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A *Phytophthora* conserved transposon-like DNA element as a potential target for soyabean root rot disease diagnosis

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A transposon-like element, *A3aPro*, with multiple copies in the *Phytophthora sojae* genome, was identified as a suitable detection target for this devastating soyabean root rot pathogen. The PCR primers TrapF1/TrapR1 were designed based on unique sequences derived from the transposon-like sequence. A 267-bp DNA fragment was amplified using this primer pair, the specificity of which was evaluated against 118 isolates of *P. sojae*, 72 isolates of 25 other *Phytophthora* spp., isolates of *Pythium* spp. and isolates of true fungi. In tests with *P. sojae* genomic DNA, detection sensitivities of 10 pg and 10 fg DNA were achieved in standard PCR (TrapF1/TrapR1) and nested PCR (TrapF1/TrapR1 and TrapF2/TrapR2), respectively. Meanwhile, PCR with TrapF1/TrapR1 primers detected the pathogen at the level of a single oospore, and even one zoospore. These primers also proved to be efficient in detecting pathogens from diseased soyabean tissues, residues and soils. In addition, real-time quantitative PCR (qPCR) assays coupled with the TrapF1/TrapR1 primers were developed to detect and quantify the pathogen. The results demonstrated that the TrapF1/TrapR1 and TrapF2/TrapR2 primer-based PCR assay provides a rapid and sensitive tool for the detection of *P. sojae* in plants and in production fields.

Keywords: *A3aPro*, *Avr3a* gene, molecular detection, PCR, *Phytophthora sojae*, transposon-like sequence

Introduction

Phytophthora sojae is currently one of the most devastating pathogens of soyabean (*Glycine max*), causing damping off to seedlings and root rot to older plants, with an annual worldwide loss of $1–2 billion (Wrather & Koenning, 2006). Since first being reported around 1950 in Indiana and Ohio (Kaufmann & Gerdemann, 1958), *P. sojae* has become widespread in many soyabean-producing countries (Schmitthenner, 1985; Erwin *et al.*, 1996). In the past few years, the disease also has caused serious soyabean losses in Heilongjiang province of China (Zhu *et al.*, 2000). Root rot caused by *Phytophthora* may be found at any stage of soyabean development. Areas that receive heavy rain may suffer plant mortality and yield losses of up to 100% in parts of affected fields. *Phytophthora sojae* is a homothallic species that produces a lot of oospores in infected soyabean tissues (Lee *et al.*, 1993). One oospore develops after an antheridium fertilizes an oogonium. Once the oospore germinates, the inner wall is absorbed and the germ tube produces either a sporangium or mycelium (Schmitthenner, 1999). *Phytophthora sojae* produces heterokont, biflagellate zoospores that are released from sporangia into water. The zoospores rapidly develop into adhesive cysts upon reaching the potential host plants. Infection occurs when cysts germinate to form appressoria or produce hyphae. The hyphae directly penetrate the plant epidermis, causing serious root rot (Enkerli *et al.*, 1997; Tyler, 2007). Growing soyabeans in continuous monoculture over several years results in increased losses to disease (Schmitthenner, 1985). Considering that increasing numbers of soyabean shipments are being traded to and from different countries and areas, root rot caused by *P. sojae* poses a serious threat to worldwide soyabean production. Therefore, rapid, accurate and sensitive detection is important for managing this disease and preventing its spread. The current protocols to regulate the spread of *P. sojae* are based on the Chinese quarantine industry standards SN/T1131-2002 and SN/T2474-2010. The detection methods of SN/T1131-2002 involve baiting from soils or isolation through selective media from infected plant tissues, followed by identification based on morphological characteristics (Canaday & Schmitthenner, 1982; Oudemans, 1999). This traditional detection method plays an important role, but the isolation and accurate identification of *P. sojae* is very complex and time-consuming. DNA-based techniques provide an effective means for identification of plant pathogens. The polymerase chain reaction (PCR) offers advantages over more traditional methods of pathogen detection because the organisms need not be cultured and the technique is rapid, with high sensitivity and specificity (Ersek *et al.*, 1994; Bonants *et al.*, 1997; Lacourt & Duncan, 1997;

