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ORIGINAL PAPER

Rapid diagnosis of Fusarium root rot in soybean caused by *Fusarium equiseti* or *Fusarium graminearum* using loop-mediated isothermal amplification (LAMP) assays

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Abstract Rapid diagnostic assays for Fusarium root rot in soybean, caused by *Fusarium equiseti* or *Fusarium graminearum* were developed using the target gene *CYP51C*. Both assays amplified the target gene in 60 min at 62 °C, after which they were assessed for specificity and sensitivity. Specificity was evaluated against other *Fusarium* spp., other fungal species, and oomycetes. A positive yellow-green color (by the naked eye) or intense green fluorescence (under ultraviolet light) was observed only in the presence of *F. equiseti* or *F. graminearum* after adding SYBR Green I, whereas other strains showed either no color change or weak fluorescence. The detection limit of the *CYP51C*-Fe-LAMP assay was 10 pg. μL^{-1} genomic DNA per reaction, and as few as four conidia per gram of soil could be detected; and the detection limit of the *CYP51C*-Fg-LAMP assay was 100 pg. μL^{-1} genomic DNA per reaction, and 40 conidia per gram of soil could be identified. The assays also detected *F. equiseti* or *F. graminearum* from inoculated soybean tissues and diseased plants in the field. These results suggest that *CYP51C*-Fe-LAMP assay and *CYP51C*-Fg-LAMP assay are effective in rapidly diagnosing soybean root rot caused by *F. equiseti* and *F. graminearum*.

Keywords Rapid diagnosis · Fusarium root rot · Soybean · *Fusarium equiseti* · *Fusarium graminearum* · LAMP

Introduction

Fusarium root rot of soybean is a worldwide disease that causes serious damage to soybean production in China. Previous studies have shown that soybean root rot involves multiple pathogens, and disease control is exacerbated by the complex involvement of numerous soilborne pathogens as well as difficulty distinguishing symptoms in the early stages of disease development (Marasas et al. 1984; Yang and Lundeen 1997; Wrather et al. 2001; Xing and Westphal 2006; Wrather and Koenning 2009). Other studies have shown that *Phytophthora sojae*, *Rhizoctonia solani*, *Pythium aphanidermatum*, *Fusarium oxysporum*, *Fusarium graminearum*, *Fusarium avenaceum*, *Fusarium equiseti*, *Fusarium solani*, and *F. solani* var. *coeruleum* can all cause soybean root rot, with *Fusarium* spp., *R. solani*, and *Py. aphanidermatum* displaying stronger pathogenicity. (Naito et al. 1993; Nelson et al. 1997; Hartman et al. 1999).

Traditional pathogen classification and identification methods are based mainly on morphological characteristics and pathogenicity testing, which is time-consuming. Additionally, many assays are not sufficiently sensitive to detect pathogens when present at low levels, and are subject to complications due to both human and environmental factors. Also, *Fusarium* varies morphologically during growth, and many traits are unstable. Identification of similar species of *Fusarium* is difficult, and distinguishing among soybean root rot pathogens is also difficult due to the similarity of symptoms among fungi and oomycete pathogens. Thus, new diagnostic techniques are needed for rapid diagnosis of Fusarium

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root rot. (Nelson et al. 1983; Lévesque et al. 1987; McGee 1992; Nelson et al. 1994; Martinelli et al. 2004) Polymerase chain reaction (PCR) with heat-stable Taq polymerase is the standard for detecting and identifying microorganisms, and the technique is useful for qualitatively and quantitatively analyzing a vast array of food-spoiling and toxinogenic species. This method has been applied by microbiologists to detect and identify *Fusarium* spp (Jurado et al. 2005). Real-time PCR using fluorescent intercalating dyes or fluorescently labeled oligonucleotide probes can be used to detect pathogen presence and quantity in the sample (Maciá-Vicente et al. 2009). However, these methods are expensive and require dedicated laboratory equipment and well-trained staff. Therefore, PCR-based analyses may be unsuitable for on-site or field-based diagnosis. As an alternative, loop-mediated isothermal amplification (LAMP) was recently described as a specific, rapid, cost-effective, and easy-to-use method for DNA amplification (Notomi et al. 2000; Nagamine et al. 2002; Caipang et al. 2004; Tomita et al. 2008). The LAMP method does not require a thermal cycler, making it suitable for on-site applications. Although this method has been applied to detect and identify bacteria (Pan et al. 2011), viruses (Niessen and Vogel 2010), and some specialized forms and races of *Fusarium* (Almasi et al. 2013; Li et al. 2013; Peng et al. 2013), few studies have applied the LAMP assay to rapidly and specifically detect *F. equiseti* or *F. graminearum*, the causative agents of Fusarium root rot in soybean.

