Characterization of a Gene Identified in Pathotype 5 of the Clubroot Pathogen *Plasmodiophora brassicae*

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ABSTRACT

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Clubroot caused by *Plasmodiophora brassicae* is an important disease of crucifers worldwide. Isolates of the pathogen can be classified into pathotypes according to their pathogenicity on differential hosts. In this study, the presence or absence of all database-available nonhousekeeping *P. brassicae* genes (118 in total) were assessed by polymerase chain reaction (PCR) analysis in isolates belonging to five *P. brassicae* pathotypes (2, 3, 5, 6, and 8 according to Williams' differential set). One gene, designated *Cr811*, was present exclusively in the isolate of pathotype 5.

This was further confirmed by dot blot hybridization and by PCR using alternative DNA preparations and primers. Reverse transcription quantitative PCR analysis indicated that in planta expression of *Cr811* was up-regulated during canola infection, especially in the stage of secondary plasmodia. Primers specific to *Cr811* could distinguish a field isolate of *P. brassicae* belonging to pathotype 5 from two other field isolates representing pathotypes 3 and 8. These findings suggest that *Cr811* is a gene that is potentially involved in clubroot pathogenesis and that it also might serve as a molecular marker for differentiation of pathotype 5 from other pathotypes.

Additional keywords: gene expression, horizontal gene transfer, resistance, virulence.

Plasmodiophora brassicae Woronin causes clubroot disease in cruciferous plants and is an emerging threat to Canadian canola (*Brassica napus* L.) production (20,39). Many strategies have been proposed for clubroot control (19,32), but the use of resistant cultivars is believed to be most effective (33). Breeding for resistance requires a better understanding of the virulence pattern of the pathogen population in areas where resistant cultivars are cultivated. Isolates of *P. brassicae* have been classified into pathotypes based on their reactions on differential hosts. Numerous sets of differential hosts have been proposed (39), including the commonly used differential sets of Williams (42), the European clubroot differential (ECD) (3) and the differential set of Somé et al. (38). Based on the results of virulence testing on the differential set of Williams, pathotype 3 (P3) has been found to be predominant in Alberta (41), although P2, P5, P6, and P8 are also present (41,43).

Pathotype differentiation via virulence tests on sets of differential hosts is time-consuming, labor intensive and can be affected by environmental conditions. Furthermore, due to the fact that different pathotypes may be present in single galls (22), complicated results may be obtained from such tests. Development of molecular markers specific to particular pathotypes would be a valuable tool to identify and monitor *P. brassicae* populations. For example, a random amplified polymorphic DNA (RAPD)-derived sequenced characterized amplified region (SCAR) marker specific to P1, as defined on the differentials of Somé et al. (38), has been developed and proven to be useful for the identification of this pathotype from field isolates (25). Our group has conducted extensive studies to characterize the

virulence pattern of *P. brassicae* populations (7,40,41) and to identify molecular markers that can be used to differentiate pathotypes.

Based on the current knowledge, the molecular determinants of a pathotype are most likely related to the presence/absence or differential expression of genes related to pathogenicity. In GenBank, there are approximately 120 nonhousekeeping genes of *P. brassicae* represented by expressed sequence tags (EST) or predicted proteins (15). In this study, 118 primer pairs specific to most of these genes were used to evaluate the presence of the corresponding genes in genomic DNA samples prepared from single-spore isolates belonging to five pathotypes as classified on the differential set of Williams (42). We hypothesized that members of these 118 genes may be specific to certain pathotypes. Thus, the objectives of this study were to (i) identify gene(s) that are differentially present in pathotypes, (ii) molecularly characterize the identified gene(s), and (iii) test the usefulness of these gene(s) as makers to differentiate unknown pathotypes. One gene, designated *Cr811*, was identified to be exclusively present in P5. The in planta expression of this gene was studied using an infection time course on both a resistant and a susceptible canola genotype. Primers specific to this gene were used to identify P5 from field isolates. The molecular function of this gene with respect to the pathogenicity of the P5 strain also is discussed.

