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Cloning and Sequence Analysis of Endopolygalacturonase Genes in *Venturia nashicola* and *Venturia pirina*

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Abstract

Genes encoding endopolygalacturonase (endoPG) were isolated from pathogens of the Asian pear scab, *Venturia nashicola* races 1, 2, 3, and 4, and European pear scab, *V. pirina*. The *Vnpgr1* gene of the *V. nashicola* race 1 consists of a 1,116-bp open reading frame, encoding a protein of 372 amino acids with an estimated molecular mass of 37.5 kDa and an isoelectric point (pI) of 6.56. The sequences of *Vnpg* genes from different races and *Vppg* gene from *V. pirina* showed high identities (95-100%). The deduced amino acid sequence of the *V. nashicola* race 1 endoPG showed 63-68% identity to the endoPG sequences of *Penicillium olsonii, Colletotrichum lindemuthianum, Cryphonectria parasitica, Fusarium oxysporum, Sclerotinia sclerotiorum,* and *Alternaria citri*. The deduced amino acid sequence of the race 1 endoPG was identical to the N-terminal amino acid sequence of the previously purified endoPG enzyme from the mycelia of this race. The results of a southern blot analysis indicated that *V. nashicola* race 1 (isolate JS-115) had a single copy of the *Vnpgr1*. Restriction fragment length polymorphism (RFLP) analysis of the polymerase chain reaction (PCR) products of the endoPG gene digested with *Hinc*II, *Bsp*EI, and *Bsr*GI was performed; thereafter, agarose gel electrophoresis yielded race-specific RFLP patterns.

Discipline: Plant disease

Additional key words: pectinase, cell wall-degrading enzyme, pear scab

Introduction

Scab is a serious disease affecting Asian pears such as the Japanese pear (*Pyrus pyrifolia* var. *culta*) and Chinese pear (*P. ussuriensis*). The ascomycete *Venturia nashicola* Tanaka et Yamamoto, the fungus that causes scab in Asian pears, is pathogenic to Japanese and Chinese pears but not the European pear[44, 51]. Some pear species and cultivars (cvs.), including the non-commercial Japanese pear cv. “Kinchaku,” are known to be resistant to *V. nashicola*[16] and have been assessed for potential use in breeding programs.

Ishii et al.[14, 18] analyzed the pathological characteristics of the *V. nashicola* and classified strains of this fungus into 4 races according to their specificities: race 1, pathogenic to the most popular but highly scab-susceptible Japanese pear cv. “Kousui” but not to the Asian pear strain “Mamenashi 12”; race 2, pathogenic to Mamenashi 12 but not on Kousui; race 3, pathogenic to both Mamenashi 12 and Kousui; and race 4, pathogenic to the Chinese pear cv. “Jingbaili” but not to either Kousui and Mamenashi 12. *Venturia pirina* causes a scab disease in European pears such as cv. “Flemish Beauty”[16, 41, 44], but not in Japanese or Chinese pears[19].

V. nashicola and *V. pirina* can be distinguished through morphological characterization of the size of conidia and ascospores[19, 44] and the internal transcribed spacer (ITS) region of the rDNA of these 2 species[2]. Amplification of a 391-bp DNA fragment within the ITS region by polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) analysis of the PCR products can enable *V. nashicola* to be dis-

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tinguished from other related fungi, including *V. pirina*[26]; however, these techniques do not identify each race of the *V. nashicola*, and pathogenicity tests are required[17] to distinguish between the races, which are time consuming and laborious.

V. nashicola infects the leaf tissues of pear by direct penetration through the intact plant surface without requiring natural openings[51], and grows on the epidermal pectin layers of the middle lamella[22, 32], without causing maceration of plant tissues. Pectic polysaccharides are the principal components of the cell wall of dicotyledonous plants, and pectin is present in high concentrations in the primary cell walls and middle lamella[2, 3, 7, 34]. The functions of pectinases produced by fungal plant pathogens for tissue maceration, penetration, nutrient acquisition, and colonization in plant tissues have been studied[1, 5, 6, 9, 24, 25, 29, 37, 50]. The importance of polygaracturonase (PG; EC. 3. 2. 1. 15) for pathogenicity has been shown in some fungi, such as *Aspergillus flavus*[42], Botrytis cinerea[45], and *Alternaria citri*[21], using targeted gene disruption. The disruption of the endoPG gene reduced the ability of these fungi to cause symptoms in host plants[21, 42, 45]. The disruption method was also used to study the role played by endo- and exoPGs in the pathogenicity of several other plant pathogenic fungi such as *A. alternata*[21], *Cochliobolus carbonum* race 1[39, 40], *Cryphonectria parasitica*[11], and *Fusarium oxysporum*[33]; however, the mutation of the endoPG genes of these species did not change their pathogenicity.

