DNA Sequence Dimorphisms in Populations of the Clubroot Pathogen *Plasmodiophora brassicae*

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Abstract

To develop genetic markers for differentiation between pathotypes of the clubroot pathogen *Plasmodiophora brassicae*, DNA polymorphisms of 85 *P. brassicae* genes were investigated by comparing the sequences of these genes from published expressed sequence tag libraries to their sequences in the two released whole genomes. A significant portion of the identified sequence differences across all polymorphic genes are between an isolate from New Zealand and the two whole-genome sequenced isolates. Four genes with a high density of polymorphisms were selected and their partial sequences were amplified by polymerase chain reaction

(PCR) from the old pathotypes 2, 3, 5, 6, and 8 (based on the Williams differential set) and the new virulent populations 3-like and 5-like. On the sequences of two of the four genes, the old pathotypes are all identical to the two whole-genome sequenced isolates and all of the new virulent populations are identical to the New Zealand isolate. Based on the dimorphism on the sequence of these two genes, an RNase H-dependent PCR protocol was developed. This protocol was demonstrated to be useful for virulent pathotype identification and may also be used to study the population dynamics of *P. brassicae* and the in planta interaction of different pathotypes.

Clubroot, caused by the protist *Plasmodiophora brassicae* Woronin, is an important threat to Canadian canola (*Brassica napus*) production (Hwang et al. 2012). In the Canadian Prairies, clubroot was first identified on canola in 2003, in a dozen fields near Edmonton, Alberta (Tewari et al. 2005). The disease has since then spread throughout central Alberta and has also been confirmed in canola fields in Saskatchewan and Manitoba (Strelkov and Hwang 2014).

Many strategies have been proposed for clubroot control (Peng et al. 2014) and, among those, breeding for resistance is believed to be most effective (Rahman et al. 2014). Several clubroot-resistant canola hybrids have been bred and introduced into the Canadian market (Strelkov and Hwang 2014). However, clubroot resistance can erode quickly in response to the selection pressure imposed by repeated cropping of a resistant cultivar (Strelkov et al. 2016). A province-wide survey for clubroot conducted in 2013 found six fields of resistant canola in Alberta in which disease severity was greater than expected for clubroot-resistant hosts (Strelkov et al. 2014). On the Williams differential hosts (Williams 1966), these field populations displayed the virulence profile of pathotype 5 but, unlike the original pathotype 5 populations, they were virulent on all registered resistant cultivars (Strelkov et al. 2016). It was apparent that the Williams set of differential hosts did not have sufficient resolution to discriminate pathotype 5 from this virulent pathotype. As a result, this new pathotype was temporarily named pathotype 5x. In 2016, significant clubroot symptoms were found on clubroot-resistant canola cultivars in more than 40 fields identified in a follow-up survey (Strelkov et al. 2017). Testing of the 2016 populations on the Williams differentials indicated that the retrieved isolates were not limited to the pathotype 5 virulence pattern but also included patterns characteristic of other pathotypes (Strelkov et al. 2016) (J. Feng, unpublished).

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With the detection of new virulent pathotypes not controlled by existing clubroot resistance in canola, the ability to rapidly differentiate pathotypes and identify new pathotypes has become extremely important for diagnosis of control failures and development of control strategies and breeding for effective resistance. To discover and develop genetic markers for pathotype differentiation, DNA polymorphisms on 85 *P. brassicae* genes were investigated by comparing the sequences of the 85 expressed sequence tags (EST) to their corresponding sequences in the two released whole genomes. Based on the sequences of two identified polymorphic genes, an RNase H-dependent polymerase chain reaction (rhPCR) protocol was developed. This protocol can be used to differentiate between two classes of *P. brassicae* pathotypes; namely, new virulent pathotypes from the old pathotypes.