MATERIALS AND METHODS

Plant materials and *P. brassicae* single-spore and field isolates. The canola cultivar Westar was used as host to maintain the *P. brassicae* single-spore and field isolates. The hybrid canola cultivars 45H26 (clubroot-susceptible) and 45H29 (clubroot-resistant) were used in the investigation of the in planta gene expression. The differential hosts of Williams (42), consisting of the cabbage cultivars Jersey Queen and Badger Shipper and the rutabaga cultivars Laurentian and Wilhelmsburger, and the ECD set, consisting of five genotypes each of *B. napus*, *B. rapa* and

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B. oleracea (3), were used in pathotype identification of unknown isolates. Five single-spore isolates of *P. brassicae* in our collection SACAN-ss3, SACAN-ss1, ORCA-ss4, AbotJE-ss1, and CDCN-ss3, which are classified as P2, P3, P5, P6, and P8, respectively (43), were used in the identification of pathotype-specific gene(s). Three field isolates of *P. brassicae* derived from canola galls caused by natural infection were collected from fields in Sturgeon County, Newell County, Athabasca County, and Alberta, Canada, and classified to pathotype on the differential hosts of Williams (42) and ECD (3). To determine whether or not the presence of *Cr811* was limited to the single-spore isolate of P5, these field isolates were subjected to PCR analysis with the *Cr811*-specific primers.

Preparation of resting spores. For plant inoculation, *P. brassicae* resting spores were prepared as previously described (17). Briefly, galls were homogenized in 10% (wt/vol) sucrose in a blender. The slurry was passed through eight layers of cheesecloth and the suspension was centrifuged at $50 \times g$ for 5 min. After transfer into a new tube, the supernatant was centrifuged at $2,000 \times g$ for 5 min. The upper layer of the resultant pellet was suspended in 5 ml of water by gentle pipetting and transferred into a new tube containing 40 ml of water. After centrifugation at $2,000 \times g$ for 5 min, the supernatant was removed. The resting spores in the pellet were resuspended in 2 ml of water, and the spore concentration was adjusted to 1×10^7 spores/ml. The resting spores were surface-disinfected with colistin sulfate at 1 μ g/ml and vancomycin hydrochloride at 1 μ g/ml at 4°C in the dark for 24 h. The spores were used as inoculum after removal of the antibiotics by washing twice with 40 ml of distilled water. For DNA extraction, the resting spores were prepared with the same protocol except that after overnight treatment with antibiotics at 4°C, the spore suspensions were further treated at 37°C with 10 units/ml of DNase I (Life Technologies, Carlsbad, CA) for 2 h to remove genomic DNA from the host plant or other organisms released into the suspension during homogenization of the galls.

Manipulation of nucleic acids. All plant and pathogen samples were ground in liquid nitrogen before nucleic acid extraction. Unless specified otherwise, *P. brassicae* DNA was extracted from the prepared resting spores following the protocol described by Feng et al. (12). DNA extraction from healthy canola roots or LB

(1% NaCl, 1% Tryptone, and 0.5% yeast extract) culture of the gall slurry, as well as from purified resting spores for Southern analysis, was performed with the CTAB protocol (35). DNA purification of PCR amplicons from agarose gels was performed using the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI). Cloning of PCR products was conducted using the pGEM-T Easy Vector System (Promega). Sequencing was done by the Department of Biological Sciences, University of Alberta (Edmonton, AB, Canada). RNA extraction from galls or healthy roots, including the DNase I treatment, was performed using the SV Total RNA Isolation System (Promega). Synthesis of the first-strand cDNA from the purified RNA was conducted using an iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA) or a GoTaq 2-Step RT-qPCR System (Promega).

