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Project Overview

Project number:	2019F023R
Project title:	Strelkov - A rapid molecular assay to identify Plasmodiophora brassicae pathotypes from plant and soil samples
Project start date:	
Project completion date:	
This is an interim report for the reporting period to	

Research Team

Principal Investigator:	
Name:	**Institution:**
Stephen Strelkov	University of Alberta
Research team members:	

Non-technical summary

Provide a summary of the project results which could be used by the funder(s) for communication to industry stakeholders (e.g., producers, processors, retailers, extension personnel, etc.) and/or the general public. This summary should give a brief background as to why the project was carried out, what were the principal outcomes and key messages, how these outcomes and key messages will advance the livestock and meat industry, how they will impact industry stakeholders and/or consumers, and the economic benefits for the industry.

Clubroot of canola, caused by the soilborne pathogen Plasmodiophora brassicae, has become a serious threat to canola production in Canada. The deployment of clubroot-resistant (CR) cultivars is the most commonly used management strategy; however, the widespread cultivation of CR canola has resulted in the emergence of new pathotypes of P. brassicae capable of overcoming resistance. Several host differential sets have been reported for pathotype identification, but such testing is time-consuming, labor-intensive, and based on phenotypic classifications. The development of rapid and objective methods that allow for efficient, cost-effective and convenient pathotyping would enable testing of a much larger number of samples in shorter times. The objective of this project was to develop rapid molecular assays to identify Plasmodiophora brassicae pathotypes from plant and soil samples.

Two molecular tests were developed: an RNase H2-dependent PCR (rhPCR) assay and a SNaPshot assay. Both could clearly distinguish between pathotype clusters in a collection of 38 single-spore isolates of P. brassicae. Additional isolates pathotyped from clubbed roots and samples from blind testing also were correctly clustered. The rhPCR assay generated clearly differentiating electrophoretic bands without non-specific amplification. The SNaPshot assay was able to detect down to a 10% relative allelic proportion in a 10:90 template mixture with both single-spore isolates and field isolates when evaluated in a relative abundance test.

The high-throughput potential and accuracy of both assays makes them promising as SNP-based pathotype identification tools for clubroot diagnostics. rhPCR is a highly sensitive approach that can be optimized into a quantitative assay, while the main advantages of SNaPshot are its ability to multiplex samples and alleles in a single reaction and the detection of up to four allelic variants per target site.

Project details

Project team

a. Describe the contribution of each member of the R&D team to the functioning of the project.
b. Describe any changes to the team which occurred over the course of the project.

The project was led by Stephen Strelkov, who supervised the research, helped interpret the data, reviewed and edited the resulting scientific papers, and contributed to the communication of the results through reports and presentations to industry, grower and government stakeholders.

The Research Associate Leo Galindo-Gonzalez (initially a postdoctoral fellow when the project began) contributed to the development of the research concept, helped design and plan the experiments and interpret the data, and reviewed and edited the scientific papers.

A graduate student, Heather Tso, helped to plan the experiments, conducted much of the lab work, interpreted the data and wrote the first drafts of the scientific papers. Troy Locke (technician) provided assistance and suggestions regarding technical aspects of the assays. Sheau-Fang Hwang provided plant pathology expertise and materials, and contributed to research discussion. Finally, a number of summer students and other technical staff provided support in conducting the experiments.

Abbreviations

Define ALL abbreviations used.

CCD = Canadian Clubroot Differential
CR = clubroot-resistant
ddNTP = dideoxynucleotide
ECD = European Clubroot Differential
FI = field isolate
rhPCR = RNase H2-dependent PCR
SNP = single nucleotide polymorphism
SSI = single-spore isolate

Background

a. Review the project background and update as needed.
b. State the related scientific and development work that has been completed to date by your team and/or others.

Clubroot, caused by Plasmodiophora brassicae, is an important soilborne disease of crucifers. In Canada, clubroot is a major constraint to canola production, with the disease managed primarily by planting clubroot-resistant (CR) cultivars. The widespread cultivation of CR canola has resulted in the emergence of multiple new pathotypes of the clubroot pathogen, many of which can overcome host

resistance. It is important to understand the distribution and occurrence of these novel pathotypes, in order to make informed crop management decisions [1-5].

Traditionally, the identification of P. brassicae pathotypes has relied on bioassays, with pathogen isolates inoculated onto a series of differential hosts, and then grouped into pathotypes based on their virulence patterns on these hosts. Various differential systems have been developed, including the hosts of Williams [6], Somé et al. [7], the European Clubroot Differential (ECD) set [8] and, most recently, the Canadian Clubroot Differential (CCD) set [4] and Sinitic Clubroot Differential set [9]. The CCD set is now the most widely used differential system in Canada, and was developed to improve the identification of resistance-breaking pathotypes from canola [2, 4]. While effective, the use of host differential sets is time-consuming, labor-intensive, and requires biosecure plant growth facilities. Molecular assays would facilitate rapid pathotype identification and testing of much larger numbers of samples.

