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TECHNIQUE

Detection of *Pantoea stewartii* from sweet corn leaves by loop-mediated isothermal amplification (LAMP)

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Abstract To improve the diagnosis of Stewart's wilt, we developed a loop-mediated isothermal amplification (LAMP) assay, based on two conserved sequences of the *cpsD* and *pstS–glmS* gene regions. The detection limit of the LAMP assay was 10^4 colony-forming units/mL. No cross reaction was observed with other *Pantoea* spp., other genera associated with sweet corn diseases, or strains isolated from the surface of maize leaves in Japan. The LAMP reaction was not inhibited by leaf contents. The simplicity of sample preparation and short processing time make this LAMP assay useful for field surveillance of Stewart's wilt.

Keywords *Pantoea stewartii* · Loop-mediated isothermal amplification (LAMP) · Diagnostics

Introduction

Stewart's wilt, caused by *Pantoea stewartii* subsp. *stewartii* (syn. *Erwinia stewartii* [Mergaert et al. 1993], hereafter abbreviated *Pnss*), is one of the most important diseases of sweet corn (*Zea mays* L. var. *rugosa*) and inbred field corn. Young seedlings infected with *Pnss* become severely wilted and die in most cases. The organism is transmitted by the corn flea beetle (*Chaetocnema pulicaria*). Seed transmission is also possible (Pataky and Ikin 2003). *Pnss* is endemic throughout a large portion of the maize-growing regions of the eastern and midwestern United States, and it occurs intermittently in Canada.

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More than 60 countries have placed quarantine regulations on maize seed produced in *Pnss*-affected regions to prevent the introduction of the pest (Pataky and Ikin 2003). Because *Pnss* is technically difficult to detect from seed at a port-of-entry inspection but can be easily detected in the field during the growing season, importation of maize seeds is approved under the condition that the plants concerned are inspected at the growing site in exporting countries (MAFF Plant Protection Station 2014). Therefore, also in noninfested countries and areas, including Japan, field surveillance is essential to eliminate the disease at an early stage. To implement field surveillance effectively, proper and rapid methods are needed to detect Stewart's wilt from questionable plant materials in the fields.

Several methods to detect and identify *P. stewartii* have been reported (Coplin et al. 2002; Lamka et al. 1991; Tambong et al. 2008; Wensing et al. 2010), including subspecies-specific methods (Gehring et al. 2014; Xu et al. 2010). Some conventional techniques, such as enzyme-linked immunosorbent assay (Lamka et al. 1991), are time-consuming and relatively insensitive compared with nucleic acid-based methods. Molecular techniques include conventional PCR (Coplin et al. 2002; Gehring et al. 2014; Wensing et al. 2010), 'miniprimer' PCR (Xu et al. 2010) and a specific real-time PCR assay (Tambong et al. 2008; Wensing et al. 2010). Some of these methods use the *cpsD* gene region and the region between the *pstS* and *glmS* genes. The *cpsD* region, part of the *cps* gene cluster, is required for the production of the exopolysaccharide stewartan, and plays an important role in pathogenicity and virulence (Coplin and Majerczak 1990). The region between *pstS* and *glmS* seems to be specific to this species (Wensing et al. 2010). However, while PCR-based methods are rapid, specific and highly sensitive, their practical use is hindered by the need for complex and expensive

thermal cycling equipment and time-consuming processes. Inhibition of PCR by contaminant plant components also makes it difficult to detect *Pnss* in plant materials.

Loop-mediated isothermal amplification (LAMP) is a new molecular diagnostic technique that is simple, rapid and sensitive (Notomi et al. 2000). It does not require expensive thermal cycling equipment because DNA amplification uses only one enzyme, *Bst* DNA polymerase, under an isothermal condition of 60° to 65 °C (Notomi et al. 2000). The LAMP reaction uses a set of four specially designed primers (FIP, BIP, F3, B3) that recognize six distinct sequences on the target (Notomi et al. 2000). The reaction is accelerated by the addition of loop primers (Nagamine et al. 2002). The LAMP reaction can be read by measuring turbidity caused by a white precipitate of magnesium pyrophosphate without the need for gel electrophoresis. Moreover, its sensitivity is less affected than that of PCR by leaf components (Kaneko et al. 2007). For these reasons, the LAMP assay has been used to detect a range of plant pathogens (Harper et al. 2010; Kubota et al. 2008; Okuda et al. 2005; Oya et al. 2008).

