A Genetic Mechanism for Emergence of Races in Fusarium oxysporum f. sp. lycopersici: Inactivation of Avirulence Gene AVR1 by Transposon Insertion

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Abstract

Compatible/incompatible interactions between the tomato wilt fungus Fusarium oxysporum f. sp. lycopersici (FOL) and tomato Solanum lycopersicum are controlled by three avirulence genes (AVR1–3) in FOL and the corresponding resistance genes (I−I3) in tomato. The three known races (1, 2 and 3) of FOL carry AVR genes in different combinations. The current model to explain the proposed order of mutations in AVR genes is: i) FOL race 2 emerged from race 1 by losing the AVR1 and thus avoiding host resistance mediated by I (the resistance gene corresponding to AVR1), and ii) race 3 emerged when race 2 sustained a point mutation in AVR2, allowing it to evade I2-mediated resistance of the host. Here, an alternative mechanism of mutation of AVR genes was determined by analyses of a race 3 isolate, KoChi-1, that we recovered from a Japanese tomato field in 2008. Although KoChi-1 is race 3, it has an AVR1 gene that is truncated by the transposon Hormin, which belongs to the hAT family. This provides evidence that mobile genetic elements may be one of the driving forces underlying race evolution. KoChi-1 transformants carrying a wild type AVR1 gene from race 1 lost pathogenicity to cultivars carrying I, showing that the truncated KoChi-1avr1 is not functional. These results imply that KoChi-1 is a new race 3 biotype and propose an additional path for emergence of FOL races: Race 2 emerged from race 1 by transposon-insertion into AVR1, not by deletion of the AVR1 locus; then a point mutation in race 2 AVR2 resulted in emergence of race 3.

Introduction

In the arms race between plants and pathogens, the pathogens can win by circumventing the immune system of host plants, e.g., by avoiding or suppressing defense mechanisms. In general, plants have two types of resistance: polygenic (horizontal), controlled by multiple genes, each with a small phenotypic effect, and monogenic (vertical), controlled by a single resistance (R) gene, which often confers a high level of resistance [1]. Monogenic resistance generates immune responses (e.g., hypersensitive reaction, HR) to particular pathogen(s) [1], and has been effective and practical to use in modern plant breeding. This resistance is described by the ‘gene-for-gene theory’ [2], which explains the relationship between pathogen races and host plant cultivars by the interaction between an avirulence (AVR) gene in the race and an R gene in the cultivar. When a race possessing an AVR gene attacks a cultivar carrying the corresponding R gene, resistance is induced in the plant and the disease does not occur. A loss of function in an AVR gene allows the pathogen to avoid induction of resistance in the cultivar, the pathogen gains pathogenicity to that cultivar, and a new pathogenic race has emerged.

The ascomycete Fusarium oxysporum Schlecht. emend. Snyd. et Hans. causes vascular diseases of many plant species, yet each strain of this fungus has strictly defined host specificity [3]. Strains that cause wilt disease only on tomato (Solanum lycopersicum L.) are classified as f. sp. lycopersici Snyd. et Hans. (FOL). Three races of FOL have been reported; their relationship with tomato cultivars is explained by the ‘gene-for-gene theory’ [4]. Original descriptions of FOL races 1, 2 and 3 appeared before 1895 in England, in 1939 in the USA and in 1978 in Australia, respectively [5]. In Japan, races 1, 2 and 3 were reported in Fukuoka in 1905, in 1966 and in 1997, respectively [6]. To date, the R genes I, I2 and I3 are known in tomato cultivars [7]; these R genes correspond to the avirulence genes AVR1, AVR2 and AVR3 in FOL, respectively (Table 1). Historically, race 1-resistant cultivars (I I2 i3), races 1 and 2-resistant cultivars (I2 i3), and races 1, 2 and 3-resistant cultivars (I2 I3) have been bred sequentially, each genotype corresponds to the emergence of a new race.

The FOL AVR genes (AVR1, AVR2 and AVR3) are unique to FOL [8,9,10] and are carried in different combinations in different FOL races (Table 1). AVR1 (= SIX) is unique to race 1 [11], whereas AVR2 (= SIX) is found in races 1 and 2. Three nucleotide substitutions (G121A, G134A and G137C) in AVR2, which cause loss of avirulence function (avr2) have been found in race 3 [9].
Table 1. Relationship between FOL races and tomato cultivars.

| FOL race (AVR gene*) | Tomato cultivar (β genea) | Ponderosa | Momotaro | Walter | Block |
|----------------------|--------------------------|-----------|----------|--------|-------|
| 1 (AVR1 AVR2 AVR3)   |                          | S         | R        | R      |       |
| 2 (– AVR2 AVR3)      |                          | S         | S        | R      | R     |
| 3 (– avr2 AVR3)      |                          | S         | S        | S      | R     |

S, compatible; R, incompatible.

