A PCR-based Assay for Distinguishing between A1 and A2 Mating Types of *Phytophthora capsici*

Ping Li and Dong Liu

College of Plant Protection, Anhui Agricultural University, 130 West of Changjiang Road, Hefei 230036, Anhui, China; and Department of Horticulture and Landscape, Anqing Vocational and Technical College, 99 North of Tianzhushan Road, Anqing 246003, Anhui, China

Min Guo, Yuemin Pan, Fangxin Chen, Huajian Zhang, and Zhimou Gao¹

College of Plant Protection, Anhui Agricultural University, 130 West of Changjiang Road, Hefei 230036, Anhui, China

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ABSTRACT. Sexual reproduction in the plant parasite *Phytophthora capsici* Leonian requires the interaction of two distinct mating types, A1 and A2. Co-occurrence of these mating types can enhance the genetic diversity of *P. capsici* and alter its virulence or resistance characteristics. Using an intersimple sequence repeat (ISSR) screen of microsatellite diversity, we identified, cloned, and sequenced a novel 1121-base pair (bp) fragment specific to the A1 mating type of *P. capsici*. Primers Pcap-1 and Pcap-2 were designed from this DNA fragment to specifically detect the A1 mating type. Polymerase chain reaction (PCR) using these primers amplified an expected 997-bp fragment from known A1 mating types, but yielded a 508-bp fragment from known A2 mating types. This PCR-based assay could be adapted to accurately and rapidly detect the co-occurrence of A1 and A2 *P. capsici* mating types from field material.

The oomycete plant pathogen, *Phytophthora capsici*, first described by Leon H. Leonian at the New Mexico Agricultural Research Station, is distributed globally and is a critical threat to vegetable production as a cause of damping-off, foliar blight, and fruit rot in solanaceous and cucurbitaceous plants (Babadoost, 2000; Erwin and Ribeiro, 1996; Granke et al., 2012; Hausbeck and Lamour, 2004; Leonian, 1922). The host range of this pathogen subsequently was expanded to include lima bean (*Phaseolus lunatus* L.), snap bean (*Phaseolus vulgaris* L.), fraser fir [*Abies fraseri* (Pursh) Poir.], and certain weeds (Davidson et al., 2002; Gevens et al., 2008; Quesada-Ocampo et al., 2009).

As a heterothallic species, *P. capsici* requires cross-fertilization by distinct mating types, A1 and A2, to complete the sexual stage of its life cycle (Savage et al., 1968). Coculture of the A1 and A2 types yields oospores, sexual spores that function in nature as the overwintering inoculum of the pathogen. Co-occurring A1 and A2 isolates of *P. capsici* were detected in New Jersey in 1981 and in North Carolina in 1990; the mating types have been identified in single-crop fields as well as within the same plant (Papavizas et al., 1981; Ristaino, 1990). Sexual reproduction augments genetic variation in *P. capsici* via allelic recombination. The resulting offspring genotypes may include acquired resistance to fungicides or antibiotics, creating new challenges for phytophthora blight management.

The genetic basis of mating-type establishment in oomycetes remains unclear. The mating types of ascomycetes and

basidiomycetes are expressed in a haploid life stage (Kües and Casselton, 1992). In contrast, oomycetes such as *P. capsici* are diploid during the vegetative stage, and mating-type alleles likely are inherited from distinct A1 and A2 parents in a heterozygous pattern. Therefore, a genetic marker for differentiating A1 and A2 mating types in *P. capsici* has been the subject of much speculation. A paucity of morphological and biochemical mutants has impeded genetic studies of *P. capsici* and other oomycetes (Judelson, 1996b). *Phytophthora infestans* (Mont.) de Bary has been used to address the genetic mechanisms of heterothallism in diploid fungi because its mating system is representative of most heterothallic oomycetes (Judelson, 1996a). In this species, mating type is determined by a single locus that displays a non-Mendelian pattern of segregation (Judelson et al., 1995).

