Characterization of Five Molecular Markers for Pathotype Identification of the Clubroot Pathogen *Plasmodiophora brassicae*

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ABSTRACT

Clubroot disease is an important disease on cruciferous crops caused by *Plasmodiophora brassicae* infections. The pathotypes have been classified based on the reactions of differential hosts. However, molecular markers of particular pathotypes for *P. brassicae* are limited. In this study, we found five genetic markers in association with different pathotypes. Different gene expression patterns among different pathotypes (P4, P7, P9, and P11) were assayed according to the transcriptome data. The assay

indicated that molecular markers *PBRA_007750* and *PBRA_009348* could be used to distinguish P11 from P4, P7, and P9; *PBRA_009348* and *Novel342* could distinguish P9 from P4, P7, and P11; and *PBRA_008439* and *Novel342* could represent a kind of P4. Polymerase chain reaction cycles ranging from 25 to 30 were able to identify the predominant pathotype in general. Therefore, these molecular markers would be a valuable tool to identify and discriminate pathotypes in *P. brassicae* population.

Clubroot disease is an important disease on cruciferous crops and is caused by *Plasmodiophora brassicae* Woronin infections. It is a soilborne, obligate, and biotrophic pathogen that leads to a subsequent reduction in the crop yield (Crisp et al. 1989; Dixon 2009). The disease is spreading rapidly in Asia and has become a problem not only in China, Japan, and Korea but also in tropical countries such as Indonesia, Thailand, and Vietnam (Dixon 2009). Now it is known to occur in more than 60 countries (Dixon 2009). In China, clubroot disease occurred in many provinces and caused serious damages to crucifer crops, and more than 20 public institutions have been making efforts to control clubroot disease (Chai et al. 2014).

However, clubroot disease is difficult to be controlled by either chemical or cultural means. Many strategies have been proposed; among them, the development of resistant cultivars is believed to be the most effective and environmentally friendly method. Breeding for a resistant cultivar requires a good understanding of the virulence pattern from the pathogen population. However, *P. brassicae* is so variable that a satisfactory method for detecting specific virulent isolates is still lacking. To learn more about identification of *P. brassicae* pathotypes and accelerate the breeding of clubroot-resistant crops, Shen et al. (2009) identified clubroot pathogens from 15 major areas in China (including Liaoning, Jilin, Shandong, and Sichuan Provinces) and discovered that P4 was the predominant pathotype in 11 areas.

A specific isolate of *P. brassicae* could not be distinguished from others by phenotypic characteristics, except for virulence. Therefore, the pathotypes are usually identified based on their impacts on different hosts (Buczacki et al. 1975; Kuginuki et al. 1999; Williams 1966). The Williams differential system is the earliest one, and is used formally in wide applications (Williams 1966). Theoretically, by using four differential hosts, 16 physiological races can be identified. Buczacki et al. (1975)

established the European clubroot differential (ECD) set, which is a currently universal identification system for clubroot pathogens in Europe. Both the Williams differential system and ECD are considered to be international identification systems and have been generally applied.