A study examining the pectin-degrading enzymes for obligate or biotrophic pathogens such as bean rust fungus *Uromyces viciae-fabae*[8], lettuce downy mildew pathogen *Bremia lactucae*[48], wheat powdery mildew fungus *Blumeria graminis* f. sp. *tritici*[43], and apple scab fungus *Venturia inaequalis*[47], has also been initiated. An exoPG from mycelia of the *V. pirina*[20] and 3 endoPGs from mycelia of the *V. nashicola* races 1, 2 and 3 were also previously purified[20]. The PGs of *Venturia* species bind non-covalently to the mycelial walls and cause only localized degradation of the host wall, indicating the biotrophic characteristics of these fungi[20]. Ultrastructural studies of infection behavior have also identified that hyphae of these *Venturia* species were localized in the pectin layers and did not damage other parts of the host cells[22, 23]. Despite these interesting findings, the molecular study of *Venturia* has stalled due to technical limitations. For example, no transformation system has been established, and the genes encoding these enzymes have not been isolated from any *Venturia* species, including *V. nashicola*, *V. pirina* and *V. inaequalis*. However, the identification of particular genes is often useful and can provide tools for the molecular identification of distinct species. Therefore, we cloned and characterized endoPG genes from various races of the *V. nashicola* and *V. pirina*, designated as *Vnpg* and *Vppg*, and developed a potential method for PCR RFLP analysis, using genes to distinguish between *V. nashicola* races.

Materials and methods

1. Fungal culture

We used laboratory stock isolates of race 1 (JS-115, Yasato 2-1-1), race 2 (Mamenashi12A Nos. 1-1 and 1-3), and race 3 (Mamenashi12B Nos. 1-1 and 1-2) of the *V. nashicola*[15, 18], and *V. pirina* (Akita FB-1, race unknown)[15] that were obtained from Japanese and European pear orchards in Oita (JS-115), Ibaraki (Yasato 2-1-1), Tottori (races 2 and 3), and Akita (*V. pirina*) prefectures in Japan. The isolate Jingbaili 4 (race 4) of the *V. nashicola*[14] was isolated from a Chinese pear orchard in Liaoning, China. All the isolates were stored at 5°C on potato dextrose agar (PDA, Difco, Detroit, MI, USA) slants.

2. DNA isolation

All isolates of the *V. nashicola* and *V. pirina* were grown at 20°C on PDA plates for 45 days. Plugs of mycelium from a 45-day-old culture were placed at 20°C in potato dextrose broth (PDB, Difco) for 1 month. The mycelial mats grown in PDB were washed with distilled water 3 times and stored at −20°C until use. The genomic DNA of these isolates was extracted from the frozen mycelial mats by the method described by Le Cam et al.[26] with some modification. The purified DNA was dissolved in 50 µl of TE buffer (10 mM Tris-HCl at pH 8.0, 0.1 mM EDTA) and stored at −20°C.

3. Isolation of the *V. nashicola* gene encoding endoPG

A pair of PCR primers of VNPG F-AY (5'-AAGA MIAAGCCMAAGTTCCT-3') (forward) and VNPG R2-AY (5'-CCRATGGAIAGRCCGTICC-3') (reverse), designed from the conserved region of deduced amino acid sequences of endoPGs from *A. citri*, *Penicillium olsonii*, *Colletotrichum lindemuthianum*, *Cryphonectria parasitica*, *Fusarium oxysporum*, and *Sclerotinia sclerotiorum*, was used for the PCR amplification of the partial region of the *V. nashicola* gene encoding endoPG. PCR was performed in a Gene Amp PCR System 9700 (Applied Biosystem, Foster City, CA, USA) with Blend Taq DNA polymerase (0.63 units; Toyobo, Osaka, Japan) for 1 cycle of 3 min cycle at 94°C; 30 cycles of 30 seconds each at 94°C, 1 min at 50°C, and 30 seconds at 72°C; and 1 cycle of 3 min at 72°C with 1 µM

primer (each), 2 mM deoxynucleoside triphosphate (dNTP; each) mixture, 100 ng of genomic DNA from *V. nashicola* race 1 (JS-115, Yasato 2-1-1), race 2 (Mamenashi12A Nos. 1-1 and 1-3), race 3 (Mamenashi12B Nos. 1-1 and 1-2) of the *V. nashicola*[15, 18], race 4 (Jingbaili 4), or *V. pirina* (Akita FB-1, race unknown)[15], and the reaction buffer supplied with the Blend Taq DNA polymerase (Toyobo).

Total RNA was isolated from frozen mycelial mats, according to the method described by Masunaka et al.[28]. Rapid amplification of cDNA ends (RACE) was used to obtain the full-length cDNAs using a GeneRacer RACE-Ready cDNA kit (Invitrogen, Carlsbad, CA, USA). Total RNA (6 μg) was reverse transcribed with an oligo (dT) primer, and second-strand cDNA synthesis and ligation of adaptors were performed with the GeneRacer kit. Thereafter 5'-RACE PCR was conducted using *V. nashicola* JS-115 cDNA, the GeneRacer 5' primer (5'- CGAC TGGAGCACGAGGACACTGA-3') (forward), and JS-115PGR2 (5'- ATGGCGAGGCAATCGTCCTGGTTTT-3') (reverse). Furthermore, 3'-RACE PCR was conducted using *V. nashicola* JS-115 cDNA, JS-115PGF1 (5' -GCT TCAGCGTAAACGGAGCCGACAA-3') (forward), the GeneRacer 3' primer (5'-GCTGTCAACGATACGCTAC GTAACG-3') (reverse). Subsequently, 3'-nested- RACE PCR was conducted using 10-fold diluted 3'-RACE PCR product, JS-115PGF2 (5'-CAGCGTAAACGGAGC CGACAACCTT-3') (forward) and the GeneRacer 3' Nested Primer (5'-CGCTACGTAACGGCATGACAGTG-3') (reverse). RACE PCR was performed to amplify the internal region under the conditions described above, except that the concentration of each of the primers was 1 μM, at 94°C for 2 mins for 1 cycle and 30 cycles of 1 min each at 94°C, 1 min at 68°C, and 2 mins at 72°C.