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Cullen *et al.*, 1999). The specificity of PCR-based detection technology is determined by the target gene sequences used, and many primers have been designed based on the internal transcribed spacer (ITS) region (Cooke *et al.*, 1995; Bonants *et al.*, 1997; Tooley *et al.*, 1997; Trout *et al.*, 1997; Liew *et al.*, 1998; Schubert *et al.*, 1999; Judelson & Tooley, 2000; Winton & Hansen, 2001; Grote *et al.*, 2002; Ippolito *et al.*, 2002). The methods of SN/T2474-2010 are based on the ITS region with primers PS1 and PS2 for molecular detection of *P. sojae*, drafted in the laboratory of the present study (Wang *et al.*, 2006). Apart from the ITS region, other genes, such as *β*-tubulin (Fraaije *et al.*, 1999), actin-coding sequences (Weiland & Sundsbak, 2000), translation elongation factor 1-alpha (EF-1*α*; O'Donnell *et al.*, 1998), cytochrome oxidase (Martin *et al.*, 2004), elicitin (Lacourt & Duncan, 1997), etc. are easy to detect. However, in other cases, sequences of these conserved genes are not sufficiently variable to distinguish between closely related species (Wang *et al.*, 2006). As a result, a target with high specificity and efficiency is needed for rapid detection of *P. sojae* in soyabean production fields and in soil and residues carried with transported soyabeans. This will be very important for controlling the dispersal of *P. sojae* and maintaining '*Phytophthora*-free' soyabean production.

To this end, this study used an identifiable target, named *A3aPro*, which is a 300-bp deletion element upstream (1·5 kb in the promoter region) of the avirulence gene *Avr3a* in *P. sojae* race 7, when compared to races 2 and 12 (Fig. S1), to develop a PCR assay for *P. sojae*. The objectives of the study were to: (i) develop species-specific primers for detecting *P. sojae* based on the identifiable target *A3aPro*; (ii) evaluate the sensitivity of the primers using PCR and real-time SYBR® Green I quantitative PCR (qPCR) assays; (iii) use the assay to detect the pathogen in diseased soyabean tissues; and (iv) determine whether the primers can be used to detect the pathogens in soil samples from different regions.

Materials and methods

Source of isolates

Phytophthora sojae isolates were obtained from diseased soyabean stems collected in different areas of Heilongjiang province in China from 2002 to 2011. All tested *P. sojae* isolates were isolated using a leaf disc baiting method from diseased soyabean plots (Jinhuo & Anderson, 1998). Using the same method, additional *P. sojae* isolates were baited from soyabean residues and soil carried by soyabeans imported from the USA, Brazil, Argentina and Canada. The *P. sojae* isolates, as well as isolates of *Phytophthora* spp., *Pythium* spp., *Fusarium* spp. and various other pathogens used in this study, are maintained in a collection in the Department of Plant Pathology, Nanjing Agricultural University, and are listed in Table 1.

Culture conditions and preparation of mycelia

Phytophthora isolates were cultured on 10% V8 media at 25°C (Erwin *et al.*, 1996). Mycelia of each *Phytophthora* and *Pythium* isolate were obtained by growing the isolates on 10% V8 media at 18–25°C (temperature-dependent isolates) for at least 3 days (Zheng, 1995). Mycelia of the other fungi were grown in potato dextrose broth (Erwin *et al.*, 1996). The mycelia were harvested by filtration and frozen at −20°C.

Source of plant samples

Plant samples exhibiting disease symptoms, collected from the field in Heilongjiang province, China in 2010, were processed at the Department of Plant Pathology, Nanjing Agricultural University. The pathogen was isolated from the diseased plant tissues into pure culture to verify the presence of *P. sojae*.

Source of soil samples

A total of 360 soil samples were collected from the soyabean production fields in several provinces (Heilongjiang, Jiangsu, Anhui, Shanghai, Xinjiang, Sichuan, Henan and Guizhou) of China from 2002 to 2010. In addition, 33 soil samples were collected from the soil carried with soyabeans imported from the USA, Brazil, Argentina and Canada and described previously (Kalinina *et al.*, 1997; Wang *et al.*, 2006). In commercial processing systems, soyabeans are typically screened to remove contaminated soil and soyabean residues. These soils and residues were collected for PCR assays and the samples evaluated are listed in Table 2.

Baiting assay

Soyabean field soil (10 g) was moistened in a Petri dish (9 cm diameter), preincubated in the light at 25°C for a week, then flooded with 25 mL sterile distilled water (SDW). Twenty leaf discs (10-mm diameter), freshly cut with a paper punch from the unifoliolate leaves of 10-day-old soyabean cv. Williams seedlings were floated immediately on the surface of the water. After 12 h, the floating leaf discs were removed, washed and placed in another Petri dish with 25 mL SDW. Sporangia emerging from the edge of the infected leaf discs, observed under stereomicroscopy after 48 h of incubation in the water, were recorded as positive results.