Various molecular markers have been explored for P. brassicae pathotyping. A random amplified polymorphic DNA marker specific to pathotype P1, as defined on the differentials of Somé et al. [7], was identified and converted into a sequence-characterized amplified region [10]. The Cr811 gene was found to be exclusive to pathotype 5 [11], as defined on the differentials of Williams [6], and hence could serve a diagnostic purpose. A region of the 18S internal transcribed spacer sequence specific to pathotype 5X, as defined on the CCD set, was used to develop a probe-based qPCR assay for detection of this pathotype [12]. Five molecular markers were found to distinguish pathotypes 4, 7, 9, and 11 [13], as classified on the differentials of Williams [6]. Recently, over 1500 SNPs were identified as differentiating two genetically distinct P. brassicae populations from Alberta, enabling development of population-specific markers [14, 15]. Two rhPCR [16] primer pairs were also developed to differentiate a new, resistance-breaking “pathotype 3-like strain” of P. brassicae from the original pathotype 3 [17]. However, neither the exact nature of this pathotype 3-like strain, nor its CCD classification, were available. To our knowledge, no rhPCR-based assays have been reported to distinguish between pathotype clusters in P. brassicae isolate collections. Similarly, there are no reports of the use of SNaPshot technology [18] for the identification of P. brassicae pathotypes.

The novel allelic discrimination technology, rhPCR, provides greater accuracy and sensitivity relative to conventional PCR [16]. Amplification with rhPCR requires perfect binding of primers to the target, allowing differentiation of samples with a single nucleotide difference. SNaPshot is a modified sequencing single base extension reaction that enables discrimination based on SNPs [18]. Differentiating SNPs are identified based on a fluorescent color corresponding to one of the four possible alleles. In this project, we developed and validated two independent assays based on rhPCR and SNaPshot technologies to differentiate between a pathotype 5X cluster and a pathotype 3H cluster of P. brassicae, as defined on the CCD set.

Objectives

a. Review the original objective(s).
b. Indicate any modifications to the objective(s) that occurred over the course of the project.

a) Main objective: To generate an effective, sensitive and rapid molecular (PCR-based) assay to identify abundance and diversity of Plasmodiophora brassicae pathotypes in soil and plant samples.
Main deliverable: A quick and reliable assay to identify P. brassicae pathotypes from soil and plant samples, to facilitate clubroot management and resistance breeding in canola.

b) In the original proposal, the focus was on developing an rhPCR assay, and this was successfully accomplished. In addition, we were also able to develop a second assay based on SNaPshot technology. This was possible because much of the genomic and sequence information we required for the SNaPshot assay became available as we developed the rhPCR assay, so that with additional effort by the team, we were able to generate two molecular assays to distinguish pathotype clusters.

Research design and methodology

In summary, describe the project design, methodology, laboratory and statistical analysis used to carry out the project. Please provide sufficient detail to determine the experimental and statistical validity of the work and give reference to relevant literature where appropriate. For ease of evaluation, please structure this section according to the objectives cited above.

SNP selection:
Thirty-eight P. brassicae single-spore isolates were included in this study. The isolation of purified genomic DNA from resting spores used in our study was previously reported [19]. The DNA was quantified with a Qubit 2.0 DNA HS Assay (Thermo Scientific, Waltham, MA, USA) and DNA quality was assessed with a TapeStation Genomic DNA Assay (Agilent Technologies, Santa Clara, CA, USA). Samples were then sent to Admera Health (South Plainfield, NJ, USA) for library preparation, next-generation sequencing, and variant calling. The sequencing library was prepared using a KAPA Hyper Prep Kit (Roche, Basel, Switzerland) as per the manufacturer's recommendations. Library quality and quantity were evaluated with a Qubit 2.0 DNA HS Assay (Thermo Scientific, Waltham, MA, USA) and TapeStation High Sensitivity D1000 Assay (Agilent Technologies, Santa Clara, CA, USA). The prepared library was then sequenced (2 x 150bp reads) on an Illumina® HiSeq X instrument. Sequencing reads were aligned to the 2015 e3 reference genome for P. brassicae [20] (European Nucleotide Archive project PRJEB8376). Variants were called from high quality aligned reads using HaploTypeCaller with filters of overall read depth equal to or larger than 15 (DP ≥ 15) and quality equal to or larger than 40 (GQ

≥ 40) to produce variant call format (vcf) files per each isolate. SOAPdenovo v2.01 was used to assemble the reads into draft assemblies.

We loaded the resulting vcf files into the Integrative Genomics Viewer to visualize polymorphisms and identify candidate SNP loci among the 11 CCD pathotypes represented in our 38 SSIs. All polymorphisms utilized for our assays came from alignments of all our isolates classified using the CCD set. To confirm the polymorphic region found in contig 1 [20] that differentiates the 5X pathotype cluster from the 3H cluster, a conventional PCR primer pair was designed to amplify the region encompassing the SNPs through Sanger sequencing. Forward primer SEQ1-43778fw 5'-GCCTGTCGAACGTCTGTT-3' and reverse primer SEQ1-43778rv 5'-ATAAAGTCTGGACACGAGAACG-3' were designed using PrimerQuest (Integrated DNA Technologies, Coralville, IA, USA) with parameters that included primer length ranging between 18-24 bases, GC content ranging between 40-60%, and melting temperature of 60°C. This set produced a 508 base pair amplicon to confirm SNPs used for both the rhPCR and SNaPshot assays. The primers were evaluated for specificity with command line BLAST v. 2.6.0 against the reference e3 P. brassicae genome [20]. The argument - task "blastn-short" was used as this task is optimized for short sequences of less than 30 nucleotides. The primers were also subjected to a BLAST search in the National Center for Biotechnology Information (NCBI) online database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to ensure specificity to P. brassicae.