However, these identification systems also have some limits. For example, the maturity level of spores can influence the pathogenicity and further affect the accuracy of pathotype identification. Pathotype differentiation via virulence tests on a differential host generally takes as long as 2 months and is time consuming and labor intensive. Moreover, during a long growing period, environmental conditions and other uncertain factors may also affect the accuracy of identification (Sharma et al. 2011). Notably, the interpretation of results is sometimes confusing because of the nonhomogeneous reactions owing to the heterogeneity of *P. brassicae* in field (Crute and Pink 1989). Therefore, classifying *P. brassicae* into physiological races at the molecular level is a pertinent research trend. Specific molecular markers in association with the pathotypes would be a valuable tool for the identification and monitoring of *P. brassicae* populations. Previously, some random amplified polymorphic DNA (RAPD) markers have been developed and applied in pathogen polymorphism analysis (Buhariwalla et al. 1995; Ito et al. 1997; Manzanares-Dauleux et al. 2000; Möller and Harling 1996; Yano et al. 1997). Manzanares-Dauleux et al. (2000) obtained a RAPD marker, OPL14₁₂₀₀, which is specific in pathotype 1 of *P. brassicae*, as classified by the method of Somé et al. (1996). Zhang et al. (2015) indicated that one gene, designated *Cr811*, was found to be exclusively present in pathotype P5. *Cr811* could distinguish pathotype 5 from pathotypes 3 and 8 according to the Williams differential set (Zhang et al. 2015). These reports showed that molecular markers could be suitable to distinguish pathotypes (Faggian et al. 1999; Klewer et al. 2001). Establishing a molecular identification system of *P. brassicae* pathotypes will be key for increasing the breeding efficiency for disease-resistant cruciferous varieties and realizing a better control of clubroot disease.

Based on current knowledge, the molecular determinants of pathotypes are most related to the presence or absence or differential expression of marker genes. Transcriptome sequencing data are a good resource for marker gene screening. In this study,

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transcriptome data of *P. brassicae* in *Brassica rapa* at different days postinoculation were analyzed. The differential gene expression patterns between pathotypes 4 and 7 were focused and further used to screen molecular markers to particular pathotypes. We hypothesized that part of these genes may be specific to certain pathotypes. The objective of this study was to identify genes that were present in certain pathotypes. The primer pairs specific to these genes were first used to evaluate the presence of corresponding genes in genomic DNA samples obtained from different pathotypes. Then, we tested the applicability of these genes as molecular makers to differentiate unknown pathotypes.

MATERIALS AND METHODS

Preparation of resting spore suspension. For preparation of plant inoculation, *P. brassicae* resting spores were prepared as previously described (Ji et al. 2014). Root galls were homogenized using 10% (wt/vol) sucrose in a blender. The slurry was filtered through eight layers of gauze and the suspension was clarified by centrifugation at 3,000 rpm for 9 min. The pellet was suspended in sterile water and transferred into a new tube containing 40 ml of water. After centrifugation at 4,000 rpm for 12 min, the resting spore precipitate was resuspended in sterile water, adjusted to a concentration of 1×10^7 spores/ml, and stored at 4°C. For pathotype determination on the Williams differential hosts, 30 seedlings of each host line were inoculated with each field isolate of *P. brassicae*. These steps were repeated three times.

Plant inoculation with *P. brassicae*. For transcriptome sequencing and semiquantitative reverse-transcription polymerase chain reaction (RT-PCR) analysis, *B. rapa* seedlings were inoculated with a 600-μl suspension of *P. brassicae* resting spores with a density of 10^7 spores/ml. The inoculated plants were maintained in a growth chamber under 16 h of light at 25°C and the soil was kept moist during the treatment period. The root of Chinese cabbage was obtained at 4, 10, 16, and 45 days postinoculation (dpi) and washed thoroughly with distilled water to remove the spores adsorbed on the surface, then frozen in liquid nitrogen and stored at -70°C.

***B. rapa* with *P. brassicae* inoculations and transcriptome sequencing.** According to the previous identification results by Williams's system, isolates DY and LX were identified as pathotype 4 and pathotype 7, respectively. In this experiment, *B. rapa* (Chinese cabbage cultivar Zaoshu-5) (San Duo Seed) seedlings were inoculated with suspensions of *P. brassicae* P4 (DY) and P7 (LX), respectively. The total RNA of clubroot was extracted and sent to Novogene Biotechnology Company for sequencing. The differential expression of genes between P4 (DY) and P7 (LX) was analyzed basing on the transcriptome data. Genes with higher expression levels in the P4 (DY) sample than the P7 (LX) sample were selected for further identification. Finally, five molecular markers were screened out. The read counts of genes in different samples and annotations are listed in Supplementary Table S1.