LAMP is an easy assay to perform if suitable primers have been designed based on the target gene (Notomi et al. 2000). However, rDNA-internal transcribed spacer (ITS) gene sequences are highly conserved in *Fusarium* spp., creating a barrier to the development of species-specific detection primers. The *CYP51* gene encoding sterol 14α-demethylase is a key enzyme in the pathway leading to ergosterol, phytosterol, and cholesterol biosynthesis in fungi, plants, and mammals, respectively. Many important human and plant pathogenic fungi carry multiple *CYP51* genes. For example, *Aspergillus* spp., *Magnaporthe oryzae*, *Rhynchosporium secalis*, and *Pyrenophora tritici-repentis*, as well as most filamentous ascomycetes, carry two copies called *CYP51A* and *CYP51B*. A third copy, *CYP51C*, has been identified in *Fusarium* spp. and appears to be unique to this genus. (Diaz-Guerra et al. 2003; Fernández-Ortuño et al. 2010) Thus, *CYP51C* has potential to be used as a phylogenetic marker to reliably identify *Fusarium* species and as a species-specific target.

In this study, we developed a rapid diagnosis method for Fusarium root rot in soybean caused by *F. equiseti* or *F. graminearum* using LAMP based on the target *CYP51C* gene.

Materials and methods

Strain sources

The *F. equiseti* and *F. graminearum* isolates were obtained from diseased soybean roots and stems (Fang 1998) collected from various provinces in China from 2009 to 2013. All isolates were identified based on their morphology and ITS sequencing. The *F. equiseti* and *F. graminearum* isolates and the strains of *Fusarium* spp., *Phytophthora* spp., and other pathogens used in this study are maintained in our laboratory (Table 1).

Culture conditions and DNA extraction

The *Fusarium* strains were cultured on potato dextrose agar (PDA) medium (Hopebio, Qingdao, China) in 9-cm Petri dishes. Mycelia of each *Fusarium* strain and other fungi—except *P. sojae*—were obtained by growing the strains in potato dextrose broth (Hopebio, Qingdao, China) at 28 °C for at least three days. *Phytophthora sojae* mycelia were grown in tomato juice broth (Zheng 1995). The mycelia were harvested by filtration and frozen at −20 °C. Mycelial DNA was isolated using a DNAsecure Plant Kit (Tiangen, Beijing, China) according to the manufacturer's protocol. DNA concentrations were determined spectrophotometrically or by quantitation on 1 % agarose gels stained with ethidium bromide, compared with commercially obtained standards, and stored at −20 °C.

LAMP primer design and selection

Two separate sets of LAMP primers were designed based on the *F. equiseti* and *F. graminearum CYP51C* target sequences, respectively (http://www.ncbi.nlm.nih.gov/nuccore/, GU785035.1; http://www.broadinstitute.org/, FGSG_04092). Briefly, we compared the *F. equiseti* and *F. graminearum CYP51C* sequence with that of other related pathogenic fungi in GenBank. (Table S1) Next, two specific primers based on the *CYP51C* sequence alignments were designed for LAMP detection of *F. equiseti* and *F. graminearum* using PrimerExplorer V4 software (http://primerexplorer.jp/e/). The forward inner primer (FIP) consisted of the F1 (F1c) and F2 complementary sequence, and the backward inner primer (BIP) comprised B1c and B2. The F3 and B3 outer primers were required to initiate the LAMP reaction. Two loop primers (LF and LB) were used to facilitate the LAMP reaction. The software provided more than one set of primers. Specificity and sensitivity of the primers were tested. Those primers lacking species specificity or whose sensitivities were not high were not used. Take the primer designing for *F. equiseti* as an example. The software recommended multiple sets of primers, and then we tested the specificity of the primers one by one by detecting *F. equiseti* from different areas, the sets of

