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Genomics-informed design of loop-mediated isothermal amplification for detection of phytopathogenic *Xanthomonas arboricola* pv. *pruni* at the intraspecific level

A. Bühlmann[a], J. F. Pothier[a], J. A. Tomlinson[b], J. E. Frey[a], N. Boonham[b]*, T. H. M. Smits[a] and B. Duffy[a]

[a]*Plant Protection Division, Agroscope Changins-Wädenswil ACW, 8820 Wädenswil, Switzerland; and* [b]*The Food and Environment Research Agency, Sand Hutton, York YO41 1LZ, UK*

The objective of this study was to develop a rapid, sensitive detection assay for the quarantine pathogen *Xanthomonas arboricola* pv. *pruni*, causal agent of stone fruit bacterial spot, an economically important disease of *Prunus* spp. Unique targets were identified from *X. arboricola* pv. *pruni* genomes using a comparative genomics pipeline of other *Xanthomonas* species, subspecies and pathovars, and used to identify specific diagnostic markers. Loop-mediated isothermal amplification (LAMP) was then applied to these markers to provide rapid, sensitive and specific detection. The method developed showed unrivalled specificity with the 79 tested strains and, in contrast to previously established techniques, distinguished between phylogenetically close subspecies such as *X. arboricola* pv. *corylina*. The sensitivity of this test is comparable to that of a previously reported TaqMan™ assay at 10^3 CFU mL^{-1}, while the unrivalled speed of LAMP technology enables a positive result to be obtained in <15 min. The developed assay can be used with real-time fluorescent detectors for quantitative results as well as with DNA-staining dyes to function as a simplified strategy for on-site pathogen detection.

Keywords: bacterial spot, comparative genomics, LAMP, *Xanthomonas arboricola* pv. *pruni*

Introduction

Biological invasions of plant pathogens, including bacteria, are an undesirable yet increasing consequence of globalized trade in plant material. While many invasive phytopathogenic bacteria have little impact, some can cause significant damage to agro-ecosystems and potentially serve as a genetic reservoir of pathogenicity (Morris *et al.*, 2009). Phytosanitary inspectors are the vanguard in preventing the introduction of invasive pathogens and controlling the spread of those already introduced. To achieve these goals, greater understanding of the biology of plant pathogenic bacteria (Allen *et al.*, 2009) is as important as the availability of rapid and accurate detection and identification methods (Alvarez, 2004).

One example of an invasive phytopathogenic bacterium is *Xanthomonas arboricola* pv. *pruni*, the causal agent of bacterial spot of stone fruit. It is a Gram-negative gamma-proteobacterium that infects a wide range of commercial, ornamental and forest *Prunus* species (Ritchie, 1995). It can persist in buds and can potentially be dispersed over large areas or whole countries by plant propagation before symptoms are noticed (Stefani, 2010). The reduced crop yields or even tree losses after outbreaks cause an increasing problem for the stone fruit industry, particularly on cultivated *Prunus* spp. (Garcin, 2000; Pothier *et al.*, 2010). Effective phytosanitary measures against *X. arboricola* pv. *pruni* require an interdisciplinary effort to gain insight into genetic relationships and introduction pathways (Boudon *et al.*, 2005), host specificity and pathogenicity of present and past pathogens (Lu *et al.*, 2008), reliable detection of the pathogen (Pothier *et al.*, 2011a), mitigation of infections (Mitre & Roman, 2008) and analysis of the economic and social impact of the epidemic (Stefani, 2010). Detection of *X. arboricola* pv. *pruni* can be achieved by visual inspection of necrotic lesions on different plant tissues such as leaves, fruits or cankers (Anonymous, 2006), but identification requires confirmation by molecular techniques. These have been difficult to develop in the past because of extremely low diversity within the *X. arboricola* species complex and missing genomic information (Vauterin *et al.*, 1995; Pothier *et al.*, 2011a). Existing methods for identification of *X. arboricola* pv. *pruni* are based on either PCR (Park *et al.*, 2010), suppression subtractive hybridization (Ballard *et al.*, 2011), duplex-PCR (Pothier *et al.*, 2011a) or qPCR (Palacio-Bielsa *et al.*, 2011). A technique that has the potential to provide fast, field-based molecular identification of pathogenic bacteria is loop-mediated isothermal amplification (LAMP; Notomi *et al.*, 2000).

*E-mail: neil.boonham@fera.gsi.gov.uk

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In contrast to PCR, LAMP does not rely on expensive and sophisticated thermal cycling instruments, making it ideal to use on smaller devices such as heat blocks and portable fluorescence readers. Several features make LAMP an attractive technology to test plant samples for the presence of bacterial pathogens. First, it has high tolerance to non-target biological contaminants (Kaneko *et al.*, 2007). The use of up to eight different primer recognition sites makes the method highly specific, so that it can even be applied for single nucleotide polymorphism (SNP) genotyping (Iwasaki *et al.*, 2003). The massive amount of DNA produced (i.e. up to 10^9 copies of target DNA in <1 h) (Notomi *et al.*, 2000) enables novel detection methods. Detection methods used with LAMP consist of conventional agarose gels, turbidity measurement (Iwamoto *et al.*, 2003), intercalating dyes such as ethidium bromide or SYBR®Safe for end-point visual detection and real-time amplification using fluorescence detectors, or even immunoassay-based detection on a lateral flow device if labelled primers are used (Tomlinson *et al.*, 2010). With this versatile set of interpretation methods, LAMP can be used for on-site detection as well as for laboratory-based confirmation of phytopathogens. The utility of LAMP assays has been demonstrated for detection of important human pathogens (Aryan *et al.*, 2009), as well as phytopathogenic bacteria (Rigano *et al.*, 2010; Temple & Johnson, 2011).

