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ORIGINAL PAPER

Rapid detection of *Colletotrichum gloeosporioides* using a loop-mediated isothermal amplification assay

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Abstract Anthracnose caused by *Colletotrichum gloeosporioides* is an economic disease that affects soybean production worldwide. This study developed a rapid, sensitive method for the detection of *C. gloeosporioides* using a loop-mediated isothermal amplification (LAMP) assay. By targeting a glutamine synthetase (*GS*) gene sequence, the *GS*-Cg-LAMP assay works most efficiently at 64°C and allows the detection of *C. gloeosporioides* DNA within 70 min based on a color change from orange to yellow-green following the addition of SYBR Green I to the LAMP reaction products. The detection limit was 1 pg μL^{-1} of genomic DNA per reaction. In specificity tests, the positive reaction (yellow-green color by the naked eye) was observed only in the presence of *C. gloeosporioides*, and none of other *Colletotrichum* spp. or fungal isolates produced a color change. Moreover, the *GS*-Cg-LAMP assay was successfully used to diagnose anthracnose caused by *C. gloeosporioides* in diseased soybean samples obtained from the field and detected the pathogen in soybean seeds bought from farmers' markets. Our study provides a simple, sensitive tool for the rapid diagnosis of soybean anthracnose caused by *C. gloeosporioides*.

Keywords *Colletotrichum gloeosporioides* · Anthracnose · Glutamine synthetase · Loop-mediated isothermal amplification

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Introduction

Colletotrichum gloeosporioides is an economically important pathogen that infects a wide range of crops causing enormous production losses each year (Jeffries 1990). The fungus causes anthracnose disease of cereals, legumes, vegetables, perennial crops, and tree fruits (Phoulivong et al. 2010). As a seed-borne disease (Manandhar et al. 1987), anthracnose caused by *C. gloeosporioides* damages soybeans commencing at the seedling stage up to harvest (Kwon et al. 2013), and the disease can affect all of the above ground parts of the plant. Irregularly shaped, brown lesions (Sugawara et al. 2009), which are the most distinctive symptom, appear on stems, leaves, and pods during the early reproductive stage. Seeds and crop residues are considered to be the primary sources of the infection (Chen et al. 2006).

In addition to *Colletotrichum gloeosporioides*, *C. truncatum*, *C. capsici*, *C. dematium*, *C. destructivum* and *C. coccodes* also cause soybean anthracnose (Ghosh et al. 2016; Lou et al. 2009; Hartman et al. 1988; Roy 1982). These species are difficult to differentiate by microscopic examination because of their morphological similarity. Moreover, the disease symptoms on soybean plants caused by the *Colletotrichum* spp. listed above and *C. gloeosporioides* are similar and difficult to differentiate. Therefore, new techniques for the rapid diagnosis of anthracnose caused by *C. gloeosporioides* are needed.

In recent years, numerous molecular methods have been developed to detect pathogens. The most common molecular detection method is based on the polymerase chain reaction (PCR). A PCR assay has been reported for the

detection of *C. gloeosporioides*, but it has intrinsic disadvantages, including low amplification efficiency and a complicated experimental process. Loop-mediated isothermal amplification (LAMP) (Tsugunori et al. 2000) of DNA, an alternative technology, can be applied for the diagnosis of viruses (Przewodowska et al. 2015), bacteria (Wen et al. 2011), and fungi (Sun et al. 2010). This assay requires a set of six primers and the large fragment of *Bst* DNA polymerase to amplify DNA quickly under isothermal conditions. The LAMP products are judged with the naked eye after the addition of SYBR Green I (Karlsen et al. 1995). Although this method has been developed for the detection of some soybean pathogens, such as *Fusarium equiseti* (Lu et al. 2015), *F. asiaticum* (Xu et al. 2017), and *Colletotrichum truncatum* (Tian et al. 2016), no method for detecting *C. gloeosporioides* has been reported.

In this study, we used the glutamine synthetase (*GS*) gene as the target sequence to develop a rapid, sensitive LAMP assay for the detection of *C. gloeosporioides*. The assay can be used for the rapid diagnosis of anthracnose disease caused by *C. gloeosporioides* by directly detecting the pathogen in diseased soybean tissues in the field.