Table 1 Strains of different fungi used to screen the primer specificity in the study

Species	Host	Source in China	Number of strains	CYP51C-Fe-LAMP assay	CYP51C-Fg-LAMP assay
Fusarium equiseti	Soybean	Jiangsu	6	+	−
F. equiseti	Soybean	Anhui	8	+	−
F. equiseti	Soybean	Sichuan	4	+	−
F. graminerum	Soybean	Jiangsu	9	−	+
F. graminerum	Wheat	Jiangsu	5	−	+
F. oxysporum	Soybean	Jiangsu	2	−	−
F. oxysporum	Soybean	Anhui	2	–	−
F. solani	Soybean	Jiangsu	1	−	−
F. proliferatum	Soybean	Jiangsu	1	−	−
F. moniforme	Rice	Jiangsu	1	−	−
F. culmorum	Soybean	Sichuan	1	−	−
F. nivale	Wheat	Unknown	1	−	−
F. avenaceum	Unknown	Unknown	1	−	−
Alternaria tenussima	Soybean	Shandong	1	−	−
Aspergillus oryzae	Unknown	Unknown	1	−	−
Colletotrichum gloeosporiodes	Soybean	Shandong	1	−	−
C. truncatum	Soybean	Shandong	1	−	−
Legionella sp.	Unknown	Unknown	1	−	−
Macrophoma mame	Soybean	Jiangsu	1	−	−
Magnaporthe oryzae	Rice	Jiangsu	1	−	−
Nigreospora sphaerica	Soybean	Sichuan	1	−	−
Phakopsora pachyrhizi Sydow	Soybean	Yunnan	1	−	−
Phomapsis longicolla	Soybean	Shandong	1	−	−
Phytophthora sojae	Soybean	Jiangsu	1	−	−

primers which revealed positive reactions were screened. Then we tested the sensitivity of those primers, those sets of primers had high sensitivity (the detection limit reached pictogram level) were retained. Finally, we tested the specificity of the sets of primers retained by detecting other *Fusarium* spp., other fungal species, and oomycetes, and screened one of the sets of primers which revealed negative reactions as the primers we used in the further research. The primers for *F. graminearum* were designed as the example. The screened primers are shown in Tables 2 and 3.

LAMP reaction

The LAMP assay was performed in a final reaction volume of 25 μL by adding SYBR Green I (Sigma-Aldrich, St. Louis, MO, USA) after amplification. A range of concentrations of $MgSO_4$ (2–8 mM), dNTPs (0.2–2 mM), primers (0.2–2 mM), betaine (0.8–1.6 M) (Sigma-Aldrich), and Bst DNA polymerase large fragments (0.32–0.64 μL^{-1}) (New England BioLabs, Ipswich, MA, USA), and different incubation times (30–90 min) were evaluated to optimize the reaction conditions. The final LAMP reaction was performed in a 25 μL reaction volume, including 0.8 μM FIP and BIP, 0.1 μM F3 and B3, 0.1 μM 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 % Triton X-100, 8 U of Bst DNA polymerase, and 4 μL of target DNA. The reactions were performed in 0.2 mL microtubes in a water bath for temperature control. The mixture was incubated at 62 °C for 60 min. After the reaction, 0.25 μL of SYBR Green I was added to each microtube. Positive and negative controls were included in each experiment, and each sample was analyzed at least three times.

Detection of the LAMP product

To assess DNA amplification, the LAMP product with SYBR Green I was directly visualized by the naked eye and under ultraviolet (UV) light. A positive yellow-green color (by the naked eye) or intense green fluorescence (under UV light) was observed only in the presence of *F. equiseti* or *F. graminearum* after the addition of SYBR Green I, whereas the other strains showed either no color change or weak fluorescence. This method was used to confirm that the test amplified the correct target.