PCR and quantitative PCR. Primers for the identification of the pathotype-specific gene(s) were derived from Feng et al. (15) with other primers used in this study listed in Table 1. The genomic locations of the primers are illustrated in Figure 1. PCR analyses were conducted in an Eppendorf Mastercycler thermal cycler (Eppendorf, Hamburg, Germany). Except in the genome walking analysis, all PCR analyses were run in a final volume of 20 μ l in the Promega master mix with 0.25 μ M of each primer and 100 ng of template; for all primers, the annealing temperature was 52°C and/or 56°C, the elongation time was 45 s, and the cycle number was 35. Quantitative PCR (qPCR) assays were conducted in a StepOne Real-Time PCR System (Life Technologies) using the GoTaq 2-Step RT-qPCR System (Promega) for RNA analysis or the Power SYBR Green PCR Master Mix (Life Technologies) for DNA analysis. The *P. brassicae Actin1* gene (GenBank accession number AAR88383) was used for normalization. Primer efficiency was verified using standard curves generated from the purified PCR product of the targeted fragments. The quantity of *Cr811* relative to *Actin1*, at the DNA or RNA level, was calculated via the delta-delta method, with relative expression or amount = $2^{-(\Delta CT_{\text{sample}} - \Delta CT_{\text{control}})}$ (36). All qPCR amplifications were performed in triplicate for each of three template preparations derived from different biological samples. Data from the qPCR were analyzed for statistical significance using the general linear model (GLM) procedure of the Statistical Analysis System (SAS Institute, Cary, NC). All data were subjected to one-way analysis of variance and, when appropriate, a multiple-range test was conducted by Fisher's least significant difference (LSD) at $P \leq 0.01$.

Sequence analyses. The EST sequence representing a part of the *Cr811* gene was retrieved from GenBank (JK747455). Based on this sequence, primer pair g/u (Fig. 1 and Table 1) was designed and used to amplify a genomic fragment. The PCR product was sequenced using both primers g and u. Genome walking primers were designed based on this sequence information. After genome walking, the genomic sequence including JK747455 and its 5' and 3' regions was obtained by manually assembling the sequences of the overlapped fragments. Primers specific to the 5' and 3' ends of the assembled sequence were designed and used to amplify fragments from cDNA synthesized from RNA of P5. The PCR products were sequenced and the open reading frames (ORF) predicted using the online program ORF Finder (<http://www.ncbi.nlm.nih.gov/gorf/gorf.html>). The coding sequence was identified according to the

TABLE 1. Primers used in this study

Primer	Sequence (5'-3')
a	TGGAACGTACGTAAAGGAAACG
b	ATACTGTGTCAGAAAGATGCAATCG
c	TGACATTAGCGCTTCACTTGAC
d	ATCTCGACGAGTATAGCCATAG
e	TACCTGCGAACCTATGCGTGTA
f	GTAAAGCTGCCGACAGAAC
g	CTGTACGTCGATAACGGTCAA
h	TAACGGTCAAATGGTAAGCTTCAA
i	TCGGAAACACACAGAGAGG
j	ACTCTATATCTAGGATGCTGGAACA
k	GAGCCGATCGATAACGTGAAGCAG
l	AGGAGGAACCGCAACATAGTG
m	AGGATCAACAATCGGTATCATGG
n	TAACGATAGCATTGCGACTCCGATC
o	GTTCTGTGCCATGAGTGTACAG
p	GAAGCTTACCATTTGACCGTTATC
q	CGACCTTCTTCAGGCGTAAC
r	GTTATTCCGAGCGTGTACTTCTTG
s	GTTATCGATCGGCTCCATCC
t	AGTCCATGATACCGATTGTTGATC
u	CAGTCCATGATACCGATTGTTG
v	GCCGCTATTCTGATGTTATTGCT
w	TCTTGGTCTTTTGAAGTTC
x	TCGTTGATGTAATCCATCTAGC
y	TCAATGTACTCCTCGACCAAAG
z	ATGTCCTTGCCTACATGTTG
ActF	GGGACATACCGACTACCTG
ActR	ACTGCTCCGAGTTGGACATC