Screening of primers and specificity analysis. Chinese cabbage (Zaoshu-5) was used as the host to maintain *P. brassicae* spores. Isolates LX and KD were classified as pathotype 7 (P7). DY, SL, LJ, and FL were the P4 pathotype according to the system of

Williams (Table 1). The total DNA of root gall samples, including root and resting spores, were used for the identification of pathotype-specific genes. Finally, five pathotype-specific genes were obtained. The sequences of primers are listed in Table 2. Two pairs of primers for each gene were designed according to the nucleotide sequences. The fragment was aligned with the transcriptome sequence data to ensure the presence of intron (Fig. 1), and the BLAST result is listed in Table 3.

In total, 40 field isolates (Supplementary Table S2) of *P. brassicae* derived from the host galls caused by natural infections were collected in China (mainly in Sichuan Province). Pathotypes were classified according to the system of Williams. The resting spores of *P. brassicae* were collected from root galls of different crucifer hosts; then, DNA of resting spores was subjected to PCR analysis with pathotype-specific genes.

All PCR analyses were run in a final volume of 10 μl: 5 μl of 2× Taq Master Mix (Takara), 0.5 μl of each primer, and 0.5 μl of DNA template. The concentration of DNA was approximately 200 to 300 ng. PCR was performed, and the first amplification cycle consisted of denaturation at 94°C for 2 min; 30 cycles of 94°C for 30 s (denaturation), 55°C for 60 s (annealing), and 72°C for 1 min (extension); and a final extension at 72°C for 7 min. Here 25, 30, and 35 cycles were used for analysis of the best amplification results, respectively.

To determine whether the host could affect primer specificity, we mixed DNA of *P. brassicae* with DNA of eight usual hosts. The crucifer cultivars included *B. rapa pekinensis*, *Raphanus sativus* L., *B. chinensis* var. *chinensis*, *B. napus* L., *B. juncea* (L.) Czern. et Coss., *B. oleracea* L., *B. oleracea* L. var. *botrytis* L. and *B. rapa* L. subsp. *chinensis*.

DNA extraction from *P. brassicae* resting spores. The resting spores were washed five times in sterile distilled water, dispersed in 100 mM MgCl₂ and 200 mM Tris (pH 7.4) buffer, and treated with DNase I (30 μg/ml) for 3 h at 37°C to eliminate host DNA. The solution was centrifuged at $2,500 \times g$ for 5 min, and the pellet was incubated for 30 min at 37°C in 5 mM EDTA, 0.5% sodium dodecyl sulfate, and 10 mM Tris (pH 7.8) containing proteinase K at 20 μg/ml. The sample was then centrifuged at 4,000 rpm for 10 min, the supernatant was discarded, and the resting spores were frozen in liquid nitrogen and stored -70°C.

P. brassicae DNA was extracted from the above prepared resting spores by cetyltrimethylammonium bromide (CTAB). DNA of root galls, including plant tissue and resting spores, was also extracted by CTAB. The samples were ground by first freezing in liquid

TABLE 1. *Plasmodiophora brassicae* isolates used for gene screening

Isolate name or number	Collection site	Host origin	Pathotype
KD	Kangding, Ganzi	Rape	7
LX	Lixian, Aba	Chinese cabbage	7
DY	Dayi, Chengdu	Rape	4
SL	Shuangliu, Chengdu	Rape	4
LJ	Luojiang, Deyang	Green vegetables	4
FL	Fuling, Chongqing	Mustard	4