Sexual reproduction is essential to the biology and epidemiology of *P. capsici*. Populations of *P. capsici* containing both A1 and A2 mating types can complete the sexual life stage to produce oospores with enhanced genetic variation and potentially higher virulence and adaptive resistance to fungicides (Parra and Ristaino, 2001; Qi et al., 2012; Satour and Butler, 1968). A rapid and facile genetic technique to evaluate the occurrence of *P. capsici* mating types in the field has implications for disease management. Intersimple sequence repeat fingerprinting (Zietkiewicz et al., 1994) is a simple and reliable assay to measure microsatellite (i.e., simple sequence repeat) frequency and infer genetic diversity (Blair et al., 1999). We applied ISSR fingerprinting to identify a genetic marker capable of distinguishing between A1 and A2 *P. capsici* mating types.

Materials and Methods

STRAINS AND MYCELIUM PREPARATION. Twenty-one *P. capsici* isolates were collected from infected peppers (*Capsicum*

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¹Corresponding author. E-mail: gaozhimou@126.com.

annuum L.) at different locations in Anhui, China, including Huainan, Hefei, Chaohu, Qianshan, Yuexi, Chizhou, Tongling, Fuyang, and Yingshang. Standard isolates with known mating types, P991 (A1) and P731 (A2), were kindly provided by Y.C. Wang of Nanjing Agricultural University, Nanjing, China (Table 1). All cultures were obtained from single zoospores (Ko, 1981). Twenty-three pieces of agar culture obtained from the advancing margin of a 3-d-old colony growing on a plate of carrot agar were inoculated into liquid Plich medium (0.5 g KH₂PO₄, 0.25 g MgSO₄·7H₂O, 1 g asparagine, 1 mg thiamine, 0.5 g yeast extract, and 25 g glucose in 1000 mL distilled water). After incubation at 25 °C on an orbital shaker at 100 rpm (THZ-300; Yiheng Co., Shanghai, China) for ≈7 d, mycelia were harvested by filtration through a sterile filter (Miracloth; Zhongfang Co., Nantong, China) and stored at -20 °C before DNA preparation.

CONVENTIONAL MATING-TYPE DETERMINATION. The mating types of the 21 isolates were determined by pairing with known A1 isolate (P991) and A2 isolate (P731) on a petri dish (9 cm diameter) containing 10% clarified V8 medium (Ko, 1981), respectively. A plug of an isolate to be tested was placed on one side of the plate with a plug of the A1 or A2 standard isolate on the opposite side. The plates were incubated at 25 °C in the dark for 14 d. Then, the formation of oospores was examined under a binocular microscope at 100-fold magnification (CC23; Olympus Corp., Tokyo, Japan).

EXTRACTION OF GENOMIC DNA. Total genomic DNA was extracted from each isolate as described previously (Wang and Zheng, 2003) with the following modifications: the air-dried pellet was resuspended in 100 µL Tris-EDTA (TE) buffer [10 mM Tris-HCl (pH 7.4), 1 mM EDTA] and 2 µL RNase (10 mg·mL⁻¹). Samples were then incubated at 37 °C for 1 h. DNA was quantified using a spectrophotometer (Nanodrop

N.D.1000; Thermo Fisher Scientific, Wilmington, DE), and it was diluted to 50 ng·µL⁻¹ with TE buffer and then stored at 4 °C.

ISSR ANALYSIS. ISSR fingerprinting was performed using 40 different primers (Sangon Biotechnology Co., Shanghai, China) on isolates of different mating types and pathogenicity. Twenty-three isolates were selected (Table 1). A 25-µL reaction mixture consisting of 2.5 µL 10X PCR buffer, 200 µM dNTPs, 0.2 µM primer, 0.5 µL template DNA, and 1.25 U Taq DNA polymerase (Takara Bio, Dalian, China) was subjected to thermal cycling in a thermal cycler (C1000; Bio-Rad, Hercules, CA). Thermal cycling parameters were as follows: initial denaturation at 94 °C for 5 min, 35 cycles of denaturation at 94 °C for 1 min, annealing at a primer-optimized temperature (48–50 °C) for 1 min, extension at 72 °C for 2 min, and final extension at 72 °C for 10 min. PCR products were electrophoresed through 1.8% agarose gels containing ethidium bromide in 10X Tris-borate-EDTA (TBE) buffer. Samples were visualized under ultraviolet illumination.