In this study, we developed a LAMP assay for the detection and diagnosis of Stewart's wilt from plant material in the field.

Materials and methods

Bacterial strains

We tested 26 strains of *Pantoea* spp., 17 strains of other genera (Table 1), and 57 unidentified strains isolated from the surface of maize leaves throughout Japan, the colonies of which all resemble *Pantoea* spp. on Luria–Bertani broth agar, Miller (Nakalai Tesque, Kyoto, Japan). All strains were used to test the specificity of the LAMP reaction. A pure culture of *Pnss* ICMP 5929 was used for optimizing the temperature for the LAMP reaction and for sensitivity and inoculation experiments. Sequence analysis of 5 strains of *Pnss* (ATCC 8199, ICMP 270, 722, 5929, 5930) was used to design LAMP primer sets. All strains were cultured in Luria–Bertani broth agar, Miller.

Sequencing analysis and primer design for LAMP

To obtain DNA sequences for designing LAMP primers, we performed conventional PCR. We used primer pair CPSL1/CPSR2c (Coplin et al. 2002) to amplify the *cpsD* region and PST3581/PST3909c (Wensing et al. 2010) to amplify the *pstS–glmS* region. To prepare template solutions, we suspended the bacteria in 1 mM 2-[4-(2-hydroxyethyl)-1-piperazinyl] ethanesulfonic acid (HEPES) solution (Nakalai Tesque). The bacterial cell suspensions (10^7 cfu/mL) were incubated at 98 °C for 10 min and then chilled on ice. The PCR using 5 μL of template solution was performed in a total volume of 20 μL containing 1 × Ex Taq buffer (20 mM Mg^{2+}; Takara Bio, Ohtsu, Shiga, Japan), 200 μM dNTP mixture, 0.5 μM each primer, and 0.5 U of TaKaRa *Ex Taq* DNA polymerase (Takara Bio). PCR conditions were as described previously (Coplin et al. 2002; Wensing et al. 2010).

Loop-mediated isothermal amplification primers were designed from regions of *cpsD* and *pstS–glmS* highly conserved among the 5 *Pnss* isolates. The aforementioned PCR products were sequenced directly with a BigDye Terminator Kit v. 3.1 on an Applied Biosystems 3130 Genetic Analyser following the manufacturer's instructions, and analysed with Sequencing Analysis v. 3.1 software (all from Applied Biosystems, Foster City, CA, USA). LAMP primers were designed from those sequences in PrimerExplore v. 4 software (Fujitsu Systems East, Tokyo, Japan). Two primer sets (PnsCps1 and PnsPst1) were designed (Table 2).

Conditions for LAMP reaction

Template solution was prepared as described for the conventional PCR. LAMP reactions using 5 μL of template solution were carried out with a Loopamp DNA amplification kit (Eiken Chemicals Co., Tokyo, Japan) in a 25-μL reaction mixture with a final concentration of 20 mM Tris·HCl (pH 8.8), 10 mM KCl, 8 mM $MgSO_4$, 10 mM $(NH_4)_2SO_4$, 0.1 % (v/v) Tween 20, 0.8 M betaine, 1.4 mM dNTPs, 8 U Bst DNA polymerase, 0.2 μM each outer primer (F3 and B3), and 1.6 μM each inner primer (FIP and BIP). To accelerate the reactions, 1.2 μM each of loop primers (loop-F, loop-B) were added to the reaction mixture (Nagamine et al. 2002).

For the surveillance of Stewart's wilt at the disease-free area, *Pnss*-positive samples must be detected with certainty. For this purpose, it is desirable to use multiple primer sets that detect different genome sequences at the same time. Because the use of two primer sets at the same time in one incubator simplifies handling, the threshold time (T_t, in min) of each primer sets was assessed at 60°, 63° and 65 °C to determine the optimal reaction temperature for both primer sets. The threshold was set for the time when the turbidity reached 0.1 (Okuda et al. 2008). The reaction mixture was incubated for up to 1 h in a Loopamp Real-Time Turbidimeter (LA-200, Teramecs, Kyoto, Japan).