TABLE 2. Sequence of primers used in this study

Primer	Sequence (5'-3')	Annealing temperature/°C	Size (bp)
Novel342-1-F	TCCTCTTGAACCGACACTGC	57	249
Novel342-1-R	CTTCTCTCGCACTAGCCAGG	...	...
Novel342-2-F	CCACGCCATATCCCGAAAG	57	666
Novel342-2-R	CAACAGGACGGCGTTGAAAG	...	...
Novel407-1-F	ATTGCGTTGCTGAACCTGCTG	54	549
Novel407-1-R	GTGCCCAATAGCAATCGCAG	...	...
Novel407-2-F	GTCGTTGTTCCGGGGAGAAGT	57	683
Novel407-2-R	GTCCATAGGTGTGGGAACCG	...	...
PBRA_007750-1-F	CTTCGTGCTGACCGATTCCCT	57	638
PBRA_007750-1-R	ATAATGCTCTGCGTCAGCCA	...	...
PBRA_007750-2-F	ATCTGTTGCTGCTCGCTGCT	57	1,034
PBRA_007750-2-R	GAGTGTACAGGCTCGTTCAG	...	...
PBRA_008439-1-F	TCGGCGACCTGAGCGAGAA	57	651
PBRA_008439-1-R	TCAACATGCGCATAGTAC	...	...
PBRA_008439-2-F	TCGAGGTATCGCAGTCGAC	60	367
PBRA_008439-2-R	GATCTCCAGTGACGAGCCGT	...	...
PBRA_009348-1-F	CACGTGCTATCGTCTCCCTGG	54	509
PBRA_009348-1-R	CCTGCAATGTTTCGCTGCAA	...	...
PBRA_009348-2-F	CCTTGCCCTGGTATTCGTC	54	260
PBRA_009348-2-R	CAACGACAACGACCAACCG	...	...
Actin-F	GGGACATCACCGACTACCTG	54	160
Actin-R	ACTGCTCCGAGTTGGACATC	...	...

nitrogen before extraction. Then, the powder was transferred into a 2-ml centrifuge tube, 700 µl of 2× CTAB (2% CTAB, 100 mM Tris-HCl [PH 8.0], 20 mM EDTA, and 1.4 M NaCl) was added, and samples were mixed by inversion or gentle shaking and stored at 65°C for 1 h. Following centrifugation at 12,000 × g for 15 min, the supernatant was transferred to a fresh tube. This step removed insoluble tissue fragments from the lysate. Then, an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) was added and centrifuged at 12,000 × g for 15 min. The suspension was transferred into a 2-ml centrifuge tube. DNA was precipitated from the lysate by the addition of 1 ml of 100% ethanol per 0.5 ml used for the isolation, stored at −70°C for at least 1 h, then centrifuged at 12,000 × g for 15 min at room temperature. The DNA precipitate was washed twice with 1 ml of 75% ethanol. At each wash, the DNA was suspended in ethanol by inverting the tubes three to six times. DNA was air dried by storing in an open tube after removing the ethanol. Then, DNA was solubilized in double-distilled H₂O and stored at −20°C.

Gene expression verify by semiquantitative RT-PCR. In this experiment, the Chinese cabbage cultivar Zaoshu-5 and *P. brassicae* isolate LJ (P4) were used. Total RNA of root samples was extracted by using TRIpure Reagent (Aidlab Biotech), according to the manufacturer’s instructions. For cDNA synthesis, 3 to 5 µg of total RNA and the M_MLV RT (Takara) was used according to the manufacturer’s instructions. For the

semiquantitative expression analysis, the *P. brassicae actin1* gene was used as the reference.

RESULTS

Five molecular markers could distinguish P4 from P7. DNA of *P. brassicae* was extracted from young roots of *B. rapa*; then, the mixture of host and parasite DNA was subjected to PCR.

TABLE 3. Alignment results of different markers^a

Marker name	Size (bp)	Identity (%) of polymerase chain reaction fragment with	
		Transcriptome data	Sequence of <i>Plasmodiophora brassicae</i> pbe3.h15 genome
<i>Novel342-2</i>	666	89.39	...
<i>Novel407-2</i>	683	91.54	...
<i>PBRA_007750-2</i>	1,034	100	100
<i>PBRA_008439-1</i>	651	100	100
<i>PBRA_009348-1</i>	509	100	100

^a The larger amplicon of each gene was sequenced for the verification. Markers *Novel342* and *Novel407* were aligned with the sequence of transcriptome data by using DNAMAN. Markers with names beginning “PBRA” were aligned with the sequence of GenBank and transcriptome data.

