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Sensitive detection of *Xanthomonas oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola* by Loop Mediated Isothermal Amplification

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Running Head: LAMP Detection of *Xanthomonas oryzae*

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ABSTRACT

Molecular diagnostics for crop diseases can enhance food security by enabling rapid identification of threatening pathogens and providing critical information for deployment of disease management strategies. Loop mediated isothermal amplification (LAMP) is a PCR-based tool that allows for rapid, highly specific amplification of target DNA sequences at a single temperature, and is thus ideal for field-level diagnosis of plant diseases. We developed highly specific primers for two globally important rice pathogens, *Xanthomonas oryzae* pv. *oryzae*, causal agent of bacterial blight (BB) disease, and *X. oryzae* pv. *oryzicola*, causal agent of bacterial leaf streak disease (BLS), into reliable, sensitive LAMP assays. In addition to pathovar distinction, two assays were developed that differentiate *X. oryzae* pv. *oryzae* by African or Asian lineage. Using these LAMP primer sets, the presence of each pathogen was detected from DNA and bacterial cells, as well as leaf and seed samples. Thresholds of detection for all assays were consistently 10^4-10^5 CFU ml^{-1}, while genomic DNA thresholds were between 1 pg to 10 fg. The unique sequences combined with the LAMP assay provide a sensitive, accurate, rapid, simple and inexpensive protocol to detect both BB and BLS pathogens.

INTRODUCTION

Severe rice diseases, such as bacterial leaf streak (BLS) caused by *Xanthomonas oryzae* pv. *oryzicola* and bacterial blight (BB) caused by *X. oryzae* pv. *oryzae*, are increasing in prevalence in parts of Asia and sub-Saharan Africa and can cause average yield losses of 20 or 50%, respectively (1). Increased incidences of BLS and BB are considered to be the result of the introduction of new susceptible rice varieties, the intensification of cultivation, the absence of adequate phytosanitary controls, and environmental changes such as rising global temperatures (2, 3). Losses incurred by these diseases could jeopardize global food security.

47

48 Documenting the extent and distribution of BB and BLS is invaluable to understanding the
49 severity of their threat on rice production. Seedborne dissemination of *X. oryzae* pv. *oryzicola* is
50 a problem in parts of Asia and presumably in Africa (4). While clean seed and quarantine
51 programs are prevalent in Asia, these are not yet developed in Africa. *X. o.* pv. *oryzae* has been
52 detected in seed, but whether or not this form of transmission is important is still controversial
53 (5–10).

54

55 High quality genome sequences of four strains of *X. oryzae* pv. *oryzae* and two strains of *X.*
56 *oryzae* pv. *oryzicola* are publicly available (11–14; Genbank accession numbers PRJNA228925
57 and PRJNA228927). These resources, along with draft genome sequences of another nine *X.*
58 *oryzae* strains, provided insights into the genetic diversity among strains within this species,
59 including a unique group of weakly pathogenic *X. oryzae* isolated in the United States ((13) and
60 V. Verdier, personal communication). In a previous study, we used a comparative genomics
61 approach to develop diagnostic primers that distinguished strains by pathovar (*X. oryzae*, *X.*
62 *oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola)*, and differentiated certain groups of strains based
63 on their geographic origin (13, 15). Multi-locus sequence and RFLP analysis have shown that *X.*
64 *oryzae* pv. oryzae is composed of two major genetic groups, the Asian and African lineages (16,
65 17). Pathovar-specific primers have been adopted for identification of *X. oryzae* pv. *oryzae* and
66 *X. oryzae* pv. *oryzicola* from field-collected leaf samples (4), and from seed samples (IRRI Seed
67 Health Unit, personal communication). However, the adoption of these primers for field-level
68 surveys or for routine screens of seed samples by quarantine officials has been limited largely

due to the high costs and requirements for sophisticated laboratories to perform the available diagnostic assays.

A recent advance for molecular diagnostics is the adaptation of the loop mediated isothermal amplification (LAMP) method for rapid, specific amplification of target DNA sequences at a single temperature (18). Incubation can be accomplished using a simple water bath without the need for expensive equipment (19). LAMP can be more sensitive and less prone to inhibitors in test samples than PCR, and it can be adapted to a simple visual discrimination of the test result without requiring electrophoresis or other equipment (20). LAMP assays have been developed for phytoplasma, viral, bacterial and fungal plant pathogens as well as the detection of genetically modified crops (21–28). Visual assays in particular are ideally suited for deployment in non-specialized laboratories with limited equipment and resources, or for incorporation into a simple-to-use diagnostic test for use in the field. The increased sensitivity of the LAMP assay coupled with a closed tube system where no addition of DNA intercalating dye is necessary post reaction, is attractive for regulatory labs. LAMP can be used in epidemiological surveys, to support microbial forensic investigations for quarantine officials.

The intent of this project was to develop and evaluate LAMP assays for *X. oryzae* pathovars to enable surveillance activities in rice fields and testing of traded materials (seeds) in regional quarantine offices. We focused on genomic regions unique for *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola* (15) to develop pathovar-specific LAMP primers that detect and differentiate strains of each pathovar. We show the effectiveness of these assays in detecting the pathovars in diverse sample preparations such as DNA, heat-killed cells or crude preparations

from plant tissue. In addition, we used draft genomic comparisons to develop LAMP assays that distinguish African and Asian lineages of *X. oryzae* pv. *oryzae*.