*-, loss of the AVR1 locus; avr2, allele containing a point mutation in the ORF [9].
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A small table that shows the relationship between FOL races and tomato cultivars, as well as the relationship between FOL races and tomato cultivars. The table includes information on the compatibility of the races with different cultivars. The races are identified by their AVR gene content, and the cultivars are identified by their β gene content. The table shows that race 1 is compatible with both Ponderosa and Momotaro, but not with Walter. Race 2 is compatible with both Ponderosa and Walter, but not with Momotaro. Race 3 is compatible with both Ponderosa and Walter, but not with Momotaro.

Results and Discussion

A race 3 isolate, KoChi-1, belongs to a different lineage from the known race 3 isolates in Japan.

A fungal isolate from the vascular tissues of diseased tomato in a greenhouse in Kochi Prefecture, Japan was identified as F. oxysporum based on morphology [19] and nucleotide sequence of the tDNA-internal transcribed spacer (ITS) region (DDBJ/EMBL/GenBank accession No. AB675383). Characteristics of the isolate, designated KoChi-1, are summarized in Table 2. In planta assays showed that KoChi-1 caused wilt disease on cvs. Ponderosa (I2 I2), Momotaro (I2 I2), and Walter (I2 I2), but not on cv. Block (I2 I2), indicating that KoChi-1 was race 3 (Table 2; Fig. 1A, B). This result was consistent with the fact that the commercial cultivar grown in the greenhouse was Momotaro-Fight (I2 I2), Takii Seed, Kyoto, Japan.

Previous studies found that all race 3 isolates obtained in Japan (representative isolate Chz1-A is presented in Table 2) grouped in the A3 clade [16] (Table 2; Fig. S2), and were MAT1-2 and VCG 0033. However, we found that KoChi-1 belongs to the A2 clade (Table 2; Fig. S2), and is MAT1-1 and VCG 0030+0032. The A2 clade has been reported to include only race 1 isolates in Japan [16]. Taken together, these characteristics suggest KoChi-1 is a novel biotype of race 3, distinct from the race 3 isolates previously reported in Japan.

KoChi-1 is the first reported race 3 isolate carrying the AVR1 locus, which itself is truncated by a transposon.

Although previously reported race 3 isolates (e.g., Chz1-A) have no AVR1 locus [8,11], Southern blot analysis using an AVR1 fragment from race 1 isolate MAFF 305121 (733 bp, nt 673–1406 bp, AB674509) as a probe presented that KoChi-1 possessed a single copy of AVR1 in its genome (Fig. 2A).

Then, we tried to amplify AVR1 from KoChi-1 using a primer set SIX46-F2/SIX46-R2 designed by Rep & Houteman to amplify AVR1 from race 1 (Table 3). The amplicon from KoChi-1 (2685 bp) was longer than that of MAFF 305121 (1924 bp) (Fig. 2B). The sequence of KoChi-1 AVR1 was deposited in DDBJ/EMBL/GenBank databases with the accession No. AB674508. In this paper, nucleotide positions are assigned according to AB674508 unless otherwise stated.

The structure of KoChi-1 AVR1 was compared with that of the race 1 isolate Fol004 (nt 326–2248 in AM234064; Fig. 2C). KoChi-1 contained a different number (13 bp, nt 30–42) of contiguous guanines and one cytosine deletion (nt 2136, AB234064) in addition to a 759-bp-insertion. This small number of polymorphisms suggests that the AVR1 locus is highly conserved. AVR1 in race 1 is composed of two exons (154 and 575 bp) and one intron (64 bp), and encodes a protein of 242 amino acids [8] (Fig. S4), but the KoChi-1 AVR1 sequence had a 759-bp-insertion (nt 1043–1801) in exon 2.

BLASTN searches in the NCBI database suggested that the 759-bp-insertion was a transposon with 15-bp terminal inverted repeats (TIRs; 5'-CAGGGTTCAATCCAGG-3'; nt 1043–1057, 1787–1801; Fig. 2C), and that both TIRs were flanked by 8-bp target site duplication (TSD; 5'-CACACCGG-3'; nt 1035–1042, 1802–1809; Fig. 2C). The sequence of the TIRs and the 5′ region of the transposon were highly homologous to the autonomous transposon Hormet1 from F. oxysporum (AF076626) [20]. These characteristics are consistent with those of the hAT family of class II DNA transposons [21]. Hence, we have designated this transposon Hormin (Hormet1 in miniature). Hormin does not encode transposases (and is therefore not autonomous) and may have emerged from Hormet1 through a series of mutations. A transposon identical to Hormin was previously reported in the alcohol dehydrogenase gene Add1 in FOL NRRL 34936 [22]. This is the first report of an F. oxysporum AVR1 gene truncated by a transposon.