Fig. 1. Diagram of genomic regions of *Novel342*, *Novel407*, *PBRA_007750*, *PBRA_008439*, and *PBRA_009348*. Lighter segments refer to intron and darker to exon. We blasted polymerase chain reaction product with transcriptome data to ensure that the gene fragment has introns.

The housekeeping gene *actin1* was used as the control (Supplementary Fig. S1). Among selected genes, five molecular markers were finally screened out for further analysis. The sequences of PCR fragments of *PBRA_007750*, *PBRA_008439*, and *PBRA_009348* had 100% identity with those in the *P. brassicae* genome pbe3.h1 in the National Center for Biotechnology Information (NCBI) website. The query covers of *Novel342* and *Novel407* had less than 6% identity with the sequences in the NCBI website. The sequences of PCR fragments of *Novel342* and *Novel407* had 89.39 and 91.54% identity with the transcriptome sequence and were located in CDSF01000088.1 and CDSF01000096.1 chromosome fragments, respectively.

The primer pairs of five molecular markers, including *Novel342*, *Novel407*, *PBRA_007750*, *PBRA_008439*, and *PBRA_009348*, amplified the expected size of amplicons from P4 (isolates DY, LJ, and SL) but not from P7 (KD and LX isolates). Thus, *Novel342*, *Novel407*, *PBRA_007750*, *PBRA_008439*, and *PBRA_009348* could distinguish P4 from P7 among the tested samples. The expected fragments could be amplified with the primer pairs of *Novel342* or *Novel407* but not with the primer pairs of *PBRA_007750*, *PBRA_008439*, or *PBRA_009348* in isolate FL (P4). This means that P4 of *P. brassicae* could be distinguished by two molecular markers. The primer pairs of *Novel407* especially produced a nontarget fragment (approximately 1,200 bp in size) from P7 (KD and LX isolates). To ensure the stability of PCR results, another primer pair of each gene was designed. Amplicons of expected size were also obtained, which were consistent with the above PCR assay (Fig. 2).

Analysis of primer specificities affected by different hosts. The intracellular life cycle of the pathogen suggests that plasmodia DNA was strongly contaminated by the host DNA. Although spores of the pathogen could be isolated free of host cell materials, contamination of host DNA could still be found in the spores. Therefore, we wanted to test whether the other host tissues could influence the PCR results. The DY isolate (P4) and LX isolate (P7) DNA and DNA of eight different hosts were mixed, then subjected for PCR identification. The primers of *Novel342*, *Novel407*, *PBRA_007750*, *PBRA_008439*, and *PBRA_009348* could

specifically amplify the target fragment from the DNA mixture of DY isolates with nine different hosts but did not produce any result or amplicons of expected size from the DNA mixture of LX isolates with nine different hosts. The results indicated that the primer specificity of five molecular markers could not be affected by the host's DNA (Fig. 3).

Five molecular markers distinguished field isolates of different pathotypes. Forty field isolates were used to further analyze the specificity of five molecular markers. Galls were collected from different fields in China, mainly in Sichuan Province, selected based on previous identification results by members of our lab and other researchers. The pathotype identification of 40 isolates was first determined with the Williams differential hosts. The resting spores of 40 field isolates were collected from mature galls and evaluated by PCR analysis with each primer pair. Primer pairs of *Novel342* and *Novel407* could produce the expected size of amplicons with genomic DNA of P4 and P11 but not with P7 and P9 (Fig. 4), which indicated that the two molecular markers could distinguish P4 (isolates 1 to 4, 6 to 18, 20 to 26, 31 to 32, 34, 36, 38, and 40) from P7 (isolates 9, 19, 28, and 30) and P9 (isolates 37 and 39) but not P11 (isolates 27, 29, 33, and 35).