The products were subcloned into a pGEM-T Easy Vector (Promega, Madison, WI, USA). The sequences of the 5'- and 3'- RACE products were obtained from both strands by the dideoxy chain termination method[36] with the use of an ABI PRISM Dye Termination Cycle Sequencing Ready Reaction Kit (Applied Biosystems) and an automated fluorescent DNA sequencer (Model 310, or Model 3100; Applied Biosystems). Another set of PCR primers, VPGUF2 (5'- CTACAGTCAATCAATC ATTCCATTCACAC-3') (forward) and VPGUR2 (5'- AG GTATCTACATGAAAACCAATCCA-3') (reverse), designed from the upper end of the 5'-RACE product and the untranslated region downstream of the 3'-RACE product was used to amplify the entire region of the endoPG gene from both genomic DNA and cDNA of either race 1 (JS-115, Yasato 2-1-1), race 2 (Mamenashi 12A Nos. 1-1 and 1-3), race 3 (Mamenashi12B Nos. 1-1 and 1-2) of the *V. nashicola*[15, 18], race 4 (Jingbaili 4), or *V. pirina* (Akita FB-1, race unknown)[15]. PCR was performed under the conditions described above for the amplification of the internal region, except that it was 2 mins at 95°C, and 30 cycles of 2 mins each at 95°C, 1 min at 50°C, and 2 mins at 72°C.

DNA sequences were aligned using the CLUSTAL W program4[6], and sequence analysis was performed using the PROSITE program[13], PSORT program[30], SMART program[38], NEB cutter V2.0 (New England Biolabs, Beverly, MA, USA), and GENETYX-Windows (Software Development, Tokyo, Japan). Homology analyses were performed using BLAST and FASTA at the DNA Data Bank of Japan (DDBJ).

4. Restriction analysis of PCR-amplified products

To confirm the sequence variability within races, amplified endoPG gene products from *V. nashicola* isolates were treated with restriction endonuleases *Hinc*II, *Bsp*EI and *Bsr*GI (New England Biolabs) and reactions were performed according to the manufacturer's recommendations. Digestions were performed in a final volume of 20 μl with 5 μl of PCR products and 10 units of each enzyme. Restriction fragments were separated on a 1.2% agarose gel and stained with ethidium bromide with OneSTEP Ladder 100 (0.1-2kbp; Nippon Gene, Tokyo, Japan) as the molecular size standard.

5. Southern blot analysis

For Southern blot analysis, a PCR digoxigenin (DIG) probe was prepared from the *Vnpgr1* gene of the *V. nashicola* JS-115 (race 1) in a pTBlue-2 T-vector as a template for labeling, by using the PCR DIG Probe Synthesis Kit (Roche, Mannheim, Germany) with primers of VPGF4 (5'-ATGATGGAACACATTTTCTGC-3') (forward) and VPGR4 (5'-CTAGCAAGTGGCGCCAGA GC-3') (reverse) according to the manufacturer's instructions.

Genomic DNA was isolated from freeze-dried mycelia of the *V. nashicola* JS-115 using another method as described previously[27]. Genomic DNA was digested with 12 units of *Eco*RI (Takara, Shiga, Japan), 8 units of *Eco*RV (Nippon Gene), 20 units of *Hin*dIII (Nippon Gene), and 15 units of *Bam*HI (Nippon Gene), according to the instructions of each manufacturer. The digested DNA was separated on 1% agarose gels. The hybridization condition for Southern blot was described previously[21]. The probe-bound DNA was detected by reaction with anti-DIG antibody-alkaline phosphatase and CDP-Star Detection Reagent (Amersham, Tokyo, Japan) and by exposing the membranes to X-ray films (Konica, Tokyo, Japan).

Results

1. Isolation and characterization of the endoPG gene from *V. nashicola*

The PCR primers VNPG F-AY and VNPG R2-AY, designed from the conserved region of known fungal endoPG sequences[20], produced a 376-bp product from the genomic DNA of the *V. nashicola* race 1 (isolate JS-115), which was used as a template. The nucleotide sequence of the product showed 70, 62, 62, 62, 58 and 56% similarities to the sequences of the endoPG gene from *P. olsonii*[49], *C. lindemuthianum*[4], *C. parasitica*[10], *F. oxysporum*[12], *S. sclerotiorum*[35] and, *A. citri*[21], respectively. The nucleotide sequence of the product had 100% similarity to *V. nashicola* race 1 (Yasato 2-1-1). In addition, the nucleotide sequence of the product showed 99% similarity to *V. nashicola* race 2 (Mamenashi12A Nos. 1-1 and 1-3), race 3 (Mamenashi12B Nos. 1-1 and 1-2) of the *V. nashicola*[15, 18], race 4 (Jingbaili 4), and *V. pirina* (Akita FB-1, race unknown)[15], respectively. Therefore, to amplify the full-length of the endoPG genes of the *V. nashicola* and *V. pirina* by RACE, the total RNA of the *V. nashicola* race 1 (JS-115) grown in PDB was used. According to the 376-bp PCR product sequence, a 706-bp product was amplified by 5'-RACE with the GeneRacer 5'-Nested Primer and JS115PGR2, and an 898-bp product was also amplified using 3'-RACE with the JS115PGF2 and GeneRacer 3'-Nested Primer. Two additional gene-specific primers, VPGUF2 (forward) and VPGUR2 (reverse), each encompassing the 5'- and 3'- untranslated regions (UTR) of the gene, were designed from the sequence of the RACE products. PCR amplification with these primers and sequencing of the product confirmed the full-length sequence of the *V. nashicola* JS-115 cDNA, designated *Vnpgr1* (DNA Data Bank of Japan (DDBJ) accession number of AB430830; Fig. 1). This cDNA consists of 1,319 bp with a 1,116-bp open reading frame (ORF) flanked by a 57-bp 5'-UTR sequence and a 146-bp 3'-UTR sequence. The primers VPGUF2 and VPGUR2 yielded a 1,416-bp product from genomic DNA, and comparison of the 2 sequences from cDNA and genomic DNA identified 48- and 49-bp introns at nucleotide positions of 655-703, and 1,025-1,074 in the genomic sequence, respectively (Fig. 1). Except for the intron region, the PCR product sequences using these primers were identical in terms of both total RNA and genomic DNA.

