Variation and Phylogeny of \textit{Fusarium oxysporum} Isolates Based on Nucleotide Sequences of Polygalacturonase Genes

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The nucleotide sequences of two endopolygalacturonase genes (\textit{pg1} and \textit{pg5}) and two exopolygalacturonase genes (\textit{pgx1} and \textit{pgx4}), which encode members of a major family of secreted cell-wall-degrading enzymes (CWDEs), were compared to detect the extent of genetic variation among isolates of \textit{Fusarium oxysporum}. The nucleotide variation rate in exons was 0.23–0.93%, higher than that in introns (0.01–0.64%) and untranslated regions (UTRs) (0.07–0.25%), resulting in 0.05–0.31% variation in amino acids. The nucleotide variation rate in exons was 0.23–0.93%, higher than that in introns (0.01–0.64%) and untranslated regions (UTRs) (0.07–0.25%), resulting in 0.05–0.31% variation in amino acids. Phylogenetic analysis of the four genes, which reside on different chromosomes, revealed different evolutionary patterns for each.

Our results suggest a biased evolution of the polygalacturonase genes of \textit{F. oxysporum}, or alternatively, that the genes were acquired at different times during evolution.

Key words: \textit{Fusarium oxysporum}, genetic variation, molecular evolution, phylogenetic tree, polygalacturonase

\textit{Fusarium oxysporum} Schlechtend.: Fr. is an economically important pathogenic fungus that damages vegetable crops worldwide. \textit{F. oxysporum} causes soilborne wilt disease by colonizing and occluding the vascular tissue of the host plant. More than 150 physiological forms (formae specialiae) have been identified based on host-specificity (3). Moreover, many formae specialiae have distinct sub-groups, so-called races, based on their specific pathogenicity in cultivars. Neither forma specialis nor race can be distinguished by morphological characteristics.

Vegetative compatibility groups (VCGs) (23), as well as the nucleotide sequences of translation elongation factor 1\(\alpha\), mitochondrial small subunit DNA (mtDNA) (4), the ribosomal DNA (rDNA) intergenic spacer (IGS) region (1), and the mating type locus (\textit{MAT1}) (2) have proven useful for resolving the intraspecific relationships of \textit{F. oxysporum}. However, phylogenetic analyses using these molecular markers have shown that each forma specialis often has multiple phylogenetic origins, and race and phylogeny do not always correlate with each other (1, 8, 17, 20, 31). Kawabe \textit{et al.} (13) determined that there were three evolutionary lineages of \textit{F. oxysporum} f. sp. lycopersici (FOL) among present isolates collected worldwide, based on the nucleotide sequences of rDNA IGS and \textit{MAT1}, as well as \textit{pg1}, and confirmed that FOL has multiple phylogenetic origins. Moreover, they found that the three races of FOL did not correlate with phylogenetic background.

Polygalacturonases are the major secreted cell-wall-degrading enzymes (CWDEs) in plant pathogenic fungi. It has been suggested that the comparison of polygalacturonase gene sequences might be a useful method for analyzing the genetic diversity of populations in a fungal species (26). Previously, Hirano and Arie (12) were able to differentiate forms and races in Japanese isolates of FOL by comparing the nucleotide sequences of \textit{pg1} and \textit{pgx4} in isolates of FOL and \textit{F. oxysporum} f. sp. radicis-lycopersici (FORL).

In this study, we analyzed the nucleotide sequences of four polygalacturonase genes (\textit{pg1}, \textit{pg5}, \textit{pgx1}, and \textit{pgx4}) from 38 \textit{F. oxysporum} isolates derived from 17 formae specialiae collected in Japan by sequence alignment to determine the extent of variation among the isolates. We also discuss the phylogenetic relationships of the isolates.

Materials and Methods

Fungal isolates

The thirty eight isolates of \textit{Fusarium oxysporum} used in this study, including seventeen formae specialiae, are listed in Table 1. The isolates were maintained on potato dextrose agar (PDA). To extract genomic DNA (gDNA), the isolates were cultured on potato dextrose broth (PDB) at 25°C for 5 days.