Fig. 1. Diagram of the genomic region of the *Cr811* gene. JK747455, GenBank accession number of the expressed sequence tag that is a part of *Cr811*; 5' region and 3' region, sequences obtained by genome walking; a to z, primers whose sequences are listed in Table 1. The white line denotes the sequenced cDNA.

criteria defined by Min et al. (28). Similarity searches of nucleic acid and deduced protein sequences of *Cr811* were performed using the Basic Local Alignment Search Tool (BLAST) against the National Center for Biotechnology Information (NCBI) database unless otherwise specified. Other sequence analyses were performed using the appropriate software as accessed from <http://ca.expasy.org>.

Genome walking. Genome walking was conducted based on the genomic DNA from the P5 single-spore isolate using the APAGene Gold Genome Walking Kit (Bio S&T, Montreal, QC, Canada). The gene specific primers required by the protocol were primers p, o, and n for 5' and k, l, and m for 3' (Fig. 1; Table 1). After the second nested PCR, three bands of each of 5' and 3' walkings were TA cloned and sequenced using the M13 forward primer.

Dot blotting and Southern analysis. Dot blotting and Southern analysis were performed with a Dig DNA Labeling and Detection Kit (Roche Applied Science, Indianapolis, IN). For dot blotting, 10 µl of genomic DNA (500 ng/µl) from each of the single-spore isolates belonging to the five pathotypes was boiled in water for 5 min to denature the DNA. After quickly chilling on ice, 2-µl aliquots of the solution were arrayed in duplicate on the positively charged nylon membranes (Roche Applied Science). For Southern analysis, 2 µg of genomic DNA from the P5 single-spore isolate was digested with *XhoI* (New England Biolabs, Ipswich, MA) at 37°C for 4 h. The digests were resolved on a 1% agarose gel and transferred onto the nylon membrane. Membranes for both dot blotting and Southern analysis were baked at 80°C for 2 h and then hybridized with a *Cr811*-specific (dot blot and Southern) and an *Actin1*-specific (dot blot) probe labeled with digoxigenin. Probes were synthesized from 1 µg and 200 ng of PCR products amplified from the genomic DNA of the P5 single-spore isolate using the primer pair g/u (Fig. 1; Table 1) and ActF/ActR (Table 1), respectively. The absence of the *XhoI* cutting site within the probe-specific genomic region was confirmed by a manual search. Hybridizations were performed overnight at 37°C. The posthybridization washes were performed twice for 5 min at 22°C with 2× SSC (1× SSC is 150 mM NaCl and 15 mM sodium citrate) and 0.1% SDS and twice for 15 min at 22°C with 0.5× SSC and 0.1% SDS. The hybridization signal was detected using anti-dig antibodies and visualized with NBT/BCIP. The time of color development was 10 min for dot blotting and approximately 2 h for Southern.

Plant inoculation. Seeds of host plants were surface-sterilized in 1% sodium hypochlorite for 5 min, washed with deionized water, and germinated on moistened filter paper for 7 days. The resulting seedlings were inoculated with the resting spore suspension (1×10^7 spores/ml) using the root-dipping method as described by Strelkov et al. (40). The inoculated plants were transferred into 2-liter plastic pots containing autoclaved Sunshine mix #4 soil (Sun Gro Horticulture, Vancouver, BC, Canada). The pots were kept in a greenhouse maintained at 24°C/18°C (day/night) with a 16-h photoperiod, and watered from the bottom every second day with tap water at pH 6.4 (adjusted with

HCl). For investigating the in planta expression of *Cr811*, root samples were collected at 0, 5, 7, 14, 21, 28, and 35 days after inoculation (dai). For pathotype determination on the Williams (42) differential hosts, the experiment was conducted as a randomized complete block design with four pots (replications) per treatment and 12 plants per pot. The clubroot assessment on individual plants was conducted at 35 dai using a 0 to 3 rating scale. The disease index of all plants in each pot was calculated as described by Strelkov et al. (40). Microscopic examination of the cortex of '45H26' and '45H29' at 35 dai was conducted with a Zeiss AX10 microscope (Carl Zeiss, Oberkochen, Germany).