Three SSIs from each cluster were selected for Sanger sequencing of the amplicons to validate the presence of polymorphisms detected using whole genome sequencing. The SSIs ST11 (5X), ST23 (5X) and SR20 (6B) were tested from the reference 5X cluster, and SSIs SL09 (2F), SS48 (3H), and SW30 (3H) were tested from the alternate 3H cluster. PCR analyses were carried out in a 20 µL final volume containing 1X reaction buffer, 2 mM $MgCl_2$, 0.2 mM dNTPs, 0.4 µM of each forward and reverse primers, 1 U Platinum Taq DNA polymerase (Invitrogen, Waltham, Massachusetts, USA), 10 ng of genomic DNA as template, and 13.7 µl nuclease-free water. All reactions were conducted in a Veriti 96-Well Thermal Cycler (Applied Biosystems, Waltham, MA, USA) under the following cycling conditions: 2 min at 94°C, followed by 40 cycles of 30 s at 94°C, 1 min at 63°C, and 1 min at 72°C, with a final 10 min extension at 72°C. Samples were held at 4°C. Four technical replications of each sample were performed. The PCR products from one replicate per each sample were resolved by electrophoresis on a 1% agarose gel to confirm the presence of specific amplification, product size and intensity. The other three replicates were combined and purified using the Wizard SV Gel and PCR Cleanup System (Promega, Madison, WI, USA) following the manufacturer's specifications. The quality and quantity of purified DNA were verified on a NanoDrop spectrophotometer (Thermo Scientific, Waltham, MA, USA), then sent for Sanger sequencing at the Molecular Biology Service Unit, Department of Biological Sciences, University of Alberta (Edmonton, AB, Canada). The resulting sequences were visualized and SNPs were confirmed with Sequencher 5.0 (Gene Codes Corporation, Ann Arbor, MI, USA).

RNase H2-dependent PCR:
The reference rhPCR primer pair was designed to amplify isolates of the 5X cluster; it was referred as the 'reference cluster' since the SNPs also belonged to the P. brassicae e3 reference genome [20]. The alternate rhPCR primer pair was designed to amplify isolates of the 3H cluster. In addition to the differentiating SNPs positioned against the ribonucleotide bases, the primers were positioned in a polymorphic region that would allow for multiple SNPs to increase specificity. There were five SNPs between the forward primers and two SNPs between the reverse primers. These sets produced a 230 base pair amplicon. The specificity of the primers was evaluated with command line BLAST v. 2.6.0 against the e3 reference genome. The primers were also subjected to a BLAST search in the NCBI online database to ensure specificity to P. brassicae.

The specificity of the primers and the rhPCR block-cleavable technology was evaluated against gBlocks gene fragments (Integrated DNA Technologies, Coralville, IA, USA), double-stranded synthetic oligonucleotides. One gBlock was designed to replicate the 5X polymorphic region sequence, and another was designed to replicate the 3H polymorphic sequence. The gBlock gene fragment contained the 230 base pair rhPCR amplicon in its entirety, plus an additional 100 base pairs upstream and downstream from the amplicon. PCR analyses were carried out in a 20 µL final volume containing 1X reaction buffer (Applied Biosystems, Waltham, MA, USA), 2 mM MgCl2, 0.2 mM dNTPs, 0.4 µM of each forward and reverse primer, 1 U Platinum Taq DNA polymerase (Invitrogen, Waltham, MA, USA), 5.2 mU RNase H2 enzyme, and 5 ng gBlock as template. The gBlock testing was run in a Veriti 96-Well Thermal Cycler (Applied Biosystems, Waltham, MA, USA) under the following cycling conditions: 2 min at 94°C, followed by 12 cycles of 10 s at 94°C and 30 sec at 70°C. Samples were held at 4°C until the PCR products were electrophoresed on a 1% agarose gel. The block-cleavable technology was also tested by repeating the PCR, but with the RNase H2 enzyme excluded from the master mix as a control.

The rhPCR primer pairs were then evaluated and optimized against the SSIs in our collection: 13 isolates belonging to the 5X cluster and 25 isolates belonging to the 3H cluster. PCR analyses were carried out in a 20 µL final volume containing 1X reaction buffer, 2 mM MgCl2, 0.2 mM dNTPs, 0.4 µM of each forward and reverse primer, 1 U Platinum Taq DNA polymerase (Invitrogen, Waltham, Massachusetts, USA), 5.2 mU RNase H2 (Integrated DNA Technologies, Coralville, IA, USA), and 10 ng genomic DNA as template. The reaction was run in a Veriti 96-Well Thermal Cycler (Applied Biosystems, Waltham, MA, USA) under the following cycling conditions: 2 min at 94°C, followed by 35 cycles of 10 s at 94°C and 30 sec at 70°C. Annealing temperatures and extension times for PCR were determined according to the primer sequence and amplicon size. Samples were held at 4°C until the amplicons were electrophoresed on a 1% agarose gel.