Materials and Methods

***P. brassicae* populations.** Terminologies for population, single-spore isolate, and pathotype were adopted from Xue et al. (2008). In this study, pathotypes characterized before the breakdown of resistance were defined as “old pathotypes” and populations virulent on resistant cultivars were defined as “new virulent populations” or “new virulent pathotypes”. Seven populations of *P. brassicae* were provided by Dr. S. F. Hwang at the Crop Diversification Centre North (CDCN). These populations included five single-spore isolates (SACAN-ss3, SACAN-ss1, ORCA-ss4, AbotJE-ss1, and CDCN-ss3), which are classified as pathotypes 2, 3, 5, 6, and 8, respectively, on Williams differential hosts (Williams 1966; Xue et al. 2008), and two populations that could be classified as pathotype 3 or 5, respectively, on Williams differential hosts but are virulent to the resistant gene carried by the resistant canola cultivar 45H29. The latter two populations had been selected on 45H29 for many generations in greenhouses and were named 3-like and 5-like, respectively, in this study. In addition, four populations were represented by field galls (namely, G1, G2, G3, and G4), which are similar to the 5-like population but have not been repeatedly selected on 45H29 or against other resistance genes. To initiate the present study, all of the above 11 populations were regenerated on canola in the Alberta Plant Health Lab, CDCN with the 5 single-spore isolates on the susceptible cultivar Westar; G1, G2, G3, and G4 on 45H29; and 3-like and 5-like on both Westar and 45H29 (Table 1). Thus, in total, 13 populations from Alberta (Table 1) were prepared and utilized in this study.

General molecular techniques. Extraction of genomic DNA was performed using the DNeasy Plant Mini kit (Qiagen Canada,

Toronto, ON, Canada). Primers (Table 2) for PCR were synthesized by Integrated DNA Technologies (Coralville, IA). All PCR were conducted in Promega PCR master mix (Promega Corp., Madison, WI) using a ProFlex 3x32-Well PCR system (Fisher Scientific Canada, Ottawa, ON, Canada). Purification of PCR product was performed using the Wizard SV Gel and PCR Cleanup System (Promega Corp.). Electrophoresis was performed on 1% agarose gels with GelGreen (VWR Canada, Mississauga, ON, Canada) staining. DNA bands were visualized using a GelDoc EZ Imaging System (Bio-Rad Canada, Mississauga, ON, Canada). DNA sequencing was conducted by the Department of Biological Sciences, University of Alberta (Edmonton, AB, Canada). The chromatograms of sequencing files were viewed by the BioEdit software (Hall 1999).

Gene screening. Sequences of 85 *P. brassicae* genes, represented by EST, were retrieved from the National Center for Biotechnology Information (NCBI) database by searching their GenBank accession numbers, available in Bulman et al. (2006, 2007). All of these genes were derived from a New Zealand isolate, named CNZ in this study. The genomic sequences of these 85 genes in the two whole-genome sequenced *P. brassicae* isolates, e3 from Germany (unknown pathotype) (Fähling et al. 2003; Schwelm et al. 2015) and ZJ-1 from China (pathotype 1 on the Williams differential set) (Bi et al. 2016), were obtained by BlastN using these 85 genes as a batch query against the NCBI whole-genome shotgun contigs organism *P. brassicae* (Tax ID 37360), a database containing only the whole-genome sequences of e3 and ZJ-1 when this study was conducted (last verified 4 February 2018). For each of these 85 genes, the EST sequence in CNZ and genomic sequences in e3 and ZJ-1 were compared by alignment using Clustal Omega (<https://www.ebi.ac.uk/Tools/msa/clustalo>). PCR primers (Table 2) were designed for four genes that have a high density of nucleotide polymorphisms among the three isolates.

Table 1. *Plasmodiophora brassicae* populations from which the DNA sequences used in this study were derived

Group ^a	Populations	Description ^b	Origin
X	Pathotype 2, 3, 5, 6 and 8	SSI; galls on Westar	Alberta
	e3	SSI, WGS	Germany ^c
	ZJ-1	SSI, WGS	China ^d
Y	CNZ	SSI	New Zealand ^e
Z	3-like R	Galls on 45H29	Alberta
	3-like S	Galls on Westar	Alberta
	5-like R	Galls on 45H29	Alberta
	5-like S	Galls on Westar	Alberta
	G1, G2, G3, and G4	Galls on 45H29	Alberta

^a Grouped in the present study.

^b SSI = single-spore isolate and WGS = whole genome sequenced.

^c References: Fähling et al. 2003 and Schwelm et al. 2015.

^d Reference: Bi et al. 2016.

^e References: Bulman et al. 2006, 2007.