RESULTS

The *Cr811* gene is present in the pathotype 5 isolate but not in other isolates tested. Against genomic DNA from isolates representing five *P. brassicae* pathotypes (P2, P3, P5, P6, and P8), PCR analysis was conducted using 118 pairs of primers, with each of primer pairs specific to a gene encoding a protein or represented by an EST. Among these primers, the primer pair f/q amplified a band from the P5 isolate but not from isolates of the other four pathotypes (Fig. 2A). This primer pair was specific to a previously reported EST (JK747455) that is hereafter named *Cr811* in this study. The exclusive presence of *Cr811* in the isolate of P5 was confirmed by an alternative primer pair g/u as well as all possible combinations between forward primers a, b, c, d, and e and reverse primers v, w, x, y, and z (data not shown). Using three pairs of qPCR primers, h/r, i/s, and j/t, reverse transcription (RT)-PCR was conducted against RNA from the isolates representing the five pathotypes. Transcript could be detected from the P5 isolate but not from the other isolates, indicating the exclusiveness of *Cr811* in RNA of the P5 isolate (data not shown). The PCR and RT-PCR described above were repeated against DNA and RNA extracted from galls produced on 'Westar' inoculated with resting spores from the original galls. A band or signal was produced only from the P5 isolate, indicating the vertical inheritance of *Cr811* in this isolate (data not shown).

Dot blotting using the *Actin1* fragment as a probe resulted in signals with similar intensities from all the five isolates. In contrast, the *Cr811* probe produced a signal from the DNA of the P5 isolate and no signal from isolates of the other pathotypes (Fig. 2B), suggesting that these other isolates lack the complete probe-specific sequence in the *Cr811* region.

***Cr811* encodes a putative peptide with unknown function.** The PCR product of primer pair g/u against genomic DNA of the P5 isolate was sequenced and TA cloned. The resulting sequence had one base pair difference with the region between the two primers on the cDNA clone JK747455. After genome walking, an 1,827-bp sequence including the 5' region, the sequence flanking JK747455 and the 3' region was obtained. This sequence was submitted to GenBank with the accession number KJ867531. A Blastn search with this sequence as a query did not produce any hit other than JK747455. Against cDNA from the P5 isolate, a PCR product was obtained using primer pair a/s and its sequence was identical with the region flanked by the two primers on the genomic sequence, indicating that this fragment contains a partial reading frame without introns. The cDNA sequence was submitted to GenBank with the accession number KJ867530. Using the ORF Finder software, a 492-bp coding sequence was predicted that is located at nucleotides 281-772 on KJ867530. A Blastx search with this cDNA sequence as a query produced three hits with an E-value smaller than 1 and aligned sequences larger than 150 amino acids: a hypothetical protein (RO3G_17302) from *Rhizopus delemar* with 30% identity and an E-value = 1×10^{-10} , an uncharacterized protein (UHOR_12604) from *Ustilago hordei* with 27% identity and an E-value = 2×10^{-4} , and an uncharacterized protein (CCA27723) from *Albugo laibachii* with 22% identity and an E-value = 0.011. The ORF finder-predicted coding sequence was in-frame with but

Fig. 2. Identification and confirmation of the presence of the *Cr811* gene solely in the pathotype 5 single-spore isolate of *Plasmodiophora brassicae*. Lanes 2 to 8, DNA from the five pathotypes. **A**, Polymerase chain reaction identification of *Cr811*. *Actin1* and *Cr811*, primer pairs ActF/ActR for the *Actin1* gene and f/q for *Cr811*. M, DNA marker. **B**, Dot blot analysis. The probe is specific to *Actin1* (left) or *Cr811* (right).

shorter at the 5' end than the portion of the cDNA sequence being aligned with each of the three Blastx hits. This suggests that the predicted coding sequence is the 3' part of a real ORF. Since all Blast hits encode uncharacterized proteins, no biological function can be inferred for *Cr811*.

