Rapid diagnosis of soybean anthracnose caused by *Colletotrichum truncatum* using a loop-mediated isothermal amplification (LAMP) assay

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Abstract A loop-mediated isothermal amplification (LAMP) assay that directly detects *Colletotrichum truncatum* in diseased soybean tissues is described, thus allowing rapid diagnosis of soybean anthracnose. Using the target gene *Rpb1* (that codes for the large subunit of RNA polymerase II), we designed and screened a set of species-specific primers allowing amplification at 62 °C over 70 min. After addition of SYBR Green I to the LAMP reaction products, a yellow-green color (visible to the unaided eye) developed only in the presence of *C. truncatum*. The detection limit of the LAMP assay was 100 pg (per μL genomic DNA). The *Rpb1*-Ct-LAMP assay has been successfully used to diagnose soybean anthracnose in field samples collected from Jiangsu, Anhui and Hubei provinces of China, and to detect *C. truncatum* in soybean seeds from farmers' markets. Our results show that the *Rpb1*-Ct-LAMP assay is a useful and convenient method for detecting *C. truncatum,* and thus for diagnosis of soybean anthracnose.

Keywords *Colletotrichum truncatum* · *Rpb1* · Soybean anthracnose · Loop-mediated isothermal amplification (LAMP) assay

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Introduction

Colletotrichum truncatum is an important pathogen that infects at least 180 host genera in 55 plant families worldwide (Mahmodi et al. 2014). The fungus causes anthracnose of pepper (Mahmodi et al. 2014), dragon fruit (Guo et al. 2014), and many leguminous species of economic importance, including lentil, soybean, and fava bean (Bhadauria et al. 2011). Soybean seed is an important oilseed crop and a major source of protein (40%, w/w) and oil (20%, w/w) for human and animal consumption. According to Wrather et al. (1997), brown spot, charcoal rot, stem canker, soybean cyst nematode, sclerotinia stem rot and anthracnose are all important diseases in major soybean-producing countries. However, *C. truncatum* is one of the most destructive and widespread seed-borne fungi affecting soybean (Ploper and Backman 1992). Soybean production in the major producing countries in 2006 were as follows: United States (83.4×10^6 t), Brazil (57.0×10^6 t), Argentina (40.5×10^6 t), China (16.4×10^6 t), India (7.0×10^6 t), and Paraguay (3.6×10^6 t), with the yield reduction caused by anthracnose estimated to be 492,900 t in United States, followed by Argentina (45,300 t), Brazil (22,000 t), China (1663,500 t), India (117,600 t) and Paraguay (300 t) (Wrather et al. 2010). The disease also commonly occurs in northeast China, north China, east China and in other regions of the world (He and Luo 2012).

As a soil-borne disease (Manandhar et al. 1987), anthracnose damages soybean commencing at the seedling stage up to harvest (Kwon et al. 2013), and the disease can affect all above-ground parts of the plant. The most typical and distinctive symptoms, irregularly shaped brown

lesions that gradually become roundish and slightly concave, appear during the early reproductive stages of soybean, developing on the stems, petioles, and pods. As the disease progresses, numerous epidermal acervuli with many setae develop in the lesions. The lesions enlarge slowly, requiring one month or more to cover an entire lamina (Sugawara et al. 2009); however, leaves, pods, and stems may also be infected without showing symptoms (Chen et al. 2006; Hartman et al. 1999).

In addition to *C. truncatum, C. gloeosporioides, C. capsici, C. dematium, C. destructivum,* and *C. coccodes* (Lou et al. 2009; Daniells et al. 1995; Riccioni et al. 1998; Roy 1982) also cause soybean anthracnose but *C. truncatum* is often the most common of the species causing disease (Ploper and Backman 1992; Chen et al. 2006). Because of the morphological similarity of *Colletotrichum* species, they are difficult to distinguish by microscopic examination. Morphological characterization is also tedious, time-consuming, and of low specificity (Daniells et al. 1995). Moreover, identification is further complicated due to symptoms on soybean differing depending on growth stage, host strain, cultivar, and weather conditions. Such diversity and complexity among symptoms contributes to difficulty in rapidly and accurately diagnosing anthracnose in soybean caused by *C. truncatum.* Rapid diagnosis is important to help manage the disease and limit spread of the pathogen. With the development of molecular technologies, the polymerase chain reaction (PCR) has been used successfully to detect *C. truncatum* (Forseille 2007), but it has intrinsic disadvantages, including low amplification efficiency and a complicated experimental process (Mori et al. 2001). A simple, rapid, and cost-effective method for detection of *C. truncatum* is therefore needed.