PCR amplification of gene fragments. Resting spores were prepared from galls of the 13 populations following the protocol described by Feng et al. (2012a). Genomic DNA was extracted from the prepared resting spores. Against each of these DNA samples, a fragment of each of the four selected genes was amplified by PCR using primers P-1, P-2, P-3, and P-4 (Table 2); with 35 cycles of denaturation at 94°C for 30 s (3 min for the initial denaturation), annealing at 47°C (primer pairs P-2 and P-3) or 50°C (primer pairs P-1 and P-4) for 45 s, and extension at 72°C for 1 min; and a final extension at 72°C for 5 min. All PCR products were purified and sequenced from both directions using the forward and reverse PCR primers.

rhPCR. For each of the four selected genes, the sequenced PCR products from the 13 *P. brassicae* populations were compared using multiple alignment. Based on the aligned sequences of two genes, two pairs of rhPCR primers, with one for one gene, were manually designed according to Dobosy et al. (2011). Duplex rhPCR was conducted using these two primer pairs on the DNA sample extracted from each of the 13 populations. Each 15-μl reaction contained 1.5 mU of RNase H2 (Integrated DNA Technologies), 0.25 μM each of the four primers, and 50 ng of template DNA. The rhPCR program consisted of 35 cycles of denaturation at 94°C for 30 s (3 min for the initial denaturation), annealing at 62°C for 45 s; and extension at 72°C for 1 min; and a final extension at 72°C for 5 min. After gel electrophoresis, the upper bands produced by G1, G2, G3, and G4 in the rhPCR products were sequenced with the primer 5'-GCTGGGTGCCGCTATACC-3' (Fig. 1). In addition, the potential use of rhPCR in quantification of the biomass of different *P. brassicae* pathotypes was evaluated by running the duplex rhPCR against DNA samples consisting of serial dilutions of DNA from pathotype 3 and the 3-like population. The ingredients (except template) of the rhPCR were same as described above. The rhPCR program was also the same as above, except that the cycle number was changed to 30.

Results

All 85 genes identified from CNZ are present in the whole-genome sequences of e3. In contrast, 83 are present in the whole-genome sequences of ZJ-1, with AM180201 and AM177649 absent. In total, 79 genes are single-copied in e3 and 72 are single-copied in both e3 and ZJ-1. Among these 72 genes, 56 carry polymorphisms on the coding sequences as defined by the length of the EST among the three isolates. Among these 56 genes, 42 have identical sequences in e3 and ZJ-1. From these 42 genes, AM180213, AM180230, AM411654, and AM411657 were selected for further studies. When these four genes in different Alberta *P. brassicae* populations were sequenced and the obtained sequences were compared with each other and with those in CNZ, e3, and ZJ-1, dimorphisms were found on the sequences of AM411654 and AM411657, in which pathotypes 2, 3, 5, 6, and 8, and isolates e3 and ZJ-1 (group X in Table 1) shared one sequence and all other tested populations (groups Y and Z in Table 1) shared another sequence. The dimorphisms of the two genes are illustrated in Figure 1. In addition, dimorphisms were also found in

Table 2. Polymerase chain reaction primers used in this study

Primer pair	Sequences of primers, forward (up) and reverse (down)	GenBank accession number	Amplicon (bp) ^a
P-1	AAGAAGACGTACTCCACGAAC TCCGGTGTAGAGTATGGGAC	AM180213 ...	214 ...
P-2	AGCTTGTCAGGCGATGATTG ACGCAYACTGTGTATCAGATG	AM180230 ...	298 ...
P-3	ATGTCAGATCGTCAACGGTAC CGAAAGAAGAAGTAGTCGCAG	AM411654 ...	490 ...
rhP-3	GCTGGGTGCCGCTATACCrGACCCC/3SpC3/ GTCGCAGGATGGGTCCCArCTCGCC/3SpC3/	AM411654 ...	337 ...
P-4	ACATCGCCTGTTTCACGGATTG TGAGGTACAAGAAGTCGGCATC	AM411657 ...	467 ...
rhP-4	AGCTGGTCCAGAGTGACrCCACGG/3SpC3/ AAGCGCCCAACCTCTTGrGAGTGG/3SpC3/	AM411657 ...	135 ...

^a Size of genomic DNA in isolate e3 embraced by the primer pair.