***Cr811* is present as a single copy in the *P. brassicae* genome.** Given the fact that it is almost impossible to obtain pure *P. brassicae* DNA without contamination from other organisms, experiments were designed to confirm the *P. brassicae*-origin of *Cr811*. One milliliter of gall slurry that contained 3.75×10^6 resting spores was prepared from the P5-infected 'Westar' and cultured in 20 ml of LB medium at 37°C. After 16 h, the medium became cloudy, indicating the proliferation of bacteria. Total DNA was extracted from the culture pellet. Against this DNA sample and the DNA from infected roots collected at 35 dai of '45H26' and '45H29' with the P5 isolate, qPCR analysis was conducted with the primer pair h/r for *Cr811* and primer pair ActF/ActR for *Actin1* as a control. The Δ CT values between *Cr811* and *Actin1* in the three DNA samples were not significantly different (Fig. 3A), indicating that the amount of *Cr811* DNA is proportional to the *P. brassicae* biomass represented by the CT value of *Actin1*, but not to the amount of total DNA. The data confirm that *Cr811* is not present in bacteria associated with *P. brassicae* or resident in the soil, and is most likely in the *P. brassicae* genome.

Southern analysis was conducted to investigate the copy number of *Cr811*. A hybridization signal was detected from a single band of P5 DNA cut with *XhoI*, indicating the single-copy nature of *Cr811* in the genome of the P5 isolate.

Expression of *Cr811* is up-regulated in infected plants. During the infection of the susceptible cultivar 45H26, the expression levels of *Cr811* in general were lower than those of the control gene *Actin1*. At 5 dai, the expression of *Cr811* was just 1% that of *Actin1*. The expression level increased at 7 dai and reached a peak at 14 dai, when it was 80% that of *Actin1*. After 14 dai, the expression decreased (Fig. 4A). In the resistant cultivar 45H29, a primary peak of expression was observed at 5 dai, in which the expression of *Cr811* was 1.5 times that of *Actin1*. The expression declined to a low level until 21 dai. At 28 and 35 dai, the expression increased dramatically to 600 times higher than *Actin1* (Fig. 4B). The roots of '45H26' and '45H29' at 35 dai were sampled and examined microscopically. The galls of '45H26' were full of resting spores. By contrast, no typical galls were obtained on '45H29'. Nonetheless, plasmodia were observed in the roots of '45H29' (Fig. 4C).

***Cr811* distinguished a field isolate of P5 from two other field isolates representing P3 and P8.** Galls caused by natural infection were collected from different field locations in Alberta, Canada. Three field isolates were obtained from the galls and evaluated by PCR analysis with the primer pairs g/u and ActF/ActR. A band was amplified by g/u from one field isolate but not from the other two isolates (Fig. 5A). The pathotype identities of these three isolates were determined by their reactions on the Williams' differential hosts and ECD hosts (Supplementary Table 1; Fig. 5B). The isolate that was able to produce the g/u band was classified as P5 on Williams hosts and 16/6/8 on ECD hosts, which are identical to the pathotyping results of the single spore isolate ORCA-ss4. The other two isolates were classified as P3 or P8 on Williams' hosts and 16/15/12 or 16/15/8 on the ECD hosts. This result suggests that *Cr811* may have potential as a marker to distinguish P5 or 16/6/8 from other pathotypes of *P. brassicae*.