Table 2 Primers used for loop-mediated isothermal amplification for *Fusarium equiseti*

Primer type	Sequence (5′-3′)	Length	Target
F3 (forward outer)	GCGTACCCGGTACCGAAT	18 nt	*CYP51C*
B3(backward outer)	GGACTGGTGACAGACTTGTT	20 nt	*CYP51C*
FIP (forward inner) (F1C + F2)	GGAGGGTCGAGGGAAGAACTCT-TAGTGCCTCCGTCCCATAC	41 mer (F1C:22; F2: 19)	*CYP51C*
BIP (backward inner) (B1C + B2)	TGGGATCCTCATCGCTGGGA-CCGAAGCCATAATCCACAGT	40 mer (B1C:20; B2: 20)	*CYP51C*
LF (loop forward)	TGCCAGGAGATGCAAGAAGT	20 nt	*CYP51C*
LB (loop backward)	CGAGCCTCTTGAGAAGAACGCC	22 nt	*CYP51C*

DNA extraction from soil containing *F. equiseti* or *F. graminearum* conidia

To detect pathogens in soil samples, 8×10^6 conidia of *F. equiseti* and *F. graminearum* were separately inoculated into 1 g of twice-autoclaved soil substrate in 1.5 mL tubes. The tubes were freeze-dried for 24 h and ground in liquid nitrogen to produce a fine powder. DNA was isolated using a FastDNA Spin Kit for Soil (MP Biomedicals, Santa Ana, CA, USA) according to the manufacturer's protocol. Finally, the DNA was suspended in 80 μL of double-distilled water at a final concentration of 10^5 conidia. Samples were stored at −20 °C if not used immediately. The same method was use to isolate DNA from twice-autoclaved soil substrate as a control.

DNA extraction from diseased tissues

Soybean seeds were planted in vermiculite and grown in a greenhouse at 28 °C until the first true leaves expanded. The *F. equiseti* and *F. graminearum* strains were grown for three–five days in PDA. A small piece of the culture (5 × 5 mm) was inoculated on soybean hypocotyls and plants were placed in a greenhouse. After five days, diseased seedling stems were cut to extract the DNA. DNA was isolated according to Wang et al. (1993). Briefly, several milligrams of diseased stem tissues (not disinfected) were placed in a 1.5 mL tube and 10 μL of 0.5 M NaOH were added per mg of tissue. The tissues were then ground by using a pestle until no large pieces remained. Next, 5 μL were transferred quickly to a new tube containing 495 μL of 100 mM Tris (pH 8.0) and mixed; 4 μL were used in the LAMP reaction. Samples were stored at −20 °C if not used immediately. The same method was used to isolate DNA from healthy soybean plants as a control.

Diseased soybean plants were also sampled from field settings. Soybean samples of suspected of having Fusarium root rot were sampled from Jiangsu Nanjing, Jiangsu Xuzhou, Anhui Suzhou and Anhui Huaiyuan provinces. DNA was extracted using a DNA Secure Plant Kit (Tiangen) according to the manufacturer's protocol in the laboratory. DNA concentrations were determined by spectrophotometric ally or by quantitation on 1 % agarose gels stained with ethidium bromide, compared with commercially obtained standards, and stored at −20 °C.

Results

Specificity of the *CYP51C*-FE-LAMP assay and the *CYP51C*-FG-LAMP assay

The specificity of the LAMP assay was tested with *F. equiseti* and *F. graminearum*, as well as other *Fusarium* spp. and strains. Positive or negative results were easily determined with the use of SYBR Green I. The color of the reaction mixture changed to yellow-green for positive amplification, whereas the original orange color was retained when nothing was amplified. Moreover, intense bright-green fluorescence was observed during positive amplification under UV light, whereas no fluorescence occurred in the no-target DNA fragment reaction (Parida et al. 2005). As shown in Table 1, the *F. equiseti* strains, which were isolated from diseased soybean plants, revealed positive reactions, whereas *F. graminearum*, other *Fusarium* spp. or fungal strains showed no color change

Table 3 Primers used for loop-mediated isothermal amplification for *Fusarium graminerum*

Primer type	Sequence (5′-3′)	Length	Target
F3 (forward outer)	GTCCAATCCACTCCATCCTC	20 nt	*CYP51C*
B3(backward outer)	CGGTCTTCTCGAGAGGTTCA	20 nt	*CYP51C*
FIP (forward inner) (F1C + F2)	GTGTGTGGGATGGTGGCACTA-CAAGTCAAATCACCCATGCG	41 mer (F1C:21; F2: 20)	*CYP51C*
BIP (backward inner) (B1C + B2)	CTTTCGTCTCCCGGCACAATGG-TCTTGTCCCAACGATGAGGA	42 mer (B1C:22; B2: 20)	*CYP51C*
LB (loop backward)	CCCGCTCAGAAGAATTCTTCC	21 nt	*CYP51C*

or fluorescence, similar to the negative control. And the *F. graminearum* strains isolated from diseased soybean plants also displayed positive reactions. At least three replicates were tested to assess the specificity of the LAMP reaction. These results indicate that our *CYP51C*-Fe-LAMP assay and *CYP51C*-Fg-LAMP assay species-specifically detected *F. equiseti* and *F. graminearum*, respectively.