Sources of DNA

DNA of mycelia was extracted from pure microbial cultures as described by Schubert *et al.* (1999). These cultures included isolates of *P. sojae*, other *Phytophthora* spp., isolates of *Pythium* spp. and isolates of true fungi, as listed in Table 1. DNA from mycelia was obtained according to a modified cetyltrimethyl ammonium bromide (CTAB) procedure (Sambrook *et al.*, 1989). DNA

Table 1 Isolates of fungi and oomycetes used to screen *Phytophthora sojae* (TrapF1/TrapR1) and universal (ITS1/ITS4) primers

Species	Isolation/origin		No. isolates	PCR product	
	Host/substrate	Source[a]		ITS1/ITS4	TrapF1/TrapR1
P. sojae (race 2)	*Glycine max*	NJAU	5	+	+
P. sojae (race 3)	*Glycine max*	NJAU	4	+	+
P. sojae (race 6)	*Glycine max*	NJAU	3	+	+
P. sojae (race 7)	*Glycine max*	NJAU	5	+	+
P. sojae (race 8)	*Glycine max*	NJAU	2	+	+
P. sojae (race 12)	*Glycine max*	NJAU	2	+	+
P. sojae (race 13)	*Glycine max*	NJAU	3	+	+
P. sojae (race 14)	*Glycine max*	NJAU	5	+	+
P. sojae (race 17)	*Glycine max*	NJAU	2	+	+
P. sojae (race 19)	*Glycine max*	NJAU	2	+	+
P. sojae (race 20)	*Glycine max*	NJAU	2	+	+
P. sojae (race 28)	*Glycine max*	NJAU	2	+	+
P. sojae (race 31)	*Glycine max*	NJAU	2	+	+
P. sojae	*Glycine max*	NJAU	79	+	+
P. boehmeriae	*Gossypium* sp.	NJAU	4	+	–
P. ramorum	Oak species	NJAU	1	+	–
P. drechsleri	*Beta vulgaris* var. *altissima*	CBS 292.35	1	+	–
P. vignae	*Vigna sinensis*	Michael D. Coffey	1	+	–
P. cambivora	*Castanea sativa*	CBS 248.60	1	+	–
P. rubi	Raspberry	CBS 967.95	2	+	–
P. cinnnamomi	*Cedrus deodara*	NJAU	1	+	–
P. melonis	*Cucumis sativus*	NJAU	8	+	–
P. brassicae	*Brassica* sp.	CBS 178.87	1	+	–
P. cactorum	*Malus pumila*	NJAU	19	+	–
	Rosa chinensis	NJAU	1	+	–
P. capsici	*Capsicum annuum*	NJAU	5	+	–
	Lycopersicon esculentum	NJAU	1	+	–
P. colocasiae	Unknown	CBS 192.91	1	+	–
P. cryptogea	*Gerbera jamesonii*	NJAU	5	+	–
P. drechsleri	*Beta vulgaris* var. *altissima*	CBS 292.35	1	+	–
P. erythroseptica	*Solanum tuberosum*	CBS 129.23	1	+	–
P. fragariae var. *rubi*	Raspberry	CBS 967.95	1	+	–
P. hibernalis	*Cirrus sinensis*	CBS 270.31	1	+	–
P. idaei	Raspberry	CBS 968.95	1	+	–
P. infestans	*Solanum tuberosum*	NJAU	2	+	–
P. medicaginis	*Medicago sativa*	ATCC 44390	1	+	–
P. melonis	*Cucumis sativus*	NJAU	8	+	–
	Cucumis sativus	NJAU	1	+	–
	Cucumis sativus	NJAU	1	+	–
	Benincasa hispida	NJAU	1	+	–
	Lagenaria siceraria	NJAU	1	+	–
Pythium ultimum	Irrigation water	NJAU	10	–	
Botrytis cinerea	*Cucumis sativus*	NJAU	1	+	–
Colletotrichum gossypii	*Gossypium* sp.	NJAU	1	+	–
C. truncatum	*Glycine max*	NJAU	8	+	–
Fusarium avenaceum	Unknown	CGMCC	1	+	–
F. culmorum	Unknown	CGMCC	1	+	–
F. equiseti	Unknown	CGMCC	1	+	–
F. graminearum	*Triticum aestivum*	NJAU	1	+	–
F. oxysporium f. sp. *vasinfectum*	*Gossypium* sp.	NJAU	2	+	–
F. oxysporium f. sp. *cucumerinum*	*Cucumis sativus*	NJAU	3	+	–
F. oxysporium f. sp. *cubense*	*Musa sapientum*	NJAU	1	+	–
F. sambucinum	Unknown	CGMCC	1	+	–
F. solani	*Gossypium* sp.	NJAU	5	+	–
	Glycine max	NJAU	15	+	–
Macrophoma kawatsukai	*Malus pumila*	NJAU	1	+	–
Magnaporthe grisea	*Oryza sativa*	NJAU	3	+	–
Rhizoctonia solani	*Gossypium* sp.	NJAU	2	+	–
	Glycine max	NJAU	3	+	–
Verticillium dahliae	*Gossypium* sp.	NJAU	3	+	–
V. albo-atrum	*Medicago sativa*	NJAU	1	+	–