The *Vnpgr1* gene of the *V. nashicola* JS-115 (race 1) consisted of a predicted protein product of 372 amino acids (Fig. 1) with an estimated molecular mass of 37.5 kDa and a pI of 6.56. Isshiki et al.[20] estimated the mo-

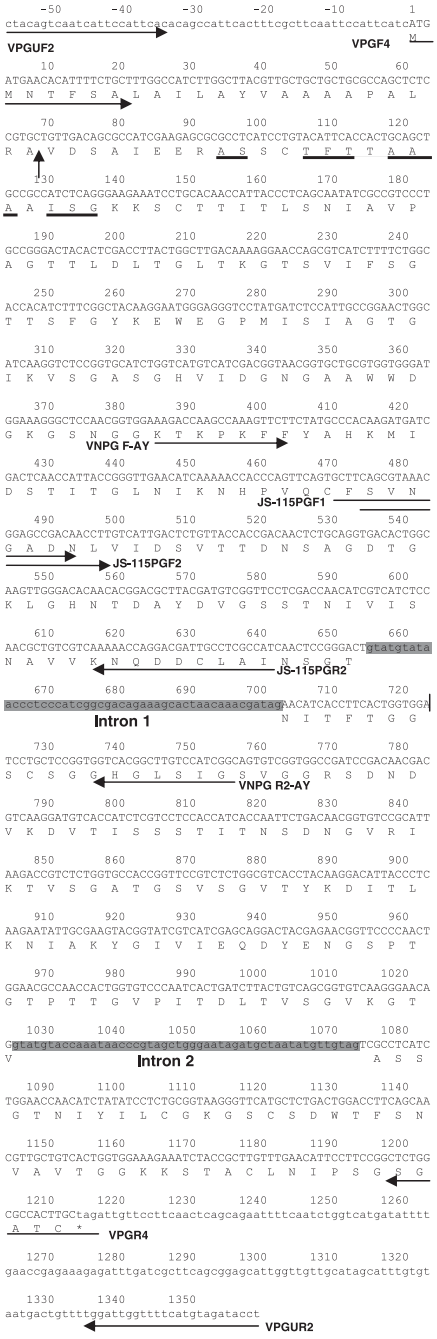

Fig. 1. Nucleotide and deduced amino acid sequence of the *Vnpgr1* gene from *Venturia nashicola* race 1

Arrows indicate the names, positions of annealing sites and extension directions of the primers used in this study. The N-terminal amino acid sequence of endpolygalacturonase (endoPG; ASxxTFTxAAAxISG), as previously identified by protein sequencing[20], is underlined. Gray boxes indicate intron regions. The vertical arrow between amino acids 23 and 24 indicates the predicted digestion site for a signal peptide. The vertical bar indicates a *Bam*HI recognition site as shown in Figure 3. The DNA Data Bank of Japan (DDBJ) accession number for Vnpgr1 of the *V. nashicola* race 1 (isolate JS-115) is AB 430830.

A)

Organism	DDBJ Accesion No.	Reference	Amino-acid sequence similarity (%)
Penicillium olsonii	**AJ243521**	**49**	**67**
Colletotrichum lindemthianum	**X89370**	**4**	**66**
Cryphonecria parastica	**U49710**	**10**	**68**
Fusarium oxyporum	**AB256848**	**12**	**65**
Scleotinia sclerotiorum	**L12023**	**35**	**63**
Alternaria citri	**AB047543**	**21**	**55**

B)