MATERIALS AND METHODS

Bacterial Strains, DNA and Plant Samples. The bacterial strains used in this study are listed in Table 1. Strains of *X. oryzae*, selected to represent the genetic and geographic diversity of the pathovars, were screened to determine assay specificity. These included 45 strains of *X. oryzae* pv. *oryzae*, 40 strains of *X. oryzae* pv. *oryzicola* and seven strains of a distinct group of *X. oryzae* isolated in the United States (13, 29). An additional 31 strains representing other plant pathogenic species and unknown bacteria isolated from rice tissue and seed were tested. Heat-killed cells, genomic DNA or crude plant exudate were used as template in LAMP reactions. Genomic DNA was isolated using either the Easy-DNA Kit (Life Technologies, Grand Island, NY) following the manufacturer's recommendations, or the DNeasy Blood and Tissue Kit (Qiagen, Inc., Valencia, CA) following the manufacturer's recommendations, except that DNA was eluted in 30 µl of water in the final step. All samples were diluted to 20 ng μl^{-1} in sterile water. Heat-killed cells were prepared from cultures grown for 24 h on peptone-sucrose agar (PSA) (30) at 28°C, diluted in sterile water to appropriate concentrations and incubated at 95°C for 10 min. Plant tissue was collected from rice plants at 0, 24, 48 and 72 h post inoculation (hpi) by syringe infiltration with either *X. oryzae* pv. *oryzae* $PXO99^A$ or *X. oryzae* pv. *oryzicola* BLS256 or MAI10 as previously reported (31). Each inoculum was adjusted to 0.2 OD_{600} diluted in distilled water (about 10^8 CFU ml^{-1}) prior to inoculation. Tissues were individually ground in a TissueLyser II (Qiagen, Inc., Valencia, CA) in one ml of distilled water and were serially

diluted. Diluted ground tissue was sampled from three independent leaves for testing in each appropriate assay and the experiment was repeated at least twice.

Primer Design and Screening. Loci PXO_00080 (conserved hypothetical protein) and Xoryp_010100019045 (putative glycosyltransferase) are unique for *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola*, respectively (15). These loci were used to develop LAMP primers that amplify all isolates within each pathovar. In addition, loci that distinguish isolates of *X. oryzae* pv. *oryzae* by geographic origin were identified by analyzing draft genomic sequence of African strains of *X. oryzae* pv. *oryzae* (GenBank accession number PRJNA228925) (Fig. S1). The locus specific to Asian populations is PXO_03925 (conserved hypothetical protein; putative lipase). Primers were designed based on all of these unique sequences using either LAMP Designer Version 1.02 (Premier Biosoft, Palo Alto, CA) or PrimerExplorer (Eiken Chemical Company, https://primerexplorer.jp/e/) and synthesized by Integrated DNA Technologies (Coralville, IA). Four primers (external primers, F3 and B3; internal primers, FIP and BIP) were designed for each assay. Loop primers were also designed for pathovar-specific *X. oryzae* pv. *oryzicola* and *X. oryzae* pv. *oryzae* assays. All oligonucleotide sequences are listed in Table 2.

Loop Mediated Isothermal Amplification. LAMP reactions (final volume of 12 µl) were performed in a CFX Connect Real-Time System (BioRad, Hercules, CA) or a Genie II (Optigene, Sussex, United Kingdom). The reaction contained 7.2 µl Isothermal Master Mix (Optigene, Sussex, United Kingdom), 32 nM outer primers (F3 and B3) and 0.32 µM inner primers (FIP and BIP). Pathovar specific assays included 0.16 µM loop primers (LoopF and LoopB). Lastly, 1 µl of template was added that was either genomic DNA (20 ng μl^{-1}), heat-killed bacterial cells, or serially diluted, ground, inoculated tissue (as described above). African and Asian *X. oryzae* pv. *oryzae* assays did not include loop primers and the remaining volume

137 was substituted with water. LAMP reactions in the CFX Connect were incubated for 60 min at
138 65°C, followed by melt curve analysis from 65°C to 95°C. Incubations on the Genie II were 30
139 or 60 min at 65°C. All LAMP assays for screening purposes were replicated at least twice and all
140 experiments included no template controls (water and no template DNA).

141 **Assay Specificity and Sensitivity.** Assay specificities were established using a pooling strategy
142 to screen large collections of negative controls after initial specificity with positive control
143 strains were confirmed. Positive controls were strains used to derive the published genome
144 sequences (PXO99^{A}, MAFF311018 and KACC10331) for *X. oryzae* pv. *oryzae* and BLS256 for
145 *X. oryzae* pv. *oryzicola* (11, 12, 14, 32). Non-target bacterial DNAs were pooled in equimolar
146 concentrations, 10 strains per pool. Each negative pool was separately spiked with 1µl of
147 positive control genomic DNA to validate detection in a mixed sample. Sensitivity of each assay
148 was determined using serial dilutions of both genomic DNA (10 ng to 1 fg) and heat killed cells
149 (10^{8} to 10^{1} CFU ml^{-1}). Initial *X. oryzae* pv. *oryzicola* assay development included loop primers,
150 but subsequent testing for specificity and sensitivities removed these primers for greater
151 consistency and to reduce the incidences of false positives. Volumes in each reaction were
152 replaced with water.

153 **Seed Detection.** A lot of clean (known to be free of *X. oryzae* pv. *oryzae* and *X. oryzae* pv.
154 *oryzicola*) *Oryza sativa* cv. IR24 seeds was disinfected using 70% ethanol, then rinsed thrice
155 with sterile distilled water and dried in a laminar flow hood. Subsamples of this lot were
156 artificially inoculated by soaking the seeds in bacterial suspensions of *X. oryzae* pv. *oryzae*
157 PXO99 and *X. oryzae* pv. *oryzicola* BLS256 for 2 h at room temperature, 2 h at 4 °C, and placed
158 in a laminar flow hood until dry. PXO99 is a Philippine strain of *X. oryzae* pv. *oryzae*, and was
159 used as a control strain in all experiments completed at the International Rice Research Institute;