According to the Broad Institute Fusarium genome database website (http://www.broadinstitute.org/annotation/genome/fusarium_group/MultiHome.html), only 2 isolates, FOL race 2 NRRL 34936 (Spain, MAT1-1, VCG 0030) and FOL race 3 NRRL 54003 (USA, MAT1-2, VCG 0033), carried Homin-
Figure 1. Virulence of KoChi-1 and its transformants. (A) KoChi-1 and its AVR1-complements were subjected to pathogenicity evaluation using four tomato cultivars, Ponderosa (I i 2 i 3), Momotaro (I i 2 i 3), Walter (I i 2 i 3) and Block (I i 2 i 3). The cv. Ponderosa does not have resistance to all FOL races, cv. Momotaro is resistant to FOL race 1 and susceptible to races 1 and 2, cv. Walter is susceptible to race 3 and resistant to races 1 and 2, and cv. Block is resistant to all FOL races. Inocula are as follows: KoChi-1 and its three transformants, K-B-b, K-2-11 and K-2-12; controls, race 1 MAFF 305121 (AVR1 AVR2 AVR3), race 2 JCM 12575 (– AVR2 AVR3) and race 3 Chz1-A (– avr2 AVR3). As a negative control, sterilized water was used (Mock). After three weeks of inoculation. (B) The disease severity of each individual was evaluated on external symptoms with 0 – 4 scale, respectively. The external symptoms were scored as follows: 0, no wilt or yellowing; 1, lower leaves are yellowing; 2, lower and upper leaves are yellowing; 3, lower leaves are...
yellowing and wilt and upper leaves are yellowing; 4, all leaves are wilt and yellowing or dead. The symptoms were evaluated after three weeks of inoculation. Four plants were used in each isolate, with three replicates.

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identical sequences (72 and 2 copies, respectively) among 13 isolates of Fusarium spp. In NRRL 34936, Homin was distributed on almost every chromosome (Fig. S3). On the other hand, F. oxysporum f. sp. raphani (NRRL 54004, pathogenic to radish and Arabidopsis), psii (NRRL 37622, pathogenic to pea), vasinfectum (NRRL 25433, pathogenic to cotton), melonis (NRRL 26406, pathogenic to melon), conglutinans (PHW808, pathogenic to cabbage), and two F. oxysporum isolates of FOSC 3-a, pathogenic to immunocompromised humans) had several Hormin-like (85.8–99.8% homology) sequences.

**kochi-1 avr1 encodes a defective protein**

The deduced amino acid sequence of KoChi-1 AVR1 with Homin revealed a chimeric protein of 175 amino acids (avr1; Fig. S4) that may be nonfunctional. Here, we designate the AVR1 gene truncated with Homin as avr1. To investigate the transcription of avr1, total RNA was extracted from tomato roots inoculated with KoChi-1 or MAFF 305121 (race 1, as a control). RT-PCR using primer set SIX4F/SIX1R (designed to amplify AVR1 including its intron) amplified a 734-bp fragment from MAFF 305121 RNA but not from KoChi-1 (Fig. 3). On the other hand, RT-PCR using primer set SIX4F with primer hornet-like2 (designed on Homin, see Table 3, Fig. 2C) generated a 440-bp fragment from KoChi-1 inoculated tomato only (Fig. 3), indicating that KoChi-1 avr1 is expressed in planta. Neither avr1 in KoChi-1 nor AVR1 in MAFF 305121 was expressed in mycelia grown on PDB or MM medium (data not shown). This expression pattern was consistent with that of AVR3 in FOL race 2 Fol007 [23].

**other kochi-1 AVR genes**

KoChi-1 avr2 contains the previously known point mutation G121A; it is one of three mutations known to cause loss of function in planta [9,24,25], are involved in the loss-of-function of AVR genes, which are found on a ca. 2.5 Mb chromosome, encoding small proteins secreted into tomato xylem on a small (ca. 2.5 Mb) chromosome together with avr2 and AVR3 (Fig. 4A, B; lane 8), which was also the case for AVR1 in race 1 isolates MAFF 305121 (1.6 Mb; Fig. 4A, B; lane 1). The small chromosome of each isolate had different size. However, although MAFF 103036 (a Japanese race 1 isolate) was found to carry AVR1 on a ca. 2.5 Mb chromosome, its AVR2 and AVR3 genes were found on a ca. 1.0 Mb chromosome (Fig. 4A, B; lane 2). In addition, apparently MAFF 103036, chromosomal fragmentation resulted in relocation of AVR2 and AVR3 to an independent small chromosome. All race 2 and race 3 isolates carried AVR2 or avr2 and AVR3 on chromosomal DNA, but none of them had the AVR1 or avr1.