Determination of sensitivity, specificity and tolerance to sweet corn leaf contents of LAMP

The sensitivity of LAMP and PCR was examined by using suspensions of *Pnss* of 10^6, 10^5, 10^4, and 10^3 cfu/mL in

Table 1 Bacterial strains used in this study and results of the loop-mediated isothermal amplication (LAMP) assay to detect *Pantoea stewartii*

Strain	Host	Origin	LAMP[a]	
			PsCps1	PsPST1
Pantoea stewartii subsp. *stewartii*				
ATCC 8200	Unknown	Iowa, USA	+	+
ATCC 29231	Insect	Connecticut, USA	+	+
ATCC 8199^T	*Zea mays* var. *rugosa*	Iowa, USA	+	+
ICMP 270	*Zea mays* var. *rugosa*	New York, USA	+	+
ICMP 722	Beetle	USA	+	+
ICMP 5929	*Chaetocnema pulicaria*	Missouri, USA	+	+
ICMP 5930	*Zea mays* var. *indentata*	Missouri, USA	+	+
Pantoea agglomerans pv. *gypsophilae*				
MAFF 301599	*Gypsophila paniculata*	Tochigi, Japan	−	−
MAFF 301600	*Gypsophila paniculata*	Tochigi, Japan	−	−
Pantoea agglomerans pv. *milletiae*				
MAFF 106593	*Wisteria floribunda*	Ibaraki, Japan	−	−
MAFF 301740	*Wisteria floribunda*	Tokyo, Japan	−	−
MAFF 301742	*Wisteria floribunda*	Chiba, Japan	−	−
MAFF 301748	*Wisteria floribunda*	Shizuoka, Japan	−	−
Pantoea ananatis				
MAFF 106649	*Oryza sativa*	Hokkaido, Japan	−	−
MAFF 301720	*Oryza sativa*	Ibaraki, Japan	−	−
MAFF 301722	*Oryza sativa*	Yamagata, Japan	−	−
MAFF811106	*Morus* sp.	Ibaraki, Japan	−	−
Pantoea sp.				
MAFF 550195	*Dactylis glomerata*	Tochigi, Japan	−	−
MAFF 550208	*Sporobolus* spp.	Okinawa, Japan	−	−
MAFF 550209	*Panicum maximum*	Okinawa, Japan	−	−
MAFF 550231	*Paspalum urvillei*	Kagoshima, Japan	−	−
MAFF 550232	*Eleusine indica*	Kagoshima, Japan	−	−
MAFF 550235	*Paspalum urvillei*	Kagoshima, Japan	−	−
MAFF 550236	*Setaria glauca*	Kagoshima, Japan	−	−
MAFF 550237	*Miscanthus sinensis*	Kagoshima, Japan	−	−
MAFF550238	*Miscanthus sinensis*	Kagoshima, Japan	−	−
Acidovorax avenae subsp. *avenae*				
MAFF 311167	*Zea mays* subsp. *mexicana*	Tochigi, Japan	−	−
Burkholderia andropogonis				
MAFF 301007	*Sorghum sudanense*	Chiba, Japan	−	−
Erwinia chrysanthemi pv. *zeae*				
MAFF 301657	*Zea mays*	Yamagata, Japan	−	−
Clavibacter michiganensis subsp. *nebraskensis*				
ATCC 27794^T	*Zea mays*	Nebraska, USA	−	−
ATCC 27795	Unknown	Nebraska, USA	−	−
ATCC 27822	*Zea mays*	Nebraska, USA	−	−
ICMP 3294	*Zea mays*	Illinois, USA	−	−
ICMP 3295	*Zea mays*	Illinois, USA	−	−
ICMP 3296	*Zea mays*	Illinois, USA	−	−
ICMP 3299	*Zea mays*	Nebraska, USA	−	−
ICMP 3300	*Zea mays*	Nebraska, USA	−	−
ICMP 3301	*Zea mays*	USA	−	−

Table 1 continued

Strain	Host	Origin	LAMP[a]	
			PsCps1	PsPST1
ICMP 5367	*Zea mays*	USA	–	–
ICMP 5368	*Zea mays*	USA	–	–
ICMP 5369	*Zea mays*	USA	–	–
ICMP 9112	Unknown	Kansas, USA	–	–
Pseudomonas marginalis pv. *marginalis*				
MAFF 106637	*Allium chinense*	Miyazaki, Japan	–	–

[a] LAMP detection: +, positive; −, negative

[T] Type strain. *ATCC*, American Type Culture Collection; *ICMP*, International Collection of Microorganisms from Plants; *MAFF*, culture collection of NIAS Genbank, National Institute of Agrobiological Sciences, which corresponds to the former culture collection of Ministry of Agriculture, Forestry and Fisheries, Japan

