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A DNA-Based Assay to Detect *Xanthomonas campestris* pv. *campestris*

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EXECUTIVE SUMMARY

The purpose of the project was to develop a new, sensitive assay for detection of the black rot pathogen, *Xanthomonas campestris* pv. *campestris* (*Xcc*), on crucifer seeds. This organism is the causal agent of one of the worst diseases of crucifers world-wide. The most effective means of control is through the use of disease-free seed, and seeds are currently assayed using techniques that are slow, expensive, and lack the sensitivity desired. This project centered on developing a new assay method utilizing the Polymerase Chain Reaction (PCR), increasing the speed and sensitivity of assays at a reduced overall cost.

In previous work a region of the *Xcc* genome was identified with specificity for pathogenic strains of the bacterium. The DNA sequence was determined and utilized to generate oligonucleotide primers for use in PCR.

Three primer pairs showed promise for use in PCR following tests of 21 primers in 32 pairwise combinations. The primer pairs reacted with 250 of 254 pathogenic strains from a geographically diverse collection, and did not react with 111 of 113 non-pathogenic strains. Using these primer pairs, a hemi-nested PCR assay was developed that is effective using either genomic DNA or intact bacterial cells. Preliminary tests using seed wash extracts from 20 cabbage seed lots showed 100% correspondence in detection of *Xcc* between traditional plating and PCR assays.

This project was conducted in collaboration with the Asian Vegetable Research and Development Center (AVRDC) in Taiwan. Dr. Wang and her technician have been trained in PCR technologies. The laboratory at AVRDC has been equipped to conduct biotechnology-based plant pathogen assays and can now serve as a regional training center for the transfer of this technology to the less developed countries that it serves. This is a more efficient transfer of technologies than U.S. sponsored training in individual countries.

Research Objectives

Cruciferous vegetables are an important nutritional source and an economic asset to developing countries. Black rot, caused by the bacterium *Xanthomonas campestris* pathovar *campestris* (*Xcc*) is the most significant disease problem of crucifers world-wide. At present, there is no effective chemical control available (10), and no seed treatment that will effectively eradicate this organism (10, 11). The most effective means of control is by the use of disease-free seed. Current methods to detect the pathogen in seeds are time consuming, expensive, and have less than desirable sensitivity. The overall aim of this project was to develop a rapid, economical, and sensitive diagnostic assay for *Xcc* using the DNA-based technology of Polymerase Chain Reaction (PCR). To reach this goal, research efforts were directed at identifying a suitable pair of oligonucleotide primers that specifically amplify DNA from pathogenic *Xcc* using PCR. The next phase was to determine the best method of seed sample preparation to allow amplification of *Xcc* DNA and optimize the PCR conditions for sensitive detection of the pathogen in crucifer seeds. This technology would have exceptional value to developing countries that must import crucifer seed. Such an assay would improve quarantine procedures in developing countries as it does not require specialized laboratories or highly trained personnel. The increased sensitivity level attained would also benefit national programs by creating more effective seed health programs, which in turn would lead to reduced crop loss, less pesticide usage, and improved quality of vegetables.

Although the molecular biology technology of DNA amplification by the PCR was in use in other detection systems, at the onset of this project its use was only beginning to be realized in the field of plant pathology (5). No system had been described using PCR for the detection of a pathogen in seeds; however, several systems have since been developed (3, 9, 12). Several offshoots from this research can be used in studies on the epidemiology of the disease, and pending completion of an optimized detection system, this PCR-based assay could serve as a model for systems to be developed for other pathogens.

Additional funding support during the project came from the American Seed Trade Association, the Puget Sound Seed Growers Association, the Washington Technologies Center, and WSU's IMPACT Center. Strains of *Xcc*, other *Xanthomonas* pathovars, and infected crucifer seeds were provided through cooperating University laboratories and private companies.

Methods and Results

Bacterial Strains

All strains of *Xcc*, and other *X. campestris* pathovars used in this study were stored as permanent stock cultures in 50% glycerol at -80C (8). Strains were grown at 28C in nutrient broth or on yeast extract-dextrose-calcium carbonate agar (YDC) (13) as needed.

Pathogenicity Tests

The pathogenicity of each *X. campestris* strain was tested on black rot susceptible cabbage (*Brassica oleracea* var. *capitata* L.) cv. 'Early Jersey Wakefield' seedlings. An excised cotyledon assay, developed by R. Morrison and W. Wiebe was used as described by Alvarez et. al. (2), except 10 seedlings were inoculated per strain, and the plants were held in growth chambers set on a 12-hr light cycle at 28C day and 21C night temperatures.

DNA Extraction

Single colonies of bacterial strains were inoculated into 7 ml of nutrient broth medium and grown overnight. Cells were harvested by centrifugation, washed with 150 mM NaCl to remove polysaccharide, and the total genomic DNA extracted using the method of Lazo, et. al. (6). Extracted DNA was purified by RNAse treatment, precipitated in isopropanol and ethanol, and then resuspended in 500 μl sterile water and stored at 4-5°C until use.