Advances in next-generation sequencing technologies have increased availability and made it relatively straightforward to sequence whole bacterial genomes, particularly when closely related taxa are available to use as templates for assembly and annotation. The aim of this study was to link a recently sequenced genome, consisting of one chromosome (Pothier *et al.*, 2011b) and one multicopy plasmid (Pothier *et al.*, 2011c), with published genomic data to demonstrate the efficiency of a novel diagnostic target identification strategy based on comparative genomics and bioinformatics. LAMP was chosen to demonstrate the high specificity of the diagnostic markers developed on the plant pathogenic bacterium *X. arboricola* pv. *pruni*.

Materials and methods

Comparative genomics and primer design

The genome information of 17 publically available *Xanthomonas*, as well as the draft genomes of *X. arboricola* pv. *pruni* strains CFBP 5530 and GBBC 2038 and *X. arboricola* pv. *fragariae* strains LMG 19145 and GBBC 2042 assembled in-house (Table 1), were used to perform a comparative genomics analysis. A list of *X. arboricola* pv. *pruni* singleton coding sequences (CDS) was generated using the program EDGAR v. 1.2 (Blom *et al.*, 2009). Further analyses were performed using standalone BLAST v. 2.2.22 (Altschul *et al.*, 1990) and BLAST searches at NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi). LAMP primer sets for true *X. arboricola* pv. *pruni* singletons were designed using PRIMEREXPLORER v. 4 software (http://primerexplorer.jp/lamp).

Bacterial strains and culture conditions

For preliminary analysis, as well as for sensitivity, specificity and qPCR assays, *X. arboricola* pv. *pruni* strain CFBP 5530 was used. A geographically and genetically representative collection of *X. arboricola* pv. *pruni* isolates (n = 28) was tested, as well as other *Xanthomonas* species and pathovars (n = 45) and bacteria of different species (n = 6) expected to co-occur on host plants (Table 2). Strains were grown on peptone yeast extract glycerol agar medium (NYGA) (Turner *et al.*, 1984) with incubation at 28°C for 24–48 h, and DNA was extracted following a standard protocol (Sambrook *et al.*, 1989). For boiled cells, *Xanthomonas* strains were grown on liquid peptone yeast extract glycerol medium at 28°C for 24–48 h. Colonies were scratched off the agar, added to H_2O and boiled at 99°C for 20 min.

Primer validation, specificity and sensitivity

Primers were synthesized at Microsynth AG. Standard PCR was performed with primer concentrations of 400 nM using 1 × HotstarTaq Master Mix (QIAGEN AG) and the following conditions: 15 min initial denaturation at 95°C; 35 cycles of 30 s at 95°C, 30 s at 58°C and 30 s 72°C; and 2 min final extension at 72°C. Sensitivity was tested in triplicate on a dilution series of boiled cells, ranging from $2{\cdot}4 \times 10^9$ to $2{\cdot}4 \times 10^2$ CFU mL^{-1}, of *X. arboricola* pv. *pruni* CFBP 5530. Additionally, apricot plant extracts of 0·1 g leaf or wooden stem material ground in 1 mL PBS buffer were spiked with *X. arboricola* pv. *pruni* CFBP 5530 boiled cells and DNA, and also assayed in triplicate. LAMP was performed in 12-μL reactions on an ABI 7500 Fast Real-Time PCR System (ABI) or on GenieII (Optigene Ltd) at 70°C for 45 min with melting curve analysis at 70–95°C. For the LAMP reaction, Isothermal Master Mix (Optigene Ltd) was used at 1 × concentration and a reaction volume of 12 μL. Primer concentrations were as follows: outer primers 160 nM, inner primers 1·6 μM and loop primers 0·8 μM. Fluorescence was detected in real-time on the FAM channel with no reference dye.

Performance comparison to qPCR

The TaqMan assay developed by Palacio-Bielsa *et al.* (2011) was used to compare the sensitivity of the newly developed assay on strain *X. arboricola* pv. *pruni* CFBP 5530. Primer concentrations were 400 nM and probe concentration was 120 nM. TaqMan Environmental Master Mix 2.0 (ABI) was used and real-time PCR reactions were performed on an ABI PRISM® 7500 Sequence Detection System using cycling conditions: 2 min at 50°C, 10 min at 95°C, followed by 45 cycles of 1 min at 95°C and 1 min at 59°C.