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                                                                      60
JS-115                MMNTFSALAILAYVAAAAPALRAVDSAIEERASSCTFTTAAAAISGKKSCTTITLSNIAV
Mamenashi 12A No. 1-3 MMNTFSALAILAYVAAAAPALRAVDSAIEERASSCTFTTAAAAISGKKSCTTITLSNIAV
Mamenashi 12B No. 1-1 MMNTFSALAILAYVAAAAPALRAVDSAIEERASSCTFTTAAAAISGKKSCTTITLSNIAV
Jingbaili 4           MMNTFSALAILAYVAAAAPALRAVDSAIEERASSCTFTTAAAAISGKKSCTTITLSNIAV
Akita FB-1            MMNTFSAFAILAYVAAGAPAFRAVDSAKEERASSCTFTTAAAAISGKKSCTTITFSNIAV
                      *******:********.***:****** ************************:*****
                                                                     120
JS-115                PAGTTLDLTGLTKGTSVIFSGTTSFGYKEWEGPMISIAGTGIKVSGASGHVIDGNGAAWW
Mamenashi 12A No. 1-3 PAGTTLDLTGLTKGTSVIFSGTTSFGYKEWEGPMISIAGTGIKVSGASGHVIDGNGAAWW
Mamenashi 12B No. 1-1 PAGTTLDLTGLTKGTSVIFSGTTSFGYKEWEGPMISIAGTGIKVSGASGHVIDGNGAAWW
Jingbaili 4           PAGTTLDLTGLTKGTSVIFSGTTSFGYKEWEGPMISIAGTGIKVSGASGHVIDGNGAAWW
Akita FB-1            PAGATLDLTGLTKGTSVIFSGTTSFGYKEWEGPMISIAGTGIKVSGASGHVIDGNGAKWW
                      ***:************************************************** **
                                                                     180
JS-115                DGKGSNGGKTKPKFFYAHKMIDSTITGLNIKNHPVQCFSVNGADNLVIDSVTTDNSAGDT
Mamenashi 12A No. 1-3 DGKGSNGGKTKPKFFYAHKMIDSTITGLNIKNHPVQCFSVNGADNLVIDSVTTDNSAGDT
Mamenashi 12B No. 1-1 DGKGSNGGKTKPKFFYAHKMIDSTITGLNIKNHPVQCFSVNGADNLVIDSVTTDNSAGDT
Jingbaili 4           DGKGSNGGKTKPKFFYAHKMIDSTITGLNIKNHPVQCFSVNGANNLVIDSVTTDNSAGDT
Akita FB-1            DGKGSNGGKTKPKFFYAHKMIDSTITGLNIKNHPVQCFSVNGADNLVIDSVTTDNSAGDT
                      ******************************************:****************
                                                                     240
JS-115                GKLGHNTDAYDVGSSTNIVISNAVVKNQDDCLAINSGTNITFTGGSCSGGHGLSIGSVGG
Mamenashi 12A No. 1-3 DKLGHNTDAYDVGSSTNIVISNAVVKNQDDCLAINSGTNITFTGGSCSGGHGLSIGSVGG
Mamenashi 12B No. 1-1 GKLGHNTDAYDVGSSTNIVISNAVVKNQDDCLAINSGTNITFTGGSCSGGHGLSIGSVGG
Jingbaili 4           GKLGHNTDAYDVGSSTNIVISNAVVKNQDDCLAINSGTNITFTGGSCSGGHGLSIGSVGG
Akita FB-1            GKLGHNTDAYDVGSSSNVVISNAVVKNQDDCLAINSGTNITFTGGSCSGGHGLSIGSVGG
                      .**************:*:****************************************
                                                                     300
JS-115                RSDNDVKDVTISSSTITNSDNGVRIKTVSGATGSVSGVTYKDITLKNIAKYGIVIEQDYE
Mamenashi 12A No. 1-3 RSDNDVKDVTISSSTITNSDNGVRIKTVSGATGSVSGVTYKDITLKNIAKYGIVIEQDYE
Mamenashi 12B No. 1-1 RSDNDVKDVTISSSTITNSDNGVRIKTVSGATGSVSGVTYKDITLKNIAKYGIVIEQDYE
Jingbaili 4           RSDNDVKDVTISSSTITNSDNGVRIKTVSGATGSVSGVTYKDITLKNIAKYGIVIEQDYE
Akita FB-1            RSDNVVKDITISSSTITNSDNGVRIKTISGATGSVSGVTYKDITLKNIAKYGIVIEQDYE
                      **** ***:******************:********************************
                                                                     360
JS-115                NGSPTGTPTTGVPITDLTVSGVKGTVASSGTNIYILCGKGSCSDWTFSNVAVTGGKKSTA
Mamenashi 12A No. 1-3 NGSPTGTPTTGVPITDLTVSGVKGTVASSGTNIYILCGKGSCSDWTFSNVAVTGGKKSTA
Mamenashi 12B No. 1-1 NGSPTGTPTTGVPITDLTVSGVKGTVASSGTNIYILCGKGSCSDWTFSNVAVTGGKKSTA
Jingbaili 4           NGSPTGTPTTGVPITDLTVSGVKGTVASSGTNIYILCGKGSCSDWTFSNVAVTGGKKSTA
Akita FB-1            NGSPTGKPTTGVPITGLTVSGVKGTVASSGTNIYLLCGKGSCSGWKFSNVAVTGGKKSTA
                      ******.********.******************:********.*.**************
                            372
JS-115                CLNIPSGSGATC
Mamenashi 12A No. 1-3 CLNIPSGSGATC
Mamenashi 12B No. 1-1 CLNIPSGSGATC
Jingbaili 4           CLNIPSGSGATC
Akita FB-1            CLNIPSGSGATC
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Fig. 2. Comparison between the sequence of fungal endopolygalacturonases (endoPGs) and the deduced amino acid sequences of *Vnpg* from *Venturia nashicola* and *Vppg* from *V. pirina*

(A) Comparison of the deduced amino acid sequences of *Vnpgr1* from *V. nashicola* JS-115 (race 1) and those of other fungal polygalacturonases. EndoPG sequences from other phytopathogenic fungi are aligned by CLUSTAL W[46]. (B) Alignment of deduced amino acid sequences of *Vnpg* from *V. nashicola* and *Vppg* from *V. pirina*. EndoPG sequences are aligned by CLUSTAL W[46], and identical residues are indicated by asterisks. The vertical arrow between amino acids 23 and 24 indicates the predicted digestion site for a signal peptide. The confirmed N-terminal amino acid sequences of endoPGs from mycelia of the *V. nashicola* JS-115 (race 1), Mamenashi12A No. 1-3 (race 2), and Mamenashi12B No. 1-1 (race 3)[20] are underlined. The gray box indicates a putative polygalacturonase active site[13].