[a]ATCC, American Type Culture Collection; CBS, Centraalbureau voor Schimmelcultures; CGMCC, China General Microbiological Culture Collection; NJAU, Nanjing Agricultural University.

Table 2 Soil samples used in this study, and numbers that were positive in the *A3aPro* PCR assay, in the ITS PCR assay and in the baiting assay

Source	No. of soil samples	No. *A3apro*-PCR positive	No. ITS-PCR positive	No. leaf-disc baiting positive
Heilongjiang	25	19	17	19
Jiangsu	29	20	20	17
Anhui	53	32	30	31
Shanghai	61	20	18	17
Xinjiang	68	18	17	17
Sichuan	60	10	9	6
Henan	46	10	9	8
Guizhou	18	2	2	2
USA	24	19	18	12
Brazil	2	1	1	1
Argentina	1	1	1	1
Canada	6	4	2	3

from oospores existing in contaminated soils was extracted by a screening method: 200-mesh (76 μm), 300-mesh (54 μm) and 400-mesh (38 μm) screens (all 20-cm diameter) were used to eliminate most of the soil, and a 600-mesh (25 μm) screen (20-cm diameter) was used to collect the oospores. A single oospore was selected under a microscope, rinsed in 100 μL SDW in a 1·5-mL Eppendorf tube and treated with a FastDNA® SPIN Kit for Soil (Q-Biogene) to extract DNA. To extract crude DNA from zoospores, hyphal tip plugs of *P. sojae* isolates were used to inoculate 30 mL sterile clarified 10% V8 broth in 90-mm Petri dishes to obtain axenically prepared mycelium. Mycelial cultures were incubated at 25°C in the dark for 3 days. Sporulating hyphae were prepared by repeatedly washing 2-day-old hyphae incubated in 10% V8 broth with SDW and incubating the washed hyphae in the dark at 25°C for 4–8 h until sporangia developed on most of them. Zoospores were filtered with Miracloth (Calbiochem) and collected by centrifugation at 2000 ***g*** for 2 min. One hundred zoospores were suspended in 100 μL double-distilled water, 0·5 g silica added, and the mixtures vortexed for 1 min, after which 1–10 μL suspension was added to the PCR reaction. DNA of *P. sojae* from plant samples was prepared using the NaOH method (Wang *et al.*, 1993), which can extract sufficient DNA in an appropriate buffer and use it directly for PCR. DNA from soil samples was extracted directly using a FastDNA® SPIN Kit for Soil (Q-Biogene) according to the manufacturer's recommendations. DNA concentrations were determined spectrophotometrically or by quantitation on 1% agarose gels stained with ethidium bromide in comparison with commercially obtained standards. DNA was stored at −20°C.

PCR

Each reaction for PCR consisted of 2·5 μL 10 × PCR buffer, 1 μL 2·5 mM dNTP, 2·5 μL 25 mM $MgCl_2$, 0·25 μL each of 20 μM primers, and 0·25 μL *Taq* DNA polymerase (5 U μL^{-1}), to which were added 1 μL template DNA and then SDW to a final volume of 25 μL. All reactions were performed in a PTC-2000 PCR instrument (MJ Research). The thermal cycling settings used were an initial denaturation at 94°C for 5 min, 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 30 s, then a final extension at 72°C for 10 min. Negative controls lacking template DNA were performed in each experiment to test for contamination. The products of PCR were electrophoresed on 1% agarose gels and stained with ethidium bromide. All of the reagents used for PCR amplification were purchased from TakaRa. PCR was repeated on independent DNA extractions. The amplification was estimated based on at least six replicates.