Table 1 *Xanthomonas* genomes used for *in silico* comparative genomic analysis with accession number, genome size and gene content

Species	Strain	Chromosome/Plasmid	GenBank	Size (bp)	Genes	Reference
Xanthomonas arboricola pv. *pruni*	CFBP 5530	Chromosome	N/A	5 066 961	3905	Pothier *et al.* (2011b)
		pXap41	FR875157	41 102	44	Pothier *et al.* (2011c)
	GBBC 2038	Chromosome	N/A	5 071 191	4179	
		pXap41	N/A	41 103	44	
X. arboricola pv. *fragariae*	GBBC 2042	Draft genome	N/A	N/A	N/A	
	LMG 19145	Draft genome	N/A	N/A	N/A	
Xanthomonas albilineans	GPE PC 73	Chromosome	FP565176	3 768 695	3172	Pieretti *et al.* (2009)
		GPE PC73 plasmid 1	FP340279.1	24 837	24	
		GPE PC73 plasmid 2	FP340278.1	31 555	38	
		GPE PC73 plasmid 3	FP340278.1	27 212	31	
Xanthomonas axonopodis pv. *citri*	306	Chromosome	AE008923	5 175 554	4374	Martins *et al.* (2010)
		pXAC64	NC_003922.1	64 920	63	
		pXAC33	NC_003921.3	33 700	41	
X. axonopodis pv. *glycines*	AG 1	pAG1	NC_010872	15 143	16	Kim *et al.* (2006)
		pXAG81	AY780632	15 143	34	
		pXAG82	AY780633	26 721	1	
Xanthomonas campestris pv. *campestris*	8004	Chromosome	CP000050	5 148 708	4368	Qian *et al.* (2005)
	ATCC 33913	Chromosome	AE008922	5 425 342	4240	da Silva *et al.* (2002)
	B 100	Chromosome	AM920689	5 079 002	4574	Vorhölter *et al.* (2008)
X. campestris pv. *musacearum*	NCPPB 4381	Chromosome	ACHT00000000	4 782 144	4510	Studholme *et al.* (2010)
X. campestris pv. *vasculorum*	NCPPB 702	Chromosome	ACHS00000000	5 425 342	4977	Studholme *et al.* (2010)
X. campestris pv. *vesicatoria*	85-10	Chromosome	AM039952	5 178 466	4606	Thieme *et al.* (2005)
		pXCV38	AM039950	182 572	44	
		pXCV183	AM039951	19 146	179	
		pXCV19	AM039949	1852	25	
		pXCV2	AM039948	1851	2	
		pXV64	U78513	64 920	2	
Xanthomonas citri		pXcB	AY228335	1315	38	El Yacoubi *et al.* (2007)
Xanthomonas gardneri	ATCC 19865	Chromosome	AEQX00000000	5 528 124	5091	Potnis *et al.* (2011)
Xanthomonas fuscans ssp. *aurantifolii*	ICPB 10535	Chromosome	ACPY00000000	5 012 633	3977	Moreira *et al.* (2010)
	ICPB 11122	Chromosome	ACPX00000000	4 879 662	3863	Moreira *et al.* (2010)
Xanthomonas oryzae pv. *oryzae*	KACC 1033	Chromosome	AE013598	4 941 439	4281	Lee *et al.* (2005)
	MAFF 31101	Chromosome	AP008229	4 940 217	4431	Ochiai *et al.* (2005)
	PXO 99A	Chromosome	CP000967	5 240 075	5131	Salzberg *et al.* (2008)
X. oryzae pv. *oryzicola*	BLS 256	Chromosome	AAQN00000000	4 831 739	4414	Bogdanove *et al.* (2011)
Xanthomonas perforans	91-118	Chromosome	AEQW00000000	5 262 127	4700	Potnis *et al.* (2011)
Xanthomonas vesicatoria	ATCC 35937	Chromosome	AEQV00000000	5 531 089	4998	Potnis *et al.* (2011)

N/A indicates information not available.

Inoculation of plants

To infect apricot plants, an inoculum solution was prepared by incubating *X. arboricola* pv. *pruni* on NYGA for 48 h. The *X. arboricola* pv. *pruni* colonies were transferred to a test tube, washed with sterile H_2O, diluted to an optical density of 0·5 at 600 nm and aliquoted to use as inoculum. Two-year-old apricot plants of an F_1 cross between cultivars Harostar × Rouge de Mauves were grown in a greenhouse under standard conditions. Plants were infected with *X. arboricola* pv. *pruni* strains CFBP 5530 and NCPPB 416 by dipping young plants (two branches per plant, 10 leaves per branch) into a previously prepared inoculum solution containing 5×10^8 CFU mL^{-1} for 5 s. At 45–50 days post-infection, 0·1 g leaf, twig or woody tissue samples with symptoms were collected, ground in 1 mL PBS (BIOREBA AG) and boiled at 99°C for 20 min. Aliquots of 1 μL boiled tissue extract were used directly as template for PCR reactions.

End-point detection using DNA-staining dyes

In order to achieve a detection method without the application of optical systems the amplification products from the sensitivity experiments were stained with different DNA-staining dyes. One microlitre of SYBR®Safe (Invitrogen), 1 μL of a 100 ng μL^{-1} ethidium bromide (Bio-Rad) stock solution or 1 μL Quant-iT™PicoGreen® Reagent (Invitrogen) was added to 10 μL LAMP products and then visualized under UV light at 312 nm.