160 in experiments completed at Colorado State University, PXO99^{A}, a 5-azacytidine-resistant
161 derivative of PXO99 (33), was used as a control strain. The remaining clean seeds were
162 subdivided into 5 g seedlots (approximately 200 seeds). Cell counts were estimated to be 1.1 x
163 10^4 CFU seed^{-1} (PXO99) and 4.6 x 10^4 CFU seed^{-1} (BLS256). To test sensitivity of the pathovar
164 specific LAMP assays to detect a 0.5% contamination of a 5 g seedlot (1 contaminated seed in
165 200 seeds), a single contaminated seed from the pool of PXO99 or BLS256 contaminated seeds
166 was added to 5 g of clean seed. Thirty samples each of PXO99-contaminated and BLS256-
167 contaminated seedlots were prepared and processed using an extraction protocol for bacteria
168 from rice seeds by sonication (Nguyen et al, unpublished). Seed extracts were stored at 0^{o}C.
169 LAMP reactions were carried out as described above with 1 µl aliquots from seed extracts as
170 template DNA. Each run included one positive DNA control, four non-target DNA controls, and
171 one no template control in which 1 µl of water was added to the reaction mix. In experiments
172 screening for *X oryzae* pv. *oryzae*, non-target controls included *X. oryzae* pv. *oryzicola* BLS175,
173 *X. oryzae* pv. *oryzicola* BLS256, *Acidovorax avenae* BPJ4821, and an uncharacterized yellow
174 non-pathogenic seed-associated bacterium named SHU199. *X oryzae* pv. *oryzae* PXO99^{A}
175 genomic DNA was used as a positive control. Experiments using the *X. oryzae* pv. *oryzicola*
176 pathovar specific primers included a positive control – *X. oryzae* pv. *oryzicola* BLS256, and four
177 non-target controls – *X. oryzae* pv. *oryzae* PXO99, X. *oryzae* pv. *oryzae* PXO349, *Acidovorax*
178 *avenae* BPJ4821 and SHU199. All DNA controls were normalized to 20 ng ul^{-1}. In analyzing the
179 *X oryzae* pv. *oryzae* contaminated seedlots, pathovar loop primers (LoopF and LoopB) were
180 used, but not in *X. oryzae* pv. *oryzicola* tests. Reactions were incubated in the Genie II
181 (Optigene, Sussex, United Kingdom) at 65^{o}C for 60 min. All LAMP tests were conducted in

triplicate. Sensitivity and specificity values were computed using the formulas discussed by Armitage et al (2002).

Visual Detection. A visual LAMP detection protocol was adapted for detection and identification of the *X. oryzae* pathovars to reduce cost and requirement for sophisticated equipment. Assays were performed in conventional thermal cyclers or a water bath at 65°C for 60 min. The 25 µl reaction mix contained 2.5 µl 10x Isothermal Amplification Buffer (New England Biolabs, Ipswich, MA), 1.4 mM dNTPs, 6 mM additional $MgSO_4$ for a final concentration of 8 mM (New England Biolabs, Ipswich, MA), 0.8 M Betaine (Sigma Aldrich, St. Louis, MO), 4 U Bst DNA polymerase large fragment or Bst DNA Polymerase 2.0 (New England Biolabs, Ipswich, MA), 0.32 µM of FIP and BIP, 32 nM of F3 and B3, and 0.16 µM LoopF and LoopB (loop primers in pathovar-specific assays only), with 1 µl of 20 ng μl^{-1} DNA, heat killed cells, or plant extract. Mineral oil (EMD Millipore, Darmstadt, Germany) was added on top of the reaction mixture (20 µl) to minimize introduction of aerosolized product in workspaces. Amplification was terminated by heat inactivation at 80°C for 3 min. Post incubation, tubes were individually opened in a separate lab and 0.5 - 1µl of Quant-IT™ Pico Green® Reagent (Invitrogen, Carlsbad, CA, USA) was added. Reactions were incubated at room temperature for 5 min and then observed under normal and UV light for either a color change from orange to green or for fluorescence.

RESULTS

Primer design, specificity and sensitivity of LAMP assays. At least five different primer sets were predicted for each unique sequence, and were used to develop specific LAMP assays for each *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola* (designated 'pathovar' primers), and for

205 geographically distinct lineages of *X. oryzae* pv. *oryzae* (African vs Asian, designated as
206 'geographic' primers). After initial screening with control DNAs (*X. oryzae* pv. *oryzae* PXO99^{A},
207 BAI3, *X. oryzae* pv. *oryzicola* BLS256), primer sets listed in Table 2 were used for testing.
208 Ratios of primer concentrations were based on previous reports and consultation with colleagues
209 (2,4,6, Bühlmann, personal communication) and did not require optimization.
210 The pathovar and geographic assays were tested for specificity and efficiency with a panel
211 comprising 44 *X. oryzae* pv. *oryzae,* 38 *X. oryzae* pv. *oryzicola,* seven *X. oryzae,* 11
212 *Xanthomonas* sp. (species unknown, but determined not to be *X. oryzae* by multiplex PCR) (15)
213 and multiple sequence alignment (Cottyn et al, unpublished) and 19 strains representing eight
214 different bacterial genera using the Isothermal Master Mix (Optigene, Sussex, United Kingdom)
215 in a CFX Connect Real-Time System (BioRad, Hercules, CA) or a Genie II (Optigene, Sussex,
216 United Kingdom) (Table 1).
217
218 Genomic DNA, diluted to concentrations ranging from 10 ng to 1 fg, was used to establish
219 sensitivities of each assay. A no template control (water) was included in each experimental
220 replication. Thresholds of detection were 10 pg for pathovar specific *X. oryzae* pv. *oryzae* (Fig.
221 1A), 1 fg for pathovar-specific *X. oryzicola* pv. *oryzicola* (Fig. 1B), 1 ng for African *X. oryzae*
222 pv. *oryzae* (Fig. 1C) and 1 pg for Asian *X. oryzae* pv. *oryzae* (Fig. 1D). As few as 10^5 CFU ml^{-1}
223 bacterial cells were detected using both pathovar-specific assays and the African *X. oryzae* pv.
224 *oryzae* assay (Fig. 2A, B, C). The Asian *X. oryzae* pv. *oryzae* assay detected as few as 10^4
225 CFU/ml, though there was more variation in the technical replicates at the lower concentrations
226 (Fig. 2D). There were no false positives for any assay in the no template control tests confirming
227 specificity of the target region and the primers. Loop primers designed for *X. oryzae* pv.