DISCUSSION

Differences in virulence among *P. brassicae* populations have been studied using various sets of differential hosts, with isolates of the pathogen classified into pathotypes (39). Although the molecular basis for differential classification of pathotypes is largely unknown, a notion accepted by most researchers is that the

difference between pathotypes ultimately reflects differences in their genetic makeup, i.e., either the presence or absence of certain pathogenicity-related genes, or differential expression of those genes. In the current study, *Cr811* was identified only in P5 isolates. The identity of *Cr811* as a gene rather than a fragment of noncoding DNA is supported by that facts that it has been repeatedly isolated from mRNA preparations and that it showed regulation patterns independent from the pathogen biomass. This gene was first identified by suppression subtractive hybridization between infected and healthy canola roots (14). In a previous study, the expression of *Cr811* in 'Westar' was found to be higher in secondary zoospores than primary zoospores (15), suggesting that it has some role in the secondary stage of clubroot infection.

In the current study, we did not intend to define the types of spores but tried to monitor the *Cr811* expression at different infection stages with a time-course. Nevertheless, the increased expression of *Cr811* in '45H26' from 0 to 14 dai observed in the current study is consistent with the findings of Feng et al. (15), indicating that the in planta regulation of *Cr811* in various susceptible genotypes is similar during the early stages of infection. On the other hand, regulation of *Cr811* expression at later infection stages in the susceptible genotype 45H26 was dramatically different from that in the resistant genotype 45H29. This may indicate that *Cr811* is specifically up-regulated in secondary plasmodia, similar to the *Y10* gene (21) and the *PbBrip9* and *PbCC249* genes (37) that have been found to be strongly expressed at stages of disease development corresponding to the occurrence of secondary plasmodia. In both '45H26' and '45H29', primary infection and secondary infection are essentially similar in manner and timing. In both genotypes, secondary infection is established at approximately 14 dai. The CT values of the *Cr811* transcript in resistant and susceptible genotypes were similar at 14 dai because similar numbers of secondary plasmodia were present in both genotypes. After 14 dai, however, the secondary plasmodia in the susceptible genotype matured and large numbers of resting spores were formed, resulting in a much lower CT value for *Actin1* than in the resistant genotype. In the resistant genotype, no resting spores were formed and secondary plasmodia continued to accumulate in the root cortex. This was observed not only in '45H29' in the current study, but also in other resistant genotypes (10,23) and even in nonhost plants (18). In resistant plants *P. brassicae* cannot progress further than the plasmodial stage, which results in a relatively high number of plasmodia that build up in the cortex. By contrast, the pathogen progresses beyond this initial plasmodial stage in susceptible plants

Fig. 3. Confirmation of the occurrence of the *Cr811* gene in the genome of *Plasmodiophora brassicae* pathotype 5 as a single copy. **A**, Quantitative polymerase chain reaction analysis of DNA derived from P5-infected plants at 35 days after inoculation. LB, overnight LB culture of gall slurry prepared from canola cultivar Westar; H26 and H29, and galls collected from canola cultivars 45H26 and 45H29, respectively. Means in the plot marked by the same letter do not differ based on Fisher's least significant difference test at $P \leq 0.01$ ($n = 3$). **B**, Southern analysis. Genomic DNA from the P5 single-spore isolate was digested with *XhoI* and hybridized with a PCR fragment specific to the *Cr811* gene. M, DNA marker. The arrow indicates that signal is evident from a single band.

and, therefore, fewer plasmodia are seen in the cortex (23). If *Cr811* is up-regulated in secondary plasmodia and down-regulated in resting spores, the different numbers of resting spores and plasmodia in '45H26' and '45H29' could explain the expression patterns observed in Figure 4A and B.

A preliminary genomic study indicated that *P. brassicae* genes are rich in spliceosomal introns (5), a finding that was supported by functional studies of individual *P. brassicae* genes. For example, the serine protease gene *PRO1* contains eight introns within the 1,847-bp ORF (13). By contrast, the 492-bp reading frame of *Cr811* does

not contain any introns. This sequence may represent one of the exons in the intron-containing *Cr811* gene or, on the other hand, *Cr811* may not contain any introns. These possibilities will be examined in a subsequent gene functional study.