We found that all P4 isolates produced the expected size of amplicons with primer pairs of *Novel342* and *Novel407*. However, only part of the P4 isolates produced the expected size of amplicons with primer pairs of *PBRA_007750*, *PBRA_008439*, and *PBRA_009348*. Because the expected fragments could be always amplified with primers of *Novel342* and *Novel407*, whereas *PBRA_008439* only worked for one P4 genotype but not the other genotypes of P4, P7, P9, and P11, the primer pairs of *PBRA_008439* and *Novel342* or *PBRA_008439* and *Novel407* should be used to differentiate the genotype of P4 from P7, P9, and P11 (Table 4).

We found that *PBRA_007750* or *PBRA_008439* could be specially detected in P11 and some genotypes of P4 but *PBRA_009348* could not be detected in P11. Therefore, we suggested that, if the target fragment could be amplified with the primers of *PBRA_007750* or *PBRA_008439* but not with *PBRA_009348*, the pathotype should be P11 but not P4.

Fig. 2. Identification of the presence of *Novel342*, *Novel407*, *PBRA_007750*, *PBRA_008439*, and *PBRA_009348* in pathotype 4 and pathotype 7 of *Plasmodiophora brassicae*. Lane M = DNA marker 2000; from top to bottom, the size of band was 2,000, 1,000, 750, 500, 250, and 100 bp, respectively.

PBRA_009348 could be specially detected in P9 and some genotypes of P4. Neither Novel342 nor Novel407 amplified the expected fragments of P9, which indicated that PBRA_009348 and Novel342 or PBRA_009348 and Novel407 could be used to distinguish P9 from P4.

These results indicated that five molecular markers had a potential to distinguish pathotypes of *P. brassicae*. The pathotypes and genotypes of *P. brassicae* may be identified by one molecular marker or several molecular markers, which was summarized in Table 4.

Effect of PCR cycles on the identification result. Different pathotypes, including dominant and rare races, might be present simultaneously in a single gall and a complicated result might be obtained. Therefore, three different PCR cycles (25, 30, and 35 cycles) were used to test the presence of rare races or complicated results in root galls. We tested five genes of resting spores in nine field isolates. The *P. brassicae actin* gene was employed as the control gene, which could be detected after 25 PCR cycles. The target amplicons from the corresponding pathotypes were only obtained between 25 and 30 cycles. However, the target amplicons could be obtained in all isolates at 35 cycles, although the amplification levels were lower from noncorresponding pathotypes. The expected PCR fragments were more obvious in isolates 9, 21, 33, and 35 than isolates 5, 17, 22, 30, and 37 with the primers of PBRA_008439 (Fig. 5). The result indicated that a mixture of different pathotypes surely existed in some clubroots and could be easily identified by PCR detection. Therefore, the optimal PCR cycles should be set between 25 and 30 cycles for the molecular identification of pathotypes to reduce false-positive results.

Expression of molecular markers in infected plants. RT-PCR was analyzed at different time points (4, 10, 16, and 45 dpi) in Chinese cabbage with isolate DY infection. The expression levels of Novel342 were visible at 4 dpi, increased at 10 dpi, and could not be observed at 16 dpi, but reached a peak at 45 dpi during

symptom information period. For other molecular markers, including Novel407, PBRA_007750, PBRA_008439, and PBRA_009348, the expression level was hardly detected until they were obvious at 45 dpi (Fig. 6). This result was consistent with the transcriptome data and suggested that these genes might contribute to gall formation.