228 *oryzicola* contributed to amplification of specific targets, however, false positive results were
229 identified more often when they were included during initial validations. To address this concern
230 in subsequent testing, loop primers were removed and the volume was replaced with water. This
231 change in protocol reduced the sensitivity and therefore the incidence of false positive results for
232 this assay by increasing time to positivity. So while these primers are reported in Table 2, we
233 recommend conducting this assay without them. Overall, the adapted primers were specific and
234 sensitive in the LAMP assay. In addition, using a comparative genomics approach with draft
235 genome sequences, we identified loci that differentiated *X. oryzae* pv. *oryzae* lineages based on
236 geographic origin.
237

238 **Detection of *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola* from seed and crude plant**
239 **extracts.** Artificially inoculated seedlots were used to assess the capacity of the pathovar-
240 specific LAMP assays to detect bacteria at a 0.5% contamination level in 5 g seed samples
241 (Table 3). Using the *X. oryzae* pv. *oryzicola* pathovar-specific LAMP primers, BLS256 DNA
242 was detected in 30 out of 30 samples, giving a sensitivity of 100%. None of the 30 seed samples
243 contaminated with *X. oryzae* pv. *oryzae* PXO99 amplified with the *X. oryzae* pv. *oryzicola*
244 pathovar LAMP primers (0% false positive detection). The sensitivity of the *X. oryzae* pv.
245 *oryzae* pathovar LAMP primers was 93.3%, with the target strain PXO99 detected in 28 out of
246 30 contaminated seedlots. Five of 30 seed samples contaminated with *X. oryzae* pv. *oryzicola*
247 BLS256 were detected as positive by the *X. oryzae* pv. *oryzae* LAMP primers (83.3%
248 specificity), i.e., the non-target organism was detected in at least two of three technical
249 replications. The trials were done with little or no optimization needed, but due to high
250 sensitivity and robustness of the primers designed for the pathovar specific *X. oryzae* pv.

oryzicola, the loop primers were excluded from the reaction mix to prevent random false positives. Representative amplification curves for each pathovar specific assay and appropriate controls are shown in Fig. 3A and B for *X. oryzae* pv. *oryzicola* and *X. oryzae* pv. *oryzae*, respectively. Pairwise inoculations were used to demonstrate consistent specificity in detection of the presence of either organism. As described above, a single contaminated seed of either *X. oryzae* pv. *oryzae* PXO99 or *X. oryzae* pv. *oryzicola* was added to 5 g of clean seed. The *X. oryzae* pv. *oryzae* PXO99 contaminated lots were tested with the *X. oryzae* pv. *oryzicola* pathovar specific assay and conversely, the *X. oryzae* pv. *oryzicola* BLS256 contaminated seed lots were tested with the *X. oryzae* pv. *oryzae* pathovar specific assay. Non-target DNAs from *Acidovorax avenae* (BPJ4821) or *Xanthomonas* sp. (SHU199) were included in each experiment as negative controls alongside no template controls and did not amplify with either assay (Table 1).

Amplifications in seed testing were delayed compared to when pure DNA was used as a template in specificity tests. The pathovar-specific *X. oryzae* pv. *oryzae* fluorescence values (RFU) detecting amplification were also higher compared to specificity and sensitivity assays. Seed detection experiments were conducted with a Genie II (Optigene, Sussex, United Kingdom) while sensitivity tests were completed on a CFX Connect Real-Time System (BioRad, Hercules, CA). We attribute the variation in these results to the two platforms used for detecting amplification as well as the nature of sample. Seed extracts may contain contaminants that slow the amplification reaction as was described in a watermelon system detecting cucumber mottle mosaic virus (35), and possibly influence fluorescence detection capabilities. However, data were evaluated for presence or absence of an exponential amplification as compared to negative

274 controls, and for these tests, the amplifications were specific for primer/DNA combinations, and
275 were consistent with all previous results.

276

277 Crude extracts from inoculated rice leaf tissue also served as viable templates for all of the
278 reported LAMP assays (Table 4). Representative data from the pathovar-specific *X. oryzae* pv.
279 *oryzicola* LAMP assay are illustrated in Fig. 4 and correlate with the threshold detected when
280 using heat killed cells (starting concentration of 10^7 CFU ml^{-1}).

281

282 Interestingly, although viable bacteria could not be recovered, *X. oryzae* pv. *oryzae* strain
283 $PXO99^A$ was correctly detected by both the *X. oryzae* pv. *oryzae* pathovar-specific and *X. oryzae*
284 pv. *oryzae* Asian lineage assays in leaf samples that were inoculated 23 years ago and stored at
285 room temperature. These samples did not amplify with the *X. oryzae* pv. *oryzicola* pathovar-
286 specific or African lineage *X. oryzae* pv. *oryzae* primers confirming that the assays are robust
287 and can detect target bacteria in diverse sample preparations (data not shown).