Results

LAMP primer design and comparative genomics

In this study comparative genomics-informed design (Fig. S1) on 21 genomes consisting of 17 published and four in-house sequenced genomes of *Xanthomonas* was applied to develop diagnostic markers discriminatory *in*

Table 2 *Xanthomonas* and other bacteria used for specificity testing

Species[a]	Strain	Origin	Host plant	LAMP[b]	TaqMan PCR[b]
Xanthomonas arboricola	CFBP 3893	Italy	*Prunus* sp.	+	+
pv. *pruni*	CFBP 3894	New Zealand	*Prunus* sp.	+	+
	CFBP 3897	Italy	*Prunus* sp.	+	+
	CFBP 3899	USA	*Prunus* sp.	+	+
	CFBP 3900	USA	*Prunus* sp.	+	+
	CFBP 3901	USA	*Prunus* sp.	+	+
	CFBP 3902	USA	*Prunus* sp.	+	+
	CFBP 3903	Italy	*Prunus* sp.	+	+
	CFBP 3904	Italy	*Prunus* sp.	+	+
	CFBP 3920	Italy	*Prunus* sp.	+	+
	CFBP 5266	France	*Prunus* sp.	+	+
	CFBP 5530	Italy	*Prunus* sp.	+	+
	CFBP 5566	France	*Prunus* sp.	+	+
	CFBP 5575	France	*Prunus* sp.	+	+
	CFBP 5716	USA	*Prunus* sp.	+	+
	CFBP 5718	USA	*Prunus* sp.	+	+
	CFBP 5719	USA	*Prunus* sp.	+	+
	CFBP 5723	Uruguay	*Prunus* sp.	+	+
	CFBP 5724	USA	*Prunus* sp.	+	+
	IVIA 2626-1	Spain	*Prunus* sp.	+	+
	IVIA 2647·1-2	Spain	*Prunus* sp.	+	+
	IVIA 2647·3-1	Spain	*Prunus* sp.	+	+
	IVIA 2826-1	Spain	*Prunus* sp.	+	+
	IVIA 2826-8	Spain	*Prunus* sp.	+	+
	IVIA 2832-10	Spain	*Prunus* sp.	+	+
	IVIA 2832-30	Spain	*Prunus* sp.	+	+
	IVIA 3161-2	Spain	*Prunus* sp.	+	+
	IVIA 3467-1	Spain	*Prunus* sp.	+	+
X. arboricola pv. *celebensis*	CFBP 3523	New Zealand	*Musa* sp.	–	–
X. arboricola pv. *corylina*	CFBP 1159	USA	*Corylus* sp.	–	+
	CFBP 6600	France	*Corylus* sp.	–	+
	CFBP 7386	Chile	*Corylus* sp.	–	+
	CFBP 7387	Turkey	*Corylus* sp.	–	+
	NCPPB 2859	Turkey	*Corylus* sp.	–	+
	NCPPB 2896	UK	*Corylus* sp.	–	+
	NCPPB 2898	UK	*Corylus* sp.	–	+
	NCPPB 3339	France	*Corylus* sp.	–	+
	NCPPB 3776	UK	*Corylus* sp.	–	+
	NCPPB 3870	Italy	*Corylus* sp.	–	+
	NCPPB 3875	Italy	*Corylus* sp.	–	+
	NCPPB 3876	Italy	*Corylus* sp.	–	+
	NCPPB 3950	Chile	*Corylus* sp.	–	+
X. arboricola pv. *fragariae*	CFBP 6771	Italy	*Fragaria* sp.	–	–
X. arboricola pv. *juglandis*	NCPPB 411	New Zealand	*Juglans* sp.	–	–
X. arboricola pv. *poinsettiicola*	LMG 5403	New Zealand	*Euphorbia* sp.	–	–
X. arboricola pv. *populi*	CFBP 3123	Netherlands	*Populus* sp.	–	–
Xanthomonas albilineans	DSMZ 3583^T	Fiji	*Saccharum* sp.	–	–
Xanthomonas alfalfae ssp. *alfalfae*	LMG 495^T	India	*Medicago* sp.	–	–
X. alfalfae ssp. *citrumelonis*	CFBP 3371^T	USA	*Citrus* sp.	–	–
Xanthomonas axonopodis pv. *axonopodis*	CFBP 4924^T	Colombia	*Axonopus* sp.	–	–
X. axonopodis pv. *citri*	CFBP 2525^P	New Zealand	*Citrus* sp.	–	–
Xanthomonas bromi	CFBP 1976^T	France	*Bromus* sp.	–	–
Xanthomonas campestris pv. *campestris*	CFBP 5241^T	UK	*Brassica* sp.	–	–
Xanthomonas cassavae	CFBP 4642^T	Malawi	*Manihot* sp.	–	–
Xanthomonas codiaei	CFBP 4690^T	USA	*Codiacum* sp.	–	–
Xanthomonas cucurbitae	CFBP 2542^T	New Zealand	*Cucurbita* sp.	–	–
Xanthomonas citri pv. *citri*	LMG 9322^T	USA	*Citrus* sp.	–	+
Xanthomonas cynarae	CFBP 4188^T	France	*Cynara* sp.	–	–

Table 2 (Continued)

