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Full Length Article

Development of a SCAR Marker to Identify *Phytophthora infestans* in Potato during the Latent Period

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Abstract

Phytophthora infestans mycelia in potato tubers represent the primary inoculum for the onset of potato late blight in the following year. It is currently very difficult to diagnose potato late blight during the latent period. In this study, we designed a primer pair (LB-3F and LB-3R) specific for a *P. infestans* genomic sequence. The primers were used to amplify genomic DNA extracted from *P. infestans*, *P. sojae*, *Alternaria solani*, *Pythium* spp., *Fusarium graminearum*, *Botrytis cinerea* and *Rhizoctonia solani*. An approximately 150-bp amplicon was produced from *P. infestans* genomic DNA, but not from the genomic DNA of the other pathogens. The amplicon was cloned, sequenced, and compared with the genome sequence of *P. infestans* A1 mating type isolates. This *P. infestans*-specific fragment was used as the probe during a Southern blot experiment involving genomic DNA extracted from *P. infestans* and selected pathogenic fungi. A hybridization band was observed only for the *P. infestans* isolates. Thus, the Southern blot and PCR amplification produced consistent results. We also used primers LB-3F and LB-3R to amplify genomic DNA extracted from *P. infestans*-infected potato leaves, stems and tubers at specific time points. These results suggest that even if obvious symptoms are not visible on the plant surface, *P. infestans* may have infected various plant tissues. A PCR–Southern hybridization experiment with the *P. infestans*-specific fragment as a probe produced results that were consistent with the PCR amplification banding patterns. The data presented herein suggest our SCAR primers may be useful for investigating *P. infestans* infections and for the rapid detection of *P. infestans*.

Keywords: *Phytophthora infestans*; Sequence characterized amplified regions (SCAR); Southern blot; Primary infection source

Introduction

Potato late blight caused by *Phytophthora infestans*, has become the most devastating disease affecting potato production (Staskawicz *et al*., 1995; Wang and Lazarovits, 2005; Jones and Dangl, 2006). The frequency, severity, and worldwide distribution of potato late blight has contributed to its adverse effects on potato yield, quality, and commercial production (Tan *et al*., 2010). Globally, China is currently the largest potato producer, but annual economic losses due to potato late blight are approximately $1 billion. Potato late blight was detected in Heilongjiang, Gansu, Shanxi and Hebei provinces as well as in other regions between 2013 and 2017. In each year, the disease occurred on more than 30% of the cultivated area, resulting in potential yield losses of 20 to 30%. However, in years with particularly severe potato late blight outbreaks, yield losses were greater than 50%, with some regions experiencing losses as high as 100% (Shen *et al*., 2013).

P. infestans can infect more than 50 plant species in the family Solanaceae, including potato and tomato. Potato late blight can spread throughout whole potato fields in a few days under low temperature and moist conditions (Jones and Dangl, 2006). *P. infestans* is a heterothallic oomycete, with positive and negative mating type strains that have a strong affinity for each other interacting to produce oospores (Hermansen *et al*., 2000). Because of the migration of the A_2 mating type, oospores have been isolated under natural conditions in a few countries in Europe, North America, Asia and other regions. The pathogen mainly overwinters in potato tubers as mycelia, and these tubers are the main inoculum sources for potato late blight. Diseased potato tubers planted in soil may become the center disease plant (Zwankhuizen *et al*., 1998). To control potato late blight outbreaks and epidemics, infected tubers must be identified before they are planted, using methods such as visual inspections, morphological observations and molecular biology-based techniques. These methods have been used for decades, but pathogenicity must be determined, the procedures are laborious, the incubation period is long and detecting the pathogen during the incubation period is difficult. Thus, because of the extensive application and development of molecular biology-based techniques, there has recently been

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considerable interest in enhancing molecular-based technologies for the detection of potato late blight (Deahl *et al*., 1993; Goodwin *et al*., 1995; Trout *et al*., 1997).

Niepold and Schöber-Butin (1995) designed their own polymerase chain reaction (PCR) primers to analyze potato tubers and leaves inoculated with *P. infestans*. Trout *et al*. (1997) detected *P. infestans* in potato tubers and tomato leaves by a PCR using the ITS5 and PINF primers, while Tooley *et al*. (1997) rapidly detected *P. infestans* in potato tubers by a PCR with the PINF2 and ITS3 primers. Additionally, Judelson and Tooley (2000) designed the 08-3 and 08-4 primers based on the 08 sequence of 33 fragments of a repeating DNA sequence in the *P. infestans* genome, and specifically amplified *P. infestans* genomic DNA using the 08-3 and 08-4 primers. The sensitivity of this primer combination was higher than that of the ITS5 and PINF primer combination. The PCR assays developed for detecting *P. infestans* are highly accurate, specific, fast and simple. Therefore, PCR technology can be applied to detect *P. infestans* during the primary infection period, and may facilitate an early diagnosis of potato late blight under field conditions and enable researchers to identify and monitor *P. infestans* (Trout *et al*., 1997).