Mobile elements, together with point mutation in the gene [9,24,25], are involved in the loss-of-function of AVR in fungal plant pathogens such as Magnaporthe oryzae and Cladosporium fulvum [26,27,28,29,30]. Generally, mobile elements play a role in duplication and translocation of the genes/genomic regions in

**Table 2. Summary of characteristics of KoChi-1 and other FOL isolates.**

| FOL Isolate | Scores of wilt disease on tomato cultivar* | AVR1 locusa | SNP in AVR2b | Polymorphism in AVR3d | VCG | MAT | Phylogenetic clade |
|-------------|------------------------------------------|--------------|--------------|-----------------------|-----|-----|-------------------|
| KoChi-1     | 3.75±0.25 (F)                            | avr1         |             |                       | 0030+0032 | 1-1             | A2                |
| Race 3 (Ch21-A, Japan) | 4.0±0.0 (F)       |             |             |                       | 0033 | 1-2 | A3                |
| Race 3 (F240, USA)      | nt                                      |             |             |                       | 0030+0032 | 1-1             | A2                |
| Race 3 (NRRL 26383, USA) | nt |             |             |                       | 0033 | 1-2 | A3                |
| Race 2 (UCM 12575, Japan) | 3.75±0.25 (F) |             |             |                       | 0031 | 1-1 | A1                |
| Race 1 (MAFF 103036, Japan) | 3.25±0.75 (F) |             |             |                       | E    | 0030+0032 | 1-1     | A2                |

*Four plants were used for each FOL isolate. The scores of external symptoms, using 0 (no symptoms) to 4 (death) scale are shown with standard error. All negative controls (inoculated with sterilized water) was 0.0±0.0 in all cultivars. These detailed results correspond to Fig. 2A, B, nt, not tested in this study.

AVR1, carrying functional AVR1 gene; avr1, carrying AVR1 truncated by Hormin; =, null.

+ wt, no SNPs; G121A indicates that 121st guanine was substituted to alanine.

Mutation at the 164 amino acid of AVR3 (E = glutamine, K = lysine).

*Corresponds to Figure 3 and previous study [16].

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Figure 2. **AVR1 in KoChi-1 genome was truncated by a transposon Hormin.** (A) Southern blot analysis to investigate the copy number of AVR1 gene. AVR1 probe was prepared using a primer set SIX4F/SIX4R (Table 3), and each gDNA was digested with restriction enzyme, EcoRV or NdeI (Fig. 2C). (B) Detection of AVR1 locus from KoChi-1 using a primer set SIX4F-F2/SIX4F-R2 (Table 3, Fig. 2C). (C) Schematic representation of AVR1 locus and AVR1 gene truncated by a transposon Hormin (avr1). The nonautonomous transposon Hormin (759 bp, shown in orange square) is inserted in the second exon of AVR1 in KoChi-1. Hormin harbors 15-bp tandem inverted repeats (TIRs, shown in blue triangle in white square) "CAGGGTTCAAATCCA" and 8-bp target site duplications (TSDs, shown with black line) "CACACCGG". Arrows show primers. E, EcoRV site; N, NdeI site.
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Table 3. Primers used in this study.