Species[a]	Strain	Origin	Host plant	LAMP[b]	TaqMan PCR[b]
Xanthomonas fragariae	CFBP 2157^T	USA	*Fragaria* sp.	–	–
Xanthomonas fuscans	CFBP 6165^T	Canada	*Phaseolus* sp.	–	–
Xanthomonas gardneri	NCPPB 881^T	Ex-Yugoslavia	*Lycopersicon* sp.	–	–
Xanthomonas hortorum pv. *hederae*	LMG 733^T	USA	*Hedera* sp.	–	–
Xanthomonas hyacinthi	CFBP 1156^T	Netherlands	*Hyacinthus* sp.	–	–
Xanthomonas melonis	CFBP 4644^T	Brazil	*Cucumis* sp.	–	–
Xanthomonas oryzae pv. *oryzae*	CFBP 2532^T	India	*Oryza* sp.	–	–
Xanthomonas perforans	NCPPB 4321^T	USA	*Lycopersicon* sp.	–	–
Xanthomonas pisi	CFBP 4643^T	Japan	*Pisum* sp.	–	–
Xanthomonas populi	CFBP 1817^T	France	*Populus* sp.	–	–
Xanthomonas sacchari	CFBP 4641^T	France	*Saccharum* sp.	–	–
Xanthomonas theicola	CFBP 4691^T	Japan	*Camellia* sp.	–	–
Xanthomonas transluscens pv. *transluscens*	CFBP 2054^T	USA	*Hordeum* sp.	–	–
Xanthomonas vasicola pv. *holcicola*	CFBP 2543^T	New Zealand	*Sorghum* sp.	–	–
Xanthomonas vesicatoria	CFBP 4645^T	New Zealand	*Lycopersicon* sp.	–	–
Agrobacterium tumefaciens	C58	USA	*Prunus* sp.	–	–
Erwinia amylovora	CFBP 1430	France	*Crataegus* sp.	–	–
Erwinia rhapontici	CFBP 3163^T	UK	*Rheum* sp.	–	–
Pantoea vagans	C9-1	USA	*Malus* sp.	–	–
Pseudomonas syringae pv. *persicae*	CFBP 1573^P	France	*Prunus* sp.	–	–
Stenotrophomonas maltophila	CFBP 3035^T	USA	Cancer patient	–	–

[a]*Xanthomonas* nomenclature follows Vauterin *et al.* (1995). Culture collections are abbreviated as CFBP (Collection Française de Bactéries associées aux Plantes), DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen), LMG (Laboratorium voor Microbiologie en Microbiele Genetica), NCPPB (National Collection of Plant Pathogenic Bacteria). Superscripts following strain names indicate ^{T} the type strain of a species and ^{P} the pathotype strain for a pathovar.

[b]LAMP using primer set XAP3806; TaqMan PCR as used in Palacio-Bielsa *et al.* (2011); +: positive reaction; –: negative reaction.

silico as well as *in planta* for X. *arboricola* pv. *pruni*, and to improve existing PCR-based detection methods (Palacio-Bielsa *et al.*, 2011; Pothier *et al.*, 2011a).

The program EDGAR (Blom *et al.*, 2009) was used to compare the X. *arboricola* pv. *pruni* CFBP 5530 genome with the other 20 *Xanthomonas* genomes delineated, because it comprises a powerful set of tools for comparative genomics and uses only annotated CDS, which allows exclusion of intergenic regions. EDGAR calls a CDS a singleton if it has no hit above 70% amino acid identity with any other annotated CDS in the selected genomes. In this step, 363 CDS of a total of 3905 CDS in the X. *arboricola* pv. *pruni* CFBP 5530 genome were identified as singletons. As EDGAR has a threshold of 70% amino acid identity, which for a reliable diagnostic marker is insufficiently stringent, a BLASTN search against all 21 *Xanthomonas* genomes was performed. This search reduced the set of singletons to 84 CDS, eliminating all CDS with hits above 50% sequence identity. The alignment to X. *arboricola* pv. *pruni* strain GBBC2038 resulted in no reduction in numbers of singletons as X. *arboricola* shows a close, almost clonal, genetic relatedness (Boudon *et al.*, 2005). As a next step, a BLASTN search against the full NCBI database resulted in a reduction to 23 truly unique regions with length greater than 200 base pairs and sequence identity of <50% on the nucleotide level to any other sequence present in the NCBI database. From these 23 unique regions, the 12 targets with the lowest E-value scores to any other sequence present in GenBank were selected for LAMP primer design.

To narrow down the list of 12 interesting target regions, specificity of the unique target regions was tested by regular PCR using outer primers F3 and B3 on a set of 28 genetically and geographically diverse X. *arboricola* pv. *pruni* strains and on 45 related *Xanthomonas* species and pathovars (Fig. S2), and other, more distantly related bacteria expected to occur on host plants of X. *arboricola* pv. *pruni*. Among these 45 strains were 13 diverse X. *arboricola* pv. *corylina* strains, as this pathovar has been responsible for most false positive results in previous studies (Pothier *et al.*, 2011a). This regular PCR resulted in one remaining primer set, XAP3806 (Table 3), which was selected for full LAMP specificity testing, while the other primer sets produced PCR fragments in non-X. *arboricola* pv. *pruni* strains, indicating that the target region is non-specific to X. *arboricola* pv. *pruni*.