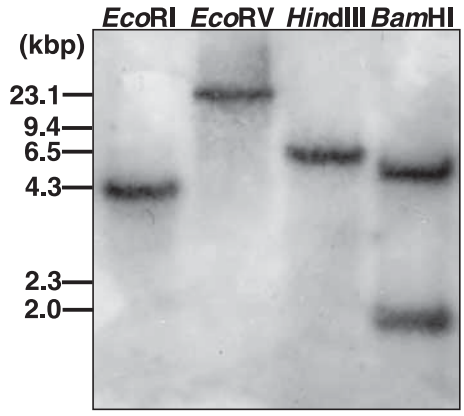

Fig. 3. Genomic Southern blot analysis of *Vnpgr1* of *Venturia nashicola* race 1

Total DNA of the isolate was digested with the restriction enzymes *Eco*RI, *Eco*RV, *Hin*dIII, and *Bam*HI and hybridized with a digoxigenin-labeled probe prepared from the polymerase chain reaction (PCR) product of *Vnpgr1*. The numbers on the left indicate the positions of the DNA size markers in kilobases.

lecular weight of the endoPGs from *V. nashicola* to be 42 kDa using sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Meanwhile, we estimated the molecular weight of endoPGs from *V. nashicola* to be 37.5 kDa using full-length deduced amino acid sequences. Both methods contained some errors due to the sugar chain. This region also has a potential N-terminal signal peptide by the PSORT program[30] with a cleavage site between 23 and 24 (Fig. 1). CLUSTAL W analysis revealed the deduced amino acid sequence of the *V. nashicola* JS-115 (race 1) endoPG to be 67, 66, 68, 65, 63 and 55% identical to the sequence of endoPG from *P. olsonii*[49], *C. lindemuthianum*[4], *C. parasitica*[10], *F. oxysporum*[12], *S. sclerotiorum*[35]and, *A. citri*[21], respectively (Fig. 2).

The copy number of the *Vnpgr1* gene in the genome of the *V. nashicola* JS-115 (race 1) was examined by genomic Southern blotting (Fig. 3). Hybridization of a probe corresponding to the full length of the *Vnpgr1* gene by using genomic DNA of the *V. nashicola* JS-115 (race 1), digested with *Eco*RI, *Eco*RV, *Hin*dIII, or *Bam*HI, resulted in the appearance of a single band from the *Eco*RI, *Eco*RV and the *Hin*dIII-digested genomic DNA, and double bands from the *Bam*HI-digested genomic DNA (Fig. 3). The sequence of the cloned *Vnpgr1* gene had 1 recognition site for *Bam*HI (Fig. 1), but not for *Eco*RI, *Eco*RV, or *Hin*dIII. These results suggested that the *V. nashicola* race 1 (isolate JS-115) had a single copy of the *Vnpgr1* gene.

The primer set of VPGUF2 and VPGUR2, also yielded 1,416 bp PCR products designated with DDBJ accession nos. of AB430831, AB430863, AB430864 and AB430865 when genomic DNA of the *V. nashicola* race 2 (isolate Mamenashi 12A No. 1-3), race 3 (Mamenashi 12B No. 1-1), race 4 (Jingbaili 4) and an isolate of European pear scab pathogen of the *V. pirina* (Akita FB-1, race unknown) were used as the templates, respectively. The PCR product sequence amplified using the same primer set determined that all genes of *Vnpgr2* from race 2 (Mamenashi 12A No. 1-3), *Vnpgr3* from race 3 (Mamenashi 12B No. 1-1), *Vnpgr4* from race 4 (Jingbaili 4) and *Vppg* from *V. pirina* (Akita FB-1, race unknown) have 2 introns in the same positions as *Vnpgr 1* from race 1. All identified genes, i.e. *Vnpgr2*, *Vnpgr3*, and *Vnpgr4*, consist of 1,116-bp ORFs and encoded predicted protein products of 372 amino acids (Fig. 4) with estimated molecular masses of 37.6, 37.5 and 37.5 kDa and pIs of 6.21, 6.56 and 7.02, respectively. *Vppg* of the *V. pirina* from isolate Akita FB-1 (race unknown) consisted of 1,116-bp ORF encoding a predicted protein product of 372 amino acids (Fig. 4) with an estimated molecular mass of 37.6 kDa and a pI of 8.49. These regions were predicted by the PSORT program[30] to also have a potential N-terminal signal peptide with a cleavage site of between 23 and 24 (Fig. 2B).

The nucleotide sequence of *Vnpgr1* obtained from the *V. nashicola* race 1 showed 99% identity to that of *Vnpgr2*, *Vnpgr3*, and *Vnpgr4* of the other race of the *V. nashicola*, and also 95% identity to the nucleotide sequence of *Vppg* from *V. pirina*. The amino acid sequences deduced from the nucleotide sequence of *Vnpgr 1* showed 99, 100, 99 and 95% identities to the sequences of *Vnpgr2*, *Vnpgr3*, *Vnpgr4* and *Vppg*, respectively (Fig. 2B).