Table 2 Nucleotide sequence of primers used for loop-mediated isothermal amplication (LAMP)

Primer	Sequence
For *cpsD* gene (PnsCps1 primer set)	
PnsCps1-FIP	5′-GGTCAGGATAATCGGCTCGACCTACAATTTCCCGTCGGGTTC-3′
PnsCps1-BIP	5′-GCACTGTTCGGCATGCTGGTCCTGTTTGGTGGAGATGGTG-3′
PnsCps1-F3	5′-GGGCGATCAGGATCTTCCT-3′
PnsCps1-B3	5′-CTTCGTAGCCGACAACCG-3′
PnsCps1-Loop-F	5′-TGTTGCAAATAGTTGGACAGACG-3′
PnsCps1-Loop-B	5′-TTCCTGCTCTATTACCTCTACTACA-3′
For intergenic region of *pstS* and *glmS* genes (PnsPst1 primer set)	
PnsPst1-FIP	5′-ACACCTTCAACATCGACGGATGCTCTGGCTATATTGGGTT-3′
PnsPst1-BIP	5′-CATGCAAATATCCTCAGTCAACTCGTGCAGGTTATTGATCGTATCC-3′
PnsPst1-F3	5′-CGCAGCTAATAACAGAGGC-3′
PnsPst1-B3	5′-TCGCTGATTTTGCGGTTAG-3′
PnsPst1-Loop-F	5′-GATTGTTTAACGGTGCCGTAAT-3′

1 mM HEPES solution. The sensitivity of LAMP was compared with that of PCR by using the *pstS–glmS* region-specific primer pair PST3899/PST4987c (Wensing et al. 2010). The conditions for LAMP were as described. The PCR reaction mixture was the same as described but with the addition of dimethyl sulfoxide to a final concentration of 5 % (v/v) and the following conditions: 94 °C for 2 min; 35 cycles at 94 °C for 20 s, 62 °C for 20 s, and 72 °C for 30 s; 72 °C for 2 min. These assays were performed independently 6 times. The specificity of LAMP was examined by using suspensions of all strains. These assays were performed independently 2 times.

To investigate the tolerance of LAMP to sweet corn leaf contents, the inhibitory effects of sweet corn leaf contents were compared using LAMP and PCR at 4 bacterial concentrations. To create a *Pnss*-free plant debris solution, a 50-mg section of a healthy leaf of sweet corn (cv. Honey Bantam) was sliced into pieces $\leq$1 mm thick, and the pieces were then soaked in 500 μL of 1 mM HEPES solution for 10 min; the supernatant was then used as the debris solution. Bacterial suspensions were diluted with the debris solution to 10^6, 10^5, 10^4, and 10^3 cfu/mL and used for LAMP and PCR assays as described above.

The usefulness of LAMP to detect *Pnss* in infected sweet corn leaves was tested. Sweet corn (cv. Honey Bantam) seedlings were inoculated at about the V4 stage by infusion of 10 μL of a suspension (10^8 cfu/mL) of *Pnss* into the base of the lowest leaf by pricking with a bundle of needles. After 2 weeks, the samples were prepared as described in the previous paragraph; we tested 40 samples that had water-soaked lesions on young, expanding leaves. Uninoculated leaves were used as negative controls. The extracts were incubated at 98 °C for 10 min and chilled on ice, then 5 μL was used as a template solution for LAMP.

Results and discussion

The five strains of *Pnss* showed 100 % nucleotide sequence identity in the amplified regions (accession numbers

AB894425–AB894434). The PnsPst 1 primer set did not include loop-B primer, due to lack of suitable region for annealing.

To optimize the LAMP assay, we investigated the optimal temperature for the LAMP reaction. The mean T_t values with the PnsCps1 primer set were increased in the order of 63 °C (19.6 min) < 65 °C (20.5 min) < 60 °C (21.7 min). Those with the PnsPst1 primer set were increased in the order of 60 °C (20.8 min) < 63 °C (21.6 min) < 65 °C (25.5 min). Analysis of variance followed by Tukey's honestly significant difference test of pairwise comparisons showed that for the PnsCps1 primer set, there was a significant difference only between 63° and 60 °C, but for the PnsPst1 primer set, there were significant differences between all three temperatures (Fig. 1a, b). Although the difference in threshold time for the PnsPst1 primer set between 60° and 63 °C was statistically significant, the difference was only 1 min and for practical use is almost the same (Fig. 1b). Therefore, we decided that 63 °C is the optimal for the reaction temperature for practical use of the LAMP detection using both primers at the same time in one incubator. Further experiments were conducted using this temperature.