Real-time PCR

Amplification was monitored in real-time using the 7500 Real-Time PCR System (Applied Biosystems). Reaction volumes (20 μL) contained 2 × SYBR® Premix ExTaq™ (Takara), primers at 10 μM each, ROX reference dye II (50 ×), 2 μL PCR control template, and SDW to the final volume. The reaction mixture was initially incubated at 50°C for 2 min, followed by 30 s denaturation at 95°C, and 40 cycles of 5 s at 95°C and 34 s at 60°C. The standard curve was constructed by plotting the log of a known concentration (10-fold dilution series from 100 ng to 10 fg) of DNA from *P. sojae* against the threshold cycle (C_t) values and melting curve. The amplification was estimated based on at least six replicates.

A3aPro-based primer design

The *A3aPro* sequence was acquired from the Joint Genome Institute (JGI) *P. sojae* genome database (http://www.jgi.doe.gov/) (position: scaffold 80: 317485–317760) and the entire *A3aPro* sequence from *P. sojae* was submitted to GenBank (accession no. JX118829). Using the *A3aPro* sequence of *P. sojae* as a bait for a BLASTN search did not show any similarity with other sequenced isolates of *P. infestans*, *P. ramorum* or *Hyaloperonospora parasitica*. Similar *A3aPro* sequences were then obtained from the genome databases for *P. infestans*, *P. ramorum* and *H. parasitica*. *Phytophthora infestans* T30-4 DNA sequence was available from the Broad Institute (http://www.broad.mit.edu/) (position: supercontig 1849: 1900–2350); *P. ramorum* DNA sequence was available from the Joint Genome Institute (JGI; http://www.jgi.doe.gov/) (position: scaffold 1220: 1–342); *H. parasitica* genome sequence was available from http://vmd.vbi.vt.edu/ (position: contig 159: 13356–13809). The *P. sojae* primers TrapF1 (5′-CGGTGGCTCTCGGCATTCGTG-3′) and TrapR1 (5′-CACCCTACTGTTATAGACACG-3′) were designed based on the *A3aPro* sequence (Fig. 1). In order to increase the sensitivity of the standard PCR, TrapF2 (5′-ATCTGACGGTGGCTCTCGGC-3′) and TrapR2 (5′-TTACACCCTACTGTTATAGAC-3′) were similarly designed for nested PCR (Fig. 1).

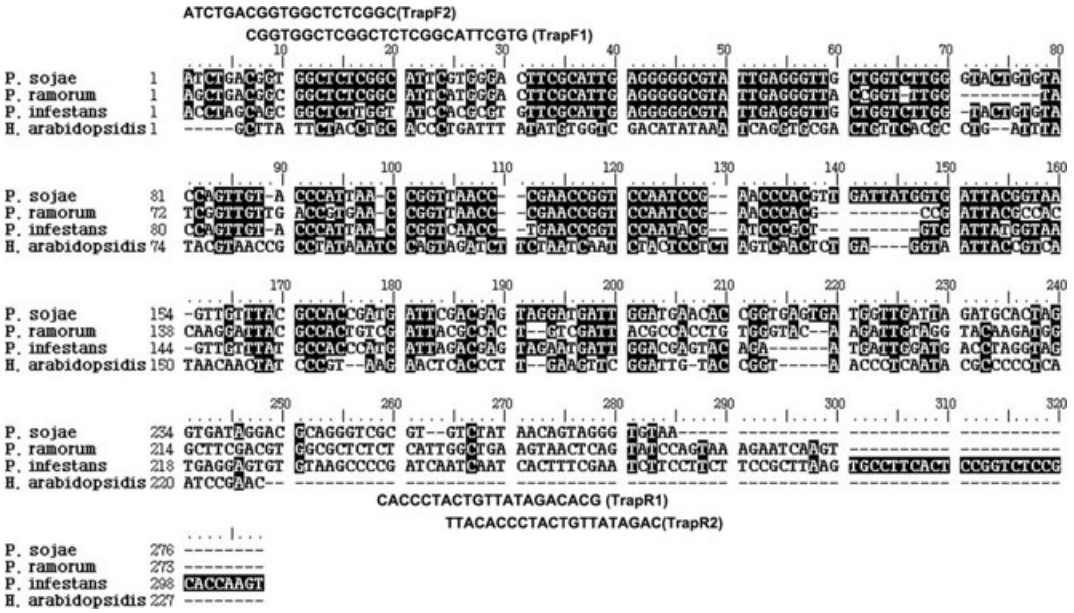

Figure 1 Nucleotide sequence alignment of the target region *A3aPro* of *Phytophthora sojae* and similar sequences of other species used for designing PCR primers. The nucleotide sequence of the sense strand of *A3aPro* DNA is shown. The schematic diagram shows the positions of the primers TrapF1/TrapR1 and TrapF2/TrapR2 for amplification.