Primer validation, specificity and sensitivity

The designed primer set XAP3806 (Table 2) was evaluated using the same set of 74 strains described above. The LAMP reaction showed outstanding specificity, with clearly positive results for the 28 genotypically representative X. *arboricola* pv. *pruni* strains and clearly negative results for all other bacteria tested. Thus, it showed an increased specificity relative to previously developed methods (Table 2). The specificity of the LAMP assay can be assessed by the characteristic amplification curve produced and additionally by performing a melt curve

Table 3 Primers designed during this study for TaqMan PCR and LAMP

Target	Primer[a]	Sequence (5′–3′)
XAP3806	B3	TGATGCCCCTCA AGAGAGG
	BIP	TACGGGATCGAG ACACCTTGGTCG GTGCATGGTAGA TCACAT
	F3	CACTGCGGATTGT TACACGT
	FIP	TCGGTGGGTCGAA TAGGTACCAGGGT GTGGAGTTGGTCGT
	loopF	AGCATGCAGAATCT GCCAGCAC
	loopR	TGCCGGGGACGCA ATGTAATGC
XAP TaqMan	Xap2F	TGGCTTCCTGACTG TTTGCA
	Xap2R	TCGTGGGTTCGCTT GATGA
	Xap2P	FAM-TCAATATCTGTG CGTTGCTGTTCTCAC GA-TAMRA

[a]F3, forward outer; R, reverse outer primer for LAMP. FIP and BIP, inner LAMP primers. loopF and loopR, forward and reverse loop primers.

analysis. Although not readily predictable, experiments have shown that LAMP products show a specific melt curve with a characteristic melting temperature peak, T_m. In the case of this described *X. arboricola* pv. *pruni* LAMP assay, the specific melting temperature was at 88 ± 0·2°C, which was observed for all positive *X. arboricola* pv. *pruni* samples.

To assess the sensitivity of the LAMP assay, the primer set XAP3806 was tested with different concentrations of boiled *X. arboricola* pv. *pruni* CFBP 5530 cells (Fig. 1) and DNA concentrations. A range of $2{\cdot}4 \times 10^9$–$2{\cdot}4 \times 10^2$ CFU mL^{-1} was tested. The LAMP reactions showed a dynamic range with quantitative amplification over six orders of magnitude from $2{\cdot}4 \times 10^9$ to $2{\cdot}4 \times 10^4$ CFU mL^{-1}. Additionally, the LAMP assay was shown to detect bacterial concentrations as low as $2{\cdot}4 \times 10^3$ CFU mL^{-1}, at which the detection limit of the assay was reached. At concentrations below $2{\cdot}4 \times 10^4$ CFU mL^{-1} the amplification became non-linear and quantification difficult to reliably interpret. The same sensitivity assay was repeated with extracted *X. arboricola* pv. *pruni* CFBP 5530 DNA. A range from 1 ng μL^{-1} to 0·1 fg μL^{-1} was tested, which, extrapolated to a *X. arboricola* pv. *pruni* genome of 5·07 Mbp, would result in an equivalent range of 2×10^9–2×10^2 CFU mL^{-1}. The detection limit was at 1 fg μL^{-1}, equivalent to 2×10^3 CFU mL^{-1}, confirming the values obtained with boiled cells.

Compared to qPCR the LAMP assay was slightly less precise, which was manifested by a drop in correlation coefficient R^2 from 0·996 in qPCR to 0·956 in LAMP when applied to a standard curve. As bacterial concentrations in field samples are to be interpreted with cautiousness and tend to fluctuate, a correlation of 0·956 is acceptable.

Plant samples

The LAMP assay was validated on plant samples spiked with *X. arboricola* pv. *pruni* CFBP 5530 DNA and boiled cells, and on infected plant material obtained from green-