Nucleic acid hybridizations are common among molecular biology-based techniques developed for the detection of specific DNA or RNA sequences (Baulcombe *et al*., 1984). These methods have also been used to analyze transgenic plants and can be applied to determine whether an exogenous gene has been incorporated into plant genomes. Because of the application and continuous improvement of the chemiluminescence substrate, the sensitivity of the digoxin (DIG) labeling system has increased so that it is now more commonly used than the radioactive isotope labeling system (Englerblum *et al*., 1993; Kruchen and Rueger, 2003), particularly during studies involving transgenic plants (Liu *et al*., 2005; Guo *et al*., 2007) and animals (Zhu *et al*., 2005; Liu *et al*., 2008) or molecular markers (Yang *et al*., 2005). Johansen *et al*. (1989) detected the bacterium responsible for potato ring rot by applying ^{32}P-tagged dCTP DNA hybridization probe technology, which can detect as little as 0.5 ng of total DNA. Cohen *et al*. (2006) simultaneously detected diverse citrus viruses by using a nucleic acid multibody probe. Du *et al*. (2007b) detected a viroid *via* a hybridization reaction on a glass substrate by using a fluorescent probe. Meanwhile, in 2011, Lü *et al*. (2011) used a cDNA probe prepared based on a PCR marker to detect a potato spindle tube viroid. The probe was able to detect 0.05 pg of total RNA.

Paran and Michelmore (1993) identified a stable marker linked to the downy mildew resistance gene in lettuce through a sequence characterized amplified regions (SCAR). This marker has subsequently been widely used for gene mapping and marker-assisted selection (Arif and Saleem, 2017). In 2006, Zhang *et al*. (2006) developed a SCAR marker associated with the *P. infestans* A1 mating type. Additionally, they observed that specific fragments were amplified by PCR in all tested *P. infestans* strains when the LB-3F and LB-3R primers were used. Milla *et al*. (2005) used bulked segregant analysis to isolate a SCAR marker linked with the gene responsible for tobacco resistance to *Peronospora hyoscyami* de Bary f. spp. *tabacina*. The SCAR marker was then applied to breed disease-resistant tobacco lines. Chen and Tong (2008) detected a specific random amplification of polymorphic DNA (RAPD) marker in five *Bacillus* spp. strains. Furthermore, Du *et al*. (2007a) applied RAPD markers developed by bulked segregant analysis to examine the metalaxyl resistance of asexual and sexual *P. infestans* progenies. They used pooled DNA from metalaxyl-resistant and -sensitive strains, and ultimately established a SCAR marker for *P. infestans* strains resistant to metalaxyl. These results provided the theoretical basis for the effective control of potato late blight and resistance to *P. infestans*. However, to the best of our knowledge, SCAR markers have not been used to detect *P. infestans*.

Therefore, in this study, we first designed a primer combination, which was based on a gene sequence reported by Zhang *et al*. (2006), for monitoring *P. infestans*. Second, we amplified genomic DNA extracted from different pathogens. Third, this primer combination was used to amplify genomic DNA extracted from potato leaves, stems and tubers collected from *P. infestans*-infected plants at specific time points after the inoculations. We also analyzed the expression of a specific *P. infestans* gene during the inoculation period. The expression results were supported by a Southern blot.

Materials and Methods

Designing a Specific SCAR Primer Pair

We used Primer 3 (version 0.6) to design a primer pair based on a specific 573-bp fragment from the *P. infestans* A1 mating type. The primers were synthesized by Shanghai Biological Engineering Company Limited. These SCAR primers (LB-3F and LB-3R) were used ina PCR to screen all fungal isolates, including *P. sojae*, *Alternaria solani*, *Pythium* spp., *Fusarium graminearum*, *Botrytis cinerea* and *Rhizoctonia solani*.

Pathogen Sources

The 20 *P. infestans* strains included in this study were isolated from potato leaflets collected at various locations in Heilongjiang province (Harbin, Mudanjiang and Jiamusi) from 2013 to 2015. The strains were isolated by placing potato tuber slices on diseased leaf fragments in Petri dishes, which were then incubated at 20°C. Mycelia were detected on the surface of the tuber slices after 5 to 7 days. Each isolate was grown by transferring hyphal tips to a tomato juice agar selective medium (200 mL tomato juice, 4.5 g calcium carbonate, 20 g agar and 800 mL distilled water)

supplemented with 500 μg/mL ampicillin, 200 μg/mL vancomycin, 50 μg/mL rifampicin, 100 μg/mL pimaricin, 35 μg/mL PCNB and 10 μg/mL benomyl. The purified isolates were maintained on tomato juice agar medium at 22°C. Other pathogens (*A. solani*, *P. sojae*, *Pythium* sp., *F. graminearum*, *B. cinerea* and *R. solani*) were provided by the Northeast Agricultural University pathology laboratory (Table 1).