Genomic DNA extraction

Thirty milligrams (fresh mass) of mycelium from each isolate was subjected to gDNA extraction using an ISOPLANT DNA extraction kit (Nippon Gene, Tokyo, Japan). The purified gDNA was dissolved in TE buffer (10 mM Tris-EDTA pH 8.2).

Polymerase chain reaction (PCR) and DNA sequencing

The primer sets endoF+endoR2, PG2F+PG2R, exoF2+exoR, and pgxF+pgxR (Table 2) were used to amplify fragments of \textit{endo}-polygalacturonase genes (\textit{pg1} and \textit{pg5}), and \textit{exopol}ygalacturonase genes (\textit{pgx1} and \textit{pgx4}), respectively. Each of the primer sets was designed to amplify a fragment that incorporated the exons, introns, stop codon, and 5'- and 3'-untranslated regions (UTRs) of the targeted gene. Primers were synthesized by Operon (Tokyo, Japan). PCR products were performed as described previously (12). Briefly, the reaction mixture (30 \uL) contained 5 \uL of purified gDNA (ca. 10 ng), 1×PCR buffer (Takara-Bio, Otsu, Japan), 167 \uM of each dNTP (Takara-Bio), 0.75 \uM of each primer, and 50 nM of each primer. Amplification was carried out using a PTC-200 instrument (Bio-Rad Japan, Tokyo, Japan). The cycling parameters consisted of denaturation at 94°C for 1 min, annealing at 52°C for 1 min, and elongation at 72°C for 2 min for 30 cycles.

PCR products were subjected to direct sequencing with a Gene

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Table 1.  *Fusarium oxysporum* isolates used in this study, and determined mating type and patterns of sequence of polygalacturonase genes

<table>
<thead>
<tr>
<th>Forma specialis, race, and isolate number</th>
<th>Mating type</th>
<th>Pattern of sequence</th>
<th>Accession number in DDBJ/GenBank database</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pg1</td>
<td>pg5</td>
<td>pgx1</td>
</tr>
<tr>
<td>f. sp. colocaiae</td>
<td>MAFF744032 MAT1-2</td>
<td>2b</td>
<td>2a</td>
</tr>
<tr>
<td>f. sp. conglutinans</td>
<td>MAFF772516 MAT1-1</td>
<td>11a</td>
<td>—</td>
</tr>
<tr>
<td>f. sp. cucumerinum</td>
<td>MAFF744001 MAT1-1</td>
<td>27a</td>
<td>—</td>
</tr>
<tr>
<td>f. sp. cucumerinum</td>
<td>MAFF103054 MAT1-2</td>
<td>13b</td>
<td>4c</td>
</tr>
<tr>
<td>f. sp. diantii</td>
<td>MAFF103072 MAT1-1</td>
<td>0a</td>
<td>4a</td>
</tr>
<tr>
<td>f. sp. fragariae</td>
<td>MAFF305946 MAT1-1</td>
<td>15a</td>
<td>10b</td>
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<td>19a</td>
<td>10a</td>
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<td>Saitama ly1</td>
<td>MAFF305122 MAT1-1</td>
<td>4a</td>
<td>2a</td>
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</tr>
<tr>
<td>f. sp. fragariae</td>
<td>MAFF103072 MAT1-2</td>
<td>9a</td>
<td>9b</td>
</tr>
<tr>
<td>f. sp. fragariae</td>
<td>02MT-1 MAT1-2</td>
<td>9a</td>
<td>6a</td>
</tr>
<tr>
<td>f. sp. melongenes</td>
<td>MAFF103051 MAT1-1</td>
<td>16a</td>
<td>3a</td>
</tr>
<tr>
<td>f. sp. melonis</td>
<td>MAFF305122 MAT1-1</td>
<td>4a</td>
<td>2a</td>
</tr>
<tr>
<td>f. sp. melonis</td>
<td>MAFF30544 MAT1-1</td>
<td>21a</td>
<td>9a</td>
</tr>
</tbody>
</table>
| 38 isolates were aligned using Clustal W (28). A gap was considered a nucleotide variation.