SNaPshot:
A conventional PCR primer pair was designed to generate the template for the SNaPshot extension

reaction. The primer sites to generate this product were conserved among the 38 SSIs and targeted a region that contained the differentiating SNP. The same forward primer SEQ1-43778fw previously designed for Sanger sequencing was used in conjunction with a newly designed reverse primer SEQ1-43778rv2 5'-CTCGAACTCTTTGTCGTCGTT-3'. This set generated a 304 base pair amplicon corresponding to coordinates 43671 to 43974 from contig 1 of the e3 reference genome [20]. The selected differentiating SNP was used earlier as one of the SNPs within the forward primer region of our rhPCR assay. A SNaPshot primer snpsht1-43778 5'-AAAAAAACGATAACGTCGTGGACGACGGCG-3' was designed upstream of the polymorphic base to distinguish pathotypes. A seven nucleotide non-homologous polyA tail was added to the 5' end to bring the length of the primer to 30 nucleotides long, the minimum length recommended for the assay (Applied Biosystems, Waltham, MA, USA). The complementary region between the primer and template was kept at 23 nucleotides, to maintain an annealing temperature of 50°C that matched the annealing temperature (50°C) of the SNaPshot control primer (Applied Biosystems, Waltham, MA, USA). The primer was subjected to reverse phase high performance liquid chromatography purification (Integrated DNA Technologies, Coralville, IA, USA).

All of the SSIs were also tested in the SNaPshot assay. Template generation was carried out in a 20 µL final volume PCR containing 1X reaction buffer, 2 mM $MgCl_2$, 0.2 mM dNTPs, 0.4 µM of each forward and reverse primer, 1 U Platinum Taq DNA polymerase (Invitrogen, Waltham, Massachusetts, USA), and 10 ng genomic DNA as template. The reaction was run in a Veriti 96-Well Thermal Cycler (Applied Biosystems, Waltham, MA, USA) under the following cycling conditions: 2 min at 94°C, then 40 cycles of 30 s at 94°C, 1 min at 63°C, and 1 min at 72°C, followed by a final 10 min extension at 72°C. Samples were held at 4°C. Four technical replications were included for each sample. The PCR products of a single replicate from each sample were resolved on a 1% agarose gel to confirm the presence of the specific amplicon and product intensity. The other three replicates were combined and purified using the Wizard SV Gel and PCR Cleanup System under manufacturer specifications (Promega, Madison, WI, USA). gBlocks corresponding to each 5X and 3H cluster were also designed and used to run control reactions in parallel. 5 ng of gBlocks were used as template instead of 10 ng genomic DNA, to reduce the copy number of this region sequence, and only 12 cycles were conducted in the PCR instead of 40 cycles, as recommended by the manufacturer (Integrated DNA Technologies, Coralville, IA, USA).

The SNaPshot Multiplex Kit (Thermo Scientific, Waltham, MA, USA) was used for the extension reaction in a 10 µL final volume containing 1X master mix (Thermo Scientific, Waltham, MA, USA), 0.2 µM SNaPshot primer, and 0.2 pmol SNaPshot template. The extension reaction was carried out in a Veriti 96-Well Thermal Cycler (Applied Biosystems, Waltham, MA, USA) under the following cycling conditions: 25 cycles of 10 s at 96°C, 5 s at 50°C, and 30 s at 60°C, then held at 4°C. Control reactions with a control template and control primers supplied by the SNaPshot Multiplex Kit (Thermo Scientific, Waltham, MA, USA) were run in parallel under the same cycling conditions. Extension reaction products were then subjected to a post-extension treatment with SAP (New England BioLabs, Ipswich, MA, USA) to remove

any unincorporated ddNTPs. One unit of SAP was added to each sample, and then incubated for 60 min at 37°C, followed by 15 min at 75°C, and held at 4°C.

Treated extension products were then prepared in a 96-well plate for capillary electrophoresis. Each injection was performed at a final volume of 10 µL containing 9 µL Hi-Di formamide (Applied Biosystems, Waltham, MA, USA), 0.5 µL GeneScan 120 LIZ size standards (Applied Biosystems, Waltham, MA, USA), and 0.5 µL extension product. The plate was incubated for 5 min at 95°C, and capillary electrophoresis was carried out in an ABI Prism 3730 Genetic Analyzer (Applied Biosystems, Waltham, MA, USA) at the Molecular Biology Service Unit, Department of Biological Sciences, University of Alberta (Edmonton, AB, Canada). The Peak Scanner v1.0 (Applied Biosystems, Waltham, MA, USA) was used to determine the SNP allele based on the resulting fluorescence peak.