Sensitivity of the *CYP51C*-FE-LAMP assay and the *CYP51C*-FG-LAMP assay

Assays were performed using serial tenfold dilutions (100 ng–10 fg) of pure *F. equiseti* and *F. graminearum* genomic DNA to determine the detection limit of the LAMP assay with the *CYP51C* primers. Decreasing concentrations of DNA are shown from left to right; the minimum detection concentration required for the *CYP51C*-Fe-LAMP assay was 10 pg. μL^{-1}; that for the *CYP51C*-Fg-LAMP assay was 100 pg. μL^{-1}.

We also determined the sensitivity of the *CYP51C*-LAMP assays by detecting the conidia in soil. When DNA samples extracted from 1 g of soil inoculated with 8×10^6 conidia of *F. equiseti* and *F. graminearum* were suspended in 80 μL of double-distilled water, the number of conidia per gram of soil was 10^5. The conidial suspension was then diluted to 10^5, 10^4, 10^3, 10^2, 10, and 1, and 4 μL were used for the LAMP reaction. Here, the *CYP51C*-Fe-LAMP assay could detect as few as four conidia of *F. equiseti* and the *CYP51C*-Fg-LAMP assay could detect as few as 40 conidia of *F. graminearum* per gram of soil.

Evaluation of the *CYP51C*-FE-LAMP assay and the *CYP51C*-FG-LAMP assay detecting *F. equiseti* and *F. graminearum* in diseased soybean tissues

DNA was extracted from diseased soybean seedling stems with *F. equiseti* and *F. graminearum* separately to evaluate the LAMP assay for detecting their presence in diseased plants. We extracted the DNA directly from the diseased plant tissues to simulate field conditions. As shown in Fig. 1a, b, the inoculated tissues showed positive reactions, similar to the positive control.

We next used these assays to detect *F. equiseti* and *F. graminearum* from diseased soybean plants collected in the field from Jiangsu Province and Anhui Province. *Fusarium equiseti* was identified in eight diseased soybean plants from 60 samples from Jiangsu Province and in 21 diseased soybean plants from 134 samples from Anhui Province. *Fusarium graminearum* was detected in one diseased soybean plant from 60 samples collected from Jiangsu Province and in 13 diseased soybean plants from 134 samples from Anhui Province. We also isolated *F. equiseti* and *F. graminearum* from those plant samples. Therefore, the *CYP51C*-Fe-LAMP assay could detect *F. equiseti* and the *CYP51C*-Fg-LAMP assay could detect *F. graminearum* in plants directly in the field. These results indicate that the assays can be used to rapidly diagnose soybean root rot caused by *F. equiseti* or *F. graminearum* in production fields.

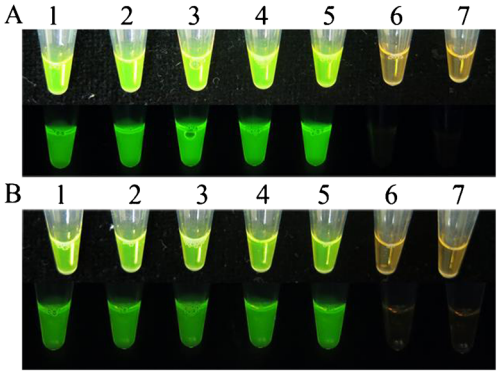

Fig. 1 Evaluation of the *CYP51C*-Fe-LAMP assay and the *CYP51C*-Fg-LAMP assay. **a** Evaluation of the *CYP51C*-Fe-LAMP assay using soybean tissues and residues. *1–4*, Tissues inoculated with a *Fusarium equiseti* isolate; *5*, DNA isolated from *F. equiseti*; *6*, healthy plant tissues; *7*, negative control. **b** Evaluation of the *CYP51C*-Fg-LAMP assay using soybean tissues and residues. *1–4*, Tissues inoculated with a *Fusarium graminearum* isolate; *5*, DNA from *Fusarium graminearum* isolates; *6*, healthy plant tissues; *7*, negative control