Sequence analysis using the PROSITE and SMART programs[13, 38] identified a PG- active site at amino acid positions 224-237 (Fig. 2B), and the N-terminal amino acid sequence identified from the endoPG enzyme purified from mycelia of race 1[20] was identical to the deduced amino acid sequences of *Vnpgr1* identified after the cleavage site of putative signal peptide in this work (Figs. 1 and 2B). The 8 deduced amino acids (VDSAIE ER) after the cleavage site of putative signal peptide were not recognized at the N-terminal amino acid sequence previously identified in the endoPG enzyme purified from mycelia of races 1, 2, and 3[20]. In addition, the deduced amino acid sequences of *Vnpgr2* and *Vnpgr 3* from races 2 and 3 after the cleavage site of the putative signal peptide also showed identity to the N-terminal amino acid sequences of previously purified endoPGs from mycelia of races 2 and 3[20] with a mis-

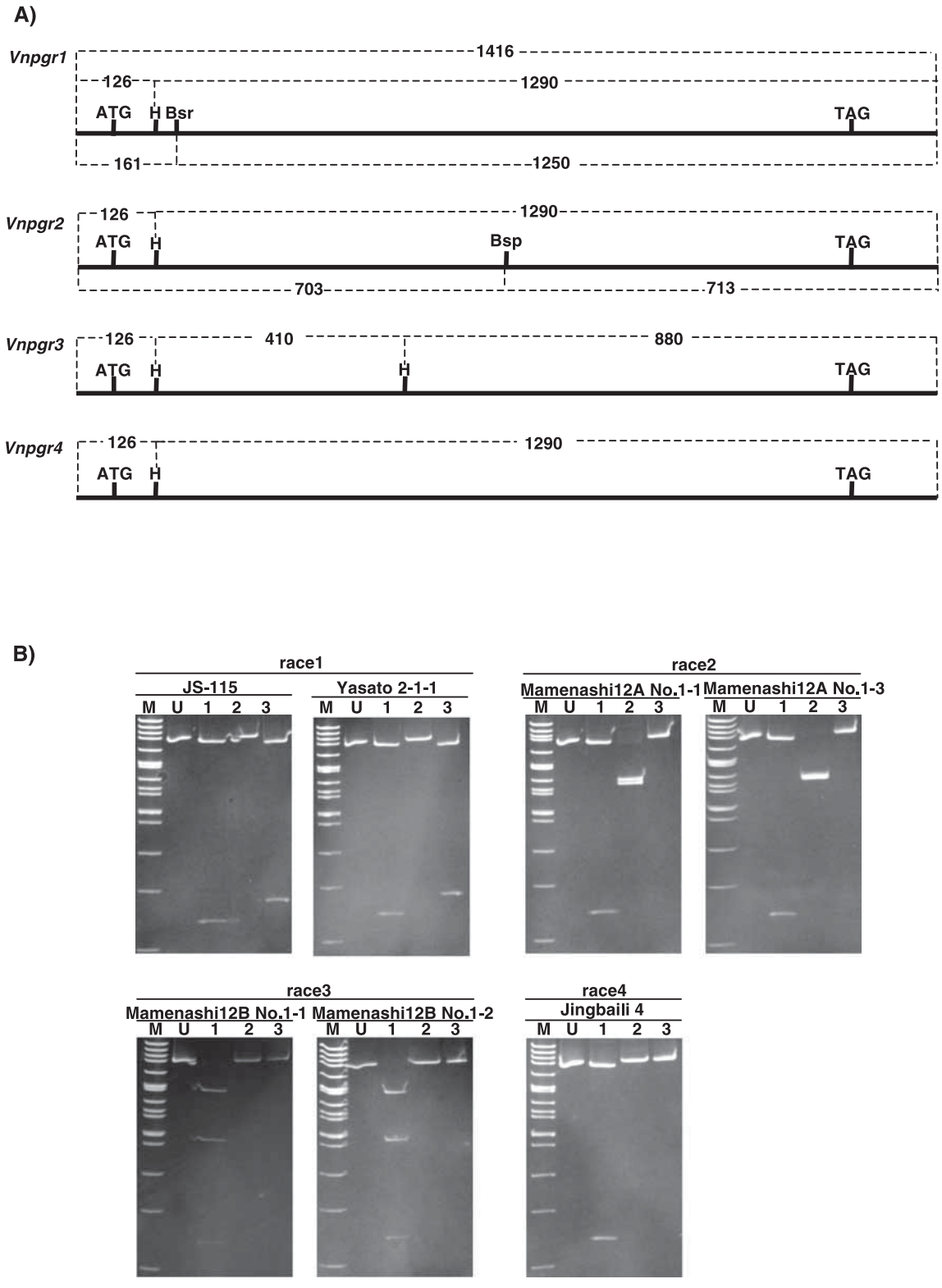

Fig. 4. Electrophoretic profiles of the PCR products amplified from 4 races of *Venturia nashicola* by using specific primers (VPGUF2 and VPGUR2) and digested with *Hinc*II, *Bsr*GI and *Bsp*EI.