Materials and methods

Source of strains

Colletotrichum gloeosporioides strains were obtained from diseased leaves, stems, and pods of soybean plants collected in the Shandong, Jiangsu, Anhui, and Hebei Provinces, China. The strains of *C. gloeosporioides*, *C. truncatum*, and *C. acutatum* were identified morphologically and by internal transcribed spacer (ITS) sequencing before use and are maintained in our laboratory. Table 1 lists the fungal species and isolates used in this study.

Culture conditions and DNA extraction from pure cultures

C. gloeosporioides and other fungi were cultured in 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 growing the strains in potato dextrose broth (200 g potato extract L^{-1}, 2% [*w*/*v*] glucose, autoclaved at 120 °C for

Table 1 The fungal species and isolates used to test the specificity of the *GS*-Cg-LAMP assay

Species	Host	Source	No. of strains	GS-Cg-LAMP assay
Colletotrichum gloeosporioides	Soybean	Shandong	1	+
		Jiangsu	5	+
		Sichuan	1	+
		Hubei	12	+
		Anhui	5	+
	Grape	Jiangsu	2	+
	Pear	Jiangsu	2	+
	Rubber	Hainan	4	+
	Navel orange	Hainan	1	+
	Fishtail palm	Hainan	1	+
C. truncatum	Peanut	Jiangsu	1	-
	Soybean	Jiangsu	1	-
C. capsici(ATCC48574)	Unknown	America	1	-
C. dematium(ATCC58684)	Strawberry	Beijing	1	-
C. destructivum(ATCC11869)	Alfalfa	America	1	-
C. coccodes(ATCC10902)	Potato	America	1	-
C. acutatum	Rubber	Hainan	2	-
Cercospora kikuchii	Soybean	Hubei	1	-
Aspergillus oryzae	Soybean	unknown	1	-
Alternaria alternata	Soybean	Jiangsu	1	-
Ascochyta sp.	Soybean	Anhui	1	-
Bipolaris maydis	Soybean	Jiangsu	1	-
Botryosphaeria dothidea	Soybean	Hubei	1	-
Epicoccum nigrum	Soybean	Suzhou,	1	-
Fusarium oxysporum	Soybean	Hubei	1	-
Macrophomina phaseolina	Soybean	Jiangsu	1	-
Nigrospora sphaerica	Soybean	Anhui	1	-
Phomopsis longicolla	Soybean	Hubei	1	-
Phialophora gregata f.sp.*sojae*	Soybean	unknown	1	-
Phytophthora sojae	Soybean	unknown	1	-
Rhizoctonia solani	Soybean	Jiangsu	1	-

+ sample tested positive using the *GS*-Cg-LAMP assay

- sample tested negative using the *GS*-Cg-LAMP assay

20 min) at 25 °C for 5 days. *Phytophthora sojae* was cultured on V8 medium and *P. sojae* mycelia were cultured in V8 juice broth. Mycelium DNA was extracted according to the CTAB protocol described by Möller et al. (Möller et al. 1992). DNA concentrations were quantified using a NanoDrop spectrophotometer ND-1000 (Thermo Fisher Scientific) and stored at −20 °C.

LAMP primer design and screening

Selection of a suitable target for LAMP primers is critical. After comparing different target gene sequences among strains of *C. gloeosporioides* and similar species at the National Center for Biotechnology Information website, the glutamine synthetase (*GS*) gene (GenBank ID: DQ792872.1) was chosen as the target DNA sequence. Glutamine synthetase is used to control nitrogen metabolism. The *GS* gene is present in fungi, plants, and animals. We compared the *GS* sequences of *C. gloeosporioides* with those of other *Colletotrichum* spp., and used a sequence specific to *C. gloeosporioides* (Fig. 1) to design LAMP primers. The LAMP primers were designed using PrimerExplorer V4 software (http://primerexplorer.jp/e/). Multiple sets of primers were designed and screened using a series of specificity and sensitivity tests. Finally, a set of four primers with high species specificity and sensitivity, targeting the *GS* sequence of *C. gloeosporioides*, was selected for further study. The loop primer (LF and LB) was used to accelerate the reaction speed. The primer sequences are shown in Table 2.