Loop-mediated isothermal amplification (LAMP) is a novel method of nucleic acid amplification in which the target nucleic acid is amplified efficiently by a single enzyme at a constant temperature in less than 80 min (Nagamine et al. 2001, 2002; Notomi et al. 2000). The LAMP method uses four primers that recognize six regions on the target nucleic acid sequence, affording a higher specificity compared to that of traditional PCR. The large quantity of amplified product and by-product (magnesium pyrophosphate) obtained via the LAMP reaction allow effective detection of DNA based on visual assessment of turbidity, or a color change developing upon addition of colour-changing reagents (Iwamoto et al. 2003; Goto et al. 2009; Mori et al. 2001; Nagamine et al. 2002); or using chromatographic

lateral flow dipsticks (LFDs) to detect labels incorporated into the products during or after amplification (Kiatpathomchai et al. 2008; Tomlinson et al. 2010a, b). The Genie I instrument (Opti-Gene, Horsham, UK) is also a portable, low-power platform for real-time fluorescence monitoring of isothermal amplification methods including LAMP that is suitable for on-site use (Tomlinson et al. 2010a, b).

We used the target sequence of the *Rpb1* gene (which codes for the large subunit of RNA polymerase II) to develop a rapid and sensitive LAMP assay for detection of *C. truncatum* in diseased soybean tissue. The addition of SYBR Green I to the LAMP product allowed the presence of fungal DNA to be confirmed by the unaided eye.

Materials and methods

Strains

Isolations of *C. truncatum* were made from diseased leaves, stems, and pods of soybean, pepper, and peanut collected in Jiangsu, Anhui, and Hubei provinces of China from 2009 to 2014. The isolates of *C. truncatum,* strains of other *Colletotrichum* spp., *Fusarium* spp., and other fungi used in this study (Table 1) were identified by morphological assessment and ITS sequencing prior to use, and are maintained in our laboratory.

Culture conditions and DNA extraction

Colletotrichum truncatum and other fungal (*C. gloeosporioides, C. acutatum, C. capsici, C. dematium, C. destructivum C. coccodes, Aspergillus oryzae, Alternaria alternata, Macrophomina phaseolina, Diaporthe phaseolorum* var. *caulivora, Diaporthe phaseolorum* var. *meridionalis, Phomopsis longicolla, Phialophora gregata* f. sp. *sojae, Fusarium oxysporum, Bipolaris maydis* in Table 1) were cultured on potato dextrose agar (PDA) (200 g potato extract L^{-1}, 2% [w/v] glucose, and 2% [w/v] agar, autoclaved at 120 °C for 20 min). Mycelia of each isolate were obtained by culture in potato dextrose broth (200 g potato extract L^{-1}, 2% [w/v] glucose, autoclaved at 120 °C for 20 min) (Erwin and Ribeiro 1996) at 28 °C for 3–5 days. *Phytophthora sojae* was cultured on V8 medium, and mycelia of *P. sojae* were cultured in V8 juice broth (Zheng 1995). The mycelia were harvested by filtration and frozen at −20 °C. Mycelial DNA was extracted using a DNAsecure

Table 1 Fungal species and isolates used to screen the specificity of the Rpb1-Ct-LAMP assay for *Colletotrichum truncatum*