Extraction of DNA from root galls for evaluating the rhPCR and SNaPshot assays:
The performance of the SNaPshot and rhPCR assays was evaluated with 12 canola root galls representing different field and single-spore isolates that had been previously pathotyped using the CCD set. The P. brassicae DNA from the galls was isolated using the cetyltrimethylammonium bromide (CTAB) extraction method [21], followed by phenol-chloroform purification. The CTAB lysis buffer was prepared with 2% CTAB (w/v), 100 mM Tris-HCl (pH 8.0), 20 mM EDTA (pH 8.0), and 1.4 mM NaCl, and the buffer was adjusted to pH 8.0 prior to sterilization in an autoclave. The galls were frozen at -80°C for 24 h, and then ground in liquid nitrogen with a mortar and pestle. The resultant ground sample (200 mg from each gall) was transferred into a 2 mL microcentrifuge tube and 600 µL of CTAB extraction buffer was added. The samples were incubated at 60°C for 20 min, during which samples were mixed by inversion every 5 min. After incubation, an equal volume of 600 µL phenol:chloroform:isoamyl alcohol (25:24:1, v/v) was added, vortexed, and centrifuged at 14,000 rpm for 5 min. The top aqueous phase (supernatant) was transferred to a new 2 mL microcentrifuge tube, and was subjected to two more rounds of phenol:chloroform:isoamyl alcohol DNA purification. The purified DNA was then precipitated in 700 µL of 100% ice-cold isopropanol; samples were mixed by inversion, placed on ice for 10 min, and then centrifuged at 14,000 rpm for 8 min. The isopropanol was discarded and the precipitated DNA pellet was washed with 500 µL of 80% ice-cold ethanol; the sample was vortexed until the pellet detached off the tube, and then centrifuged at 14,000 rpm for 3 min. The ethanol was discarded and the remaining pellet was left at room temperature to air dry. Once dried, the DNA was dissolved and resuspended in 100 µL sterile nuclease-free water. The concentration and purity of each sample were determined with a NanoDrop 1000 spectrophotometer (Thermo Scientific, Waltham, MA, USA), and DNA integrity was assessed by loading 200 ng per sample onto a 1% agarose gel. Samples were then diluted to a working concentration of 5 ng/µL with sterile nuclease-free water and stored at -20°C. The samples were tested in the SNaPshot and rhPCR assays under the same conditions as described above.

Testing of relative abundance:

Different proportions of mixed isolates were tested to assess the capacity of the SNaPshot assay to determine relative abundances. Three different two-isolate mixtures were evaluated with the different proportions of 10:90, 20:80, 30:70, 40:60, and 50:50. Mixtures were prepared prior to template generation to simulate conditions where a root gall developed from a mixed infection by more than one pathotype. Ten ng of total genomic DNA was used for the PCR. The entire SNaPshot assay procedure from template generation to capillary electrophoresis followed the same protocol as described earlier.

Blind testing:
Blind testing was conducted with the rhPCR and SNaPshot assays. While the isolates corresponding to the galls had been previously pathotyped, the experiment was conducted without knowledge of pathotype designations in a single-blind experiment. P. brassicae DNA from 16 blinded galls was extracted according to the CTAB method following the same procedure as described earlier. Blinded samples were subjected to both rhPCR and SNaPshot assays under the conditions described above.

Results, discussion and conclusions

Present the project results and discuss their implications. Discuss any variance between expected targets and those achieved. Highlight the innovative, unique nature of the new knowledge generated. Describe implications of this knowledge for the advancement of agricultural science. For ease of evaluation, please structure this section according to the objectives cited above.

RESULTS

RNase H2-dependent PCR:
We designed two sets of rhPCR primer pairs to distinguish P. brassicae isolates belonging to either one of the pathotype clusters. The specificity of the primers and the rhPCR block-cleavable technology was tested with gBlocks gene fragments. The amplification of the gBlocks with rhPCR generated bands of the expected 230 base pair amplicon. The primer pair rh1-43812R was specific to the gBlock designed to replicate the reference polymorphic sequence, and yielded no visible PCR products with the alternate gBlock. The primer pair rh1-43812A was specific to the gBlock designed to replicate the alternate polymorphic sequence, and yielded no visible PCR products with the reference gBlock. This confirmed that the rhPCR primer pairs were specific to the targeted polymorphic sequences. No amplification occurred with the no RNase H2 enzyme control.

Amplification of P. brassicae pathotype single-spore isolates (SSIs) with our rhPCR primer pairs generated strong bands of the expected 230 base pair size when using 10 ng of purified genomic DNA template. The primer pair rh1-43812R was specific to pathotypes of the reference cluster. Amplification of all 13 SSIs from the reference cluster using the reference primer pair produced single bands and yielded no visible PCR products with the alternate primer pair. In contrast, the primer pair rh1-43812A

was specific to pathotypes of the alternate cluster. Amplification of all 25 SSIs from the alternate cluster using the alternate primer pair produced bands, while no visible PCR products were obtained with the reference primer pair. The only exception was with the alternate cluster SSI ST40 classified as pathotype 3A, which produced bands of equal intensity with both primer pairs. The same 230 base pair amplicons were observed when the two primer pairs were tested against DNA extracted from 12 root galls to evaluate the specificity of the rhPCR assay against additional samples, and each sample only amplified with one primer pair. The sensitivity of the rhPCR assay with DNA extracted from the galls matched that of the DNA from the original SSIs, as the bands were of comparable intensity.

SNaPshot:
We designed a SNaPshot primer, snpsht1-43778, to identify the clustering polymorphic allele in the discriminative SNP position. The SNaPshot primer correctly produced fluorescence peaks of the expected color for all 38 SSIs, with green corresponding to the reference cluster and blue corresponding to the alternate cluster. The SSI ST40, classified as pathotype 3A, yielded both green and blue peaks, showing the existence of both alleles (A and G) in the targeted SNP position. This result is consistent with the results of the rhPCR assay, where amplification of ST40 occurred with both primer pairs, and suggests that this was due to an issue with isolate purity rather than to an error of primer specificity.

