Loop-mediated isothermal amplification (LAMP) for rapid detection of *Rhodococcus fascians* on ornamentals

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Introduction

Virulent strains of *Rhodococcus fascians (Rf)* have the potential to cause fasciation, leafy gall (Fig. 1a) and shoot proliferation (Fig. 1b) in susceptible host plants by interfering with the plant's hormone balance (2). This gram-positive bacterium (Fig. 1c) is of increasing concern to ornamental growers due to its wide host range and the lack of effective control methods. The only sure option for growers is to destroy infected plants. However, it is almost impossible to determine whether a plant intended for propagation is infected until it is too late and the next generation plants are showing symptoms. Current detection methods, including polymerase chain reaction (PCR), are expensive and not readily transferable to growers. Loop-mediated isothermal amplification (LAMP) has been successfully applied to the detection of numerous microbial pathogens (3). The LAMP reaction uses 4 to 6 primers, each with sequence complementary to a selected region of pathogen DNA (Fig. 2).

Figure 1a. Leafy gall on *Scabiosa*, 1b. Shoot proliferation and leafy galls on *Physocarpus* and 1c. *Rhodococcus fascians* colonies.

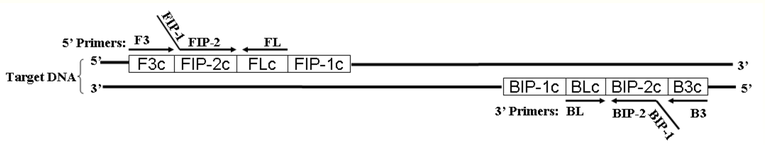

Figure 2: LAMP primer annealing sites. Target DNA with sequence complementary to specific primer as denoted with a 'c'. For example, F3c is complementary to primer F3.

Our objective was to develop a LAMP assay to detect *Rf* on plants sensitive enough to detect the minimum number of cells (2.5 x 10^4 CFU*)* capable of causing disease (1). It also has to be specific so that there is no amplification of DNA from other common plant bacterial pathogens, in particular *Agrobacterium tumefaciens,* another problematic bacterium common in nurseries and capable of producing similar symptoms to that of *Rf* (2). Finally the method needs to be rapid and cost-effective, with potential application in nurseries.

Materials and Methods

Initially, 20 primer sets were developed using PrimerExplorer V3 software, which is freely available through the Eiken genome website at http://primerexplorer.jp/e. Each set were screened for amplification of DNA extracted from a virulent *Rf* isolate (A44a), originally isolated from Veronica. DNA was extracted by boiling a 48-hr old *Rf* colony for 10 minutes. The standard LAMP reaction was set up as given in Figure 3. After mixing reagents thoroughly, 10.9 µl of cocktail was added to 14.9 µl of water. Then 1.2 µl of *Bst* polymerase (New England BioLabs) was added to each reaction tube, followed by 3.0 µl of template. Reactions were incubated in a water bath (65ºC) for 70 min, then stopped by placing the product in a frozen storage tray. Finally, 5 µl of the LAMP product was added to 1 µl Quant-it PicoGreen dsDNA reagent (Invitrogen) and analyzed on an agarose gel.

Cocktail mix:	µl
10x Bst buffer	3.0
Betaine	2.4
MgSO4 (100µM)	1.2
dNTP mix (10µM)	3.0
FIP primer	0.35
BIP primer	0.35
F3 primer	0.30
B3 primer	0.30
TOTAL	10.9

Figure 3. Cocktail mix for LAMP reaction prior to addition of molecular grade water, *Bst* polymerase and template.

One primer set (Rf16) was selected for further testing. The LAMP products from reactions using Rf16 were cloned, sequenced and compared to the GenBank database for *Rf*. In order to determine the specificity of Rf16, **17 virulent and 6 avirulent strains** of *Rf* as well as several other bacterial genera common on plant surfaces were tested with our LAMP protocol. In addition, a turbidimeter (Eiken Genomics, Japan) was used to visualize reactions in real time, and to optimize $MgSO_4$ in the reaction mix and LAMP reaction time. Rf16 specificity was tested by running the LAMP reaction with **six avirulent *Rf* strains and nine non-*Rf* bacterial species**. Sensitivity of Rf16 was tested by preparing a 1:10 dilution series (10^{-1} to 10^{-7}) from a 48-hr old A44a culture. Colony forming units (CFU) were determined by plating; DNA from aliquots **(100 ml)** for all dilutions was extracted. Separate aliquots were spiked into petunia tissue samples, and total DNA was extracted using a Fast-DNA Spin Kit (Qbiogene, CA). Sensitivity of LAMP was compared to that of PCR (virulent *Rf* diagnostic p450 and FAS primer sets), using 3.0 µl of each DNA extract as template. Subsequently, a set of loop primers (FL and BL) were added to the Rf16-LAMP to determine whether they could further improve sensitivity and reduce reaction time.