DISCUSSION

Pathotype classification of the isolates was always performed according to the disease symptoms of different hosts (Somé et al. 1996). However, the differentiating PCR markers and the molecular basis for classification of *P. brassicae* pathotypes are still limited. In this experiment, we focused on genes which expressed differently between four pathotypes of *P. brassicae* in transcriptome data. Novel342, Novel407, PBRA_007750, PBRA_008439, and PBRA_009348 were screened out as markers to identify *P. brassicae* pathotypes. Interestingly, Novel342 and Novel407 were not found in the *P. brassicae* pbe3.h15 genome (Schwelm et al. 2015), suggesting its absence in this strain. A notion accepted by most researchers is that the different pathotypes have an inner connection with their genetic makeups, such as the absence or presence of certain pathogenicity-related genes, or differential expression of those genes (Zhang et al. 2015). Among tested pathotypes P4, P7, P9, and P11, we found that Novel342, Novel407, PBRA_007750, PBRA_008439, and PBRA_009348 genes were present in P4 of *P. brassicae* but not in P7. To make more accurate identification of pathotypes, we suggest that the combination of two genes should be used to ensure a certain pathotype. Furthermore, we should notice that the same pathotype is usually present in different genotypes. For example, PBRA_007750, PBRA_008439, and PBRA_009348 detected two different genotypes of P4, indicating a potential method to identify new strains of *P. brassicae*.

The practicality of molecular markers was confirmed by using the field isolates in Sichuan Province. The results indicated that P4 was

Fig. 3. Analysis of primer specificity affected by different hosts. DY isolate (lines 1 to 8) and LX isolate (lines 9 to 16) mixed with hosts *Brassica rapa pekinensis*, *Raphanus sativus* L., *B. chinensis* var. *chinensis*, *B. napus* L., *B. juncea* (L.) Czern. et Coss., *B. oleracea* L., *B. oleracea* L. var. *botrytis* L., and *B. rapa* L. subsp. *chinensis*, respectively.

Fig. 4. Identification of five molecular markers in 40 field isolates of *Plasmodiophora brassicae*. Isolate 1 to 4, 6 to 18, 20 to 26, 31 to 32, 34, 36, 38, and 40 = pathotype 4; isolates 5, 19, 28, and 30 = pathotype 7; isolates 37 and 39 = pathotype 9; isolates 27, 29, 33, and 35 = pathotype 11; and CK = double-distilled H₂O. The *P. brassicae* gene *actin1* was used as a positive control of the existence of *P. brassicae*.

the predominant race, and mainly distributed around the Chengdu plain. The Jialing River, Minjiang River, and Tuojiang River flow through Chengdu plain and might facilitate the long-distance spread of *P. brassicae*. P7 is mainly distributed in cool areas such as Xichang, Aba, and Kangding. These results were consistent with previous identification using the Williams system in our lab. Therefore, the PCR assay designed in this study could be a convenient tool for identifying and monitoring the distribution of *P. brassicae* pathotypes in Sichuan Province. This result should be further confirmed by testing on larger collections of isolates in broader geographical regions with more pathotypes.

Horizontal gene transfer (HGT) usually provides a means for pathogens to create new races or pathotypes (Mehrabi et al. 2011; Ochman et al. 2000). HGT is very common in protists. We suggested that *P. brassicae* may be an HGT-amiable species (Richards et al. 2003). The presence of a gene in a certain isolate but complete absence in some other isolates suggests that this gene may be a result of HGT. For example, we found that the two primer pairs of *Novel407* could amplify 549- and 683-bp fragments from genomic DNA of P4 and P11, respectively, and two 1,100- and 1,200-bp nontarget fragments in P7. An approximately 200-bp nucleotide sequence in the nontarget fragments could be blasted with the transcriptome sequence of P4 (isolate DY) and genome of *P. brassicae* in the NCBI website, which means that an HGT might occur in P7.