**Sequence alignment and display pattern**

The nucleotide sequences of *pg1* (1,585–1,587 bp), *pg5* (1,873 bp), *pgx1* (1,794–1,813 bp) and *pgx4* (1,375 bp) from 38 isolates were aligned using Clustal W (28). A gap was considered a nucleotide variation.

The dominant nucleotide at each position was defined, and a consensus sequence composed of the dominant nucleotide at each position was determined for each polygalacturonase gene. Differences in nucleotide sequences among isolates were determined for each gene. Subsequently, the number of nucleotides different from the consensus sequence was counted and the sequence was designated with the number. If variations were found between or among sequences with the same number, each sequence was assigned a letter. Finally, the pattern of sequence of each gene was determined with a combination of numbers and letters (Table 1). The extent of variation for each isolate was calculated by dividing the number of nucleotides that differed from the consensus sequence by the total number of nucleotides in the consensus sequence.

**Phylogenetic analysis**

Based on the nucleotide sequences of the four polygalacturonase genes (*pg1*, *pg5*, *pgx1*, and *pgx4*), or their predicted amino acid sequences, the phylogenetic relationship of the 38 isolates was analyzed by methods of neighbor-joining (NJ) (25), maximum-parsimony (MP) (9), and minimum-evolution (ME) (24) using the MEGA 3.1 program (18). Evolutionary distance was computed using the two-parameter distance method (14), and then used to construct a phylogenetic tree. The stability of groups was assessed using 1,000 bootstrap replications of the data set.
and stop codon. The total variation rate, for each gene. position, was defined (Fig. 1). In this report, we describe the each gene, composed of the dominant nucleotide at each

localized to the untranslated regions (UTRs), introns, exons, into 26 patterns (Table 1, Fig. S1A). Nucleotide variations nucleotide variation was detected at 84 positions, which grouped

sequence times 100, was 0.69% (416/60,253 bp). Variation
time was divided by the total number of nucleotides of the 38

acid (Fig. S1A).

Variation of the DNA sequencing and displaying alignment

The nucleotide sequences of the endopolygalacturonase genes (pg1 and pg5) and the exopolygalacturonase genes (pgx1 and pgx4) from 38 F. oxysporum isolates were determined and aligned. A consensus nucleotide sequence for each gene, composed of the dominant nucleotide at each position, was defined (Fig. 1). In this report, we describe the nucleotide position (nt.) according to the consensus sequence for each gene.

Among the 38 pg1 sequences (1,585–1,587 bp), nucleotide variation was detected at 84 positions, which grouped into 26 patterns (Table 1, Fig. S1A). Nucleotide variations localized to the untranslated regions (UTRs), introns, exons, and stop codon. The total variation rate, i.e., the number of nucleotides that differed from the consensus sequence divided by the total number of nucleotides of the 38 pg1 sequence times 100, was 0.69% (416/60,253 bp). Variation rate, i.e., the number of nucleotides in each region different from the consensus sequence divided by the total number times 100, was 0.20% in the 5'-UTR, 0.25% in exons, 0.19% in introns, 0.003% in the stop codon, and 0.05% in the 3'-UTR, respectively. Of the 38 nucleotide variations that were located in exons, five (0.23%) resulted in a change in amino acid (Fig. S1A).

When we carried out PCR with the primer set for pg5, we were unable to generate amplicons from f. sp. conglutinans (MAFF 727516 and MAFF 744001) and an isolate (MAFF 305124) of f. sp. raphani, even upon repeated trials. Among the 35 remaining pg5 sequences (1,375 bp each), nucleotide variation was present at 43 positions, which grouped into 15 patterns (Table 1, Fig. S1B). Nucleotide variations localized predominantly to exons. The total variation rate was 0.35% (170/48,125 bp). The variation rate was 0.06% in the 5'-UTR, 0.28% in exons, 0.01% in introns, and 0.01% in the 3'-UTR. Among the 31 variations that localized to exons, six (0.17%) resulted in a change in amino acid (Fig. S1B).