Fungal species	Host	Source	Number of isolates tested
Colletotrichum truncatum	Soybean	Xuzhou, Jiangsu	7
		Jiangyan, Jiangsu	6
		Taixing, Jiangsu	11
		Jurong, Jiangsu	1
		Nanjing, Jiangsu	18
		Huaiyuan, Anhui	4
		Wuhan, Hubei	20
	Pepper	Nanjing, Jiangsu	2
	Peanut	Nanjing, Jiangsu	1
C. gloeosporioides	Rubber	Hainan	2
	Soybean	Sichuan	1
	Soybean	Jiangsu	3
C. acutatum	Rubber	Hainan	2
C. capsici (ATCC48574)	*Capsicum annuum*	America	1
C. capsici (ACCC36172)	Unknown	Henan	1
C. dematium (ATCC58684)	Strawberry	America	1
C. dematium (ACCC37048)	Unknown	Beijing	1
C. destructivum (ATCC11869)	Alfalfa	America	1
C. coccodes (ATCC10902)	Potato	America	1
Cercospora kikuchii	Soybean	Wuhan, Hubei	1
Aspergillus oryzae	Soybean	Unknown	1
Alternaria alternata,	Soybean	Xuzhou, Jiangsu	1
Macrophomina phaseolina	Soybean	Nanjing, Jiangsu	1
Diaporthe phaseolorum var. *caulivora*	Soybean	Unknown	1
Diaporthe phaseolorum var. *meridionalis*	Soybean	Unknown	1
Phomopsis longicolla	Soybean	Wuhan, Hubei	1
Phialophora gregata f. sp. *sojae*	Soybean	Unknown	1
Phytophthora sojae	Soybean	Unknown	1
Fusarium oxysporum	Soybean	Wuhan, Hubei	1
Bipolaris maydis,	Soybean	Nanjing, Jiangsu	1
Epicoccum nigrum	Soybean	Suzhou, Anhui	1

plant kit (Tiangen, Beijing, China). DNA concentrations were determined spectrophotometrically or via quantitation on 1% (*w/v*) agarose gels stained with ethidium bromide, with comparison to commercial standards. All samples were stored at −20 °C.

LAMP primer design and screening

Selection of a suitable target for LAMP primers is critical. After comparison and screening, the *Rpb1* gene was chosen as the target DNA sequence. We compared the *Rpb1* sequences of *C. truncatum* with other

Colletotrichum spp., and selected a unique 200–400-bp sequence of *C. truncatum* to design LAMP primers using PrimerExplorer V4 software (http://primerexplorer.jp/e/) (Fig. 1). The specificities and sensitivities of several sets of LAMP primers were tested. Primers lacking species specificity or exhibiting insufficiently high sensitivities were discarded. Finally, a set of four primers exhibiting high species specificity and sensitivity, targeting the *Rpb1* sequence of *C. truncatum*, were selected for further study. The forward inner primer (FIP) consisted of the sequence complementary to F1c and F2; and the reverse inner primer (BIP) to B1c and B2. The outer primers F3 and
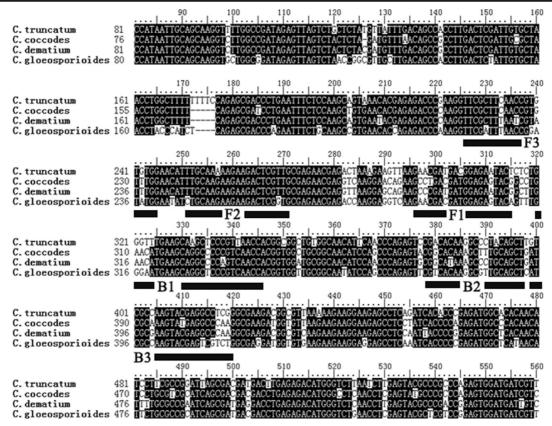
Fig. 1 Nucleotide sequence alignment of the target *Rpb1* region used for designing the LAMP primers. The nucleotide sequence of the sense strand of *Rpb1* DNA is shown. DNA sequences used for primer design are shown by bold lines

B3 were used for initiation of the LAMP reaction. The loop primer (LF) was used to accelerate reaction speed. Primer sequences are presented in Table 2 and Fig. 1.

LAMP reaction and product detection

Each LAMP assay was performed in a 25 μl reaction volume containing 0.8 μM of the primers FIP and BIP, 0.1 μM of the primers F3 and B3, 0.1 μM of the primers LF and LB, 0.8 M betaine, 1.4 mM dNTPs, 20 mM Tris-HCl (pH 8.8), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 6 mM

$MgSO_4$, 0.1% (v/v) Triton X-100, 8 U of Bst DNA polymerase (New England BioLabs, Ipswich, MA, USA), and 4 μL of the target DNA sample. The amplification reaction was run at 62 °C for 70 min. Each reaction included a positive control (a sample with DNA template of *C. truncatum* identified by morphological assessment and ITS sequencing) and a negative control (a sample to which no template was added), and each sample was analyzed at least three times.