Application of a SCAR Primer Pair to Amplify Genomic DNA Extracted from Different Pathogens

All *P. infestans* isolates were grown in rye broth medium (Caten, 1974), while the other pathogens were grown in potato dextrose broth. The mycelia were harvested and freeze-dried. Total genomic DNA was extracted from each isolate according to a slightly modified version of a method described by Goodwin *et al*. (1992). The concentration of the RNase-treated DNA samples was determined spectrophotometrically. The DNA was amplified in a GeneAmp PCR System in a 25 μL reaction volume consisting of 1× DNA polymerase buffer (10 m*M* Tris-HCl and 1.5 m*M* $MgCl_2$, pH 9.0), 0.2 m*M* dNTP, 1 U *Taq* polymerase, 0.4 μM primers and 20 ng genomic DNA. The PCR program was as follows: 94°C for 5 min; 35 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 30 s; 72°C for 7 min. Amplicons were separated in 1% agarose gels containing 0.5 μg/mL ethidium bromide. The electrophoretic separation was conducted at a constant 6 V/cm in 1× TBE buffer for 90 min at room temperature. The DNA fragments were visualized at 302 nm using a UV transilluminator (Ultra-Violet Products Ltd., U.K.) and the images were captured by autoradiography.

Subcloning and Sequencing of the *P. infestans*-specific Amplicon

An approximately 150-bp *P. infestans*-specific DNA fragment from isolate JMS-12 was excised from the agarose gel and purified using an Agarose Gel DNA purification kit (Sangon Biotech. Co., Ltd., Shanghai, China). The purified DNA fragment was subcloned into the pCR Ⅱ-TOPO cloning vector, which was then inserted into competent *Escherichia coli* DH5α cells. The recombinant plasmids were extracted using an Accuprep™ Plasmid Extraction Kit. After confirming the inserted DNA was the correct size, the fragment was sequenced with the Accuprep™ DNA sequencing kit. The resulting sequence was used as a query for a BLAST search of the National Center for Biotechnology Information database.

Identification of *P. infestans* with the SCAR Marker during the Incubation Period

P. infestans zoospores were collected from colonies grown on tomato juice agar medium in Petri dishes incubated at 22°C for 14 days. To induce zoospore production, Petri dishes were filled with cold (4°C) sterile distilled water and incubated for 2 to 3 h at 4°C. A 20 μL inoculum suspension (about 1×10^5 zoospores/mL) was used to inoculate mature potato leaves, stems and tubers. Mature potato tubers (cultivar 'Atlantic Ocean') were collected from plants grown in a greenhouse for 6 weeks in August and September under natural light conditions (10 h daylight). Inoculated leaves, stems, and tubers were kept in sterile Petri dishes in an incubator at 20°C. We collected diseased samples (1 cm diameter) with a hole puncher at specific time points after inoculations (0, 4, 8, 12, 24, 48, 72, 96 and 120 h). Samples (1 g) were placed in separate EP tubes, which were then stored in a cryogenic refrigerator. Genomic DNA was extracted from the frozen samples according to a slightly modified version of the CTAB method described by Junghans and Metzlaff (1990). The genomic DNA was used as the template for PCR amplifications with the SCAR primer pair.

Analysis of Potato Tissues with the SCAR Marker

The PCR amplifications were completed in a 25 μL reaction volume containing 1× DNA polymerase buffer (10 m*M* Tris-HCl, 40 m*M* KCl and 1.5 m*M* $MgCl_2$, pH 9.0), 0.2 m*M* dNTP, 1 U *Taq* DNA polymerase, 0.4 μM primers and 20 ng DNA from *P. infestans*-infected potato tissue. The PCR program was as follows: 94°C for 5 min; 35 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 30 s; 72°C for 7 min. Amplicons were analyzed by agarose gel electrophoresis.

Southern Blot

A Southern blot was completed using the DIG High Prime DNA Labeling and Detection Starter Kit (Roche). The manufacturer-recommended procedure was slightly modified. Genomic DNA was extracted from *P. infestans* and used as the template for a PCR amplification with the SCAR primer pair as described. A random primer method was used to label the probe, after which the efficiency of the labeling was assessed. The methods used for extracting the genomic DNA from *P. infestans* and the pathogenic fungi and for determining the concentration of the purified DNA were as described by Goodwin *et al.* (1992). We selected commonly used restriction endonucleases to digest the purified genomic DNA at 37°C for 48 h in a 20 to 50 μL reaction volume comprising 5 to 15 μg DNA and 2 to 5 μg/μL restriction endonuclease. Samples were analyzed by agarose gel electrophoresis to confirm the DNA was completely digested, after which they were purified and mixed with 6× loading buffer, which contained bromophenol blue. The digested DNA samples were separated by 1% agarose gel electrophoresis at 25 V overnight until the bromophenol blue migrated about two-thirds of the gel length. The agarose gel was stained with ethidium bromide and analyzed under ultraviolet light to ensure the electrophoresis was complete. The gel was then