LAMP reaction and product detection

The LAMP assay was performed in a reaction volume of 25 μL, which contained 0.8 μM each of FIP and BIP, 0.1 μM each of F3, B3, LF, and LB, 1.4 mM dNTPs, 0.8 MBetaine, 0.8 M Tris-HCl (pH 8.8), 0.4 mM KCl, 0.4 mM $(NH_4)_2SO_4$, 4% Triton X-100, 8 U *Bst* DNA polymerase, and 4 μL of DNA sample. The amplification reaction was run at 64 °C for 70 min. Each lamp assay included positive (a sample with *C. gloeosporioides* DNA) and negative (a sample to which no template was added) controls and each sample was analyzed at least three times.

After the reaction, a LAMP product was sought directly by the unaided eye after adding 0.25 μL of SYBR Green I (10,000× concentrate in dimethyl sulfoxide; Life Technologies, Carlsbad, CA, USA). With a positive reaction, a yellow-green color was clearly observed with the naked eye, whereas the orange color remained with a negative reaction.

DNA extraction from diseased soybean tissues

Soybean seeds were planted in vermiculite and grown in a greenhouse at 25 °C. Plugs of agar (5X5) with *C. gloeosporioides* mycelia were inoculated on soybean leaves in vitro and incubated at 25 °C for 5 days (24 h in the dark, followed by a 12 h photoperiod). DNA was extracted from the diseased leaves using the DNAsecure Plant Kit (Tiangen, Beijing, China) according to the manufacturer's protocol. As a control, the same method was used to extract DNA from non-inoculated healthy soybean leaves.

To clarify the feasibility of the LAMP assay for the rapid diagnosis of anthracnose caused by *C. gloeosporioides*, suspected diseased soybean samples were collected from the field. DNA from the tissues of diseased leaves, pods, and stems was extracted in the laboratory using a DNAsecure plant kit (Tiangen) according to the manufacturer's protocol. All samples were stored at −20 °C.

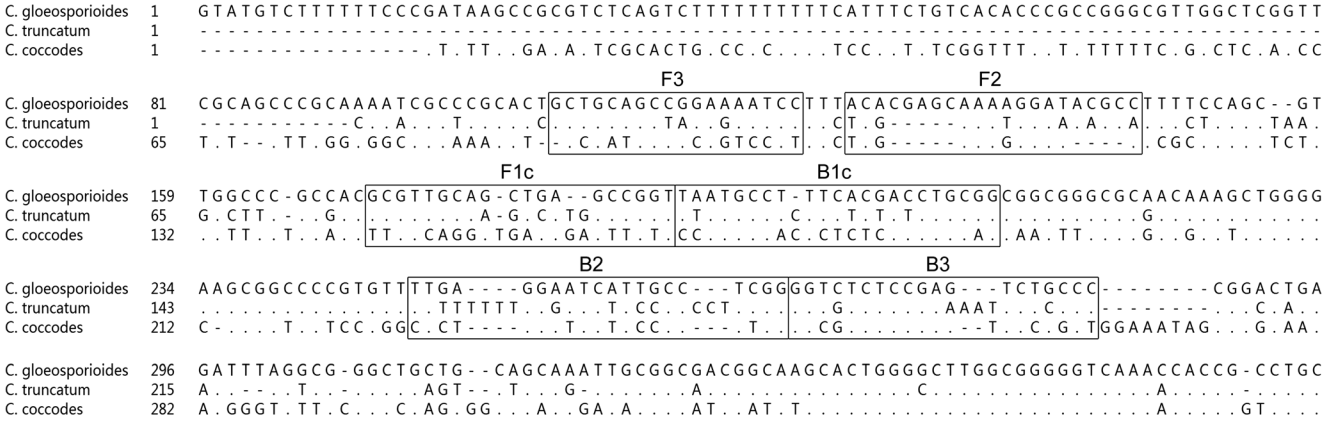

Fig. 1 Nucleotide sequence alignment of the target GS region used to design the loop-mediated isothermal amplification (LAMP) primers. The DNA sequences used for primer design are indicated by bold lines