CLONING AND SEQUENCING OF SPECIFIC DNA FRAGMENTS. Before cloning, amplification products were excised and purified using an AxyPrep DNA Gel Extraction Kit, according to the manufacturer's instructions (Axygen Bioscience, Union City, CA). Purified PCR products were ligated into pMD18-T Simple Vectors (Takara Bio) and were transferred into *Escherichia coli* (Migula) Castellani and Chalmers according to the supplier's instructions. The clone specific to FY2 isolate was sequenced (Sangon Biotechnology Co.).

SYNTHESIS OF PRIMERS FOR DISTINGUISHING MATING TYPES OF *P. capsici*. Based on the sequence of a specific 1121-bp fragment from the FY2 isolate, forward and reverse primers that probably distinguish between A1 and A2 isolates were designed using Primer Premier version 5.0 (Premier Biosoft,

Palo Alto, CA). A 25-µL reaction mixture was prepared consisting of 20 ng genomic DNA, 2.5 µL 10X PCR buffer, 200 µM dNTPs, 0.1 µM each forward and reverse primers, and 1.25 U Taq DNA polymerase (Takara Bio). The following PCR profile was used as follows: initial denaturation at 94 °C for 5 min, 30 cycles of denaturation at 94 °C for 30 s, annealing at 54 °C for 30 s, extension at 72 °C for 1 min, and final extension at 72 °C for 10 min. PCR products were electrophoresed through 1.0% agarose gels containing ethidium bromide in 10X TBE buffer. Samples were visualized under ultraviolet illumination.

DETERMINATION MATING TYPE OF *P. capsici* IN FIELD MATERIALS BY PCR. For detection of the mating type in infected field plants, 1-month-old susceptible cultivar Bianjiao NO.1 (Fengle Seed Co., Hefei, China) was inoculated with four isolates of *P. capsici* Mat-A1 and 7 isolates of Mat-A2 by applying mycelium block stem wound inoculation. Inoculated seedlings were incubated at 25 °C and

Table 1. Collection location in China, collection years, hosts, and mating types of the *Phytophthora capsici* isolates used in this study.

Isolate no.	Location	Yr of collection	Host	Mating type
HN1	Huainan	2006	<i>Capsicum frutescens</i> L.	A1
HN2	Huainan	2006	<i>C. frutescens</i>	A2
HN3	Huainan	2006	<i>C. frutescens</i>	A1
HN4	Huainan	2006	<i>C. frutescens</i>	A2
HN5	Huainan	2006	<i>C. frutescens</i>	A2
HF2	Hefei	2007	<i>C. frutescens</i>	A2
HF3	Hefei	2007	<i>C. frutescens</i>	A2
HF5	Hefei	2007	<i>C. frutescens</i>	A1
CH1	Chaohu	2009	<i>C. frutescens</i>	A2
CH2	Chaohu	2009	<i>C. frutescens</i>	A2
CH4	Chaohu	2009	<i>C. frutescens</i>	A1
QS1	Qianshan	2008	<i>C. frutescens</i>	A2
QS2	Qianshan	2008	<i>C. frutescens</i>	A2
YX1	Yuexi	2008	<i>C. frutescens</i>	A2
YX2	Yuexi	2008	<i>C. frutescens</i>	A2
CZ1	Chizhou	2009	<i>C. frutescens</i>	A2
CZ2	Chizhou	2009	<i>C. frutescens</i>	A2
TL1	Tongling	2010	<i>C. frutescens</i>	A2
FY2	Fuyang	2009	<i>C. frutescens</i>	A1
YS1	Yingshang	2009	<i>C. frutescens</i>	A1
YS2	Yingshang	2009	<i>C. frutescens</i>	A1
P991	—	—	—	A1
P731	—	—	—	A2

100% humidity for 12 h and then the genomic DNAs were extracted from infected pepper stems according to Silvar et al. (2005). DNAs from infected pepper plants showing symptoms and healthy plant were amplified using Pcap-1/Pcap-2 primer pair set according to the PCR protocol described previously.