S1 ATGTCAGATCGTCAACGGTACCCTGAACATG**T**TCCGGTCTTGATAGTCGGCTGTCGGTGG
S2 ATGTCAGATCGTCAACGGTACCCTGAACATG**A**TCCGGTCTTGATAGTCGGCTGTCGGTGG
S1 ATAACGTCCAACCGGGAACGTTGGGCAACGGCATCATGGAGGACGACATCCACGCAATGC
S2 ATAACGTCCAACCGGGAACGTTGGGCAACGGCATCATGGAGGACGACATCCACGCAATGC
S1 TGGCCAGCATTGACGAAGC**GCTGGGTGCCGCTATACC****G**ACCCGGACGTCTCCGCCTCCTG
S2 TGGCCAGCATTGACGAAGC**GCTGGGTGCCGCTATACC****A**ACCCGGACGTCTCCGCCTCCTG
S1 CCGCGAGCTGGGCGAA**A**CCCGACCGTCCAGCAGTCACTCGGT**C**GCCGAACCAACT**G**CCAG
S2 CCGCGAGCTGGGCGAA**G**CCCGACCGTCCAGCAGTCACTCGGT**C**ACCGAACCAACT**C**CCAG
S1 TGTGCCATATGACAGACGACGATCTCGACCAGATG**C**TGCTAGATATCGATGCCGACCTGG
S2 TGTGCCATATGACAGACGACGATCTCGACCAGATG**T**TGCTAGATATCGATGCCGACCTGG
S1 ACACGGCCGC**A**C**C**CGACGCCCC**G**CTGTCTACACCTG**G**CAAGGGC**A**ACTCAGGGTTTCGGA
S2 ACACGGCCGC**T**C**T**CGACGCC**C**CACTGTCTACACCTG**T**CAAGGGC**G**ATTCAGGGTTTCGGA
S1 AGTGCACCGTCGTCATGCTAGGTCAGACACC**A**GCCGCATGCTCCAACCTACGATGTACCA
S2 AGTGCACCGTCGTCATGCTAGGTCAGACACC**G**GCCGCATGCTCCAACCTACGATGTACCA
S1 TGTGCGACCACG**T**CGTCGTGCGCATTGTCAAAC**G**CGAG**T**GGGACCCATCCTGCGACTACT
S2 TGTGCGACCACG**C**CGTCGTGCGCATTGTCAAAC**A**TGGGACCCATCCTGCGACTACT
S1 TCTTCTTTTCG
S2 TCTTCTTTTCG

S1 ACATCGCCTGTTTACGGATTGGTACCAGCGCGTGCACATCGGCAGGGCGAATGCAAACA
S3 ACATCGCCTGTTTACGGATTGGTACCAGCGCGTGCACATCGGCAGGGCGAATGCAAACA
S4 ACATCGCCTGTTTACGGATTGGTACCAGCGCGTGCACATCGGCAGGGCGAATGCAAACA
S1 AGTGGATTACAATCGG**G**GGATCATACCCGGCGCTCTGGCAGCATGGTCTGCGGTCCATC
S3 AGTGGATTACAATCGG**A**GGATCATACCCGGCGCTCTGGCAGCATG-----
S4 AGTGGATTACAATCGG**A**GGATCATACCCGGCGCTCTGGCAGCATGGTCTGCGGTCCATC
S1 GGCTCTCTGCCCAATGG**T**CAGACTCTGAATTCTGAATCGTCAGGTATCGCCTGAAGTA**C**CC
S3 -----GTATCGCCTGAAGTA**T**CC
S4 GGCTCTCTGCCCAATGG**A**CAGACTCTGAATTCTGAATCGTCAGGTATCGCCTGAAGTA**T**CC
S1 GCATCTGACAGCAGGCGC**A**CTGGCATCGAGCGCTGTGGTTGCCCCGTTTGACAGAGTTCCC
S3 GCATCTGACAGCAGGCGC**G**CTGGCATCGAGCGCTGTGGTTGCCCCGTTTGACAGAGTTCCC
S4 GCATCTGACAGCAGGCGC**G**CTGGCATCGAGCGCTGTGGTTGCCCCGTTTGACAGAGTTCCC
S1 TGAATTCGACGA**G**CAAGTGGCAT**C**ATCAGCTGGTCCAG**C**GTGCAC**A**CACGCGCTGCAAGA
S3 TGAATTCGACGA**A**CAAGTGGCAT**T**ATCAGCTGGTCCAG**A**GTGCAC**C**CACGCGCTGCAAGA
S4 TGAATTCGACGA**A**CAAGTGGCAT**T**ATCAGCTGGTCCAG**A**GTGCAC**C**CACGCGCTGCAAGA
S1 CGTAATGTGTACA**A**ATG**G**CCGT**G**TGGTGTGATGTGAATTTGACGCCAGTGCTCGAAGAT
S3 C-----AT
S4 CGTAATGTGTACA**G**ATG**T**CCGT**C**TGGTGTGATGTGAATTTGACGCCAGTGCTCGAAGAT
S1 CACTGCAATGGTGGAAAGGTGCACT**T**CAAGAGGGTGGGCGCTTGGCTGACGAAATGAAGGC
S3 CACTGCAATGGTGGAAAGGTGCACT**C**CAAGAGGGTGGGCGCTTGGCTGACGAAATGAAGGC
S4 CACTGCAATGGTGGAAAGGT**G**CACT**C**CAAGAGGGTGGGCGCTTGGCTGACGAAATGAAGGC
S1 CCTGTTTTTCGTGCTCGCAATTGTCGGATGCCGACTTCTTGTACCTCA
S3 CCTGTTTTTCGTGCTCGCAATTGTCGGATGCCGACTTCTTGTACCTCA
S4 CCTGTTTTTCGTGCTCGCAATTGTCGGATGCCGACTTCTTGTACCTCA