Table 2 Primers used for the LAMP assay to detect *C. gloeosporioides*

Primer type	Sequence (5′ -3′)	Length
F3	GCTGCAGCCGGAAAATCC	18 nt
B3	GGCAGACTCGGAGAGACC	18 nt
FIP (F1c + F2)	ACCGGCTCAGCTGCAACGC-ACACGAGCAAAAGGATACGC	39 nt
BIP (B1c + B2)	TAATGCCTTTCACGACCTGCGG-CCGAGGCAATGATTCCTCAA	42 nt
LF	CGGGCCAACGCTGGAAAA	18 nt
LB	GGCGCAACAAAGCTGGG	17 nt

DNA extraction from seeds containing *C. gloeosporioides* conidia

Ten soybean seed samples were purchased from different farmers' markets. To detect *C. gloeosporioides* in the samples, 50 g of seeds from each sample were soaked and shocked with 100 mL of deionized water and 3–5 drops of 20% (*v*/v) Tween in 250-mL Erlenmeyer flasks for 30 min. The mixture was filtered through a sieve and the filtrate collected and centrifuged at 6500 rpm for 10 min. The DNA was extracted from the precipitate using a Power Soil® DNA Isolation Kit (MOBIO) according to the manufacturer's protocol. The DNA was stored at −20 °C.

Assays were also performed by adding conidia of *C. gloeosporioides* to soybean seed samples that were negative by LAMP detection to determine the detection limit of the *GS*-Cg-LAMP assay. Spore suspensions of *C. gloeosporioides* containing various numbers of conidia (0, 10, 50, 100, 1000, and 10,000) were added to 50 g of soybean seeds. DNA was extracted using the method described above and stored at −20 °C.

Results

Specificity of the *GS*-cg-LAMP assay

The strains of *C. gloeosporioides* isolated from different hosts and different areas, several other *Colletotrichum* spp., and isolates of other fungi were used to confirm the specificity of the *GS*-Cg-LAMP assay. As shown in Fig. 2 and Table 1, only the samples containing *C. gloeosporioides* gave positive reactions as indicated by a visible yellow-green color, whereas reactions containing other *Colletotrichum* spp. or other fungi isolates were negative (orange color). Assays were performed using serial tenfold dilutions (ranging from 100 ng to 1 fg) of pure *C. gloeosporioides* genomic DNA to determine the detection limit of the *GS*-Cg-LAMP assay. The minimum concentration of *C. gloeosporioides* DNA detected with the *GS*-Cg-LAMP assay was 1 pg μL^{-1}. This indicated that the *GS*-Cg-LAMP assay can be used to detect *C. gloeosporioides* specifically.

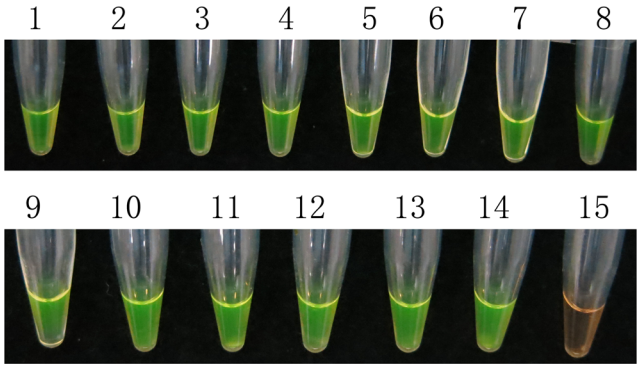

Fig. 2 Generality of the *GS*-Cg-LAMP assay for different *Colletotrichum gloeosporioides* isolates. The *C. gloeosporioides* isolated from different hosts and different areas were showed as follows: 1, soybean of Shandong; 2, soybean of Jiangsu; 3, soybean of Sichuan; 4, peanut of Hubei; 5–6, grape of Jiangsu; 7–8, pear of Jiangsu; 9–12, rubber of Hainan; 13, C. fishtail palm of Hainan; 14, navel orange of Hainan; 15, negative control

Detection of *C. gloeosporioides* in diseased soybean tissues

We extracted the DNA from diseased soybean leaves inoculated with *C. gloeosporioides* to simulate field conditions. All the inoculated soybean tissues reacted positively, similar to the positive control.