Discussion

Few reports describing the application of the LAMP assay to rapidly and specifically detect *F. equiseti* and *F. graminearum* are available. The *CYP51C*-LAMP assays reported here could specificity detect *F. equiseti* or *F. graminearum*; the detection limit was 10 pg. μL^{-1} for *F. equiseti* and 100 pg. μL^{-1} for *F. graminearum*. We developed a method for the rapid diagnosis of Fusarium root rot in soybean caused by *F. equiseti* or *F. graminearum* using LAMP and *CYP51C*, a specific identifiable target gene. The *rDNA-ITS* gene is often used as the detection target (Abd-Elsalam et al. 2004; Zhang et al. 2005; YongYang et al. 2010); however, *rDNA-ITS* sequences from closely related species are highly conserved, hindering the development of species-specific detection primers (Martinez-Culebras et al. 2003; Tang et al. 2005). The *CYP51C* gene was identified in *Fusarium* spp. and appears to be unique to this genus (Deng 2007). *CYP51C* is conserved and is universal in *F. equiseti* and *F. graminearum*; therefore, the *CYP51C* gene sequence is a better target than *rDNA-ITS* or the *β-tubulin* gene sequence for identifying *F. equiseti* and *F. graminearum* (Fernández-Ortuño et al. 2010; Niessen and Vogel 2010).

The in-tube detection of an amplified product can be achieved by directly staining double-stranded DNA with intercalating dyes such as SYBR Green I and SYTO-9 (Njiru et al. 2008), or by precipitating the LAMP product with a fluorescently labeled cationic polymer (Mori et al. 2006). Indirect in-tube detection of the LAMP product has been

achieved using pyrophosphate, a specific by-product of enzymatic DNA synthesis. Visual detection was possible in our assay due to the high specificity and amplification efficiency of LAMP (Iwamoto et al. 2003) and the high binding affinity of SYBR Green I to DNA (Karlsen et al. 1995). Only 0.25 μL of SYBR Green I was required to visualize the reaction products; a distinct yellow-green color or intense green fluorescence indicated a positive result, whereas orange or weak fluorescence specified a negative result (Parida et al. 2005; Soliman and El-Matbouli 2006).

Fusarium root rot of soybean is a worldwide disease and one that severely damages soybean production in China (Marasas et al. 1984; Yang and Lundeen 1997). Soybean root rot caused by *Fusarium* spp. and other fungal and oomycetes often has similar symptoms; the xylem of the stem base browns, the plants grow weak, and the whole plant can die. Distinguishing the pathogen is both time-consuming and difficult, but the *CYP51C*-LAMP methods reported here could detect *F. equiseti* and *F. graminearum* among other *Fusarium* spp. and other fungi or oomycete pathogens (Naito et al. 1993; Nelson et al. 1997; Hartman et al. 1999). The *Fusarium* spp. that cause soybean root rot are soilborne pathogens (Nelson et al. 1993; El-Kazzaz et al. 2008); they can survive for long periods in the soil, and their incidence and prevalence are difficult to predict. As few as four conidia of *F. equiseti* per gram of soil or 40 conidia of *F. graminearum* could be detected using our *CYP51C*-LAMP methods.

LAMP tests haven been developed for rapid detection (2 h) of *P. sojae* (Dai et al. 2012), *P. ramorum*, *F. oxysporum* (Lu et al. 2015), *F. graminearum* by GaoA-LAMP (Niessen and Vogel 2010) or other pathogens from diseased plant tissues. From the diseased soybean plants sampled from Jiangsu Province and Anhui Province, we identified *F. equiseti* and *F. graminearum* using our *CYP51C*-LAMP methods, and *F. oxysporum* was also detected using a previously reported method (Lu et al. 2015). Thus, the *CYP51C*-Fe-LAMP assay and the *CYP51C*-Fg-LAMP assay developed in this study can be used to rapidly diagnose Fusarium root rot in soybean, and may facilitate control of the dispersion of these pathogens on soybean plants.

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