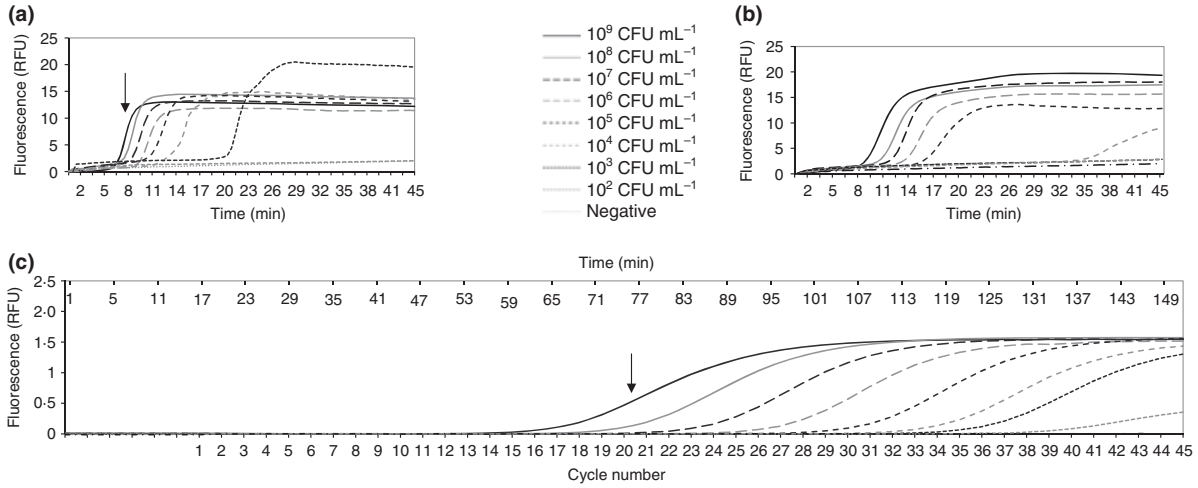

Figure 1 Comparison of sensitivity of (a) LAMP assay with *Xanthomonas arboricola* pv. *pruni* DNA samples, (b) LAMP assay with samples of apricot tissue spiked with *X. arboricola* pv. *pruni* cells and DNA, and (c) qPCR (time shown on top axis to facilitate comparison with LAMP). The *y*-axis values differ between LAMP and qPCR graphs because EvaGreen was used for fluorescence detection used in LAMP and FAM dye was used in qPCR. Over a range of six orders of magnitude the LAMP reaction shows a quantitative amplification signal. Arrows in (a) and (c) indicate time of first positive detection at 8 and 76 min, respectively, highlighting the speed of the LAMP method.

house experiments, for possible inhibitory effects of plant phenolic compounds. The same standard curve as before, ranging from $2{\cdot}4 \times 10^9$ to $2{\cdot}4 \times 10^2$ CFU mL^{-1} was assayed. Indeed, the sensitivity was one order of magnitude lower compared to using pure DNA isolated from cultured bacteria in the sample with ground wood material, but not when using leaf material. This indicates that, without purification of samples, a loss in sensitivity has to be accepted. Additionally, plant tissue from artificially infected apricot plants, with or without symptoms, was tested. Tissue with symptoms, such as leaves, twigs or stems with canker, always tested positive, with time to positive values of 11·7–13·5 min, corresponding to 10^7–10^6 CFU g^{-1} infected tissue. Of the inoculated but symptomless plant samples, only one out of five could be confirmed positive, with a calculated value of 4×10^3 CFU g^{-1} plant tissue. At such low values, the standard curve is no longer linear and impossible to conclusively interpret. The number of bacteria is thus below the threshold for quantification for *X. arboricola* pv. *pruni* on symptomless plant samples, but a positive sample can be scored.

End-point detection using dyes

As a less expensive alternative to using a fluorescence recording platform such as the GenieII, results from LAMP reactions can be resolved using DNA-staining dyes, as reported previously. LAMP products were successfully stained with three different commercially available DNA-staining dyes. However, ethidium bromide had to be added at such a high concentration to enable a visual detection that even the negative control samples showed a colour change to orange, so it is not an optimal staining dye. The SYBR®Safe and RiboGreen® dyes gave superior performance, as shown in Figure 2. The sensitivity of the end-point detection method, at 10^3 CFU mL^{-1} *in vitro* and 10^4 CFU mL^{-1} with spiked plant samples, was essentially the same as with fluorescence detection.

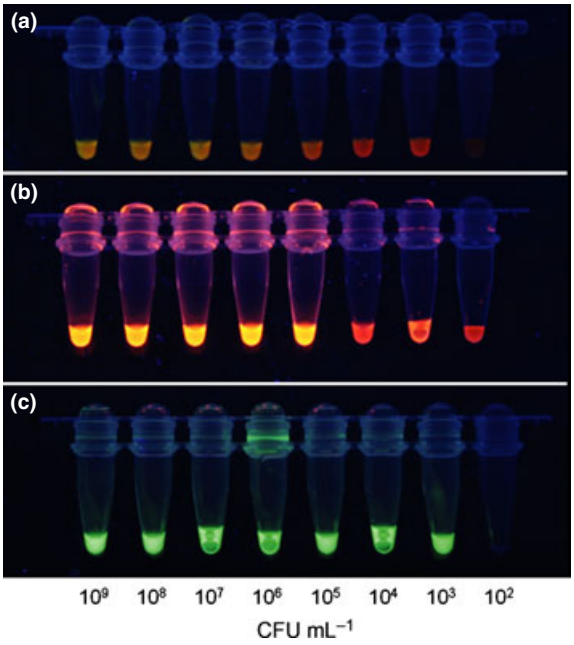

Figure 2 Series of 10-fold dilutions of *Xanthomonas arboricola* pv. *pruni* boiled cells, amplified with LAMP and stained with (a) 1 μL SYBR®Safe, (b) 10 ng μL^{-1} ethidium bromide, or (c) 1 μL^{-1} PicoGreen®. Picture was taken under UV light to increase visibility but results could also be visualized under normal light. The strips represent the same dilution series used for the LAMP and qPCR assays in Figure 1.

Discussion

This study applied a comparative genomics approach to identify targets for molecular diagnostics and was able to streamline this previously tedious process. The identification process also allows selection of targets based on presence of singleton genes, which, compared to target identification approaches chosen recently based on housekeeping genes (Parkinson *et al.*, 2009) or sequences identified by suppression subtractive hybridization (Ballard *et al.*, 2011), enables much more flexibility because of the increased amounts of potential targets to apply. Additionally, housekeeping genes or unidentified gene sequences are diverse and discriminatory between species but often fail to reliably identify bacteria at the intraspecific level. Plasmid genes were excluded from the study because of the high plasticity and fluidity of mobile elements, which can introduce uncertainty in diagnostic assays (Llop *et al.*, 2008), as reported with a recently developed LAMP assay targeting a plasmid region of the fire blight pathogen, *Erwinia amylovora* (Temple & Johnson, 2011). Moreover, copy-number variation of plasmids interferes with accurate quantification of bacterial cells.