Oligonucleotide Primer Screening

The sequence of a 2.9 Kb insert of *Xcc* DNA from a recombinant clone showing specificity for *Xcc* (pXC233), identified in previous work (1; Aline et. al., unpublished), was utilized to generate 21 oligonucleotide primers (Fig. 1). Using all reasonable pairings of these primers, 32 pairs were tested in PCRs at a variety of annealing temperatures using a test panel of 2 *Xcc* DNAs and the *incanae* and *carotae* pathovar DNAs. The first three primers were made 20 nucleotides (nts) long with GC contents ranging from 60-70%. In PCRs, these primers produced extraneous amplification products which could not be eliminated by increased annealing temperatures. The remaining 18 primers were made 16 nts long with GC contents of 50 or 55%. Of these 18 primers, 5 pairs (primers 4 and 5, 4 and 9, 4 and 14, 8 and 14, and 5 and 16) were found suitable for more extensive testing. Three of these pairs (primers 4 and 5, 4 and 14, and 5 and 16), were tested using 367 *Xanthomonas* strains (Table 1). Of these, 250 out of 254 pathogenic *Xcc* strains (isolated from a wide variety of world-wide locations) tested positive with the three primers pairs. All 47 strains of the related *armoraciae* and *raphani* pathovars (also crucifer pathogens) tested negative. Of the 66 other non-pathogenic Xanthomonads isolated from crucifers or crucifer seed, 64 tested negative and two tested positive (false positives).

Subsequent testing with other primers found the four *Xcc* strains that did not react with the three primer pairs above would react with primer pairs 8 and 14, or 11 and 15, but not with any primers covering the central portion of the pXC233 region (Fig. 1). To determine whether a deletion or insertion was present in the region, the four strains were subjected to restriction fragment length polymorphism analysis using the recombinant clone pXC233 as the probe. Results showed the BamH I site in this region is missing in these strains, suggesting (based on band sizes) that there is a deletion that has removed the region covering primers 4 and 5. Since these four strains were all isolated from the same or a neighboring field, they may represent reisolates of the same individual strain.

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PCR Assays

Using the three primer pairs, a hemi-nested PCR assay was developed. In first round amplifications, either the primer pairs 4 and 14, or 5 and 16, were used to amplify target DNA added as either 1 μl extracted genomic DNA, 1 to 5 μl of whole cell suspensions, or 1 to 5 μl of a concentrated crucifer seed wash extract. Seed wash extracts were prepared by shaking 10,000 seeds in either sterile water or 0.85% saline (2.5 ml/g seed) for one hr, followed by concentration by centrifugation and resuspension of pellets in 2 ml of water or saline. One μl of first round product was used as target DNA in the second round of amplification using the primer pair 4 and 5. All PCRs were performed in 25 μl volumes containing 10 mM tris-HCl (pH 8.3); 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% Tween 20, 0.01% NP-40, 0.01% (w/v) gelatin, 200 mM each dNTP, 1 mM each primer, and 2 units *Taq* DNA polymerase. Mineral oil was added (25 μl) to prevent evaporation. First and second round amplifications were carried out in a Hybaid TR1 thermal reactor (Hybaid Limited, U.K.) with the following thermal profile: an initial denaturation at 94C for 30 sec, a 65C "hot start" step (4) for 5 min to add the DNA polymerase, followed by 26 cycles of denaturation, annealing, and DNA extension at 94C for 30 sec, 58C for 30 sec, and 70C for 1.5 min, with the extension time increased to 5 min in the final cycle. The amplified DNA fragments were loaded directly onto 0.7% agarose gels in 0.5 x Tris-Boric acid-EDTA buffer (7), electrophoresed, and viewed with ultra-violet light following ethidium bromide staining. The assay was effective using either extracted genomic DNA or whole cell suspensions of *Xcc*. Dilutions of *Xcc* cells, plated onto YDC medium to ascertain actual cell concentration, were used to define the sensitivity threshold of the assay. One to two colony forming units of *Xcc* were detectable in a PCR sample volume of one μl. The assay was also capable of detecting the pathogen directly in samples of concentrated crucifer seed wash extracts. In tests of 20 cabbage seed lots, results between the PCR assay and the traditional plating assay corresponded completely, indicating that for these seed lots, the associated seed microflora do not interfere with the PCR assay.

Impact, Relevance, and Technology Transfer

Both Dr. Wang and her technician have been trained in PCR technology in order to maintain expertise in this area at the AVRDC. Dr. Wang visited WSU-Puyallup in December, 1993 to discuss the project, the needs of the lab at AVRDC, and the training of the technician. The technician was trained for two months at WSU-Puyallup in seed diagnostics, strain pathogenicity assessment, and PCR technologies during December 1993 and January 1994. In June 1994, Dr. Aline visited the AVRDC for three weeks to help set up the lab for PCR work, determine the supplies still needed for the research, and finalize the research plan for the AVRDC portion of the project. He also toured cabbage fields and collected black rot samples for later *Xcc* isolation.