Fig. 1. DNA sequence dimorphism of GenBank accession numbers AM411654 (up) and AM411657 (down) among *Plasmodiophora brassicae* populations. S₁, S₂, S₃, and S₄, populations correspondent to the groups X (pathotypes 2, 3, 5, 6, and 8, and isolates e3 and ZJ-1), Y (CNZ), and Z (3-like R, 3-like S, 5-like R, 5-like S, G1, G2, G3, and G4), with S₁ = X, S₂ = Y + Z, S₃ = Y, and S₄ = Z. Dimorphic nucleotides are shown in bold. The solid arrow lines indicate the RNase H-dependent polymerase chain reaction (rhPCR) primers and the dotted arrow line indicates the sequencing primer for the upper bands produced by G1, G2, G3, and G4. From a DNA sample derived from a mixture of S₁ and S₂ populations, the rhPCR primer pair for AM411654 amplified a fragment of 337 bp from the S₁ populations only and the primer pair for AM411657 amplified a fragment of 135 bp from the S₂ (i.e., S₃ and S₄) populations only. A 29-bp fragment is boxed in the upper panel.

AM180213 and AM180230, in which pathotypes 2, 3, 5, 6, and 8, and CNZ shared one sequence and all other tested populations shared another sequence (data not shown). Among the four dimorphic genes, AM411657 was the only one that carried introns on its genomic sequences defined by the primer pair P-4 (Table 2). Thus, the genomic sequence of AM411657 in populations belonging to groups Y and Z (Table 1) represented a new sequence and, therefore, was submitted to GenBank with accession number MH084665.

Two rhPCR primer pairs specific to AM411654 and AM411657, respectively, were designed (Fig. 1; Table 2). The primer pair rhP-3 amplified a band at 337 bp from populations belonging to group X but not groups Y and Z, whereas the primer pair rhP-4 amplified a band at 135 bp from populations belonging to groups Y and Z but not group X. Duplex rhPCR using the two primer pairs amplified the 337-bp band from pathotypes 2, 3, 5, 6, and 8, and the 135-bp band from the two 3-like and two 5-like populations (Fig. 2A). The four field populations (G1 to G4) produced both bands, suggesting that they were mixtures of different isolates, which was not consistent with the sequencing results (Fig. 2B). The 337-bp bands from these four populations were purified and sequenced. The resultant sequences are identical to each other and to the corresponding sequence in the populations belonging to group X.

When mixtures of pathotype 3 and 3-like DNA were tested with the duplex rhPCR, both the 337- and 135-bp bands were obtained (Fig. 3). The 337-bp band became stronger when the proportion of pathotype 3 DNA in the DNA sample increased and, similarly, the 135-bp band became stronger with the increased proportion of the 3-like DNA (Fig. 3), indicating the potential usage of this duplex rhPCR technique in evaluating the purity of *P. brassicae* populations.

Discussion

Sequence polymorphisms were found between a high proportion of genes among the *P. brassicae* isolates e3, ZJ-1, and CNZ. Interestingly, the sequences of most of these genes in e3 and ZJ-1 are identical, suggesting that the polymorphisms are likely dimorphisms. When the four selected genes in Alberta *P. brassicae* populations were sequenced, the dimorphic nature of the polymorphisms was confirmed. For all four genes, the new virulent populations share one sequence and the old pathotypes share the other sequence. These dimorphisms suggest that the new virulent pathotypes and the old pathotypes may belong to different lineages. The extensiveness of the polymorphisms across the tested genes also suggests that the new virulent pathotypes might have gone through a lengthy

coevolution with the hosts, and their emergence in Alberta was not simply because of the loss of an avirulence gene or mutations on a few genes.