(A) Comparison of the restriction maps of the PCR products amplified from 4 races of the *V. nashicola* by using specific primers (VPGUF2 and VPGUR2). The abbreviations H, Bsr, Bsp indicate *Hinc*II, *Bsr*GI and *Bsp*EI restriction sites, respectively, with numbers indicating the sizes of the restriction fragments. ATG, start codon; TAG, stop codon. (B) Restriction enzyme digests of the *Vnpg* gene amplified with specific primers (VPGUF2 and VPGUR2) from 4 races of the *V. nashicola* using lane 1: *Hinc*II, 2:*Bsp*EI, 3: *Bsr*GI. Lane M: OneSTEP Ladder 100 (0.1-2 kbp), U: undigested PCR products.

match of a few amino acids, which might be caused by an error in the N-terminal sequencing analysis owing to the insufficient purity or quantity of the enzymes[20] (Fig. 2B). These results suggested that *Vnpgr1*, *Vnpgr2* and *Vnpgr3* were identical to the endoPG enzyme purified from mycelia of races 1, 2 and 3, respectively.

2. PCR-RFLP analysis using endoPG genes from different races of the *V. nashicola*

Although sequences of endoPG genes from *Venturia* species are highly similar, sequence analysis showed that digestion by the restriction enzymes *Hinc*II, *Bsp*EI and *Bsr*GI generated different digestion fragment patterns among the isolates belonging to the 4 distinct races of the *V. nashicola* (Fig. 4A). A single recognition site for *Hinc*II was present in the sequences of the endoPG gene from *V. nashicola* race 1, 2 and 4 isolates, but 2 recognition sites were present in race 3 isolates (Fig. 4A). One recognition site for *Bsp*EI was present in the sequence of race 2 isolates (Fig. 4A) but not in those of other races. Moreover, the 1 *Bsr*GI recognition site was present in the endoPG gene sequence of the *V. nashicola* race 1 isolates (Fig. 4A). Digestion patterns of the PCR products from respective races, generated by the restriction enzymes, are consistent with sequence analysis predictions, and no polymorphisms were observed within the isolates of each race tested using these 3 restriction enzymes (Fig. 4B).

Discussion

In this study, we cloned the gene encoding endoPG of the *V. nashicola* races 1, 2, 3, 4, and *V. pirina*. The endoPG gene existed as a single copy in the genome of the *V. nashicola* race 1 (JS-115). The deduced amino acid sequences from the nucleotide sequence of *Vnpgr1*, *Vnpgr2* and *Vnpgr3* had the same N-terminal sequence as the endoPG enzymes purified from the mycelia of the *V. nashicola* races 1, 2, and 3, respectively[20]. We showed that amino acid sequences deduced from the nucleotide sequence of *Vnpgr1*, *Vnpgr2*, *Vnpgr3*, *Vnpgr4* and *Vppg* were highly identical, and shared a putative PG active site[13]. The pI of the endoPG isolated from *V. nashicola* (6.56) differed slightly from the pI of endoPGs from *C. parastica* (7.2)[10], but was approximately equal to that of endoPGs of *P. olsonii* (6.8)[49]. Meanwhile, the molecular weight of 37.5 kDa of the endoPG isolated from *V. nashicola* was approximately equal to that of endoPGs of *C. lindemuthianum* (38 kDa)[4], but differed slightly from the molecular weight of endoPGs from *C. parastica* (42 kDa)[10]. However, all the N-terminal amino acid sequences deduced from the nucleotide sequence of the *V. nashicola*, *V. pirina*, *C. lindemuthianum*, and *C. parastica* contained a consensus motif of ASxxxTFTxAAAxxxG.

Comparison of the nucleotide sequence showed changes at 14 nucleotide positions, which represented almost synonymous substitutions. The nucleotide positions used for PCR-RFLP in this study were located in this region. These base substitution mutations might not be associated with specific mutation in the 4 races. However, the role played by these genes in the race specificity or pathogenicity of *Venturia* species remains uncertain. Because no effective transformation system has yet been developed for *Venturia* species, a regular methodology for molecular approaches, such as targeting or silencing, is not suitable for the evaluation of endoPG in the pathogenesis of the disease caused by this fungus. However, the establishment of an *in situ* PCR and a real-time PCR method is progressing, with the goal of analyzing the expression patterns of these genes in the respective *Venturia* races during infection of different pear cultivars.

The PCR-RFLP method, using *Venturia* endoPG gene sequences employed in this study, may be an effective and rapid method for distinguishing between races, as compared to the pathogenicity tests. Le Cam et al.[26] also previously developed a PCR- based method using a combination of PCR and RFLP analysis with ITS regions of rDNA for identification of the *V. nashicola* and *V. pirina*[26]. The nuclear rDNA repeat unit is a useful area of the genome to identify polymorphisms because of the tandem repeat sequence, including conserved and variable regions and its high copy number[31]. The endoPG gene exists as a single copy in the genome of the *V. nashicola* JS-115 (race 1). Therefore, the ITS region and endoPG gene might differ in terms of the genetic stability of RFLPs. However, the use of additional methodologies using multiple target genes is always helpful to accurately identify pathogens, and we believe that the use of PCR- RFLP methods using the target regions of both the endoPG gene and ITS will be useful in distinguishing between the races of fungi that cause major Asian pear diseases, including scab caused by *V. nashicola*. The number of isolates tested in this study was insufficient to determine races, hence further research is need to answer this question.

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