Results

Rf16 consistently gave positive results for A44a and negative results for the water controls (Figs. 4a and 4b). Sequencing of the LAMP products confirmed that Rf16 amplifies the fasR region of *Rf,* which is required for virulence.

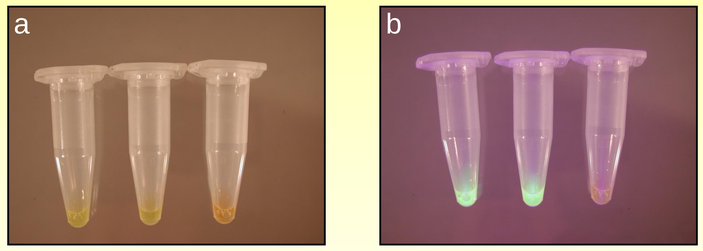

Figure 4a. The addition of PicoGreen to Rf16-LAMP products results in a yellow color change in samples positive for *Rhodococcus fascians* (two tubes on left) and orange for negative samples (tube on right). Figure 4b. The same tubes as in Fig. 4a viewed under ultra-violet light, with bright fluorescense confirming the presence of virulent *R. fascians*.

Turbidimeter. The optimal concentration of $MgSO_4$ was 6mM (Fig. 5a). At 4mM $MgSO_4$, a banding pattern was produced on a gel and a positive PicoGreen reaction achieved (Fig. 5b), while there was very little precipitate detected by the turbidimeter (Fig. 5a). This indicates that PicoGreen was at least as sensitive as detection by the turbidimeter. Optimal reaction time without loop primers was 70 minutes. With the addition of loop primers, we could reduce reaction time to 50 minutes (Fig. 6), at which point, without loop primers, amplification was just beginning.

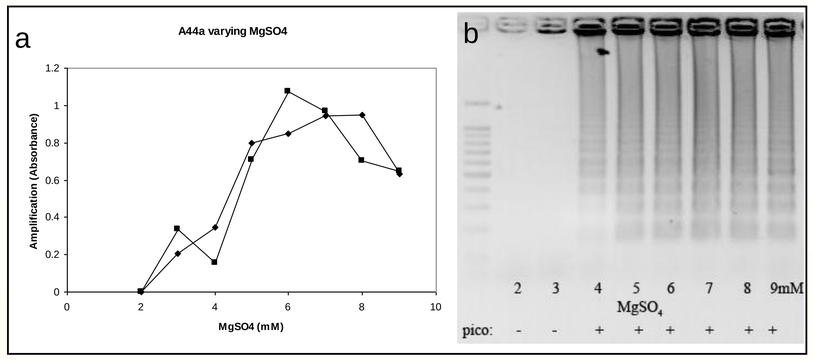

Figure 5. Rf16-LAMP reactions were as in Fig. 1 except [$MgSO_4$] was varied between 2 and 10 mM, and reactions were incubated at 65ºC in a turbidimeter for real-time detection of precipitate. (a) The final turbidity (at 70 min) of duplicate experiments. (b) Aliquots of reactions from a single experiment in (a) were analyzed by an agarose gel and PicoGreen (pico).