| Name       | Sequence (5′-3′) | Targeting gene/Region                          | Reference          |
|------------|------------------|-----------------------------------------------|--------------------|
| ITS1       | TCCGTAGTGAAACCTGCGG | Ribosomal DNA internal transcribed spacer (ITS) region | [39]               |
| ITS4       | TCTCCCGCTTTTATGAGAC | Ribosomal DNA internal transcribed spacer (ITS) region | [39]               |
| FIGS11     | GTAAGCCGTCCCTCAGGCTCG | Ribosomal DNA intergenic spacer (IGS) region | [16]               |
| FIGS12     | GCAATAATTCAATGATGGC | Ribosomal DNA intergenic spacer (IGS) region | [16]               |
| SIX4F      | ACTCGTGTTAGTCTCGG  | AVR1 (SOX) gene                              | This study         |
| SIX4R      | CCAAGGTAAGAAGGAAGCATA | AVR1 (SOX) gene                              | This study         |
| SIX3-F1    | CAGCGCAAAGGCCAGTTT | AVR2 (SOX) gene                              | [12]               |
| SIX3-R2    | GGAATTTACACTCTCGC | AVR2 (SOX) gene                              | [12]               |
| FP962      | TGAGCGGGCTGGCAAATTC | AVR2 (SOX) gene                              | [46]               |
| FP963      | CAATACTCTGAAGAGATAGAAG | AVR2 (SOX) gene                              | [46]               |
| P12-F1     | CCGGAATTGAGGTGAAG | AVR3 (SOX) gene                              | [10]               |
| P12-F2     | GTATCCTCCGATTTGACG | AVR3 (SOX) gene                              | [10]               |
| P12-R1     | AATAGAGGCTCGAAAGGTAG | AVR3 (SOX) gene                              | [10]               |
| SIX4-F2    | GTCGACTTAgGTTATCTCC | AVR1 locus (5′ flanking region) | Rep & Houterman (personal communication) |
| SIX4-F2    | ACTTAATTAAATGCTGTGGT | AVR1 locus (3′ flanking region) | Rep & Houterman (personal communication) |
| SIX4-in1   | CCACTACCTCTCTCCTCTCT | AVR1 locus (5′ flanking region) | This study         |
| SIX4-in2   | CTATCCGAAGACGGCATT | AVR1 locus (exon 2) | This study         |
| Gfm1a      | GTCCTACAAAGGCAGCGGATT | MAT1-1-1 alpha-box (MAT1-1) | This study         |
| Gfm1b      | TAAGCGGTCCTTTAAGCCCTC | MAT1-1-1 alpha-box (MAT1-1) | This study         |
| GfhMGI1    | TACGGTAGGACGGGTAC | MAT1-2-1 alpha-box (MAT1-2) | This study         |
| GfhMGI2    | GTACTGCTGGCAGATTTCC | MAT1-2-1 HMG-box (MAT1-2) | This study         |
| hornet-like2 | CGTGAATTAGGTTAAGTGG | Transposon Hormin in avr1 | This study         |
| FP157      | ATGAACTACCTCCGCTACC | FEM1                                           | [46]               |
| FP158      | GGTGAAAGTGAAAGACTCC | FEM1                                           | [46]               |
| Actin-f    | AGGCCAAGAGTAGGTTAGT | actin (S. lycopersicum) | [47]               |
| Actin-r    | AGCAACTCGAAGCTTCTTTG | actin (S. lycopersicum) | [47]               |

The genome [20,31], sometimes they cause genetic mutations. AVR genes often locate on mobile element-rich regions in fungal plant pathogens, such as M. oryzae [32], Lepidiothrix maculans [24], Blumeria graminis [33], and F. oxysporum [34]. In Phytophthora infestans, more than five hundreds of potential avirulence genes carrying RxLR motif located in mobile element-rich genomic regions [35]. Moreover, in FOL NRRL 24936 (race 2), a large amount of mobile elements are located on the linkage specific (LS) chromosomes such as Chr03, Chr06, Chr14 (2.2 Mb), the small chromosome carrying AVR2 and AVR3 and Chr15. Of the 72 Hormin elements, 37 are located on LS chromosomes of NRRL 34936 (Fig. S3).

Unlike other fungal isolates, it is easy to speculate how races emerged sequentially in FOL due to its simple combination of AVR genes and the small number of races. Based on the arms race model [36], FOL and its races are considered to have emerged as follows [9] (Fig. S6): First, a nonpathogenic F. oxysporum isolate acquired a small chromosome carrying AVR1, AVR2 and AVR3, and became FOL race 1. The deletion of the AVR1 locus in race 1 resulted in the emergence of race 2 (~AVR2 AVR3), and the point mutation in AVR2 (shown as avr2) in race 2 resulted in the emergence of race 3 (~avr2 AVR3). Refer to Table 2 for relationships among AVR genes, where phylogenetic groups, MAT and VCG of each isolate are also indicated. This study presented an alternative model: AVR1 in a race 1 isolate (AVR1 AVR2 AVR3) lost its function by a transposon insertion, resulting in the emergence of race 2 (avr1 AVR2 AVR3), and race 3 (avr1 avr2 AVR3) emerged from the race 2 as a result of the point mutation (G121A) in AVR2 (Fig. S6). If this scenario describes how KoChi-1 emerged, then where might it have happened? Soilborne pathogens are often carried with seed [1]. KoChi-1 may have been imported on tomato seeds from a production field because we have not found race 2 isolates carrying AVR1 truncated by Hormin, so far, in Japan. There still is the possibility that KoChi-1 evolved via race 2 from a race 1 isolate belonging to the A2 clade in a particular field in Kochi Prefecture. Analysis of more isolates from Kochi, and seed production fields, will be necessary to test these hypotheses.

Materials and Methods

Fungal and plant materials

We sampled diseased tomato (cv. Momotaro-Fight) at a greenhouse in Hidaka, Kochi Prefecture, Japan (latitude, N33°31'35.7"; longitude, E131°57'3.0"; altitude, 52 m) on 4 Feb. 2009. Sampling was permitted by the owner of the private land and greenhouse. No other specific permits were required for the described field study. Moreover, the field study did not involve endangered or protected species. All of the isolates obtained from
diseased individuals at the field were identified as *F. oxysporum* based on morphological characteristics [19]. In addition, all isolates showed identical phenotypes including virulence, mating type (MAT), vegetative compatibility (VC), combination of avirulence genes (AVR) and sequence of rDNA-IGS and rDNA-ITS regions. One representative isolate (KoChi-1) was chosen for this study.