cut and washed twice with double-distilled water. The gel pieces were then submerged in an appropriate volume of HCl (0.25 *M*) to depurinate the DNA fragments, after which the degeneration solution (1.5 *M* NaCl and 0.5 *M* NaOH), neutralizer (1.5 *M* NaCl and 0.5 *M* Tris-HCl, pH 7.5) and 20× SSC buffer were added. The DNA fragments were then transferred to a nylon membrane for 48 h at room temperature. The gel was stained with ethidium bromide to confirm the transfer was complete. The DNA was fixed (120 MJ/cm^2) by UV irradiation. The membrane was added to a preheated DIG prehybridization solution and then incubated in a hybridization oven. The probe was denatured in boiling water and then used for the hybridization step at 42°C for 16 to 20 h in a hybridization oven. The membrane was then washed and fixed in a cassette in a darkroom to generate an autoradiographic image at 15 to 25°C for 15 to 25 min (*i.e.*, development, rinsing and fixation).

Verification of the Utility of the *P. infestans*-specific SCAR Marker by PCR–Southern Blot Hybridization

Genomic DNA extracted from *P. infestans*-infected potato tissues at specific postinoculation time points was used as the template for a PCR amplification with primers LB-3R and LB-3F. The amplicons were separated by agarose gel electrophoresis (100 V, 30 min). The hybridization procedure was the same as that used for the Southern blot.

Results

Development of a *P. infestans*-specific SCAR Primer Pair

Using published sequences of specific fragments in the *P. infestans* A1 mating type genome, we designed a pair of primers with Primer 3 (version 0.6). The primer sequences were as follows:

LB-3F: 5′ -TTAATTCTTTCGGTCGGATA-3′
LB-3R: 5′ -GCGAGTACTTCCTGCTCTAA-3′

Selection of *P. infestans*-specific Fragments

Primers LB-3F and LB-3R were used for PCR amplifications, with genomic DNA from 12 pathogens serving as the template. We detected a 150-bp amplicon specific to *P. infestans* (Fig. 1). Moreover, these primers were unable to amplify the same sequence when genomic DNA from another *Phytophthora* species (*P. sojae*) was used as the template. These results suggest that primers LB-3F and LB-3R were specific for *P. infestans* DNA, and may be useful for detecting and identifying *P. infestans*.

The recombinant plasmid DNA including the inserted DNA fragment was digested by *Eco*RI/*Pst*I, which generated a fragment smaller than 200 bp. A subsequent PCR amplification with primers LB-3F and LB-3R produced an approximately 150-bp fragment (Fig. 2).

Sequencing of the *P. infestans*-specific Fragment and Comparison with other A1 Mating type Sequences

A comparison between the digested fragment sequences and published *P. infestans* A1 mating type sequences revealed that the *P. infestans*-specific fragment was 156 bp long. Additionally, there was a 99% similarity between the 156-bp fragment and the middle genomic region (259 to 414 bp) of a 573-bp *P. infestans* A1 mating type fragment.

001 GATGACCGCCTAGGTCCGCGTGCGGAAATAATCGTTCTGGGAGATCGCGA
051 TTACTTCGCATTCCTTCACTATTGCACCGGACGAACTGGGGCCGCTATTA
101 TCATGACGAGGATGGAAGGTATTCAAAGCTCATGGGAGCCATCACAAAAC
151 TCCAAAAGTGAGGTCCAAGCTTTGGTGTCGATTCGACACTGTGAAGTATG
201 CGTTGATGAATGGCGTTGATGAACAGCAGGGAGATAAACTAATTTGTTGG
251 TTATACTTTAATTCTTT GGTCGGATACAGATTAGTTTCGTCTCACCCTCGTAATTCTTTCGGTCGGATACAGATTAGTTTCGTCTCACCCTCG
301 ACGCCTCTCAGATTGCGACTGCGATGCGACGGTGTGCACTTATAGTGAGCACGCCTCTCAGATTGCGACTGCGATGCGACGGTGTGCACTTATAGTGAGC
351 TGTATGTTGGTTGAATAGAGCGCGTTCATCGAGAGCAGCAGGTTTAGAGCTGTATGTTGGTTGAATAGAGCGCGTTCATCGAGAGCAGCAGGTTTAGAGC
401 AGGAAGTACTCGCTGCTGCACTGGCACTTACAAGCATCATGCCGATGCCGAGGAAGTACTCGCA
451 TATGTTAACTCTCTTTTCGCCTCACGAGGAGTTTTAAATAAAGTCTCTGC
501 TTAGGAATCACGCAATGCCAATACTACTACAAAATCGGTCGCTCTGCTTT
551 GTATCTCGTGTCTGGCGGTCATC

Temporal Effects on *P. infestans* Pathogenicity on Potato Plants

We inoculated different potato plant tissues with a *P. infestans* zoospore suspension and assessed *P. infestans* pathogenicity under controlled environmental conditions (Fig. 3). Minor disease symptoms were detected on leaves at 8 h after the inoculation, while symptoms were observed on the stems at 24 h after the inoculation. Small and irregularly shaped spots were initially present on the leaves and stems, but at later time points, the spots became fuscous and formed mildew, before turning into water-soaked lesions. Additionally, white mildew was detected on the back of tuber slices at 24 h after the inoculation. The *P. infestans* migrated through the tuber slices by 48 h after the inoculation. Thus, the leaves were more susceptible than the stems and tubers under the artificial inoculation conditions used in this study.