The rhPCR technique relies on primers specific to the DNA sequences carrying the single nucleotide polymorphism between individuals (Dobosy et al. 2011). In this study, the duplex rhPCR was able to differentiate new virulent pathotypes from the old avirulent pathotypes. However, this was only limited to the populations accessible by the Alberta Plant Health Lab. We did not test more field populations in addition to G1, G2, G3, and G4, partially due to our speculation that many field populations with a history of canola production were likely composed of both old and new pathotypes. In these circumstances, it would be difficult to use molecular markers for pathotype differentiation on field galls. Thus, based on the data obtained from the present study, we would not recommend using the rhPCR protocol for molecular pathotyping of field populations before repeated selection on resistant hosts or the achievement of single-spore isolation.

Duplex rhPCR produced two bands from the populations G1, G2, G3, and G4. This is because each of the four populations was likely a mixture of new virulent pathotypes with a small proportion of old pathotypes. In such a mixture, the majority could overwhelm the minority in a sequencing reaction but could not do so in an rhPCR. For example, duplex rhPCR detected pathotype 3 from a template consisting of 1% pathotype 3 DNA and 99% 3-like DNA. From such a mixture, sequencing may not be able to make the same detection. Therefore, the duplex rhPCR developed from the present study could be used to verify the purity of new virulent populations, especially if the population is represented by the original galls collected from the field.

The importance of determining the purity of populations was also emphasized by other studies. Jones (1980, cited after Dixon 2009) reported that more than one pathotype occurred within a spore suspension prepared from a single gall. Isolates of both old and new virulent pathotypes were obtained by single-spore isolation from single galls of both susceptible and resistant canola cultivars (S. E. Strelkov, unpublished). The coexistence of old and new virulent pathotypes, especially the persistence of old pathotypes in galls of resistant plants caused primarily by the new virulent pathotypes, suggested that there may be collaboration between the two pathotypes. Previous microscopic studies observed primary infection (root hair infection) on resistant (Diederichsen et al. 2009) and nonhost plants (Feng et al. 2012b). This infection might be caused by the virulent individuals coexisting with the avirulent population. Another possibility is that, as proposed by Feng et al. (2013), the primary infection of *P. brassicae* is indeed unspecific and the pathogenicity of a *P. brassicae* population is only determined in the postprimary infection stage. Studies on the interaction between a virulent pathotype and an avirulent pathotype during the coinfection process would provide important information on our understanding of clubroot pathogenesis and *P. brassicae* population dynamics. However, such studies have been difficult, due to the absence of a reliable method for pathotype purification and differentiation. The rhPCR protocols developed in this study, for the first time, provide a tool for the differentiation between the tested pathotypes. More importantly, the same rhPCR principle can be used in quantitative PCR (qPCR). Nucleotide dimorphisms present on all four genes investigated in this study (and likely on other

Fig. 2. Differentiation of *Plasmodiophora brassicae* populations by analyzing the dimorphic sequences of two selected genes. **A**, RNase H-dependent polymerase chain reaction (PCR) assay. Lanes 2, 3, 5, 6, and 8 = single-spore isolates (SSI). Lanes G1, G2, G3, and G4 = four field populations that are virulent on the resistant canola 45H29. Lane M = Promega 100-bp DNA ladder. Lanes 3xS and 3xR = the 3-like populations on the susceptible canola Westar and 45H29, respectively, and lanes 5xS and 5xR = the 5-like populations on Westar and 45H29, respectively. **B**, Sequencing chromatogram of a 29-bp fragment from population G2, which illustrates that the RNase H-dependent PCR could not identify the contamination of new virulent populations by old populations. The PCR product was amplified by primer pair P-3 and sequenced using the forward primer of P-3. The resultant sequence after sequencing is listed on the top, which is identical to the sequences of the corresponding fragments in isolate CNZ and the new virulent isolates. The sequence of the corresponding fragments in isolates e3 and ZJ-1 and the old pathotypes is listed at the bottom, with the dimorphic nucleotides underlined.

Fig. 3. RNase H-dependent polymerase chain reaction assay against DNA mixtures. Row 3 = DNA from the isolate of pathotype 3 and row 3x = DNA from the 3-like population. Proportions of the two DNA for each reaction are listed on the top. The left-most line is the Promega 100-bp DNA ladder.

polymorphic genes) allowed the design of rhPCR primers that can be used for qPCR analyses. Currently, two quantitative rhPCR primer pairs, one specific to pathotype 5 and the other one to the 5-like population, are being evaluated in our lab for their usefulness on biomass quantification from canola roots infected by a mixture of the two *P. brassicae* populations. By a time-course analysis, information on the dynamics of a virulent population and an avirulent population during pathogenesis will be revealed for the first time.

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