The SNaPshot clustering of the DNA samples extracted from canola root galls was consistent with the results of the rhPCR testing. Isolates classified as pathotypes 2F (SACAN-ss3), 3A (F3-14, F185-14, F189-14) and 3H (SACAN-ss1) belonging to the alternate cluster produced blue fluorescence peaks, and isolates of pathotype 5X (LG-1, LG-2, LG-3) belonging to the reference cluster produced green fluorescence peaks. Pathotypes 5I (ORCA-ss3), 6M (AbotJE-ss1), 8E (F187-14) and 8N (CDCN-ss1) were identified as part of the alternate cluster due to their blue fluorescence. This confirmed that the differentiating SNPs selected for assay development occurred beyond our SSI collection. Furthermore, the sensitivity of the SNaPshot primer with the galled root collections matched that of the DNA from the SSIs as fluorescence peaks were of comparable strengths.

The capacity of the SNaPshot assay to determine the relative abundance of different isolates was assessed with three two-isolate mixtures. The first mixture consisted of isolates from our original SSI collection, and the second and third mixtures consisted of DNA extracted from root galls. The assay was able to detect a 10% relative allelic proportion in a 10:90 template mixture. However, relative peak strengths were not always proportional to the abundance ratio of the two isolates within each mixture, and therefore this does not represent a quantitative assay

Blind testing:
The rhPCR and SNaPshot assays were validated in a single-blind study with 16 blinded samples. Samples were assigned into either the reference or alternate clusters based on the results of the rhPCR

amplification and SNaPshot fluorescence peaks. The rhPCR primer pairs produced the expected 230 base pair amplicon, and band intensity was comparable with earlier testing. The SNaPshot primer produced either green or blue fluorescence peaks of comparable strength. After completing the assays, samples 5, 6, 11, 13, and 16 were revealed to be the same SSIs as in our original collection, while samples 1, 3, 7, 8, 12, and 14 were revealed to be the same isolates we had previously used for DNA extraction from root galls. Sample 6, which was revealed as SSI ST40 classified as pathotype 3A, again produced amplicons with both primer pairs in the rhPCR assay and both blue and green peaks with the SNaPshot assay.

DISCUSSION

The aim of this study was to develop P. brassicae pathotyping assays for clubroot diagnostics using discriminating polymorphic regions that differentiate pathotype clusters. Molecular pathotyping of P. brassicae has been limited up to this point, as only a few assays and molecular markers have been reported. The rhPCR and the SNaPshot assays developed in this study are much faster than the use of the CCD or any other host differential set, generating same day results once DNA is extracted. The technologies behind these two assays show strong potential to be specific and reliable for molecular pathotyping. The SNPs used as molecular markers for the development of the assays were tested and confirmed to be specific to the pathotype clusters from which they were designed. Isolate origin had no effect, since all of the SSIs in our original collection and the DNA extracted from the root galls resulted in the same level of specificity with both the rhPCR and SNaPshot assays, and yielded the same 230 base pair amplicon with the rhPCR assay. This suggests that the polymorphic region selected here is consistent among all isolates.

Unlike a previously reported rhPCR assay [17], the assay reported here was developed using a collection of P. brassicae isolates that had been pathotyped on the CCD set. This allows for a more distinct and potentially relevant clustering of pathotypes from Canada, with the ability to link this clustering to the virulence phenotypes of the pathotypes on the hosts of the CCD. The two rhPCR primer pairs simultaneously and specifically amplified the expected pathotype clusters and produced no amplification of pathotypes of the opposing cluster, demonstrating their high specificity for the SNPs in the selected polymorphic region.

The SNaPshot assay is the first of its kind in clubroot diagnostics, as no single base extension assay for the purpose of P. brassicae pathotyping has been reported. Template generation with the conventional PCR primer pair produced an amplicon suitable for the extension reaction. The primer sites were conserved among all the SSIs in our original collection and across the pathotyped galls, with the primers consistently producing the expected 305 base pair amplicon that is used as template for the SNaPshot reaction (see Methods). Our selected differentiating SNP and the target site of the SNaPshot primer was

adequately situated within the amplicon, as indicated by the successful extension of the SNP. The SNaPshot primer accurately produced green fluorescence peaks for pathotypes of the reference cluster and blue fluorescence peaks for pathotypes of the alternate cluster. The assay also was shown to be sufficiently sensitive to detect both pathotypes in two-isolate mixtures in the relative abundance testing.

The rhPCR and SNaPshot assays were able to differentiate pathotypes of the reference 5X cluster from pathotypes of the alternate 3H cluster; however, the one exception was the SSI ST40 classified as pathotype 3A. With this isolate, amplification of products of comparable intensity was observed with both the reference and alternate rhPCR primer pairs, and extension of the SNaPshot primer produced both blue and green peaks. These mixed results from the SSI ST40 were further confirmed in the single-blind study, where ST40 was revealed to be blinded sample 6, for which amplification occurred with both rhPCR primer pairs, and both blue and green peaks appeared with the SNaPshot primer. This indicates that allelic variants of both pathotype clusters are present in the template. In the report where ST40 was first described, it was indicated that while this SSI most closely resembled pathotype 3A, it also displayed characteristics similar to pathotypes 3H, 5X and 6B [2]. As such, the authors of the original study decided to eliminate SSI ST40 from further testing. Since SSI ST40 was supposedly produced from a single-spore, its heterogeneity could reflect an error in the initial single-spore isolation process (e.g., two resting spores attached together), or perhaps mixing during propagation under greenhouse conditions.