We next used the *GS*-Cg-LAMP assay to detect *C. gloeosporioides* in samples suspected of having soybean anthracnose collected from the field in the Jiangsu, Anhui, and Hubei Provinces. Of 152 samples analyzed, 65 tested positive in the LAMP assay. Of these samples, 22 were identified using traditional isolation methods (Table 3). The results show that our *GS*-Cg-LAMP assay was both rapid and accurate compared with traditional isolation methods. The identification of isolates based on morphological characteristics and ITS sequence alignment was consistent with the assay results. The *GS*-Cg-LAMP assay reported here can be used to diagnose soybean anthracnose caused by *C. gloeosporioides* in the field rapidly.

Detection of *C. gloeosporioides* in soybean seeds bought from farmers' markets

The *GS*-Cg-LAMP assay was used to detect *C. gloeosporioides* in ten samples of soybean seeds obtained from farmers' markets. *C. gloeosporioides* was identified in four samples from the Jiangsu, Shandong, and Zhejiang Provinces.

To evaluate the efficiency of the LAMP assay for detecting *C. gloeosporioides* in soybean seeds, conidia of *C. gloeosporioides* were added to pathogen-free samples of soybean seeds manually. The *GS*-Cg-LAMP assay was positive when there were ten or more conidia in 50 g of soybean seeds.

Table 3 Detection of *C. gloeosporioides* in diseased soybean tissues from the field

Collection site	Suspect samples	*GS*-Cg-LAMP positive samples	Isolation method
Jiangsu province	55	16	5
Hubei province	54	36	12
Anhui province	43	13	5
Total	152	65	22

Discussion

This study developed an effective method for detecting *C. gloeosporioides* from diseased soybean samples and rapidly diagnosing soybean anthracnose caused by the pathogen in the field. The *GS*-Cg-LAMP assay can also detect *C. gloeosporioides* in soybean seeds efficiently.

The LAMP system that we developed for the detection of *C. gloeosporioides* uses four specific primers and two loop primers, the designs of which were based on the sequence of the glutamine synthetase gene (*GS*), which is common to fungi, plants, and animals. A blast search indicated that the *GS* gene sequence was conserved in different *C. gloeosporioides* strains but had rich polymorphisms among related *Colletotrichum* spp. Consequently, the *GS* gene has the potential to be used as a species-specific target to detect *C. gloeosporioides*.

A PCR-based detection assay for *C. gloeosporioides* using a forward primer (*CgInt*) from a conserved rDNA region and a universal primer (ITS4) has been reported. However, false-positive reactions can still occur because the ITS sequence is highly conserved in closely related species. The attempted detection of pathogens in samples may produce erroneous results due to nonspecific binding of the primer pairs. Moreover, the amplified PCR products must be separated using gel electrophoresis, strained with ethidium bromide, and examined with ultraviolet light, which requires at least six hours to complete.

Compared with PCR and traditional methods of detecting *C. gloeosporioides* in diseased soybean tissues, the *GS*-CG-LAMP assay has several advantages. First, high specificity is afforded by the use of four primers that recognize six distinct regions of the template DNA, unlike the PCR method, which uses only two primers defining two regions of the template. Second, the *GS*-Cg-LAMP assay is simple to perform, with only 2 h needed to detect the pathogen, and the results can be visualized with the unaided eye. Third, the *GS*-Cg-LAMP assay is very efficient, as *C. gloeosporioides* can be detected directly and specifically in a mixture containing the DNA of the host plant, *C. gloeosporioides*, and saprophytic microbes. The assay can be used directly on samples obtained from diseased soybean tissues from the field or soybean seeds purchased in markets.

In conclusion, we developed a LAMP assay for the specific detection of *C. gloeosporioides* in diseased soybean tissues, allowing the rapid diagnosis of soybean anthracnose caused by the pathogen. We also used the assay to detect *C. gloeosporioides* strains from other diseased hosts, including grapes, pears, and rubber, and all strains produced positive reactions with the *GS*-Cg-LAMP assay.

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