Among the 38 pgx4 sequences (1,873 bp each), nucleotide variation was detected at 64 positions, which grouped into 17 patterns (Table 1, Fig. S1D). Nucleotide variations localized to the UTRs, introns, and exons. The total variation rate was 0.42% (302/71,174 bp). The variation rate was 0.07% in the 5'-UTR, 0.23% in exons, and 0.12% in introns. Of the 42 variations that were located in exons, four (0.05%) resulted in a change in amino acid (Fig. S1D).

Results

Mating type determination

The mating type (either MAT1-1 or MAT1-2) of each isolate was determined by PCR, according to the protocol of Arie et al. (2). The primer set Falpha1 and Falpha2 amplifies an approximately 370-bp fragment, while the primer set FHMG11 and FHMG12 amplifies an approximately 190-bp fragment (Table 2). The PCR conditions were as described by Arie et al. (2).

Nucleotide sequence accession numbers

The polygalacturonase genes sequences obtained in this study were submitted to the DDBJ/GenBank and accession numbers were described to Table 1.

Table 2. Primers used in this study to amplify fragments of polygalacturonase genes and MAT1 genes

<table>
<thead>
<tr>
<th>Primer</th>
<th>Nucleotide sequencea)</th>
<th>Position</th>
</tr>
</thead>
<tbody>
<tr>
<td>pg1 (endopolygalacturonase)</td>
<td>5'-CCAGAGTGCGCATACGCATT-3'</td>
<td>nt. 458–478 of U96456</td>
</tr>
<tr>
<td>endoF</td>
<td>5'-GCTTAYGAACAKGAGT-3'</td>
<td>nt. 2082–2064 of U96456</td>
</tr>
<tr>
<td>pg2</td>
<td>5'-AGATGCGAGCGCAGTGT-3'</td>
<td>nt. 118–137 of AF078156</td>
</tr>
<tr>
<td>pg2R</td>
<td>5'-TCCATGATCTTCTACC-3'</td>
<td>nt. 1536–1517 of AF078156</td>
</tr>
<tr>
<td>pgx1 (endopolygalacturonase)</td>
<td>5'-TCGTGGGGGTAAAGCAGTT-3'</td>
<td>nt. 586–604 of AF083075</td>
</tr>
<tr>
<td>pgxF</td>
<td>5'-TTAAGTGGTGACGATT-3'</td>
<td>nt. 2434–2415 of AF083075</td>
</tr>
<tr>
<td>exoR</td>
<td>5'-ACCGCAACCCCTTCACTT-3'</td>
<td>nt. 2277–2259 of AF136444</td>
</tr>
<tr>
<td>MAT1-1</td>
<td>Falpha1 5'-CGGTCAAGGATCTTCTG-3'</td>
<td>nt. 1575–1556 of AB0011379</td>
</tr>
<tr>
<td>Falpha1</td>
<td>5'-GATGAGTAGGAGGTCTCA-3'</td>
<td>nt. 1200–1219 of AB0011379</td>
</tr>
<tr>
<td>MAT1-2</td>
<td>FHMGI1 5'-TACCAGGAGGTCACCC-3'</td>
<td>nt. 30–48 of AB00541</td>
</tr>
<tr>
<td>FHMGI2</td>
<td>5'-TTYWYCTATSACGSMKHWSCTT-3'</td>
<td>nt. 218–196 of AB00541</td>
</tr>
</tbody>
</table>

a) Y=C and T; M=A and C; K=G and T; S=C and G; W=A and T; H=A, C, and T.

When we carried out PCR with the primer set for pg5, we were unable to generate amplicons from f. sp. conglutinans (MAFF 727516 and MAFF 744001) and an isolate (MAFF 305124) of f. sp. raphani, even upon repeated trials. Among the 35 remaining pg5 sequences (1,375 bp each), nucleotide variation was present at 43 positions, which grouped into 15 patterns (Table 1, Fig. S1B). Nucleotide variations localized predominantly to exons. The total variation rate was 0.35% (170/48,125 bp). The variation rate was 0.06% in the 5'-UTR, 0.28% in exons, 0.01% in introns, and 0.01% in the 3'-UTR. Among the 31 variations that localized to exons, six (0.17%) resulted in a change in amino acid (Fig. S1B).