To confirm the effectiveness of the loop primers, we compared the reaction times with and without the loop primers. The addition of loop primers significantly shortened the mean T_t values from 38.5 to 17.6 min (PnsCps1) and from 33.5 to 21.2 min (PnsPst1) (paired Student's t test, $P < 0.05$; Fig. 2a, b).

The methods for the diagnosis should satisfy the requirements of International Standards for Phytosanitary Measures No. 27 for diagnostic protocols (IPPC 2006). Methods should be selected on the basis of their sensitivity, specificity and reproducibility. In addition, simple and quick methods are better for on-site inspection.

We also confirmed the LAMP sensitivity. The LAMP assays with both primer sets could detect all bacterial suspensions of 10^4 cfu/mL, with mean $T_t = 26.5$ min

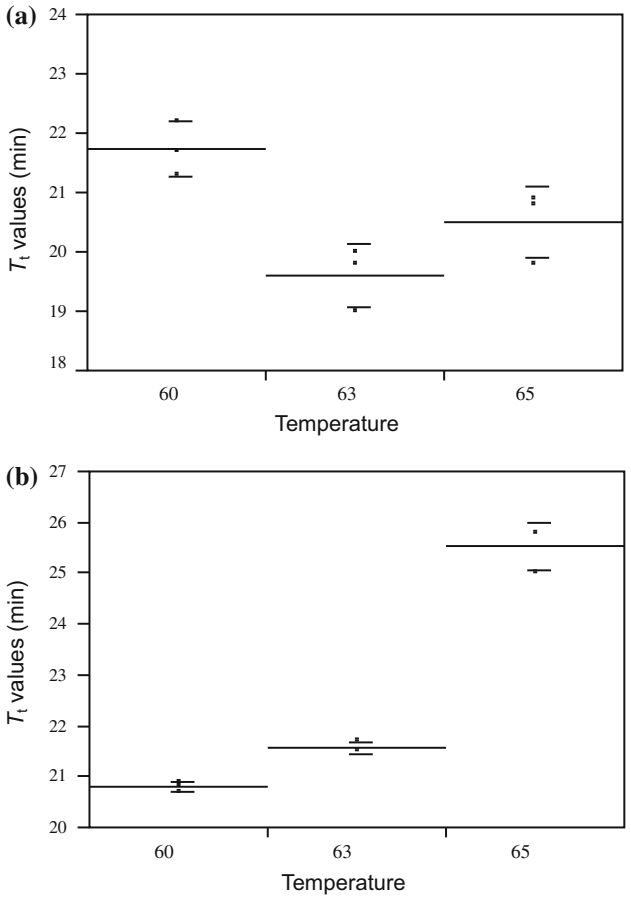

Fig. 1 Threshold time (T_t) values (time until turbidity = 0.1) (min) at 3 LAMP reaction temperatures (60°, 63°, 65 °C). **a** LAMP reaction with PsCps1 primer set. **b** Reaction with PsPst1 primer set. The *long middle line* is the group mean ($n = 3$); *top and bottom lines* represent the standard deviation