We observed differences in the disease symptoms of samples incubated under controlled (*i.e.*, laboratory) and field conditions because of differences in the weather. However, when the humidity reached a certain level in the field, the resulting mold on the leaves and stems was similar to that observed under controlled conditions.

Table 1: The pathogens used in this experiment

pathogen	Number of isolates	Years	Gathering place	Host	Mating types	Resistant to Metalaxyl
P.infestans	Heb-13-1	2013	Harbin	potato	A_1	HR
	Heb-13-14	2013	Harbin	potato	A_1	HR
	Heb-13-23	2013	Harbin	potato	Self fertile	HR
	Mdj-13-9	2013	Mudanjiang	potato	A_1	HR
	Mdj-13-10	2013	Mudanjiang	potato	A_1	HR
	Mdj-13-11	2013	Mudanjiang	potato	A_1	HR
	Jms-13-7	2013	Jiamusi	potato	A_2	HR
	Jms-13-11	2013	Jiamusi	potato	A_1	HR
	Jms-13-33	2013	Jiamusi	potato	A_1	HR
	Heb-14-3	2014	Harbin	potato	A_1	HR
	Heb-14-5	2014	Harbin	potato	A_1	HR
	Heb-14-6	2014	Harbin	potato	A_2	HR
	Mdj-14-2	2014	Mudanjiang	potato	A_1	HR
	Mdj-14-8	2014	Mudanjiang	potato	A_1	HR
	Jms-14-12	2014	Jiamusi	potato	A_1	HR
	Heb-15-6	2015	Harbin	potato	A_1	HR
	Heb-15-7	2015	Harbin	potato	A_1	HR
	Mdj-15-3	2015	Mudanjiang	potato	A_1	HR
	Jms-15-5	2015	Jiamusi	potato	Self-fertile	HR
	Jms-15-10	2015	Jiamusi	potato	A_1	HR
Other	*A. solani*	2015	Harbin	potato	MAT1	HR
pathogens	*P. sojae*	2015	Harbin	soybean	—	HR
	P. sojae	2015	Harbin	soybean	—	HR
	Pythium spp.	2015	Harbin	melon	—	—
	F.graminearum	2015	Harbin	wheat	—	—
	B. cinerea	2015	Harbin	tomato	—	—
	R. solani	2015	Harbin	rice	—	—

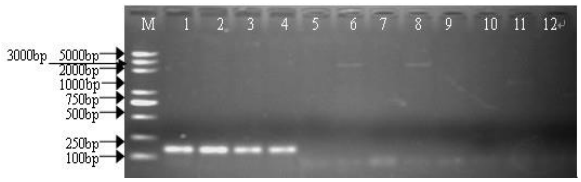

Fig. 1: Electrophoretic profile of amplicons produced by primer pair LB-3F/LB-3R and genomic DNA extracted from different pathogens. Lanes 1 to 4: *Phytophthora infestans*; lanes 5 to 7: *Phytophthora sojae*; lane 8: *Alternaria solani*; lane 9: *Pythium* spp.; lane 10: *Fusarium graminearum*; lane 11: *Botrytis cinerea*; lane 12: *Rhizoctonia solani*

Amplification by PCR with SCAR Primers and Genomic DNA Extracted from Infected Potato Plants at Different time Points

The SCAR primers were used to amplify genomic DNA extracted from infected potato leaves, stems and tubers. The expected 156-bp fragment was amplified from genomic DNA extracted from leaves, stems, and tubers at 4, 48 and 24 h after the inoculations, respectively (Fig. 4). Therefore, even if the surface of the potato tissues lacked obvious disease symptoms, *P. infestans* had already infected the plants. Consequently, the SCAR primers may be useful for determining whether potato plants are infected by *P. infestans* even before visible symptoms develop.

Validation of the *P. infestans*-specific SCAR Marker by a Southern blot

Assessment of probe-labeling efficiency: We gradually diluted the *P. infestans*-specific probe concentration for a

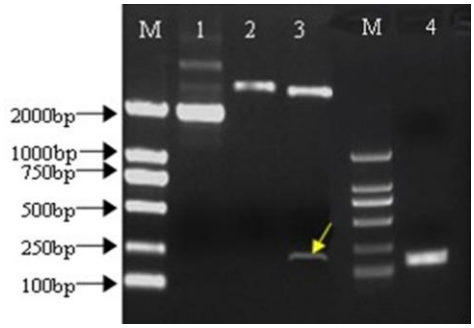

Fig. 2: Cloning of *P. infestans*-specific fragments. Lane 1: Plasmid DNA extracted from transformed *Escherichia coli* DH5α cells; lane 2: DNA digested by *EcoR* I ; lane 3: inserted *P. infestans* DNA digested by *EcoR* I and *Pst* I; lane 4: amplicon generated with primers LB-3F and LB-3R