Results

MATING-TYPE FREQUENCIES. Twenty-one isolates of *P. capsici* from Anhui province (China) were paired with isolates P991 (A1) and P731 (A2) to determine mating type. If oospores were produced from an A1 pairing, then the unknown isolate was designated A2; if the A2 pairing yielded oospores, the isolate was designated A1. Of the 21 isolates, 7 were A1 and 14 were A2 (Table 1).

MATING-TYPE-SPECIFIC ISSR MARKER. Genomic DNAs were extracted from 23 isolates of *P. capsici*. Forty ISSR primers were then screened to identify candidates that could distinguish between *P. capsici* mating types. Primer UBC821 (5'-GTGTGTGTGTGTGTGT-3') reliably generated an 1121-bp fragment specific to the A1 mating type at a primer-optimized annealing temperature of 48 °C (Fig. 1). This fragment was further investigated for the development of a specific biomarker assay to distinguish between A1 and A2 isolates.

CLONING AND SEQUENCING OF THE *P. CAPSICI* A1-SPECIFIC FRAGMENT. The 1121-bp A1-specific DNA fragment was isolated, ligated into a T-vector, and transformed into *E. coli*. The cloned DNA was then sequenced using a T-vector universal

primer pair (Fig. 2). The sequence was searched against deposited nucleotide sequences using the National Center for Biotechnology's Basic Local Alignment Search Tool (BLASTn) and against nonredundant protein sequences using BLASTx (U.S. National Library of Medicine, Bethesda, MD). The sequence also was input to the database of the U.S. Department of Energy's Joint Genome Institute (Walnut Creek, CA). No significant hits were detected, suggesting that the candidate biomarker is novel.

A PCR ASSAY OF MATING-TYPE IN *P. CAPSICI*. A PCR primer pair was designed from the 1121-bp A1-specific sequence to differentiate between *P. capsici* mating types. The sequence of the forward primer was 5'-ACGAGTACGAGTGCTTGGT-3' [Pcap-1 (52% G+C)]. The sequence of the reverse primer was 5'-TGAGTCTCGAGACAGAGAG-3' [Pcap-2 (52% G+C)]. Eight A1 and 15 A2 isolates of *P. capsici*, of which 21 isolates from infected peppers and 2 standard isolates, subsequently were assayed by PCR using Pcap-1 and Pcap-2 primers. A 997-bp fragment was specifically amplified from all A1 mating types, whereas a 508-bp fragment was only amplified from A2 isolates (Fig. 3).

ANALYSIS OF SPECIFIC SEQUENCE OF MATING TYPE OF A1 AND A2 ISOLATES. The clones specific to isolates YS1, CH2, and TL1 were excised, purified, and sequenced. Then, sequences of isolates FY2, YS1, CH2, and TL1 were analyzed by MegAlign (DNASTAR, Madison, WI). The result showed that FY2 homology with YS1 was up to 99.9%, whereas isolate CH2 and TL1 showed 100% sequence identity with each other (Fig. 4). The alignment of specific fragment between A1 and A2

Fig. 1. Intersimple sequence repeat (ISSR) fingerprints of *Phytophthora capsici* generated using primer UBC821. The black arrow indicates the 1121-bp genomic DNA fragment derived from the A1 mating type. The white arrows indicate the specific ISSR product (1121 bp) derived from *P. capsici* Mat-A1 (lane M = DL 2000 marker, lane CK = no DNA template).

Fig. 2. Nucleotide sequence of the *Phytophthora capsici* A1-specific DNA fragment. The numbers on the left refer to the nucleotide sequence. Boxes identify the forward (Pcap-1) and reverse (Pcap-2) primers used to differentiate *P. capsici* A1 and A2.

mating types revealed higher homologous region, with bases insertion, deletion, or substitution.

DETERMINATION OF MATING TYPE OF *P. CAPSICI* IN FIELD PLANTS. In this study, DNA obtained from the target *P. capsici* (Mat-A1 and Mat-A2) infected stems and health plants was amplified by PCR with the primer set Pcap-1/Pcap-2. Consequently, the predicted fragment of 997 and 508 bp was observed in stems from four isolates of A1 mating type and seven isolates of A2 mating type, whereas no amplification products were obtained for samples that were healthy (Fig. 5). Therefore, the use of the primer pair set will make it possible to quickly diagnose the mating type of *P. capsici* isolates in field material of the pepper blight.