The evolution of pathogens and their hosts is often interpreted as an arms race, in which both hosts and pathogens can respond to changes that occur in their opponents. This arms race is accelerated after a pathosystem is moved from a natural ecosystem to agroecosystem (2). New pathotypes or races are generated in the pathogen population at a higher speed, usually achieved by mutations in single

Fig. 4. Expression of *Cr811* during plant infection. Canola cultivars **A**, 45H26 and **B**, 45H29 were inoculated with pathotype 5 of *Plasmodiophora brassicae* and the infected root samples were collected at 0 to 35 days after inoculation. The primers used for quantitative polymerase chain reaction analysis were ActF/ActR for the *Actin1* gene and h/r for *Cr811*. Means ± standard deviation in the plot annotated by the same letter do not differ based on Fisher's least significant difference test at $P \leq 0.01$ ($n = 3$). **C**, Root cortex of canola cultivars 45H26 and 45H29 at 35 days after inoculation. Bars = 10 μ m.

effector genes (8). In addition, horizontal gene transfer (HGT) also provides a means for pathogens to create new virulent forms (races or pathotypes) or to broaden their host range (27,31). HGT is very common in protists (1,34) and thus *P. brassicae* may be a HGT amiable species. Phylogenetic and systemic studies suggested that clubroot is a disease of cultivation (9) and *P. brassicae* likely became a pathogen of crucifers by means of cross-kingdom host shifts (30), increasing the likelihood that HGT has been involved in the coevolution of *P. brassicae* and cruciferous plants. The amenability of *P. brassicae* for HGT has been supported by the successful genetic transformation of this pathogen (16) and by the observation that the same EST sequences were identified from both *P. brassicae* (6) and the host plants (24). The ability to accept foreign DNA may also be related to the unusual variability seen in *P. brassicae* field populations (26,38,43). In the current study, the presence of the *Cr811* gene in P5 isolates of *P. brassicae* and its complete absence in isolates representing other pathotypes suggests that this gene may be a result of HGT. Furthermore, the absence of an intron in the 492-bp reading frame strengthens the possibility that *Cr811* is a result of HGT.

The relationship between diversity in pathogenicity (races and pathotypes) and DNA polymorphisms in *P. brassicae* populations has been studied, mainly by RAPD marker analysis (4,11,25,29,44). Yano et al. (44) reported that RAPD analysis was able to clearly differentiate Williams' race 9 populations from populations of other races. Similarly, Manzanera-Dauleux et al. (25) developed an RAPD-derived SCAR marker specific to P1, as classified on the differentials of Somé et al. (38) of *P. brassicae*. The *Cr811* gene identified in the current study may prove useful as another marker to differentiate *P. brassicae* pathotypes. Nonetheless, the specificity of this marker for P5 will have to be confirmed by testing a larger collection of isolates with broader geographic origins. With optimized techniques for *P. brassicae* DNA purification and single-spore isolation, along with the accumulation of publicly available *P. brassicae* sequences, more genetic markers able to differentiate pathotypes of the parasite are expected to be found.

In summary, the current study indicates that the *Cr811* gene is present in several P5 isolates of *P. brassicae*, but not in isolates representing P2, P3, P6, or P8, and therefore may serve as a genetic marker to identify P5 from isolates of other pathotypes. It has not escaped our notice that the exclusive presence of *Cr811* in the tested P5 isolates provides a useful tool in studies on pathotype dynamics under the selection pressure of various resistance genes. The gene is highly expressed in secondary plasmodia, suggesting a potential role in pathogenicity during this stage of infection.

Fig. 5. Identification of pathotype 5 in three field isolates of *Plasmodiophora brassicae* **A**, by polymerase chain reaction analysis using *Cr811*-specific primers and **B**, by pathogenicity tests on the Williams and the European clubroot differential (ECD) hosts. *Actin1* and *Cr811*, primer pairs ActF/ActR for the *Actin1* gene and g/u for *Cr811*; M, DNA marker. Pathogenicity data are derived from four replications of the experiment, each consisting of 12 plants.

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