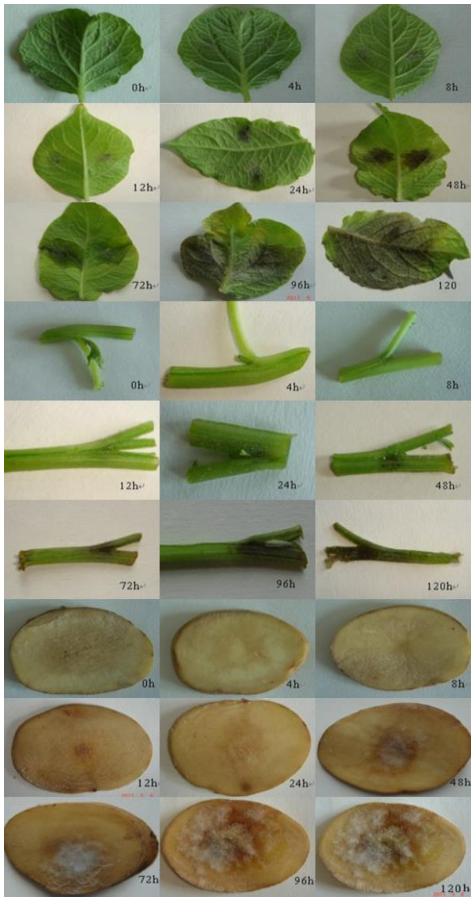

Fig. 3: Pathogenicity of *P. infestans* on potato leaves, stems and tubers

comparison with a control DIG-labeled DNA probe whose concentration was known. We also assessed the probe-labeling efficiency. The reaction between the CSPD substrate and alkaline phosphatase produced a fluorescent signal that formed a black spot on the exposed film. The first *P. infestans*-specific probe (Fig. 5B) was the brightest,

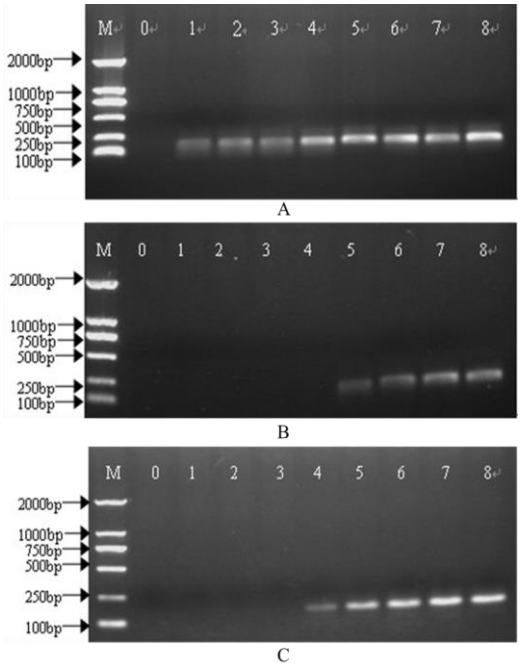

Fig. 4: Electrophoretic profile of amplicons produced by primer pair LB-3F/LB-3R and genomic DNA extracted from infected potato (A) leaves, (B) stems and (C) tubers. M: DL2000 Marker; 0: 0 h; 1: 4 h; 2: 8 h; 3: 12 h; 4: 24 h; 5: 48 h; 6: 72 h; 7: 96 h; 8: 120 h

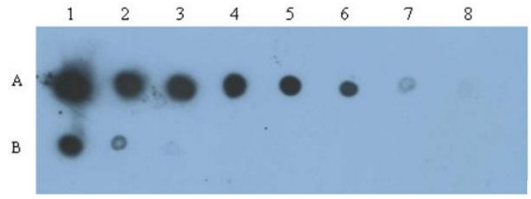

Fig. 5: Determination of probe-labeling efficiency. (**A**) DIG-labeled control DNA probe. (**B**) DIG-labeled *P. infestans*-specific probe

with a fluorescent signal similar to that of the fifth control DIG-labeled DNA probe (Fig. 5A). The concentrations of the control DIG-labeled DNA probe were as follows: 1 ng/μL, 10 pg/μL, 3 pg/μL, 1 pg/μL, 0.3 pg/μL, 0.1 pg/μL, 0.03 pg/μL and 0.01 pg/μL. Thus, the concentration of the *P. infestans*-specific probe was estimated as 0.3 pg/μL.