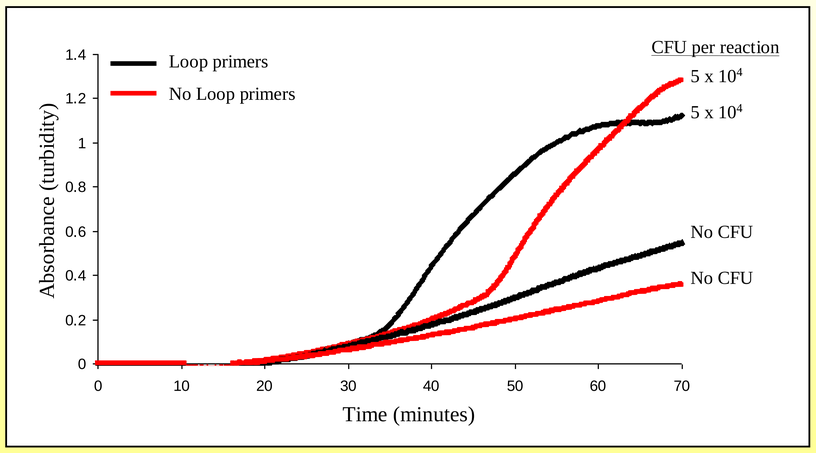

Figure 6. Rf17-LAMP with and without loop-primers.

Specificity of Rf16-LAMP. Seventeen known virulent *Rf* strains were isolated from different plant hosts from different regions of the U.S. All 17 gave a positive reaction with Rf16-LAMP, while the six avirulent strains tested negative. *Xanthomonas campestris* pv. *campestris, Xanthomonas axonopodis* pv. *pruni, Xanthomonas hortorum* pv. *carotae, Erwinia amylovora, Erwinia carotovora, Pantoea agglomerans, Pseudomonas fluorescens, Pseudomonas syringae* and *Agrobacterium tumefaciens* were all negative with Rf16-LAMP.

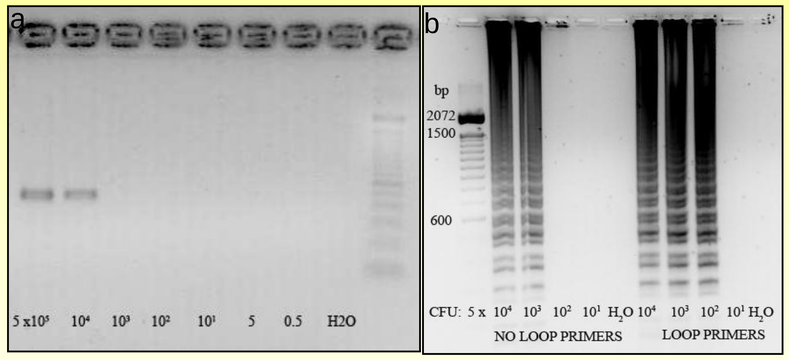

Figure 7a. Sensitivity of PCR (with FAS primers), and (b) Rf16-LAMP without and with loop primers. CFU per reaction is indicated below each lane.

Sensitivity of Rf16-LAMP. The Rf16-LAMP is 10-fold more sensitive than PCR, detecting approximately 5 x 10^3 CFU per reaction when DNA was extracted directly from bacteria by the colony boil method (Figs. 7a and 7b). As shown in Fig. 7b, the addition of loop-primers to the Rf16-LAMP increased sensitivity 10-fold, to approximately 5 x 10^2 CFU per reaction. When *Rf* was added to petunia leaves and the DNA extracted, Rf16-LAMP was 10-fold less sensitive than direct detection of *Rf* (boil method), but still more sensitive than PCR (date not shown).

Conclusions

Rf-LAMP amplifies a portion of fasR, an *Rf* gene necessary for pathogenicity, located on a linear virulence plasmid. A set of 6 primers (Rf16 plus loop primers) was developed and successfully used to detect virulent *Rf* strains on several different plant hosts from various US states. We were able to show that our method does not detect avirulent *Rf* strains, nor other bacterial species commonly found on plant surfaces. These are fundamental requirements as Rf-LAMP is intended for use on field samples.

The turbidimeter was very useful in optimizing our Rf-LAMP reaction, but is not an essential piece of equipment. The addition of PicoGreen eliminates the need for a turbidimeter.

Rf16-LAMP is more sensitive than PCR, and has the added advantage that it does not require a thermocycler – only a water bath. By adding loop primers to our assay, we reduced reaction time and increased sensitivity, allowing *Rf* detection at levels lower than what is required for disease development (1). Quantification of detection is only approximate, since the proportion of virulent *Rf* within a population will vary.

Our LAMP assay is a sensitive, specific, rapid and cost-effective method to detect *Rf*-infected plants, with potential application in nurseries. We are continuing to test the assay by using it with additional virulent and avirulent *Rf* strains from diverse geographic locations.

References

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Acknowledgements

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