR marker primers, which target DNA between the long terminal repeat insertion sites of retrotransposons, were designed based on specific DNA fragments that displayed inter-retrotransposon amplified polymorphism. The IR-SCAR marker primers helped identify VCG0421 and VCG0425 of *F. oxysporum* f. sp. *cepae* isolated from South Africa and Colorado (32). Both PCR products (392 and 244 bp) were detected only in the *F. oxysporum* f. sp. *cepae* isolates belonging to the IGS-clade H. However, four isolates (AC140, AC150, AC205, and TA) generated only a 392-bp band. Furthermore, *F. oxysporum* f. sp. *cepae* isolates belonging to the IGS-clade H were grouped into the same VCG. Taken together, these results suggest that *F. oxysporum* f. sp. *cepae* isolates belonging to the IGS-clade H are not a monoclonal group, although they are genetically very similar.

Transposable elements, including retrotransposons, can travel throughout the genome and can cause genetic changes due to insertion or excision (15). This mechanism may have provided genetic variation and evolution of new phenotypes of fungi (30). For example, Inami et al. (13) reported a new mechanism underlying the emergence of *F. oxysporum* f. sp. *lycopersici* race 3 in which the *AVR1* gene (*SIX4*) was disrupted by the transposon *Hormin*. In this context, it is interesting that the four isolates (AC140, AC150, AC205, and TA) that did not generate the 244-bp fragment exhibited high virulence in the onion cultivars used as hosts. Target sequences of the IR-SCAR marker primers are included in the retrotransposon sequences as described above, suggesting that the genomic region containing retrotransposon sequences may be absent among these four isolates. Thus, genome regions flanking the 244-bp DNA might be involved in virulence, such as virulence via the production of an effector protein. In addition, Southern blot analysis revealed that target sequences of IR-SCAR markers as well as *SIX3* homologs were located on the same 4 Mb-sized chromosome. In *F. oxysporum* f. sp. *lycopersici*, most of the *SIX* genes are known to be located on chromosome 14 (the pathogenicity chromosome), which is enriched in the transposable elements (24). Thus, it is plausible that the 4 Mb-sized chromosome of *F. oxysporum* f. sp. *cepae* could be a pathogenicity chromosome, similar to chromosome 14 of *F. oxysporum* f. sp. *lycopersici*, despite their size difference (chromosome 14 of *F. oxysporum* f. sp. *lycopersici* is 2 Mb).

We observed that *F. oxysporum* f. sp. *cepae* isolates belonging to IGS-clade H contain *SIX3*, *SIX5*, and *SIX7* homologs, and that Welsh onion isolates and nonpathogenic onion isolates of

F. oxysporum f. sp. *cepae* do not contain any *SIX* genes. To our knowledge, this is the first report stating that *SIX3* and *SIX5* homologs are present in a forma specialis other than f. sp. *lycopersici*. These results strongly suggest that *F. oxysporum* f. sp. *cepae* isolates belonging to IGS-clade H are genetically different from *F. oxysporum* isolated from Welsh onions. Further investigation is required to determine whether the *SIX* homologs of *F. oxysporum* f. sp. *cepae* are involved in inducing virulence in onion.

In the inoculation test, most of the fungal isolates, other than those belonging to the IGS-clade H, displayed low or no virulence in onion seedlings (Table 2). Although the virulence of these isolates in Welsh onion is unclear at present, these isolates may have adapted to Welsh onion as their host since there is a long history of cultivation of Welsh onion in Japan. However, some isolates from Welsh onion (AF88, AF91, and AF95) were highly virulent in onion seedlings. These isolates may provide new insights into the mechanisms of development of virulence in onion among indigenous *F. oxysporum* f. sp. *cepae* populations in Japan.

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