After the reaction, a LAMP product was sought directly by the unaided eye after addition 0.25 μL of

Table 2 Primers used for the loop-mediated isothermal amplification to detect *Colletotrichum truncatum*

Primer type	Target	Sequence (5′–3′)	Length
F3	*Rpb1*	ACGGAGAATACTCTCTGGGT	20
B3	*Rpb1*	AGGATGTTGTGTGCCATCTC	20
FIP(F1c + F2)	*Rpb1*	GCCTTGTGTCGGACTCTGGG-GCAAGCTCCCGTTAACCA	38
BIP(B1c + B2)	*Rpb1*	ACAGCTTGTCGCCAAGTACGAG-GGGTGTGATCTGAGGCTCTT	42
LF	*Rpb1*	TGAATGTTGCCACAGCCGC	19

SYBR Green I (10,000X concentrate in DMSO, Life Technologies, USA). With a positive reaction, a yellow-green color was clearly observed, whereas, with a negative reaction, the color remained orange.

DNA extraction from diseased tissues of soybean inoculated with *C. truncatum*

Soybean seeds were planted in vermiculite and grown in a greenhouse at 28 °C until the first true leaves had expanded. Cultures of *C. truncatum* were grown for 3–5 days on PDA. Plugs of agar (5 × 5 mm) with mycelia were inoculated onto soybean leaves in vitro and incubated at 28 °C for 5 days (24 h in the dark, followed by a 12 h photoperiod). DNA was extracted from the diseased leaves using a DNAsecure plant kit (Tiangen) according to the manufacturer's protocol. Samples were stored in TE at −20 °C if not used immediately. The same method was used to extract DNA from leaves of healthy soybean plants, which served as negative controls.

DNA extraction from naturally diseased tissues were collected from field-grown soybean plants in Jiangsu, Hubei, and Anhui provinces. DNA from the tissues of diseased leaves, pods, and seedlings were extracted in the laboratory using a DNAsecure plant kit (Tiangen) following the manufacturers protocol. DNA concentrations were determined spectrophotometrically or via quantitation on 1% (w/v) agarose gels stained with ethidium bromide, via comparison with commercial standards. All samples were stored at −20 °C.

Isolation and culture of *C. truncatum* from diseased tissue from the field

We isolated *C. truncatum* from soybean samples from the field and identified them to compare results with the LAMP assay. Firstly, typical diseased tissue was selected, the samples cut into small segments (1 cm × 1 cm), and immersed in sterile water to remove debris from the surface. The segments were immersed in 4% sodium hypochlorite for 2-3 min and 70% alcohol for 60 s successively and rinsed three times in sterile water. Finally, the samples were dried with sterile absorbent paper, and placed on PDA in a Petri-dish sealed with parafilm and stored at 20–25 °C in the dark. Fungal growth was typically observed after 2-3d. Individual isolates were transferred by removing a section of agar with hyphal tips using a sterile loop, and transferring the isolate to a fresh PDA plate. In some cases, to obtain a pure culture, hyphal tip capture was repeated until the colony was deemed pure following macroscopic and microscopic examination. Depending on the culture and fungal strain, growth was observed after 2–3 d. DNA of the pure strains was extracted for ITS sequencing after 1–2 weeks incubation.

Detection of *C. truncatum* in soybean seeds

Assays were performed by addition of conidia of *C. truncatum* to soybean seed samples that presented negative LAMP detection to determine the detection limit of the *Rpb1*-Ct-LAMP assay. Spore suspensions of *C. truncatum* containing different numbers of conidia (0, 10, 50, 100, 1000, 10,000) were separately added to 50 g of soybean seeds in 250-mL Erlenmeyer flasks containing 100 mL of sterile water. After addition of 3–5 drops of 20% (v/v) Tween, the samples were washed in a table concentrator (220 rpm, 30 min) and filtered through a steel sieve (200 mesh). Filtrates were centrifuged at 6500 rpm for 10 min and the pellets subjected to DNA extraction using a PowerSoil DNA isolation kit (MoBio, USA) optimized for soil samples according to the manufacturer's protocol. If not used immediately, the samples were stored at −20 °C. The same method was used to isolate DNA from pathogen-free soybean seeds (negative controls).