The project results can currently only be used on a trial basis, except for the use of pXC233 as a hybridization probe to identify pathogenic *Xcc* strains. Anticipated uses of the PCR-based diagnostic assay, when completed, include testing of all commercial crucifer seed lots for the presence of *Xcc*, quarantine testing of imported seed, and testing of soils to insure

that the planting fields are "clean". With this knowledge, the application of the principle of "clean seed, clean ground" can be effectively used to combat black rot in Asian countries.

By transferring PCR technologies to personnel at the AVRDC, modern diagnostic technologies can be utilized by a variety of Asian less developed countries (LDCs). The AVRDC plans to serve as a regional training center for the transfer of this type of technology to all of the LDCs that it services. This project has brought the expertise and physical plant of the AVRDC up to the level necessary for this technology transfer to occur routinely and efficiently.

Project Activities/Output

Meetings

Dr. Robert Aline Jr. traveled to the 6th International Congress of Plant Pathology held in Montreal from July 27 - August 6, 1993. Afterwards, he traveled to Ottawa for the International Seed Testing Association Symposium on Seed Health Testing from August 9 - 11. He presented a poster at both meetings as well as a short oral presentation in Montreal on the project.

Training

The AVRDC technician traveled to WSU-Puyallup for two months of training from December 1, 1994 - January 31, 1994 and Dr. Wang visited WSU-Puyallup December 12 - 18, 1993.

Dr. Aline traveled to the AVRDC in June 1994 for three weeks to help set up and assess the laboratory for PCR research.

Patents

A U.S. Patent was filed on the DNA sequence of pXC233. The patent was denied and is not being pursued by WSU.

Publications

Aline, R. Jr., Gabrielson, R.L., and Bradley, R.D. (1993). PCR detection of *Xanthomonas campestris* pathovar *campestris*. (Abstr.) 6th International Congress of Plant Pathology, p. 46.

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Project Productivity

The project did not complete all of the proposed goals. Although we have developed a preliminary assay system based on nested-PCR using the three primer pairs, the optimization

of conditions and validation using a large number of seed samples was not accomplished. This was due to the unexpected problems with certain Taiwanese strains of *Xcc*. This necessitated an in-depth examination of the pXC233 region in these strains using RFLP analysis. In addition, hoped-for industrial funding to add personnel to the research team did not come through, thus slowing the expected research progress.

Future Work

No future work is contemplated at this time.

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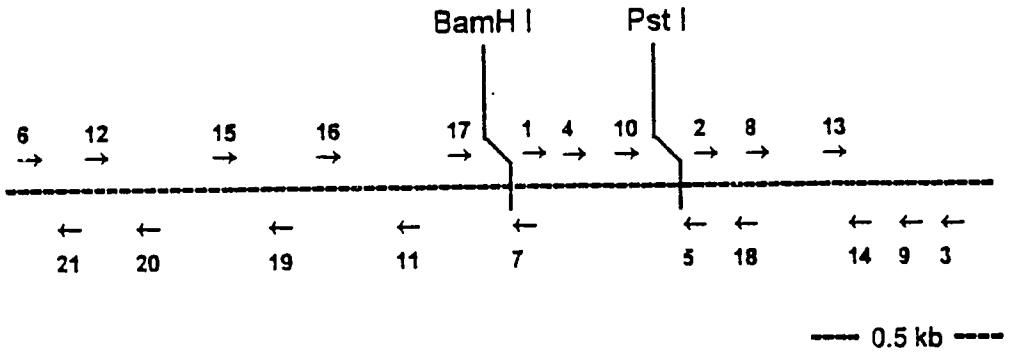

Total length of insert = 2868 nt

Oligos 1 - 3: 20 nt, 60 - 70% GC

Oligos 4 - 21: 16 nt, 50 - 55% GC

Figure 1. Oligonucleotide primers from pXC233. Relative position of each primer and direction of extension is shown. Cleavage sites of restriction endonucleases BAM HI and Pst I are given.

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TABLE 1. Comparison of cabbage pathogenicity and polymerase chain reaction assay results for strains of *Xanthomonas campestris*.

Pathovar	No. Strains	Pathogenicity[b]	PCR[c]
campestris	250	++	+
	4	++	-
(campestris)[a]	2	-	+
	64	-	-
armoraciae	28	+	-
raphani	4	+	-
(raphani)	15	-	-

[a] pathovar designator in ( ) based on host isolation and/or fatty acid methyl ester analysis (FAME).

[b] based on results in excised-cotyledon assay:

- \- = no symptoms apparent or vascular discoloration only at inoculation site;
- \+ = black lesions girdling stems of plants in 4-7 days;
- ++ = symptoms ranging from vascular discoloration extending from inoculation site to tissue collapse, chlorosis, vein blackening, and death of plant after 7 days.

[c] polymerase chain reaction assay results using either of three oligonucleotide primer pairs (4-14, 5-16, or 4-5):

- \+ = ethidium bromide-stained band present and
- \- = no band present