Among the 38 pgx4 sequences (1,873 bp each), nucleotide variation was detected at 64 positions, which grouped into 17 patterns (Table 1, Fig. S1D). Nucleotide variations localized to the UTRs, introns, and exons. The total variation rate was 0.42% (302/71,174 bp). The variation rate was 0.07% in the 5'-UTR, 0.23% in exons, and 0.12% in introns. Of the 42 variations that were located in exons, four (0.05%) resulted in a change in amino acid (Fig. S1D).

Among the 38 pgx1 sequences (1,793–1,813 bp each), nucleotide variation was detected at 170 positions, which grouped into 19 patterns (Table 1, Fig. S1C). Nucleotide variations localized to the UTRs, introns, and exons. The total variation rate was 1.7% (1153/68,815 bp). The variation rate was 0.08% in the 5'-UTR, 0.93% in exons, 0.64% in introns, and 0.03% in the 3'-UTR. The rate of amino acid variation was 0.31% (54/17,290 aa.). In pgx1, at nine positions (nt. 297, 544, 581, 699, 742, 1,104, 1,287, 1,597, and 1,624), there were several different variations, and the variation at nt. 1,597, in particular, resulted in two different amino acid changes (from asparagine to serine or isoleucine) (Fig. S1C).

Polymorphisms were observed among isolates in each forma specialis, and for example, ff. sp. cucumerinum, fragariae, melonis, phaseoli, and tracheiphilum seemed to be rich in diversity (Table 1, Fig. S1).
Phylogenetic analysis

Phylogenetic trees were constructed based on the nucleotide sequences of each polygalacturonase gene (Fig. 2). Phylogenetic trees based on the combined sequences of each gene were also constructed using the NJ method (Fig. S2). The tendency of phylogenetic relationships did not differ among the NJ, MP, and ME methods (data not shown). In all trees, isolates of each race of *F. oxysporum f. sp. lycopersici* formed a cluster, which was consistent with previous reports (12, 13).

Mating type determination

The mating type of each isolate was determined is shown in Table 1 and Fig. 2. In several formae specialis, including *conglutinans*, *fragariae*, and *tulipae*, both isolates were of a single mating type. On the other hand, in *F. oxysporum f. sp. cucumerinum*, *lycopersici*, *melonis*, *niveum*, *phaseoli*, and *radicis-lycopersici*, isolates of both mating types were determined (Table 1, Fig. 2). Isolates of different mating types in a forma specialis tended to show distinct patterns of sequence and phylogenetic position (i.e. *f. sp. cucumerinum MAFF 103054 and 744005*).

Discussion

Mutation as well as horizontal gene transfer would generate diversity among individuals of a species, and diversity is distributed and/or accumulates through sexual and parasexual recombination. It has been generally assumed that in the asexual ascomycete *F. oxysporum*, a strain that acquired specific host-adaptability arose accidentally, and became established as the viable pathogen for the particular host plant (6, 7, 19, 27). Baayen *et al.* (4) and Namiki *et al.* (21) showed that each of the ff. sp. *lilii*, *tulipae*, *cucumerinum*, *lagenariae*, and *niveum* had monophyletic origin. However, most of the other formae specialis, such as, *cubense*, *melonis*, *asparagi*, *dianthi*, *gladioli*, and *lycopersici*, have been revealed to...
Variation of F. oxysporum PG Genes

have multiple evolutionary origins; in other words, they are polyphyletic (4, 10, 13, 22). No stable correlation between phylogeny and pathogenic-group has been reported for F. oxysporum (5, 11, 13, 16, 30). These findings have raised the question as to how isolates of different origins have acquired pathogenicity towards the same host (29).