In addition, 10 soybean seed samples were collected from farmers' markets in Liaoning, Anhui, Jiangsu, Shandong, Hubei, Zhejiang, and Heilongjiang provinces. DNA was extracted using the method described above and stored at −20 °C until tested using the LAMP assays.

PCR amplification and ITS rDNA sequencing

The internal transcribed spacer (ITS) regions of the nuclear ribosomal DNA (nrDNA) gene of *C. truncatum*, other *Colletotrichum* spp., *Fusarium* spp., and the other pathogens used in this study were amplified and sequenced. The ITS universal primer pair, ITS1 (5′-TCCGTAGGTGAACCTGCG G-3′) and ITS4 (5′-TCCTCCGCT TATTGATATGC-3′), was used (White et al. 1990). The PCR reaction mix for amplification of the ITS region include 0.1 mM dNTPs, 0.1 μM primers, 1 U rTaq DNA Polymerase, 10X PCR buffer with 1.5 mM MgSO4, and 1 μL of target DNA. Sterile distilled water was added to make a final volume of 25 μL. The PCR amplification was performed using a

PCR Thermal Cycler Dice (Takara Bio Inc., Japan): 5 min at 94 °C for initial denaturation followed by 32 cycles consisting of 5 min of denaturation at 94 °C, 30 s of annealing at 56 °C, and 45 s of DNA synthesis at 72 °C. An extension time of 10 min at 72 °C was added at the end of the last cycle. Amplified products of DNA were separated by electrophoresis in 1% agarose gels and subsequently purified using Takara MiniBEST Agarose Gel DNA Eaction Kit Ver.4.0.

The purified PCR products were sequenced by the Genscript Company (Nanjing, Jiangsu, China). Sequences of the amplified products were subjected to a blast search in the nucleotide sequence database (GenBank) to identify the sequences with the greatest homology.

Results

Specificity of the *Rpb1*-Ct-LAMP assay

Strains of *C. truncatum*, *C. gloeosporioides*, *C. acutatum*, *C. capsici*, *C. dematium*, *C. destructivum*, *C. coccodes* and other fungi pathogenic on soybean were used to confirm the specificity of the *Rpb1*-Ct-LAMP assay. Only samples containing *C. truncatum*, isolated from different hosts and different areas, yielded positive reactions indicated by the visible color change, whereas reactions containing either other *Colletotrichum* spp. or other fungal strains were negative (Table 3). The results showed that the *Rpb1*-Ct-LAMP assay specifically detected only *C. truncatum*.

Sensitivity of the *Rpb1*-Ct-LAMP assay

The detection limit of the *Rpb1*-Ct-LAMP assay was determined via amplification of 10-fold serial dilutions (from 100 ng to 10 fg) of purified DNA of *C. truncatum*. Based on visual evaluation after addition of SYBR Green I, the minimum concentration of *C. truncatum* DNA detected in the LAMP assay was 100 pg μL^{-1} (Fig. 2).

Using the *Rpb1*-Ct-LAMP assay to detect *C. truncatum* in inoculated soybean

DNA was extracted from diseased leaves of soybean inoculated with *C. truncatum* to evaluate the ability of the *Rpb1*-Ct-LAMP assay to detect the presence of the fungus in diseased plants. DNA was extracted directly

Table 3 Results of screening different fungal species using the *Rpb1*-Ct-LAMP assay designed to detect *Colletotrichum truncatum*

Fungal species	Number of isolates tested	*Rpb1*-Ct-lamp assay [a]
Colletotrichum truncatum	70	+
C. gloeosporioides	6	-
C. acutatum	2	-
C. capsici (ATCC48574), *C. capsici* (ACCC36172), *C. dematium* (ATCC58684), *C. dematium* (ACCC37048), *C. destructivum* (ATCC11869), *C. coccodes* (ATCC10902), *Cercospora kikuchii*, *Aspergillus oryzae*, *Alternaria alternata*, *Macrophomina phaseolina*, *Diaporthe phaseolorum* var. *caulivora*, *Diaporthe phaseolorum* var. *meridionalis*, *Phomopsis longicolla*, *Phialophora gregata* f. sp. *sojae*, *Phytophthora sojae*, *Fusarium oxysporum*, *Bipolaris maydis*, *Epicoccum nigrum*,	1 each	-

[a] '+' sample tested positive using the the *Rpb1*-Ct-LAMP assay; '-' sample tested negative using the *Rpb1*-Ct-LAMP assay

from diseased plant tissues to simulate field conditions. All the inoculated samples were positive (Table 4).