**Figure 3.** Gene expression of *AVR1*, *avr1*, *AVR2* (avr2) and *AVR3*.

Eight days after inoculation with race 1 MAFF 305121 (*AVR1* *AVR2* *AVR3*), race 3 KoChi-1 (*avr1* *avr2* *AVR3*) and the three transformants (*avr1* *AVR1* *avr2* *AVR3*; K-B-b, K-2-11 and K-2-12), total RNA was extracted from the roots of tomato (cv. Ponderosa) and investigated the transcription of genes *AVR1*, *avr1*, *AVR2* (avr2), *AVR3*, *FEM1* and *Actin* with the primer sets SIX4F/SIX4R, SIX4F/hornet-like2, FP962/FP963, P12-F1/P12-R1, FP157/FP158 and Actin-f/Actin-r, respectively (Table 3). *FEM1* and *Actin* are used as controls for constitutively-expressed genes in fungal and plant tissues, respectively. Sterilized water is used as a negative control.

**Figure 4.** Localization of *avr1*/*AVR1*, *AVR2* and *AVR3* on the chromosomes of KoChi-1 and other FOL isolates. (A) Karyotype of FOL isolates by CHEF-gel electrophoresis. Electrophoresis was performed in 1.0% Sea Kem gold agarose gel with CHEF Mapper XA Pulsed Field Electrophoresis System, as following condition; 260 hours run at 8 ℃, 1200-4800 s switch time at 1.5 V/cm. MAFF 305121 (*AVR1* *AVR2* *AVR3*, Japan); MAFF 103036 (*AVR1* *AVR2* *AVR3*, Japan); 73 (*AVR1* *AVR2* *AVR3*, Italy); Ita3 (*AVR1* *AVR2* *AVR3*, Italy); JCM 12575 (– *AVR2* *AVR3*, Japan); NRRL 34936 (– *AVR2* *AVR3*, Spain); Chz1-A (– *avr2* *AVR3*, Japan); KoChi-1 (avr1 *avr2* *AVR3*, Japan). The chromosomes of *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* were used as CHEF DNA size markers. (B) Southern blot analysis probed with *AVR1* (upper), *AVR2* (middle) and *AVR3* (bottom). Probes to detect *AVR1* (avr1), *AVR2* (avr2) and *AVR3* were prepared using primer sets SIX4F/SIX4R, SIX3-F1/SIX3R2 and P12-F2/P12-R1, respectively (Table 3).

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from H. Hosobuchi, Sakata Seed, Japan) were used for vegetative compatibility group (VCG) determination. OSU-451B and MN-66 were imported to Japan under special permission of Ministry of Agriculture, and Forestry and Fisheries of Japan. All isolates were stored in 25% glycerol at -150°C.

Four race differential cultivars of tomato; Ponderosa (Noguchi Seed, Saitama, Japan), Mormonaro (Takii seeds, Kyoto, Japan), Walter (gifted from National Institute of Vegetable and Tea Science, Mie, Japan) and Block (Sakata Seed, Yokohama, Japan) were used. Ponderosa (i 2 12) is susceptible to all FOL races, Mormonaro (I 2 12) is resistant to FOL race 1 but susceptible to races 2 and 3, Walter (I 2 12) is resistant to races 1 and 2 but susceptible to race 3, and Block (I 2 12) is resistant to all races.

Pathogenicity assay
Race differential tomato cultivars were used to evaluate FOL pathogenicity. Each isolate was cultured on potato sucrose broth (PSB) for 5 days at 25°C and 120 rpm, and conidial suspensions (1.0 × 10⁷ conidia/ml) were prepared. Two seeds of each cultivar were sown to soil (Kureha Soil, Kureha, Iwaki, Japan) in a plastic pot (7 cm-diam.), and were maintained in a growth chamber (16 hours light at 28°C/8 hours dark at 25°C). Roots of 15-day-old tomato were injured, dipped in a conidial suspension for 5 min, and replanted to well-moistened soil. Two weeks later, external symptoms of each plant were evaluated as follows: 0, no wilt or yellowing; 1, lower leaves yellowing; 2, lower and upper leaves yellowing; 3, lower leaves yellowing and wilting and upper leaves yellowing; 4, all leaves wilted and yellowing or dead.