Discussion

P. capsici is a soilborne plant pathogen of global significance, particularly with regard to vegetable crops. Because *P. capsici* requires cross-fertilization between disparate A1 and A2 mating types to sexually reproduce and enhance its genetic diversity, the virulence and resistance characteristics of this

Fig. 3. Polymerase chain reaction profiles demonstrating mating type determination of *Phytophthora capsici* isolates using Pacp-1/Pcap-2 primers. Lane M, DL 2000 marker; lanes 1–8, *P. capsici* A1; lanes 9–23, *P. capsici* A2 (lane CK = no DNA template). Isolates of *P. capsici* are listed in Table 1.

Fig. 4. Alignment of mating-type-specific sequences corresponding to different isolates of *Phytophthora capsici* including FY2, YS1, CH2, and TL1. Shade residues with solid black represent them match the consensus exactly. Dashed lines indicate absence of sequence. Numbers on the right refer to the nucleotide sequences.

Fig. 5. Mating type determination of *Phytophthora capsici* in artificially infected plants using Pacp-1/Pcap-2 primers (lane M = DL 2000 marker, lanes 1–11 = artificially infected plants, lane CK = no DNA template). Isolates of *P. capsici* are listed in Table 1.

pathogen depend on the geographical codistribution of its mating types. We sought to develop an accurate and rapid assay to identify *P. capsici* mating types in the field and prevent substantial crop losses from this pathogen. Traditional mating-type assays include visual examination of pheromone production (Ko, 1988) and attempted cross-fertilization with known mating types. These methods are time-consuming and may require microscopic observation. Furthermore, this mating test often failed because the mating type of *P. capsici* could change in response to mechanical damage, growth condition, and the like (Zheng, 1997). The availability of an improved method for the rapid and accurate determination of mating type of *P. capsici* becomes very important. Various PCR-based

molecular methods have been developed to detect and differentiate among fungal plant pathogens using minute quantities of DNA and minimal handling times (Lacourt and Duncan, 1997; Schena et al., 2002). Although molecular detection of different mating types had been reported for *P. infestans* (Kim and Lee, 2002; Kim et al., 2005), no such methodology was available for *P. capsici*. In this study, in contrast to traditional mating-type determination, a PCR-based assay is easily used to detect mating types in vitro and in planta even before they develop typical symptoms. Also, further studies are needed to test the sensitivity of the primer pair linked to Mat-A1 and Mat-A2 of *P. capsici* in the soil before infecting peppers.

Using a primer set designed against an 1121-bp region in the *P. capsici* A1 genome (Pcap-1 and Pcap-2), we report a PCR assay that distinguishes between *P. capsici* mating types. PCR with the primer set amplified a 997-bp amplicon and a 508-bp amplicon from A1 and A2 isolates of *P. capsici*, respectively. The 508-bp fragment possibly was a deletion of the 997-bp fragment.

In the present study, both A1 and A2 mating types of *P. capsici* were detected in a single-pepper field. Similar results have been reported in squash (*Cucurbita pepo* L.), pepper, processing pumpkin (*Cucurbita moschata* L.), and other Cucurbitaceae fields (Islam et al., 2005; Lamour and Hausbeck, 2000; Ristaino, 1990). The co-occurrence of both mating types in pepper fields increases the potential for sexual reproduction, oospore production, and genetic variation of *P. capsici* (Castro-Rocha et al., 2016; Li et al., 2012). The broadleaf weed, common purslane (*Portulaca oleracea* L.), colonized by *P. capsici* could harbor *P. capsici* during the off-season (Ploetz and Haynes, 2000), facilitating the survival of this pathogen. Crop rotation with nonhost species may be an ineffective disease management strategy in regions which A1 and A2 *P. capsici* mating types were colocalized with this weed. The PCR strategy described here can be applied to rapidly and accurately identify codistribution of these mating types in the field. With this assay, it may be possible to monitor the mating type effectively, and we take measures to delay the spread of the two mating types to other areas through strengthening seed transportation management, timely detection of the disease, and more timely fungicide applications.

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