288

289 **Visual detection of LAMP products.** A visual detection protocol was adapted and tested for all
290 LAMP primers. Chemistries, including hydroxynapthol blue, Gel Red (Biotium, In., Hayward,
291 CA), and ethidium bromide (data not shown) did not perform as reliably or clearly as the SYBR
292 stain Quant-IT™ Pico Green® Reagent (Life Technologies, Grand Island, NY, USA) added post
293 incubation. SYBR green was able to detect DNA directly in heat killed cells to the same
294 threshold as the Isothermal Master Mix using a thermal cycler. SYBR green stained reactions are

295 shown for the *X. oryzae* pv. *oryzae* pathovar specific assay in Fig. 5. A water bath was
296 successfully used for incubation and crude inoculated plant extract amplified in each specific
297 primer set designed (data not shown).
298
299 **DISCUSSION**
300 Adaptation of previously designed specific conventional PCR primers to LAMP resulted in a
301 reliable, sensitive, specific and robust test to detect and differentiate *X. oryzae* pv. *oryzae* and *X.*
302 *oryzae* pv. *oryzicola*. The primers and LAMP assays were validated on a wide diversity of
303 bacterial strains, including a large collection of both *X. oryzae* pathovars as well as other
304 *Xanthomonas* species and other genera of bacteria, to demonstrate primer specificity and assay
305 reliability. The pathovar-specific *X. oryzae* pv. *oryzicola* primers provided the most sensitive
306 assay. The Asian *X. oryzae* pv. *oryzae* primers, pathovar-specific *X. oryzae* pv. *oryzae* primers,
307 and the African *X. oryzae* pv. *oryzae* primers were slightly less sensitive but still detected 1ng of
308 genomic DNA. Differences in assay sensitivity are not likely due to copy number of the target,
309 because, where sequences are known, all loci are present in single copies. Therefore, we predict
310 that the inherent efficiency of each primer set in annealing causes this variation. Regardless,
311 sensitivity thresholds among the four assays developed were consistent with those previously
312 reported for other plant pathogenic bacteria ranging from 10 fg to 0.01 ng genomic DNA and 10^3
313 to 10^4 CFU ml^{-1} (22, 36–38) and correlate to the equivalent range of 10^3 to 10^6 genome copies
314 based on the 5.2 Mbp *X. orzyae* pv. *oryzae* $PXO99^A$ and the 4.8 Mbp *X. orzyae* pv. *oryzicola*
315 genomes (11, 12).
316

317 We previously used comparative genomic approaches with whole genome (15) and draft genome
318 (13) sequences for the design of pathovar-specific primers. In this study, we mirrored this
319 approach, and took advantage of compared draft genomes (authors, unpublished) that identified
320 sequences differentiating geographically distinct populations of *X. oryzae pv. oryzae*, i.e., from
321 Africa or Asia. The ability to differentiate geographic origin of a strain could facilitate
322 epidemiological and surveillance studies along with the monitoring of imported seed at
323 quarantine stations. The specificity for all primers after adaptation to LAMP-based assays was
324 confirmed and no cross reactivity to other Xanthomonads or bacterial genera was detected (Table
325 1). Furthermore, no false positive amplification occurred in water (no template) controls. These
326 results confirm the utility of draft sequence for development of unique primers and the
327 streamlined adaptation of conventional PCR to LAMP. Because of their specificity and
328 sensitivity, the primers and assays will be useful as forensic tools in quarantine offices,
329 epidemiological studies and seed certification.
330
331 Using available public databases such as GenBank (http://www.ncbi.nlm.nih.gov/genbank/), the
332 Pathosystems Resource Integration Center (PATRIC) (http://patricbrc.org/), and the
333 Comprehensive Phytopathogen Genomics Resource (CPGR) (http://cpgr.plantbiology.msu.edu),
334 we searched for relationships of loci used for primer development with annotated genes in
335 existing curated genomes. The *X. oryzae* pv. *oryzicola* specific locus (Xoryp_010100019045)
336 was the only gene with available annotation. It encodes a predicted glucosyltransferase with
337 sequence similarity to rhamnose-glucose polysaccharide assembly protein F50 (RgpF) of
338 *Streptococcus mutans* and shows high similarity to a recently reported *X. oryzae* pv. *oryzicola*
339 virulence factor, *wxocB* (39). This locus is flanked by a methyltransferase on one side and a

cluster of ABC O-antigen lipopolysaccharide (LPS) transporters on the other side, consistent with clustering of LPS gene islands (40).

PXO_00080, unique to all *X. oryzae* pv. *oryzae* and absent in *X. oryzae* pv. *oryzicola*, encodes an uncharacterized protein. The *X. oryzae* pv. *oryzae* African locus was identified in draft sequence of NAI8 (authors, unpublished), a strain originally from Niger (16), and bears no similarity to any publicly available sequence. Its presence was confirmed in draft genome sequence of MAI1 and BAI3 (authors, unpublished). The *X. oryzae* pv. *oryzae* Asian locus also is annotated as a hypothetical protein, but has similarity to the lipase family of proteins. *X. oryzae* pv. *oryzae* loci PXO_00080 have sequence similarity to plasmids found in two related species of *Xanthomonas* including *X. arboricola* pv. *pruni* CFBP 5530 plasmid pXap41 (FR875157) and *X. axonopodis* pv. *citri* 306 plasmid pXAC33 (AE008924). For example, four mismatches occur between locus PXO_00080 and the plasmid sequences in question, and two of our primers include those mismatches. While the presence of mismatches does not prove the primer would not amplify the non-target sequences, and we did not test the specific strains containing these plasmids, our use of a highly diverse and comprehensive panel of negative control strains for testing primers increases our confidence that the assays are specific for *X. oryzae* pv. *oryzae*. Furthermore, the likelihood of *X. arboricola* pv. *pruni* and *X. axonopodis* pv. *citri* being present on rice tissue or seed is unlikely.