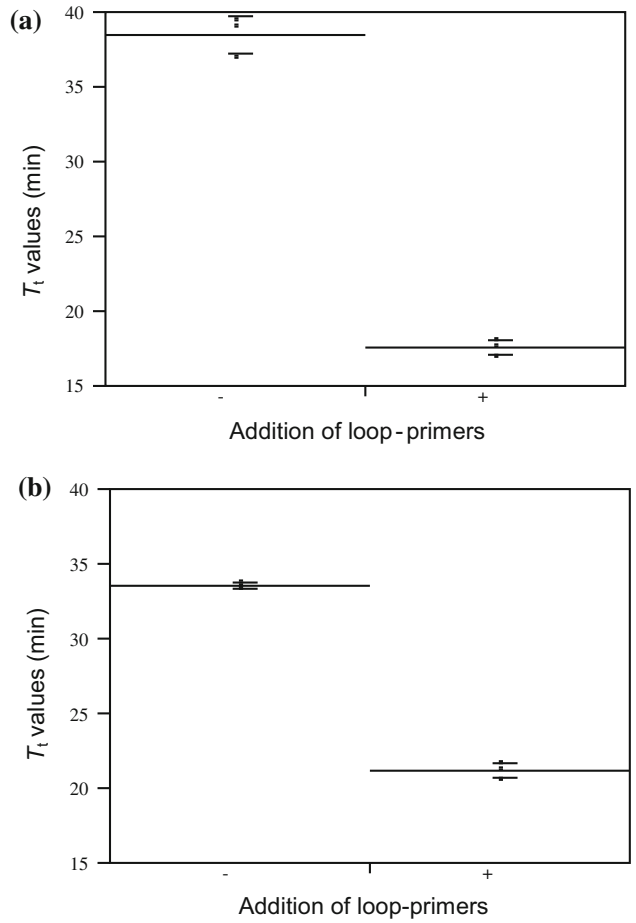

Fig. 2 Threshold time (T_t) values (time until turbidity = 0.1) (min) with and without loop primers for LAMP reaction. **a** LAMP reaction with the PsCps1 primer set. **b** Reaction with the PsPst1 primer set. The *long middle line* shows the group mean ($n = 3$); *top and bottom lines* represent the standard deviation

Table 3 Sensitivity of loop-mediated isothermal amplification (LAMP) assays and polymerase chain reaction (PCR) assays to detect *Pantoea stewartii* in serial dilutions

Dilution (cfu/mL)	LAMP		PCR (Wensing et al. 2010)
	PsCps1	PsPst1	
10^6	6/6[a]	6/6	6/6
10^5	6/6	6/6	6/6
10^4	6/6	6/6	6/6
10^3	3/6	4/6	1/6

[a] No. positive samples/total samples

Table 4 Tolerance to maize leaf components of loop-mediated isothermal amplification (LAMP) assays and polymerase chain reaction (PCR) assays to detect *Pantoea stewartii*

Dilution (cfu/mL)	LAMP		PCR (Wensing et al. 2010)
	PsCps1	PsPst1	
10^7	12/12[a]	12/12	0/6
10^6	12/12	12/12	0/6
10^5	12/12	12/12	0/6
10^4	16/16	16/16	0/6
10^3	13/16	11/16	0/6

[a] No. positive samples/total samples

(PsCps1) and 32.5 min (PsPst1), and maximum values of 28.7 min (PsCps1) and 33.3 min (PsPst1). On the other hand, DNA amplification from suspensions of 10^3 cfu/mL was not reliable (Table 3). Therefore, the detection threshold of the template bacterial suspension in this method was 10^4 cfu/mL. Conventional PCR could also detect as few as 10^4 cfu/mL. Thus, LAMP is at least as sensitive as conventional PCR. In the presence of leaf contents, LAMP could detect *Pnss* down to 10^4 cfu/ml, but conventional PCR failed to detect *Pnss* at any concentration (Table 4). This difference suggests that LAMP is better suited than conventional PCR for field surveillance of Stewart's wilt. The tolerance of LAMP to the sweet corn leaf components obviates the need for pre-treatment of the template, reducing the time of the entire process to only 2 h.

The LAMP assay was specific to all *Pnss* strains, but it was negative for other *Pantoea* spp. strains and other genera associated with maize diseases (Table 1). In addition, no cross reaction was observed with 57 strains isolated from the surface of maize leaves in Japan. Another subspecies, *P stewartii* subsp. *indologenes* (*Pnsi*) (Mergaert et al. 1993), is commonly isolated from maize (Gehring et al. 2014), but it is a pathogen of millet and not virulent on maize (Mergaert et al. 1993). It is difficult to differentiate these subspecies by molecular methods. Unfortunately, we could not import strains of *Pnsi*, so we could not examine the specificity of the test for *Pnss*. But because *Pnsi* does not cause disease on maize, the LAMP assay is useful for identifying *Pnss* from leaves with disease symptoms. The identification of pure culture isolates or detection from asymptomatic leaves will still require additional tests such as virulence assays on seedlings, indole assays or subspecies-specific PCR (Gehring et al. 2014).

All 40 samples of tissue suspensions from fresh water-soaked lesions on young sweet corn seedling leaves on inoculated plants reacted positively with the two primer sets, but the samples from uninoculated leaves were negative. These results verify the reproducibility of the LAMP assay. They suggest that LAMP is suitable for on-site diagnosis and for high-throughput screening of Stewart's wilt.

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