Results

Specificity of primers TrapF1 and TrapR1

The specificity of the *P. sojae* primers TrapF1 and TrapR1 was evaluated using 118 isolates of *P. sojae* and 136 other isolates of fungi and oomycetes (Table 1). The 118 isolates representing *P. sojae* were able to amplify a unique DNA sequence of 267 bp (Fig. S2c) with primers TrapF1 and TrapR1. However, isolates representing all of the other *Phytophthora* species and the other species tested yielded no amplification products (Fig. S2a,b) with primers TrapF1 and TrapR1. All of the DNA preparations from fungi and oomycetes amplified a product with the ITS universal primers ITS1/ITS4 (Table 1), proving that the DNA preparations were suitable for PCR amplification.

Sensitivity of TrapF1 and TrapR1

To determine the detection sensitivity of the standard PCR, DNA from *P. sojae* P6497 was serially diluted from 1 ng to 100 ag, and 1 μL of each dilution was used as a template for PCR using the conditions previously described. A nested PCR protocol using the primers (TrapF1/TrapR1 and TrapF2/TrapR2) was also assessed for sensitivity: the serially diluted template DNA was initially amplified using the outer primers TrapF2/TrapR2, as previously described; then 1 μL of the products from PCR with primers TrapF2/TrapR2 was used as a template for secondary amplification with the TrapF1/TrapR1 primers. The detection limit of the standard PCR was 10 pg μL^{-1} (Fig. 2a). The nested PCR could detect as little as 10 fg DNA μL^{-1} (Fig. 2b) (9·3 copies in genome equivalents) (http://www.uri.edu/research/gsc/resources/cndna.html). Other *P. sojae* isolates (R3, R20, R31) were also tested; the results showed the same sensitivity (data not shown). At least six replicates of each dilution were evaluated to assess the sensitivity of the *A3aPro* PCR reaction.

Crude DNA from zoospores and oospores was used as template for testing the sensitivity of primers TrapF1 and TrapR1. Ten concentrations of zoospores and oospores were tested, ranging from 1000 to 1 μL^{-1}. With a 1 μL^{-1} concentration, DNA could be extracted from one to 10 zoospores or oospores as template in a 25-μL reaction volume, all resulting in amplification of the 267-bp band

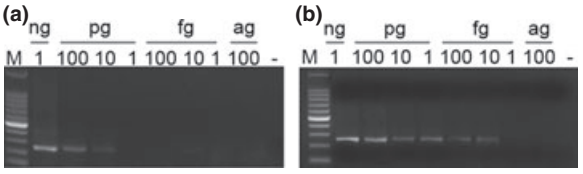

Figure 2 Sensitivity of the *A3aPro* PCR method using serially diluted genomic DNA (1 ng–100 ag) with *Phytophthora sojae* isolate P6497 as template. M, DL100 bp DNA ladder marker. –, no template DNA. (a) Standard PCR using the primers TrapF1/TrapR1; detection limit 10 pg μL^{-1}. (b) Nested PCR using the product of primers TrapF2/TrapR2 as template and TrapF1/TrapR1 as the primers; detection limit 10 fg μL^{-1}.

(Fig. S3). Therefore, DNA extracted from a single oospore or even one zoospore was also sufficient for PCR detection. At least six replicates of each dilution were evaluated to assess the sensitivity of the *A3aPro* PCR reaction.

Sensitivity of TrapF1 and TrapR1 using real-time PCR

The suitability of the species-specific primers TrapF1/TrapR1 for qPCR was demonstrated by a pilot study using SYBR Green chemistry and validated using standard *P. sojae* isolate P6497. A qPCR using DNA extracted from pure cultures of standard *P. sojae* isolate P6497 produced an amplicon with template concentrations from 100 ng (average C_t = 11·9) to 10 pg (average C_t = 25·3). Electrophoresis on 1% agarose gels showed that the amplicons were the predicted size (267 bp) (data not shown). The standard curve showed a linear correlation between the logarithm of the concentration and the C_t values, with a correlation coefficient (R^2) of 0·9991. The relationship between C_t (y) values and the log of DNA concentration (x = log DNA concentration) for *P. sojae* was expressed by the equation $y = -3{\cdot}382x + 18{\cdot}689$ (Fig. S4). At least six replicates of each dilution were evaluated to assess the sensitivity of the *A3aPro* qPCR reaction.