Out of 90 assays (30 independently inoculated seedlots tested in triplicate), only five false positive results were generated using pathovar-specific *X. oryzae* pv. *oryzae* primers. This 83.3% error rate is consistent with previous reports for LAMP assays detecting bacteria (22). These data

363 further emphasize the importance of reliable positive controls and replicated experiments to
364 validate all results. For critical samples, further testing to confirm results using a multiplex PCR
365 or individual PCRs reported in Lang et al 2010 (15) targeting different unique loci is
366 recommended. False positives were detected more often when using the loop primers designed
367 for the pathovar-specific *X. oryzae* pv. *oryzicola* assay. While in some assays loop primers play
368 an important role in enhancing sensitivity, their removal can reduce the false positive rates,
369 presumably by reducing the assay sensitivity, resulting in a more reliable and robust assay.
370
371 An advantage of LAMP assays over conventional PCR assays is that sophisticated equipment is
372 not required for the LAMP protocols. We detected *X. oryzae* pv. *oryzicola* and *X. oryzae* pv.
373 *oryzae* directly from leaf tissue ground in water (data not shown) using the SYBR green
374 detection system demonstrating the feasibility of using the assay on field samples. Because the
375 reaction is isothermal, a water bath or thermos with hot water could be used for reactions,
376 simplifying the process for use in the field. We estimate the current cost for a single 12 µl
377 reaction is 1 USD. The availability of improved DNA polymerases was a key factor in improving
378 the efficiency and accuracy of LAMP technology (41). New formulations of the enzyme, Bst 2.0,
379 including one requiring a warm start (Bst 2.0 Warm Start, New England Biolabs, Ipswich, MA)
380 have improved efficiency and multiplexing capabilities in custom developed reaction mixes.
381 Because of the sensitivity of the LAMP assays, extreme care must be taken to avoid
382 aerosolization of the amplified products. This is particularly important when using post reaction
383 detection methods. We added mineral oil to the top of each reaction to avoid contamination when
384 tubes are opened to add the detecting dye. Liang et al (42) recently developed a technology to

separate DNA intercalating dye from the samples during incubation using a temperature sensitive wax. We did not test this approach, but it could be very useful for field-based LAMP assays.

In agriculture, the ability to work with crude extracts from plants and minimal equipment enhance the value of LAMP technology for rapid detection for etiological or epidemiological studies or for regulatory purposes. In this study, we demonstrate the utility with leaf and seed extracts. Most recently, Gurinder et al (25) reported detection of genetically modified organisms using LAMP based on promoter sequences. Researchers will be able to quickly determine sources of contaminated material or outbreaks in the field using LAMP. In future work, surveys conducted using the reported LAMP assays will provide a reliable picture of the presence of these diseases across a region or country.

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FIGURE LEGENDS

FIG. 1 Pathovar-specific *X. oryzae* pv. *oryzae* (A), pathovar-specific *X. oryzae* pv. *oryzicola* (B), African *X. oryzae* pv. *oryzae* (C) and Asian *X. oryzae* pv. *oryzae* (D) LAMP assay sensitivity tests. Each diluted DNA was tested at least three times.

FIG. 2 Standard curves showing sensitivity of detection by LAMP using dilutions of heat killed cells. Pathovar-specific *X. oryzae* pv. *oryzae* primers with *X. oryzae* pv. *oryzae* PXO99^{A} heat killed cells (A), pathovar-specific *X. oryzae* pv. *oryzicola* primers with *X. oryzae* pv. *oryzicola* BLS256 cells (B), African *X. oryzae* pv. *oryzae* primers using *X. oryzae* pv. *oryzae* BAI3 cells (C) and Asian *X. oryzae* pv. *oryzae* using *X. oryzae* pv. *oryzae* PXO99^{A} cells (D). Each dilution was tested three times. Bars represent +/- standard deviation of the mean and associated R^2 value after linear regression analysis.

FIG. 3 LAMP detection of *X. oryzae* pv. *oryzicola* (A) and *X. oryzae* pv. *oryzae* (B) using pathovar specific assays and seed extracts of 5 g seedlots with 0.5% contamination (1 seed carrying 10^4 CFU out of the 200 seeds) and from DNA extracts (20 ng μl^{-1} in TE buffer). Mean fluorescence data for seed extracts were obtained from the amplification curves of 10 seedlots per isolate against each primer set. *X. oryzae* pv. *oryzae* (*Xoo*) PXO99 DNA and *X. oryzae* pv. *oryzicola* (*Xoc*) BLS256 DNA, no template controls (NTC) consisting of water and non-target DNAs served as negative controls and failed to amplify in any run with

either primer set in at least three independent runs. Data were collected on a Genie II (Optigene, Sussex, United Kingdom) and normalized to background fluorescence.

FIG. 4 Sensitivity of pathovar-specific *X. oryzae* pv. *oryzicola* primers in a LAMP assay using ground tissue inoculated with *X. oryzae* pv. *oryzicola* MAI10, sampled at 48 hpi and then serially diluted.

FIG. 5 Visual detection of *X. oryzae* pv. *oryzae* PXO99^{A} heat killed cells using the pathovar-specific LAMP assay. A dilution series was tested consisting of 10^8 (1), 10^7 (2), 10^6 (3), 10^5 (4), 10^4 (5), 10^3 (6), 10^2 (7), 10^1 CFU ml^{-1} (8), *X. oryzae* pv. *oryzicola* BLS256 (9) and a no template control (10). Products were detected using 1µl Quant-IT™ Pico Green® Reagent (Life Technologies, Grand Island, NY, USA) under visual light (A), where a positive result changes from orange to green; ultra-violet light (B) where a positive result fluoresces or by 1.5% agarose gel electrophoresis (C), where a positive result is a laddered product.

587 **TABLE 1.** Bacterial strains used in this study to validate specificity of each assay[a,b,c].