The greatest important advantage is that the PCR method could be performed rapidly and directly from clubs without purification of resting spores. Host DNA would not affect the result; therefore, it may serve as a molecular marker to identify pathotypes with total DNA from a tiny gall. Because a mixture of different pathotypes might exist in a root nodule, as reported by Somé et al. (1996) and Kuginuki et al. (1999), it is not an ideal way to apply too many PCR cycles. An appropriate number of cycles would give optimal results. With the molecular markers, we could detect what kind of

pathotype may hold in a field and which pathotype is predominant. Also, the method is useful for *P. brassicae*-resistant cultivar breeding.

Among five molecular markers, the expression level of *Novel342* was visible at early and late infection periods. However, its functions have not been annotated. We predicted the function on the SMART protein website (<http://smart.embl-heidelberg.de/>). The *Novel342* protein was predicted with four domains, including CXC, ANATO, DEFSN, and CRAL_TRIO_N domains. Anaphyla toxin homologous (ANATO domain) C3a, C4a, and C5a anaphyla toxins are protein fragments generated enzymatically in serum during activation of complement molecules C3, C4, and C5. Complement components C3, C4, and C5 are large glycoproteins that have important functions in the immune response and host defense (Fritzinger et al. 1992). Defensins (DEFSN domain) are 2- to 6-kDa, cationic, microbicidal peptides active against many Gram-negative and Gram-positive bacteria, fungi, and enveloped viruses (White et al. 1995). *PBRA_008439* was annotated, which includes an ankyrin repeat and SEC7 domain containing protein SecG. SecG translocates both periplasmic and outer membranes through the cytoplasmic membrane and is coassembled into lipids with SecA to yield highly active translocons (Sugai et al. 2007). Here, we suggest that *PBRA_008439* might be related to translocation of defense proteins, especially in the late infection of *P. brassicae*. The roles of five molecular markers need to be further verified.

Selection of certain pathotypes by host plants has implications for agricultural practice, because the infection potential in a certain field is dependent on the cultivar varieties. New pathotypes may be generated by mutations in one or some key pathogenic genes and these processes may be accelerated in artificial cultivars. Molecular markers may be used to gain a better understanding of genetic variability. This set is also useful for studying the correlations among molecular markers, virulence, and recombination in a progeny. Establishing a molecular identification set needs less time and space than the traditional Williams identification system. Future studies may include a larger number of isolates which come from different hosts and geographic origins and add more markers to find more representative genes for different pathotypes.

TABLE 4. Identification of tested pathotypes by one molecular marker or several molecular markers

Pathotype	Molecular markers ^a
P4	
Distinguish P4 and P11 from P7 and P9	<i>Novel342</i> (Y) or <i>Novel407</i> (Y)
Distinguish a genotype of P4 from P7, P9 and P11	<i>PBRA_008439</i> (Y) + <i>Novel342</i> (Y) <i>PBRA_008439</i> (Y) + <i>Novel407</i> (Y)
P9	
Distinguish P9 from P4, P7, and P11	<i>PBRA_009348</i> (Y) + <i>Novel342</i> (N) <i>PBRA_009348</i> (Y) + <i>Novel407</i> (N)
P11	
Distinguish P11 from P4, P7, and P9	<i>PBRA_007750</i> (Y) + <i>PBRA_009348</i> (N) <i>PBRA_008439</i> (Y) + <i>PBRA_009348</i> (N)

^a Y means that the target gene could be amplified and N means that the target gene could not be amplified.

Fig. 5. Results of different polymerase chain reaction cycles for identification of pathotypes by *PBRA_008439*. Isolates 5 and 30 = pathotype 7; isolates 9, 17, 21, and 22 = pathotype 4; isolate 37 = pathotype 9; isolates 33 and 35 = pathotype 11; and CK = double-distilled H₂O.

Fig. 6. Expression of *Novel342*, *Novel407*, *PBRA_007750*, *PBRA_008439*, and *PBRA_009348* during the different infection periods. *Plasmodiophora brassicae actin1* was used as the reference gene.

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