Detection in diseased soyabean tissues

DNA samples of *P. sojae* from different regions of Heilongjiang province in 2010 were prepared using the NaOH method. A 267-bp DNA fragment was amplified using the specific primer pair TrapF1/TrapR1, while no PCR product was amplified in the negative controls (Fig. 3). The presence of *P. sojae* was verified by isolating the pathogen from the tissue into pure culture. In addition, to confirm that no PCR inhibitors were present in healthy soyabean tissues, the primers ITS1 and ITS4, which amplify a 700-bp DNA band (data not shown), were used in PCR analyses as a control. The PCR amplifications were repeated at least three times per sample.

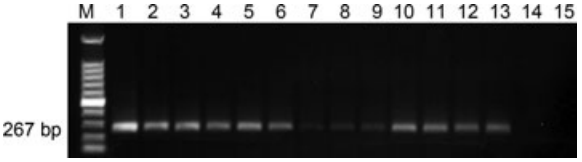

Figure 3 Amplification of *Phytophthora sojae* DNA from diseased soyabean tissues (at least six replicates) using TrapF1/TrapR1 primers. M, DL100 bp DNA ladder marker. Lane 1, positive control, 10 ng *P. sojae* DNA; lanes 2–13, amplified products using DNA of diseased soyabean tissues from different regions of Helongjiang province (2, Haerbin; 3, Jiamusi; 4, Qiqihaer; 5, Mudanjiang; 6, Jixi; 7, Daqing; 8, Yichun; 9, Hegang; 10, Shuangyashan; 11, Heihe; 12, Wuchang; 13, Qitaihe); lane 14, healthy plant tissues; lane 15, no template DNA.

Detection in infested field soils

To evaluate the *A3aPro* PCR assay for detection of *P. sojae*, 360 soil samples collected from different regions of China from 2002 to 2010, plus 33 samples from the USA, Brazil, Argentina and Canada, were tested by the *A3aPro* PCR assay and the ITS PCR assay, which has been previously described (Wang *et al.*, 2006). Using the *A3aPro* PCR-based method, 156 of the 393 samples (39·7%) produced 267-bp bands (Fig. S5), compared with 144/393 (36·6%) using the ITS PCR assay and 134/393 (34%) using the baiting method (Table 2). Hence, the *A3aPro* PCR assay reported here may be used for detection of *P. sojae* in plants and production fields.

Comparison of previously published primers PS1/PS2 and the newly developed primers TrapF1/TrapR1

The new developed primers TrapF1 and TrapR1 produce a product of 267 bp. Isolates representing all of the other species tested yielded no amplification product with primers TrapF1 and TrapR1 (Fig. S2). Wang *et al.* (2006) reported detection of *P. sojae* with primers PS1 and PS2 down to 1 fg DNA in an ITS-based PCR assay, but several other *Phytophthora* species, such as *P. melonis* and *P. drechsleri*, cross-reacted in the assay (Fig. S6a); only when the annealing temperature was increased to more than 66°C was the amplification of a specific 330-bp PCR product from *P. drechsleri* and *P. melonis* prevented (Fig. S6b).

Discussion

Research on the interaction between soyabean and *P. sojae* will greatly increase understanding of the disease mechanisms and help to control crop damage. The avirulence gene *Avr3a* from *P. sojae* was recently identified (Qutob *et al.*, 2009), and elucidation of its structure has improved understanding of the evolution and characteristics of *Avr* genes. This will also aid in identification of additional *Avr* genes in the future. Given this research background, a DNA transposon-like sequence of *Avr3a*, designated *A3aPro*, which has a high copy number in the genome of *P. sojae* and is species-specific, was used to develop the specific and sensitive PCR primers TrapF1/TrapR1 for rapid detection of *P. sojae*.

The *A3aPro*-based primers TrapF1 and TrapR1 for *P. sojae* designed here had higher specificity than the previously reported (Wang *et al.*, 2006) primers PS1 and PS2 based on ITS sequences. There was a 97% similarity in ITS sequences between *P. sojae* and *P. melonis* or *P. drechsleri*. Only an increase in annealing temperature to 66°C or more could distinguish among these *Phytophthora* species. However, a high annealing temperature may reduce the sensitivity of PCR reactions. Thus, increasing the annealing temperature did not resolve this

problem. One approach to improving the discrimination between similar species is to design a new assay based on a different region of the genome. Thus, the primers TrapF1 and TrapR1, based on an identifiable target, *A3aPro*, were designed specifically for *P. sojae* in the present paper. A 267-bp DNA fragment from the genome of all 118 analysed isolates of *P. sojae*, regardless of origin, was produced using this primer pair; isolates of other species, including *P. melonis* and *P. drechsleri*, amplified no PCR product (Fig. S2).