Species	Strain	Origin	Host	Source	Xoc LAMP	Xoo LAMP	African Xoo	Asian Xoo
Xanthomonas oryzae pv. *oryzae*	R-3	Australia	*Oryza sativa*	I. Buddenhagen	-	+	-	+
X. o. pv. *oryzae*	BAI3	Burkina Faso	*O. sativa*	V. Verdier	-	+	+	-
X. o. pv. *oryzae*	BAI4	Burkina Faso	*O. glaberrima*	V. Verdier	-	+	+	-
X. o. pv. *oryzae*	BAI1	Burkina Faso	*O. sativa*	V. Verdier	-	+	+	-
X. o. pv. *oryzae*	BAI2	Burkina Faso	*O. sativa*	V. Verdier	-	+	+	-
X. o. pv. *oryzae*	CFBP1948	Cameroon	*O. sativa*	J.L. Notteghem	-	+	+	-
X. o. pv. *oryzae*	CFBP1952	Mali	*O. sativa*	J.L. Notteghem	-	+	+	-
X. o. pv. *oryzae*	94 (35)	China	*O. sativa*		-	+	-	+
X. o. pv. *oryzae*	A3857	India	*O. sativa*	J.E. Leach	-	+	-	+
X. o. pv. *oryzae*	IXO16	Indonesia	*O. sativa*		-	+	-	+
X. o. pv. *oryzae*	MAFF311018	Japan	*O. sativa*	A. Bogdanove	-	+	-	+
X. o. pv. *oryzae*	KACC10331	Korea	*O. sativa*		-	+	-	+
X. o. pv. *oryzae*	Xoo197	Korea	*O. sativa*	S.H. Choi	-	+	-	+
X. o. pv. *oryzae*	Xoo199	Korea	*O. sativa*	S.H. Choi	-	+	-	+
X. o. pv. *oryzae*	MXO90	Malaysia	*O. sativa*		-	+	-	+
X. o. pv. *oryzae*	MXO92	Malaysia	*O. sativa*		-	+	-	+
X. o. pv. *oryzae*	CFBP1949	Mali	*O. sativa*	V. Verdier	-	+	+	-
X. o. pv. *oryzae*	CFBP1951	Mali	*O. sativa*	V. Verdier	-	+	+	-
X. o. pv. *oryzae*	MAI1	Mali	*O. sativa*	V. Verdier	-	+	+	-
X. o. pv. *oryzae*	MAI9	Mali	*O. sativa*	V. Verdier	-	+	+	-
X. o. pv. *oryzae*	R-13	Myanmar	*O. sativa*	I. Buddenhagen	-	+	-	+
X. o. pv. *oryzae*	NXO537	Nepal	*O. sativa*	Tika Adhikari	-	+	-	+
X. o. pv. *oryzae*	NXO544	Nepal	*O. sativa*	Tika Adhikari	-	+	-	+
X. o. pv. *oryzae*	NXO588	Nepal	*O. sativa*	Tika Adhikari	-	+	-	+
X. o. pv. *oryzae*	NAI2	Niger	*O. sativa*	V. Verdier	-	+	+	-
X. o. pv. *oryzae*	NAI5	Niger	*O. sativa*	V. Verdier	-	+	+	-
X. o. pv. *oryzae*	NAI6	Niger	*O. sativa*	V. Verdier	-	+	+	-
X. o. pv. *oryzae*	NAI7	Niger	*O. sativa*	V. Verdier	-	+	+	-
X. o. pv. *oryzae*	NAI8	Niger	*O. sativa*	V. Verdier	-	+	+	-
X. o. pv. *oryzae*	NAI9	Niger	*O. sativa*	V. Verdier	-	+	+	-
X. o. pv. *oryzae*	PXO69	Philippines	*O. sativa*	C.M. Vera Cruz	-	+	-	+
X. o. pv. *oryzae*	PXO99A	Philippines	*O. sativa*	A. Bogdanove	-	+	-	+
X. o. pv. *oryzae*	PXO86	Philippines	*O. sativa*	C.M. Vera Cruz	-	+	-	+
X. o. pv. *oryzae*	PXO111	Philippines	*O. sativa*	C.M. Vera Cruz	-	+	-	+
X. o. pv. *oryzae*	PXO121	Philippines	*O. sativa*	C.M. Vera Cruz	-	+	-	+
X. o. pv. *oryzae*	PXO130	Philippines	*O. sativa*	C.M. Vera Cruz	-	+	-	+
X. o. pv. *oryzae*	PXO132	Philippines	*O. sativa*	C.M. Vera Cruz	-	+	-	+
X. o. pv. *oryzae*	PXO172	Philippines	*O. sativa*	C.M. Vera Cruz	-	+	-	+
X. o. pv. *oryzae*	PXO183	Philippines	*O. sativa*	C.M. Vera Cruz	-	+	-	+
X. o. pv. *oryzae*	PXO344	Philippines	*O. sativa*	C.M. Vera Cruz	-	+	-	+