The accuracy and sensitivity of the rhPCR and SNaPshot assays should facilitate the analysis of P. brassicae field populations for the presence of heterogeneity. For example, the field isolates LG-1, LG-2, and LG-3, all of which were classified as pathotype 5X [1, 4], presented miniscule but notable blue peaks in addition to the expected green peaks with the SNaPshot assay. This indicates the presence of another pathotype of the 3H cluster (in much smaller proportions) within the 5X field isolates, likely reflecting the coexistence of multiple pathotypes in one field gall [2]. The virulence patterns of FIs on differential hosts often reflect the ‘predominant’ pathotype found in a root gall, and may not capture the extent of heterogeneity in P. brassicae populations from the field.

Initially, our intention was to develop assays to distinguish pathotype 5X from pathotype 3H. However, we found that the discriminatory polymorphic region we selected for our analysis could group many other pathotypes into one of these two main clusters. It is possible that the two pathotype clusters observed in this study correspond to the two genetically distinct populations of P. brassicae identified in an earlier reported study [14], with the 5X and 3H clusters correlating with their “virulent” and “avirulent” populations, respectively. Additional testing will be necessary to confirm this hypothesis.

During the initial primer design stage of this study, we were limited to the whole-genome SNP profiles of the 38 SSIs in our collection. Additional pathotypes for which we did not have sequencing reads were

only later classified into the clusters, based on the results of the rhPCR and SNaPshot assays. Specifically, the isolates ORCA-ss3, AbotJE-ss1, F187-14 and CDCN-ss1, corresponding to pathotypes 5I, 6M, 8E and 8N, respectively, were tested without prior knowledge of which cluster they grouped with, as they were not originally considered nor did we have their corresponding whole-genome sequences. This consideration would also apply to any new P. brassicae pathotypes identified and tested in the future, as the primers were not initially designed to target their variants. If based on these two assays exclusively, clustering of new pathotypes would depend on the allelic variant in the discriminatory SNP positions, which might or might not be consistent with their CCD designation(s) based on virulence phenotype(s).

Since rhPCR is a PCR-based approach, the capacity to adapt rhPCR primers into a quantitative assay is an advantage of this technique [22]; primers and rhPCR components can be incorporated in a dye-based or probe-based PCR. For a dye-based qPCR (SYBR-green), the mix of rhPCR components and dye is sufficient. For a probe-based qPCR, an additional polymorphic region for the probe is needed between the primers, or one of the polymorphic regions of one of the primers would have to be used as probe-binding region, displacing the position of one of the primers. The application of qPCR also provides greater sensitivity for detection of low frequency DNA, since the initial amount of target DNA is directly correlated with an early or late exponential curve of amplification [23,24]. A multiplex quantitative rhPCR assay would require the design of additional primer pairs and labeling of probes with distinct fluorophores for each amplicon. The main advantage of the SNaPshot technology is its capacity to detect up to four alleles per targeted site by means of fluorescent ddNTPs variants. It would therefore be ideal if a SNaPshot primer is designed against a polymorphic SNP that distinguishes four distinct pathotype clusters (although this level of polymorphism is unlikely for a single site). In addition, SNaPshot is scalable through a multiplex reaction, where discriminatory SNPs from several different genomic regions can be examined concurrently. This would facilitate efficient and rapid testing. Differential primer lengths for each targeted SNP are required, however, since the length of the primer determines the product size of the fluorescence peak.

The rhPCR and SNaPshot assays in this project can only distinguish pathotypes of the 5X cluster from the 3H cluster, since the rhPCR primer pairs target only one set of allelic variants and the SNaPshot primer targets one SNP. To be able to distinguish isolates within the clusters further (ideally down to their individual CCD pathotype designations), multiple primers targeting various differential SNPs would need to be designed and multiple reactions would have to be carried out in parallel or multiplexed. In this case, the development of a multiplex reaction would increase efficiency. The sequencing reads of the SSIs in this study were assembled against the 2015 e3 reference genome [20]. We are currently re-aligning the SSI sequencing reads against the 2019 re-assembled e3 reference genome. The 2019 genome is more accurate and reliable than its 2015 counterpart, containing an improved genome assembly with longer continuous sequences. Moving forward, we will be using the re-aligned whole-genome SNP profiles from our isolates for assay development.

CONCLUSIONS
This project resulted in the development of two independent rapid and sensitive technologies for P. brassicae pathotyping, an rhPCR and a SNaPshot assay. The high-throughput potential and accuracy of both assays makes them promising as SNP-based pathotype identification tools for routine testing of P. brassicae pathotypes. The rhPCR technology is a highly sensitive approach that can be optimized into a quantitative assay, using widely available lab equipment, while the main advantage of SNaPshot is its ability to multiplex samples and alleles in a single reaction. To our knowledge, this is the first report of an rhPCR assay for the detection of P. brassicae pathotype clusters as classified by the CCD set, and the first single-base extension assay for the purpose of P. brassicae pathotyping.

Tables, graphs, manuscripts, etc., may be included as appendices to this report.

Literature cited

Provide complete reference information for all literature cited throughout the report.