DNA extraction and standard PCR
Fungal genomic DNA (gDNA) was extracted using the protocol described earlier [37,38] with modifications. Fragments of rDNA-ITS (521 bp) and IGS (598 bp) regions were amplified using primer sets ITS1/ITS4 [39] and Figs11/FIGS12 [16], respectively (Table 3). We also amplified fragments of ca. 800 bp of AVR1, ca. 300 bp of AVR2 and ca. 900 bp of AVR3 using primer sets SIX4F/SIX4R, SIX3-F1/SIX3-R2 and P12-F2/P12-R1, respectively (Table 3). Each reaction mixture of 20 µl contained 20 ng of gDNA, 2.0 µl of 10×buffer (Takara Bio), 1.6 µl of 2.5 mM (each) dNTPs (Takara Bio), 8 µl of each primer, and 0.5 µl of Ex-Taq polymerase (Takara Bio). Thermal conditions were as follows: One incubation at 94°C for 2 min; 30 cycles of: denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and elongation at 72°C for 30 s; and a final extension at 72°C for 7 min. To amplify the fragment (ca. 2.0 kb) of the AVR1 locus by SIX4F2/SIX4R2 (Table 3), we modified the annealing temperature and extension time to 45°C and 2 min, respectively.

Sequencing
PCR amplicons purified with EXOSAP-IT (USB, Cleveland, USA) or 100 ng of plasmids were subjected to sequencing reaction using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and analyzed with a 3130xl Genetic Analyzer (Applied Biosystems). Sequence was arranged with GENETYX ver. 13 (Genetyx, Tokyo, Japan).

Phylogenetic analysis
Nucleotide sequences of the rDNA-IGS fragment from KoChi-1 were aligned with those from other FOL isolates using CLUSTAL X 2.0 [40]. We constructed the phylogeny by the neighbor joining (NJ) method [41] based on Kimura’s two-parameter model [42], using MEGA v. 4 [43]. The statistical reliability of each node was assessed using 1000 bootstrap iterations. F. sacchari (synonym, Gibberella sacchari; mating population B of the G. fujikuroi-species complex) FGSC 7610 was used as an outgroup. All sequence data except for KoChi-1 were cited from the NCBI database.

Mating type (MAT) and vegetative compatibility group (VCG) determination
Mating type, MAT1-1 or MAT1-2, was determined by PCR using Gfmat1a/Gfmat1b or GfHMG11/GfHMG12, respectively (Table 3). The reaction mixture was prepared as described in the section of Standard PCR, reaction conditions were set as follows: One incubation at 94°C for 2 min; 30 cycles of: denaturation at 94°C for 30 s, annealing at 58°C for 30 s, and elongation at 72°C for 45 s; and a final extension at 72°C for 6 min.

We also identified the VCG type of each isolate. To date, four vegetative compatibility groups (VCGs), 0030+0032, 0031, 0033 and 0035, have been reported in FOL [5]. The complementation test was performed using the tester isolates, OSU-451B (VCG 0031), MN-66 (VCG 0030+0032) and H-1-4 (VCG 0033). Each nitrate nonutilizing (nid) mutant of each isolate (nid1 and NidM) was prepared, and a compatibility test was performed following the procedures described previously [44].

Gene expression analysis
Tomato cv. Ponderosa was inoculated with F. oxysporum as described in the section entitled “Pathogenicity assay”. Eight days after inoculation, we vigorously washed the tomato roots with sterile water. After drying with paper towels, roots were crushed in liquid nitrogen and total RNA was extracted with the SV Total RNA Isolation System (Promega) following the manufacturer’s manual. From the extracted total RNA, cDNA was synthesized using TaKaRa RNA PCR Kit (AMV) Ver. 3.0 (TaKaRa Bio). Expression of target genes was examined with 5 ng of cDNA. To investigate expression of AVR1, avr1, AVR2, AVR3, FEM1 and the tomato actin gene, primer sets SIX4F/SIX4R, SIX4F/SIX4R, SIX4F/hornet-like2, FP962/FP965, and FP157/FP158, and Actin-f/Actin-r (Table 3) were used for PCR, respectively. FEM1 [45,46] and Actin [47] were used as controls for fungal and plant genes, respectively. Negative controls substituted sterile water for conidial suspension. Reaction mixtures were prepared as described above. Thermal conditions were as follows: One incubation at 94°C for 2 min; 35 cycles of: denaturation at 94°C for 30 s, annealing at 57°C for 30 s, and elongation at 72°C for 30 s; and a final extension at 72°C for 7 min.

Complementation with AVR1 using Agrobacterium tumefaciens-mediated transformation (ATMT)
The AVR1 gene of FOL race 1 Fol004 was integrated into the KoChi-1 genome ecologically by the ATMT method. Transformation using the binary vector pHSIX4r+ (carrying about 2.0 kb of AVR1 locus and phleomycin resistance gene) [9] was carried out following the procedure described earlier [10] with minor modifications. To suppress the growth of Agrobacterium after transformation, we used 25 µg/ml Melopen (Dainippon Sumitomo Phama, Osaka, Japan) and 50 µg/ml Zeocin (Invitrogen, San Diego, USA), respectively.