In this study, we investigated genetic variation in F. oxysporum by analyzing the sequences of four polygalacturonase genes. For each gene we determined a consensus nucleotide sequence, composed of the dominant base at each nucleotide position, using 38 isolates from 17 formae speciales of F. oxysporum. Although the total number of isolates of f. sp. lycopersici used in this study was larger than the number of isolates from other ff. sp. (usually two for each), which may have introduced some bias into the consensus nucleotide sequences, the phylogenetic trees indicated that f. sp. lycopersici carried relatively many variations from the consensus sequence in the four genes, suggesting that f. sp. lycopersici is not the representative member in F. oxysporum.

In all four polygalacturonase genes, the highest level of variation was in the exons. Of the 170 nucleotide variations in pgx1, 92 (54%) localized to exons. Similarly, 45% (38/84), 72% (31/43), and 66% (42/64) of the variation in pg1, Fig. 1. Continued.

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Fig. 2. Phylogenetic analysis of the nucleotide sequences of the four polygalacturonase genes (A, pg1; B, pg5; C, pgx1; D, pgx4) of *F. oxysporum* using the neighbor-joining (NJ) method. Numbers of nodes represent bootstrap values estimated from 1,000 replications of the data set. Bars indicate the isolates carrying the sequence closest to the consensus sequence. The mating type of each isolate is represented with ● (MAT1-1) and ▲ (MAT1-2).
pg5, and pgx4, respectively, was concentrated in exons. Of the four genes examined, pgx1 was richest in diversity in the exons. However, while there was a great deal of nucleotide variation in exons, relatively few of the variations resulted in amino acid changes. Of the exon-specific variations, 7 of 92 (pg1), 6 of 31 (pg5), 5 of 38 (pgx1) and 3 of 42 (pgx4) resulted in changes in amino acids (Fig. S1). Four of the variations in pgx1 that were located at the first codon position (nt. 591, 645, 749, and 1,255) resulted in no change in the amino acid sequence. Relative synonymous codon usage (RSCU) resulted in fewer amino acid changes for leucine in pgx1 as compared to pg1, pg5, and pgx4 (data not shown).

Isolates NBRC 9969, MAFF 103059, and MAFF 235725 had identical pg1 sequences; MAFF 744032, MAFF 744035, MAFF 727510, NBRC 9969, MAFF 235727, and MAFF 235725 had identical pg5 sequences; MAFF 305544 and MAFF 235105 had identical pgx1 sequences; and MAFF 727510, MAFF 103051, MAFF 305122, and MAFF 305543 had identical pgx4 sequences, even though all of these isolates were from different formae speciales (Table 1, Fig. S1). Moreover, F. oxysporum f. sp. spinaciae (MAFF 103059 and MAFF 731044) had an 18-bp gap (corresponding to 6 amino acid residues) in the exon adjacent to the stop codon in pgx4.

The chromosomal location of each polygalacturonase gene was investigated using the web site http://www.broad.mit.edu/annotation/genome/fusarium_group/. The four genes localized to different chromosomes. The pg1 locus was on chromosome (chr) 12; the pg5 locus was on chr 9; the pgx1 locus was on chr 2a; and the pgx4 locus was on chr 5. The phylogenetic trees of the four polygalacturonase genes revealed different phylogenetic relationships among isolates (Fig. 2). For example, F. oxysporum f. sp. lycopersici (FOL) races 2 and 3 were included in a single cluster in the pg1 tree, whereas these races formed different, independent clusters, in the pg5 and pgx4 trees (Table 1, Fig. 2). Of the four trees, the pgx1 tree was most similar to that of rDNA-IGS, which was created by Kawabe et al. (13). These results suggested that each polygalacturonase gene has evolved independently. Several studies have provided evidence of the horizontal transfer of pathogenically-related genes, such as those involved in toxin-biosynthetic pathways, in several plant pathogenic fungi (15). van der Does et al. (29) suggested that the host specificity-determining genomic locus (SIX), where there are several putative transposable elements, has horizontally transferred between isolates in FOL.

In this study, we investigated the genetic origin of the polygalacturonase genes of F. oxysporum. While we do not have sufficient data to discuss the evolution of these four genes, the different levels of nucleotide sequence variation among them suggests either a biased-evolution of these genes, or their acquisition at different points in time.

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