The comparative genomics approach was restricted to annotated CDS sequences because genes are expected to be more conserved and less likely to mutate or recombine than intergenic regions with no or unknown biological functions. The CDS which were identified as singletons were mostly annotated as hypothetical proteins or assigned dubious annotation. The fact that they are singleton CDS unique to *X. arboricola* pv. *pruni* indicates that little or no similarity to other known genes exists, and then annotation relies solely on functional annotation tools such as SIGNALP (Petersen *et al.*, 2011), PFAM (Finn *et al.*, 2010), and INTERPRO (Hunter *et al.*, 2012). Preliminary data from an RNA-Seq transcriptomic experiment using Illumina sequencing in the laboratory of the present study (data not shown), where singleton CDS were screened for expression under standard culture conditions, demonstrated that 91% of the 23 target CDS sequences were expressed, while 50% of those 23 sequences showed a medium to high expression value, among which was the (draft) CDS_Xap3806. This fact opens up the opportunity for LAMP to be used on RNA to confirm viability of the detected bacteria.

The measured detection limit of 10^3–10^4 CFU mL^{-1} is slightly higher than the detection limit of the previously

developed qPCR assay (Palacio-Bielsa *et al.*, 2011), but is one order of magnitude lower than that of regular PCR (Pothier *et al.*, 2011a) and two orders of magnitude lower than that of immunoassays on lateral flow devices. This reinforces the potential for LAMP as a tool that can be developed for simple, routine use in on-site field diagnostics, where current methods hold less promise. A major, practical advantage of LAMP is the speed of the reaction, enabling a positive test result to be obtained in as little as 15 min. However, the speed of amplification comes with a slight reduction in applicability for quantification because differences in concentration of one order of magnitude differ only by 1 min until a positive amplification is obtained. Reducing the speed of the assay would improve the resolution of the quantification, although this may reduce end-point sensitivity.

The described validation confirmed the specificity of the designed LAMP assay, enabling reliable discrimination of even very closely related *Xanthomonas* pathovars, and with no cross-reactions to other bacteria associated with host plants of *X. arboricola* pv. *pruni*. The performance of this new LAMP assay was superior to that reported for any of the other diagnostic methods currently available, and does not have the main drawback common to other methods, namely that of potential false positives resulting from cross-reaction with closely related species such as *X. arboricola* pv. *corylina* (Pothier *et al.*, 2011a), or even less closely related taxa such as *Xanthomonas citri* ssp. *citri* (Palacio-Bielsa *et al.*, 2011). The present study also demonstrated the utility of the LAMP assay for obtaining efficient and sensitive pathogen detection directly from plant samples.

The simplicity of LAMP and advancements in equipment technology such as the GenieII, a portable, battery powered, isothermal heating device with fluorescence detection, suggest the possibility of user-friendly on-site detection in the near future. Because LAMP produces a large amount of DNA and has a robust mode of action it could be suitable amplification chemistry for implementing in a range of detection scenarios with a range of platform types. For example, LAMP could be miniaturized and implemented on microfluidic chips (Fang *et al.*, 2010), which has the potential of greatly improving throughput and perhaps reducing pipetting, which would improve field applicability. Moreover, if needed as a very low-cost assay, the LAMP reaction can be applied with visual end-point detection using DNA-staining dyes (although a UV light source would be needed), increasing turbidity as a result of precipitation of magnesium pyrophosphate inherent to a positive reaction, or by incorporating ligands into the amplification primers and using disposable lateral flow devices. However, although the latter methods are simple and less expensive, they are also heterogeneous assay formats (i.e. not closed-tube), requiring additional pipetting steps, which open the possibility of postamplification contamination and false positive results, which are mostly mitigated using the GenieII platform.

Echoing a genomics-informed approach (Lang *et al.*, 2010), this study developed and validated a rapid, simple, diagnostic tool with unprecedented specificity and sensitivity, demonstrating the power conferred by increasingly accessible next-generation sequencing technologies. Coupled with advances in equipment (GenieII) which make LAMP tests more portable and simple, and with advances in enzyme development (faster strand-displacement polymerases) enabling cheaper and faster detection, the test for *X. arboricola* pv. *pruni* will satisfy the need for fast and accurate detection and identification of the plant pathogen. As part of integrated pest management systems it can help to prevent spread of infections in single orchards caused by incautious handling of infected plant material or equipment, save antibiotic or copper compounds used in mitigation by delivering accurate values of infection state and prevent spread of disease via infected seedlings sent around the world by nurseries. Moreover, these combined efforts can inhibit the spread of the pathogen to new areas and may be a valuable tool in the fight against the bacterial spot disease.

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Supporting Information

Additional Supporting Information can be found in the online version of this article at the publisher's web-site:

Figure S1 Genomics-informed LAMP assay development approach followed in this study.

Figure S2 Phylogenetic tree obtained using MEGA v. 4.0 (Tamura *et al.*, 2007) using neighbour-joining method.

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