Using the *Rpb1*-Ct-LAMP assay to detect *C. truncatum* in naturally diseased soybean tissues from the field

The *Rpb1*-Ct-LAMP assay was used to detect *C. truncatum* in samples suspected of having soybean anthracnose collected from the field in Jiangsu, Anhui, and Hubei provinces. *C. truncatum* was detected in 61 diseased soybean leaves and pods from 154 suspect diseased samples using the *Rpb1*-Ct-LAMP assay
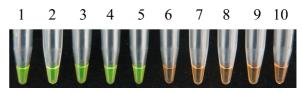

Fig. 2 Sensitivity of the *Rpb1*-Ct-LAMP assay. Sensitivity was determined using serially diluted genomic DNA (100 ng to 10 fg) of an isolate of *Colletotrichum truncatum* as the template (multiple isolates were tested). The detection limit was 100 pg μL^{-1}. The sensitivity of the LAMP method was determined using SYBR Green I. A positive reaction was indicated by the development of a yellow-green color, visible to the naked eye. A negative reaction was indicated by an orange color. 1, positive control; 2–9, serially diluted genomic DNA (100 ng to 10 fg); 10, negative control

Table 4 Results of the Rpb1-Ct-LAMP assay in detecting *Colletotrichum truncatum* in inoculated soybean plants

Sample	Source	*Rpb1*-Ct-LAMP assay [a]
01	Positive control (spiked with *C. truncatum*)	+
02–07	Soybean tissues inoculated with a *C. truncatum* isolates	+
08	Healthy plant tissues	−
09	Negative control (no *C.truncatum* or plant tissue)	−

[a] '+' sample tested positive using the the *Rpb1*-Ct-LAMP assay; '-' sample tested negative using the *Rpb1*-Ct-LAMP assay

(Table 5). When compared to traditional isolation and culture, the *Rpb1*-Ct-LAMP assay was more accurate: among these 61 samples, 40 were isolated and cultured by the traditional method, but only 29 strains of *C. truncatum* were identified. The results show that, compared to the traditional isolation method, our *Rpb1*-Ct-LAMP assay was both rapid (only about 2 h) and accurate. Identification of isolates based on morphological characteristics and ITS sequence alignment was consistent with the assay results. The results demonstrate that the *Rpb1*-Ct-LAMP assay could detect *C. truncatum* from anthracnose-suspect, symptomatic soybean plants collected directly from the field.

Detection of *C. truncatum* in soybean seeds from famers' markets

The efficiency of the *Rpb1*-Ct-LAMP assay in detecting *C. truncatum* in soybean seeds was determined by inoculating pathogen-free samples of soybean seed with conidia of the *C. truncatum*. The *Rpb1*-Ct-LAMP assay was positive when there were 10 or more conidia in 50 g soybean seed (Fig. 3).

The assay was also used to detect *C. truncatum* in 10 samples of soybean seeds obtained from farmers' markets from several Chinese provinces (Table 6). *C. truncatum*

was identified in samples from five provinces (Liaoning, Anhui, Jiangsu, Hubei, and Shandong Provinces).

Discussion

Soybean anthracnose caused by *C. truncatum* is an important seed-borne disease that is common in China. The *Rpb1*-Ct-LAMP assay for *C. truncatum* described in this report allows accurate, direct detection of the pathogen in diseased soybean tissues and seeds.

The LAMP system we developed for the detection of *C. truncatum* uses four specific LAMP primers and a loop primer, the design of which were based on the sequence of the novel target gene *Rpb1* (the large subunit of RNA polymerase II). *Rpb1* is the largest and principal functional subunit of RNA polymerase II, which plays an important role in the initiation and elongation of mRNA during transcription (Li et al. 2007). *Rpb1* is highly conserved among strains of the same species from different sources but exhibits a high degree of variation among different species of the genus *Colletotrichum*. Thus, *Rpb1* was an appropriate and highly specific target for the design of LAMP primers aimed at the detection of *C. truncatum*.