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Benefits to the industry

a. Describe the impact of the project results on Alberta's agriculture and food industry (results achieved and potential short-term, medium-term and long-term outcomes).
b. Quantify the potential economic impact of the project results (e.g., cost-benefit analysis, potential size of market, improvement in efficiency, etc.).

a) With new virulent pathotypes overcoming the resistance in clubroot resistant canola cultivars [1], growers are at risk of losing the most effective clubroot management tool at their disposal. Other strategies, such as the application of soil fumigants, liming to increase soil pH, longer rotations with alternate non-host crops (e.g., wheat, barley) and equipment sanitization, can help to prolong the effectiveness of resistance but do not provide the same level of clubroot control. As such, new tools are needed to quickly and easily identify and distinguish P. brassicae pathotypes, helping to guide crop management decisions and the development and deployment of resistant cultivars. The pathotype detection systems established in this project represent such tools.

The rhPCR assay could be readily implemented in the same commercial and government labs that have been using PCR/qPCR-based clubroot detection tests. Since our assay is also PCR-based (like previous tests) and requires similar equipment, it can be easily adapted by these facilities. Because of the adaptability of the protocol, both detection and quantification of pathotypes should be possible, providing a monitoring system over time and space if necessary. The SNaPshot assay is highly reliable and scalable, although it requires equipment that may not be available in all diagnostic laboratories. This limitation may not be an issue in well-equipped labs or those having access to complementary facilities, and the assay may also facilitate pathogen surveillance activities. This project presented novel approaches for pathotyping P. brassicae, and will serve as the foundation for a recently approved follow-up study that will use DNA metabarcoding as a step forward for pathotyping multiple samples and polymorphic genomic regions at the same time.

b) Canola contributes $26.7 billion annually to the Canadian economy. According to a report based on models from a 12-year rotation projected from 2003 to 2014 in Alberta [25], economic losses in canola due to clubroot can reach $76 per acre and yield losses could reach 75%. The research conducted here provided additional tools to inform the application of improved clubroot management strategies. Among other things, detection of the abundance and diversity of dominant or mixed pathotypes can help to: i) suggest the need for alternative rotation crops, ii) determine if different canola cultivars can be introduced, iii) study if new pathotypes are confined to specific patches or widespread, iv) assess how resistance management practices can be altered. Early and rapid detection of new pathotypes will mitigate economic loss, not only because yield should increase, but also because in the end the effectiveness of resistance sources will be prolonged.

Performance Measures

Collaboration and partnerships

# of Industry partners:	1
# of Public partners:	1
# of international partners:	0

Training of Highly Qualified Personnel (HQP)

# of Undergraduate students trained:	3
# of graduate students trained:	1
# of postdoctoral fellows trained:	1
# of research associates trained:	1
# of technicians trained:	2
Others (Such as Visiting scientist):	1

Technology Transfer & Commercialization

# of Peer reviewed scientific publications:	2
# of scientific presentations, posters and abstracts:	4

# of industry communications:	2
# of patents and licences:	0
# of new innovations / products / practices:	2

Project resources

Statement of revenues and expenditures

In a separate document certified by the organisation's accountant or other senior executive officer, provide a detailed listing of all cash revenues to the project and expenditures of project cash funds. Revenues should be identified by funder, if applicable. Expenditures should be classified into the following categories: personnel; travel; capital assets; supplies; communication, dissemination and linkage (CDL); and overhead (if applicable).

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Total Files: 1

Provide a justification of project expenditures and discuss any major variance (i.e., ± 10%) from the budget approved by the funder(s).

The expenditures in this project were consistent with the original budget and included personnel costs (Graduate Student and Postdoctoral Salaries, Other Salaries including Technical and Summer Students), as well as materials, supplies and related charges. The only major variance was for travel expenses; originally, a total of $3,000 had been budgeted for travel, but no travel costs were incurred. This reflected COVID-19-associated travel restrictions, which were in place for most of the life of this project, and the cancellation or movement of meetings to an online format.

Resources

Provide a list of all external cash and in-kind resources which were contributed to the project.

Sources	Amount	Percentage of total project cost
Funder(s)	$436,800	51.63%
Other government sources: Cash	$0	0.00%
Other government sources: In-kind	$300,000	35.46%
Industry Cash	$109,200	12.91%
Industry In-kind	$0	0.00%
Total project cost	$846,000	100.00%

External sources of funding for the entire project.

Clearly indicate any changes to confirmed sources of funding as well as any new sources of funding.

Government Sources

Name (no abbreviations unless stated in Section 3)	Amount cash	Amount in-kind
University of Alberta	$0.00	$300,000.00
	$0.00	**$300,000.00**

Industry Sources

Name (no abbreviations unless stated in Section 3)	Amount cash	Amount in-kind
Alberta Canola	$109,200.00	$0.00
	$109,200.00	**$0.00**

Attachments

Attachments

Please attach any supplemental documents

2021_-_Tso_et_al__Pathotyping_platforms_for_Pbr_-_Plants_10__1446.pdf
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2022_-_Tso_et_al__rhPCR_and_SNaPshot_assays_to_distinguish_Pbr_-_Plant_Methods.pdf
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Total Files: 2

Does your agreement with RDAR include funds from the Canadian Agricultural Partnership (CAP) Program?

No