The detection sensitivity of the TrapF1/TrapR1 primer pair was demonstrated to be 10 pg μL^{-1} in a standard PCR and increased 1000-fold to 10 fg μL^{-1} in a nested PCR (Fig. 2). The higher sensitivity of the *A3aPro*-based primers developed here compared to ITS-derived primers is probably the result of the higher abundance of the *A3aPro* target, which is more than 200 copies in the JGI database (data not shown). Meanwhile, in tests to assess the sensitivity, real-time PCR combined with fluorescent SYBR Green I dye was used to quantify *P. sojae* directly from purified DNA, and could detect 10 pg μL^{-1} pure DNA per 25-μL reaction volume (Fig. S4). Although this was not an improvement on standard PCR, it had the advantage of allowing pathogen levels to be quantified, which may be important for studies investigating levels of pathogen occurrence or the inoculum levels in the field. The PCR detection limit is a single oospore or even one zoospore for the TrapF1/TrapR1 primers (Fig. S3). For *P. sojae*, the PCR detection sensitivity of a single oospore or one zoospore is biologically useful and would allow the pathogen to be detected at low levels in the field. However, this theoretical limit assumes that there is no inhibition in the PCR reaction.

Combined with rapid NaOH lysis, the PCR assay could be used to detect *P. sojae* in less than 3 h in infected soyabean tissues (Fig. 3). This is especially important for the import and export trade of soyabeans, or when the target concentration is low. The absence of amplification from healthy roots removed any doubt about the specificity of this primer pair.

Furthermore, the assay allows the rapid detection of the pathogen using primers TrapF1/TrapR1 in soil samples (Fig. S5). In this study, 393 soil samples from China and other countries were tested by the *A3aPro* PCR assay, ITS PCR assay, and a leaf-disc baiting method for comparison (Table 2). Comparing the positive-sample ratios of the three methods, the newly developed *A3aPro* PCR assay improved the detection efficiency and was more specific than the ITS assay. Increasing numbers of soyabean shipments are traded to and from different countries, including more than 56 million tons imported into China (where *P. sojae* is a quarantine pathogen) annually. Therefore, rapid detection of *P. sojae* in the soil carried with the transported soyabeans is not only important for the soyabean trade between China and other countries, but also for controlling the spread of *P. sojae* within China. The newly developed PCR detection technology with TrapF1/TrapR1 primers offers an effective way to do this and, in view of its speed and efficiency, it is especially suitable for quarantine situations. It has, in fact, been used in some Chinese Entry–Exit Inspection and Quarantine Bureaus in the past few years.

In conclusion, the *Phytophthora* conserved transposon-like DNA element is a potential target for soyabean root rot disease diagnosis and the TrapF1/TrapR1 and TrapF2/TrapR2 primer-based PCR assay provides a rapid and sensitive tool for the detection of *P. sojae* in plant tissues and in soils from production fields. Moreover, this assay could control the dispersal of *P. sojae* and increase '*Phytophthora*-free' soyabean production.

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Supporting Information

Additional Supporting Information may be found in the online version of the article:

Figure S1. The position of the *A3aPro* element in the *Avr3a* gene.

Figure S2. 1% agarose gel electrophoresis of polymerase chain reaction (PCR) products using *Phytophthora sojae*-specific primers TrapF1/TrapR1 (at least 6 replicates). (a) PCR amplification products from *P. sojae* strain and isolates of *Phytophthora* spp. (b) PCR amplification products from *P. sojae* strain and isolates of true fungi. (c) PCR amplification products from different *P. sojae* strains.

Figure S3. Sensitivity of standard PCR with *Phytophthora sojae* DNA from zoospores (upper row) and oospores (lower row). PCR with TrapF1/TrapR1 primers detected the pathogen at the level of a single oospore.

Figure S4. Correlation coefficients assessed for *Phytophthora sojae* using real-time PCR amplification with primers TrapF1 and TrapR1 (at least 6 replicates).

Figure S5. Nested PCR with TrapF1/TrapR1 and TrapF2/TrapR2 primers amplification of DNA extracted from infested field soils (at least 6 replicates).

Figure S6. Polymerase chain reaction (PCR) products using previously published primers PS1/PS2 of *Phytophthora sojae* (at least 6 replicates). Annealing temperature: (a) 60°C; (b) 66°C.

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