Detection of a *P. infestans*-specific Genomic DNA Fragment

Genomic DNA was extracted from various pathogens and digested with a restriction enzyme. A Southern blot with the amplicon produced by primers LB-3R and LB-3F used as the DIG-labeled probe revealed the expected bands for only *P. infestans* (Fig. 6). These results implied that the *P. infestans* genome had at least one fragment that was absent in the genomes of the other tested pathogens. Moreover, the LB-3R and LB-3F primers may be useful for specifically detecting *P. infestans*. Furthermore, banding pattern differences were observed for the four examined *P. infestans* strains, which were obtained from diverse locations. There were at least three copies of the *P. infestans*-specific genomic sequence in each strain. Our findings suggested there is genetic diversity among the four analyzed *P. infestans* strains.

Verification of the *P. infestans*-specific SCAR Marker by PCR–Southern Blot Hybridization

Genomic DNA was extracted from inoculated potato leaves, stems and tubers at various time points after the inoculation by *P. infestans*. The purified DNA served as the template for PCR amplifications. The amplicons separated by 1% agarose gel electrophoresis were transferred to a nylon membrane for a Southern blot with a *P. infestans*-specific genomic fragment as the probe. The expected band (in terms of size) was detected for genomic DNA extracted from infected leaves at 4 h after the inoculation. The size of the band increased as the incubation period progressed. In contrast, the expected band was not observed for genomic DNA extracted from stems and tubers until 48 and 24 h after the inoculations, respectively (Fig. 7). These observations were consistent with the PCR amplification results. Thus, primers LB-3R and LB-3F may be useful for detecting *P. infestans* in infected potato tissues.

Discussion

Potato late blight is a devastating disease worldwide, and is responsible for considerable yield losses. This disease can spread relatively quickly at 12 to 22°C, under relatively high humidity. Late blight infections can be divided into the following three stages: (1) the central diseased plant appears, with only the upper leaves exhibiting disease symptoms; (2) late blight disease symptoms begin to spread, with most leaves exhibiting symptoms after approximately 10 days and (3) plants in the whole field exhibit late blight disease symptoms as well as decreased productivity, while there is also a sharp increase in the number of dead plants (Jin *et al*., 2005). Detecting the central diseased plant and preventing the spread of the responsible pathogen during the first stage are the keys to controlling potato late blight. The seedling is the most important source of potato late blight inoculum. Therefore, inhibiting the spread of *P. infestans* prior to sowing tubers is an important disease control measure. Traditional methods for preventing late blight involve visual inspections and morphological analyses to detect disease symptoms. Although visual inspections are simple and fast, there are considerable limitations (*e.g.*, lack of visible symptoms during the latent period). Morphological analyses, which can be complex, require the detection of specific pathogen characteristics. However, the incubation period for late blight disease is long and similar symptoms may be

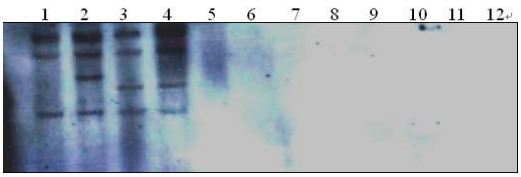

Fig. 6: Southern blot profile of genomic DNA extracted from diverse pathogens. Lanes 1 to 4: *P. infestans*; lanes 5 to 7: *P. sojae*; lane 8: *A. solani*; lane 9: *Pythium* spp.; lane 10: *F. graminearum*; lane 11: *B. cinerea*; lane 12: *Rhizoctonia solani*

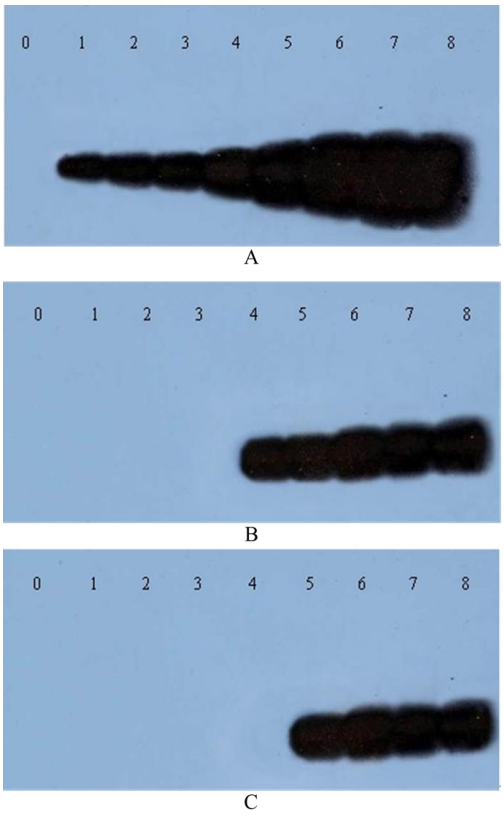

Fig. 7: Genomic DNA PCR–Southern blot results for *P. infestans*-infected (A) potato leaves, (B) stems, and (C) tubers. 0: 0 h; 1: 4 h; 2: 8 h; 3: 12 h; 4: 24 h; 5: 48 h; 6: 72 h; 7: 96 h; 8: 120 h