Contour-clamped homogeneous electric field (CHEF)-gel analysis
In addition to KoChi-1, we used several race 1 isolates; MAFF 305121 (Japan), MAFF 103036 (Japan), 73 (Italy; gift from Corby H. Kistler) and Ia3 (Italy; gift from Giorgia Ferro, The Regional
Center For Agricultural Experimentation and Assistance, Italy): race 2 isolates; NRRL 24936 (Spain; gift from A. Di Pietro, University of Cordoba, Spain) and JCM 12575 (Japan) and race 3 isolate, Chz1-A (Japan). Protoplasts were prepared following [48] with slight modification; we used enzyme solution containing 1.0% Lysing enzymes (Sigma, St. Louis, USA) and 1.0% Driselase (ASKA Pharmaceutical, Tokyo, Japan) for digestion of fungal cell wall, and Proteinase K (Nakarai Tesk, Kyoto, Japan) was used for plug purification.

CHEF gel electrophoresis was performed in 1.0% Sea Kem gold agarose gel (FMC BioProducts, Rockland, USA) with CHEF Mapper® XA Pulsed Field Electrophoresis System (BioRad, Hercules, USA). The condition to separate chromosomes was as described earlier [34] with slight modification; 260 hours run at 3°C, 1200–4800 s switch time at 1.5 V/cm. The running buffer 0.5xTBE was refreshed every 2 days. Chromosomes of *Schizosaccharomyces pombe* (BioRad) and *Saccharomyces cerevisiae* (BioRad) were used as DNA size markers. The gel was stained with ethidium bromide to visualize chromosomes after running electrophoresis.

**Southern blot analysis**

Probes for **AVR1/avr1, AVR2/avr2** and **AVR3** were prepared using SI64F/SI64R, SI63-F1/SI63-R2 and P12-F2/P12-R1, respectively. For genomic Southern hybridization, 8.0 μg gDNA were digested with NdeI and BstHII, and incubated overnight at 37°C. The following procedure was performed as described earlier [49], note that Whatman Nytran SuPerCharge (SPC) nylon blotting membranes (Sigma) was used in this study.

The CHEF-gel was treated with 0.25 N HCl for 30 min, followed by denaturation buffer (0.5 M NaOH, 1.5 M NaCl), and the digested chromosomes were transferred to a nylon membrane (Hybond N+; Amersham, Amersham, UK) washed in 0.4 M NaOH for about 72 hours. The following procedure after transfer was performed as early study [49]. For stripping the hybridized probe, the used membrane was washed twice, for 15 min each, with 0.2 M NaOH, 0.1% SDS at 37°C, then the membranes were soaked in 2×SSC for 5 min and dried.

**Supporting Information**

**Figure S1 Fusarium wilt of tomato caused by *F. oxysporum* f. sp. lycopersici in Kochi, Japan.** (A) Location of the wilt disease emerged. Asterisk at the tip of bar presents Hidaka, Kochi Prefecture, Japan (latitude, 33°31′33.0″; longitude, 148°31′21.3″; altitude, 32 m). (B) Diseased tomato cultivar Momotaro-Fight (*F. oxysporum* f. sp. lycopersici) in a greenhouse in Hidaka, Kochi Prefecture, Japan. The diseased tomato plants wilted and the color of the leaves turned yellow. Severely diseased plants did not survive and white hyphae were observed on the lower part of their stems. (TIF)

**Figure S2 Phylogenetic relationship of tomato wilt fungus (FOL) isolates in Japan.** KoChi-1 and other FOL races 1~3 isolates obtained in Japan were used. Race, the source, mating type (MAT) and vegetative compatibility group (VCG) were described in parentheses at the end of the isolates name. A hyphen indicates incompatible isolates with VCG testers. *Gibberella fujikuroi* strain FGSC 7610 was used as the outgroup. The phylogeny was constructed based on Kimura’s two-parameter [42] as nucleotide substitution model using MEGA v. 4 [43]. Bootstrap iterations are 1000 replications, the values are indicated at tree nodes. Bootstrap values greater than 70% are shown beside nodes. The FOL clades A1, A2 and A3 are consistent with the previous study [16]. All sequence data are in the DDBJ/EMBL/GenBank databases; KoChi-1 (AB674508), MAFF 100343 (AB106032), JCM 12575 (AB106027), SUF 1330 (AB106035), MAFF 100306 (AB106031), MAFF 305121 (AB106021), MAFF 100306 (AB106020), MAFF 727501 (AB106022), Chz1-A (AB373019), F-1-1 (AB106037) and FGSC 7610 (AB106061). (TIF)

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**Author Contributions**

Conceived and designed the experiments: KI TA. Performed the experiments: KI CYA TA. Analyzed the data: KI TA. Contributed reagents/materials/analysis tools: YM MY. Wrote the paper: KI TT TA.

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