Microscopic observation of the causal pathogen is important in the diagnosis of plant fungal diseases.

Table 5 Detection of *Colletotrichum truncatum* using the *Rpb1*-Ct-LAMP assay in diseased soybean tissues collected from the field in different provinces in China

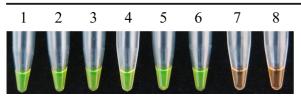| Collection site	Diseased tissue	Suspect samples (n)	*Rpb1*-Ct-LAMP positive samples (n)
Jiangsu province	Seedlings	39	7
	Pod	16	7
	Leaves	6	1
Hubei province	Leaves	31	25
	pods	19	8
Anhui province	Leaves	39	12
	pods	4	1

Fig. 3 Sensitivity of the *Rpb1*-Ct-LAMP assay in detection of *Colletotrichum truncatum* in soybean seeds infested with different numbers of conidia. Decreasing numbers of conidia are shown from left to right (10,000, 1000, 100, 50 and 10 conidia, respectively). 1, DNA from *C. truncatum* isolates; 2–6, seeds containing different numbers of *C. truncatum* conidia in sequence; 7, DNA from noninfested seeds as a negative control; 8, negative control (no seed)

However, because of morphological similarities, it is difficult to distinguish *C. truncatum* from other *Colletotrichum* species using microscopy alone. Diagnosis is further complicated by the fact that disease symptoms vary under different weather conditions, among host plants, and between infected organs. PCR, based on sequence variations within the ITS1 and ITS2 regions, has been used to detect *C. truncatum* with a sensitivity of ≥100 pg DNA μL^{-1} (Chen et al. 2006), but the amplified products must be separated using gel electrophoresis, stained with ethidium bromide, and examined under UV light, which requires at least 4 h to complete testing.

In contrast to PCR and traditional methods of pathogenic fungal detection in diseased soybean tissues, the *Rpb1*-Ct-LAMP assay has several advantages. First, high specificity is afforded by the use of four primers

recognizing six distinct regions on the template DNA (Begum et al. 2010), unlike the PCR approach, which uses only two primers defining two regions on the template. Second, the *Rpb1*-Ct-LAMP is simple, and does not require expensive equipment. Only the primers, reagents, and a temperature-controlled water bath or vacuum cups are needed to perform the LAMP reaction. Third, the results can be visualized with the unaided eye following the addition of 0.25 μL of a solution of SYBR Green I. A distinct yellow-green color indicates a positive result and an orange color a negative result (Parida et al. 2005; Soliman and El-Matbouli 2006). Fourth, the assay is fast, with only 2 h needed to detect a species, in contrast to the 4 h required when using conventional PCR. Fifth, the assay procedure simplifies DNA extraction, as it is more tolerant of inhibitors and thus has the potential to be deployed in field. Sixth, the *Rpb1*-Ct-LAMP assay is highly efficient, as *C. truncatum* can be detected directly and specifically in a mixture containing the DNA of the host plant, *C. truncatum*, and saprophytic microbes present in diseased tissues; thus the assay can be used directly on samples obtained from soybean production fields.

This is the first report describing a LAMP assay for the specific detection of *C. truncatum* in diseased soybean plants. We also used the assay to detect *C. truncatum* strains in other diseased hosts, for example, pepper and peanut, and all strains yielded a positive reaction when using the LAMP assay (Tables 1 and 3).

Table 6 Detection of *Colletotrichum truncatum* in soybean seed using the *Rpb1*-Ct-LAMP assay. Seed was obtained from markets in different provinces in China

Sample	Source (city, province)	*Rpb1*-Ct-LAMP assay [a]
01	Dalian, Liaoning	+
02	Fuyang, Anhui	+
03	Xuzhou, Jiangsu	+
04	Wuhan, Hubei	+
05	Weifang, Shandong	+
06	Dezhou, Shandong	+
07	Zibo, Shandong	−
08	Mudanjiang, Heilongjiang	−
09	Jiaxing, Zhejiang	−
10	Taizhou, Jiangsu	−

[a] '+' sample tested positive using the the *Rpb1*-Ct-LAMP assay; '-' sample tested negative using the *Rpb1*-Ct-LAMP assay

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