induced by other pathogens that are difficult to culture separately for assessments of biological characteristics. Consequently, these methods may be inefficient for determining whether potato plants and seed potatoes are infected with *P. infestans.* Xu *et al*. (2004) isolated brown plaques from plant material infected with late blight, but were unable to isolate *P. infestans*. Thus, their method is not appropriate for testing whether a seed potato is infected by *P. infestans*. The extensive application and development of molecular biology-based techniques has compelled researchers to focus on the relevance of molecular detection technology for identifying plants infected with potato late blight. For example, PCR-based methods for identifying pathogens are fast, sensitive and reliable. In this study, we developed a SCAR marker specific for *P. infestans* and PCR primers (LB-3F/LB-3R) were designed and used to test different *P. infestans* strains as well as other potato pathogens. A 156-bp fragment was amplified from *P. infestans* genomic DNA, but not from the genomic DNA extracted from the other pathogens (including *P. sojae*). Therefore, the *P. infestans*-specific SCAR marker may be stable and useful for detecting potato late blight. Our rapid and accurate PCR-based detection method may be able to overcome the deficiencies of traditional detection methods. The data presented herein may be relevant for the future development of a molecular diagnostic kit for detecting *P. infestans.*

In this study, we validated the utility of specific primers using potato plants and seed potatoes. Potato leaves, stems and tubers inoculated with *P. infestans* were analyzed at specific time points. Late blight symptoms were visible on the leaves, stems, and tubers at 8, 24 and 24 h after the inoculations, respectively. However, similar and more typical late blight symptoms (dark green water-like spots as well as a sparse layer of white mold) were detected on the leaves, stems, and tubers at 12, 48 and 48 h after the inoculations, respectively. A PCR amplification with primers LB-3F and LB-3R produced the expected bands for genomic DNA extracted from the leaves, stems and tubers at 4, 48 and 24 h after the inoculations, respectively. These results imply that even if disease symptoms were not clearly visible, *P. infestans* may still have infiltrated the tested plant tissues. Furthermore, this specific primer pair may be used to detect *P. infestans* in potato plants and seed potatoes.

The PCR system established in this study may be applied to qualitatively detect *P. infestans*. Quantitative real-time (qRT)-PCR assays can be used to quantify target gene expression levels. A qRT-PCR technique may enable the detection of changes in the abundance of plant pathogenic bacteria in the host tissue in real time, thereby providing relevant temporal data for disease prevention and control (Gao *et al.*, 2011). To date, qRT-PCR technology has been used for the quantitative detection of plant pathogenic fungi, including *P. infestans* (Cullen *et al*., 2002; Lees *et al*., 2002; Luchi *et al*., 2005). Therefore, qRT-PCR assays may be important for the development of effective potato late blight control measures.

To further verify the accuracy and stability of PCR amplifications with primers LB-3F and LB-3R, genomic DNA extracted from *P. infestans* and other pathogens were included in a Southern blot with a *P. infestans*-specific fragment used as the probe. The Southern blot results were consistent with the PCR amplification banding patterns. The four *P. infestans* strains examined in this study were collected from Pingshan county, Xiangfang district, Mudanjiang city, and Jiamusi city. We detected differences in the copy number and location of the 156-bp *P. infestans*-specific fragment in the genomes of the collected strains. Additionally, there were at least three copies of this

fragment in the genomes of all examined strains, but there was some genetic variability among the four *P. infestans* isolates. Thus, this fragment may be useful for investigating the genetic diversity of *P. infestans* strains. These genome-level differences should be studied in more detail.

The advantages of molecular detection methods include high sensitivity, specificity, simplicity, speed, accuracy, affordability and the fact they can be used to analyze low-abundant samples. Thus, these methods are commonly used. For example, primers have been designed for the PCR-based detection of pathogens in cotton, tomato, watermelon, and other crops (Wang, 2007). Laterally related areas like human and animal medical diagnosis and food safety industries are more advanced than detecting plant pathogens and insect pests (He *et al*., 2009). However, molecular detection methods for detecting plant pests will change the situation. It will have a broader space for development, thanks to the advances and widely uses in PCR technology. It is possible that in the near future, plant diseases will be diagnosed primarily using methods applying molecular detection technology.

Conclusion

The amplicons produced for the infected potato leaves, stems and tubers were analyzed in a PCR–Southern hybridization experiment, and the results were consistent with those of the PCR amplifications. The SCAR primers detected the target sequence in genomic DNA extracted from potato leaves at 4 h after the inoculations. The band intensity for the P. infestans-specific DNA fragment increased over time. In contrast, the P. infestans-specific DNA fragment was detected in infected stems at 48 h after the inoculations, with increasing band intensity over time. Meanwhile, the target DNA fragment was detected in tuber genomic DNA extracted at 24 h after the inoculation, with the most intense band observed at 120 h after the inoculation. Our data confirmed the specificity of the LB-3F and LB-3R primers as well as the accuracy of the PCR assay. Therefore, for sustainable commercial potato production, SCAR marker-based PCR assays completed during the latent period as well as the tuber storage period may be useful for the early detection of potato late blight.

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