X. o. pv. *oryzae*	CL-1	Sri Lanka	*O. sativa*	I. Buddenhagen	-	+	-	+
X. o. pv. *oryzae*	Xoo1	Thailand	*O. sativa*	J.E. Leach	-	+	-	+
X. o. pv. *oryzae*	Xoo3	Thailand	*O. sativa*	J.E. Leach	-	+	-	+
X. o. pv. *oryzae*	RS61	China	*O. sativa*	J.S. Wang	-	+	-	+
X. o. pv. *oryzicola*	RS85	China	*O. sativa*	J.S. Wang	+	-	-	x
X. o. pv. *oryzicola*	CFBP2286	Malaysia	*O. sativa*	V. Verdier	+	-	-	-
X. o. pv. *oryzicola*	MAI4	Mali	*O. sativa*	V. Verdier	+	-	-	-
X. o. pv. *oryzicola*	MAI5	Mali	*O. sativa*	V. Verdier	+	-	-	-
X. o. pv. *oryzicola*	MAI6	Mali	*O. sativa*	V. Verdier	+	-	-	-
X. o. pv. *oryzicola*	MAI7	Mali	*O. sativa*	V. Verdier	+	-	-	-
X. o. pv. *oryzicola*	MAI8	Mali	*O. sativa*	V. Verdier	+	-	-	-
X. o. pv. *oryzicola*	MAI10	Mali	*O. sativa*	V. Verdier	+	-	+	-
X. o. pv. *oryzicola*	BLS46	Philippines	*O. sativa*	C.M. Vera Cruz	+	-	-	x
X. o. pv. *oryzicola*	BLS96	Philippines	*O. sativa*	C.M. Vera Cruz	+	-	-	x
X. o. pv. *oryzicola*	BLS98	Philippines	*O. sativa*	C.M. Vera Cruz	+	-	-	-
X. o. pv. *oryzicola*	BLS105	Philippines	*O. sativa*	C.M. Vera Cruz	+	-	x	-
X. o. pv. *oryzicola*	BLS106	Philippines	*O. sativa*	C.M. Vera Cruz	+	-	x	x
X. o. pv. *oryzicola*	BLS111	Philippines	*O. sativa*	C.M. Vera Cruz	+	-	-	x
X. o. pv. *oryzicola*	BLS114	Philippines	*O. sativa*	C.M. Vera Cruz	+	-	x	-
X. o. pv. *oryzicola*	BLS123	Philippines	*O. sativa*	C.M. Vera Cruz	+	-	-	x
X. o. pv. *oryzicola*	BLS125	Philippines	*O. sativa*	C.M. Vera Cruz	+	-	x	-
X. o. pv. *oryzicola*	BLS170	Philippines	*O. sativa*	C.M. Vera Cruz	+	-	x	x
X. o. pv. *oryzicola*	BLS175	Philippines	*O. sativa*	C.M. Vera Cruz	+	-	-	x
X. o. pv. *oryzicola*	BLS179	Philippines	*O. sativa*	C.M. Vera Cruz	+	-	x	-
X. o. pv. *oryzicola*	BLS220	Philippines	*O. sativa*	C.M. Vera Cruz	+	-	x	x
X. o. pv. *oryzicola*	BLS256	Philippines	*O. sativa*	A. Bogdanove	+	-	-	x
X. o. pv. *oryzicola*	BLS276	Philippines	*O. sativa*	C.M. Vera Cruz	+	-	x	-
X. o. pv. *oryzicola*	BLS281	Philippines	*O. sativa*	C.M. Vera Cruz	+	-	x	x
X. o. pv. *oryzicola*	BLS291	Philippines	*O. sativa*	C.M. Vera Cruz	+	-	x	x
X. o. pv. *oryzicola*	BLS294	Philippines	*O. sativa*	C.M. Vera Cruz	+	-	x	x
X. o. pv. *oryzicola*	BLS333	Philippines	*O. sativa*	C.M. Vera Cruz	+	-	-	x
X. o. pv. *oryzicola*	BLS346	Philippines	*O. sativa*	C.M. Vera Cruz	+	-	x	-

X. o. pv. *oryzicola*	BLS354	Philippines	*O. sativa*	C.M. Vera Cruz	+	-	x	x
X. o. pv. *oryzicola*	BLS356	Philippines	*O. sativa*	C.M. Vera Cruz	+	-	-	x
X. o. pv. *oryzicola*	BLS377	Philippines	*O. sativa*	C.M. Vera Cruz	+	-	-	-
X. o. pv. *oryzicola*	BLS404	Philippines	*O. sativa*	C.M. Vera Cruz	+	-	x	-
X. o. pv. *oryzicola*	BLS413	Philippines	*O. sativa*	C.M. Vera Cruz	+	-	x	x
X. o. pv. *oryzicola*	BLS415	Philippines	*O. sativa*	C.M. Vera Cruz	+	-	-	x
X. o. pv. *oryzicola*	BLS417	Philippines	*O. sativa*	C.M. Vera Cruz	+	-	-	-
X. o. pv. *oryzicola*	BLS420	Philippines	*O. sativa*	C.M. Vera Cruz	+	-	-	-
X. o. pv. *oryzicola*	BLS421	Philippines	*O. sativa*	C.M. Vera Cruz	+	-	x	x
X. o. pv. *oryzicola*	BLS468	Philippines	*O. sativa*	C.M. Vera Cruz	+	-	-	x
X. oryzae	X1-8	USA	*O. sativa*	C. Gonzalez	-	+	-	-
X. oryzae	X8-1A	USA	*O. sativa*	C. Gonzalez	-	+	-	-
X. oryzae	X211-2	USA	*O. sativa*	C. Gonzalez	-	+	-	-
X. oryzae	X4-2C	USA	*O. sativa*	C. Gonzalez	-	+	-	-
X. oryzae	RU87-17	USA	*O. sativa*	C. Gonzalez	-	+	-	-
X. oryzae	X11-5A	USA	*O. sativa*	C. Gonzalez	-	+	-	-
X. oryzae	X1-10	USA	*O. sativa*	C. Gonzalez	-	+	-	-
Xanthomonas sp.	97M	Philippines	*O. sativa*	V. Verdier	-	-	-	-
Xanthomonas sp.	M136	Mali	*O. sativa*	V. Verdier	-	-	-	-
Xanthomonas sp.	SHU36	Philippines	*O. sativa*,seed	C.M. Vera Cruz	-	-	-	-
Xanthomonas sp.	SHU50	Philippines	*O. sativa*,seed	C.M. Vera Cruz	-	-	-	-
Xanthomonas sp.	SHU100	Philippines	*O. sativa*,seed	C.M. Vera Cruz	-	-	-	-
Xanthomonas sp.	SHU147	Philippines	*O. sativa*,seed	C.M. Vera Cruz	-	-	-	-
Xanthomonas sp.	SHU178	Philippines	*O. sativa*,seed	C.M. Vera Cruz	-	-	-	-
Xanthomonas sp.	SHU199	Philippines	*O. sativa*,seed	C.M. Vera Cruz	-	-	x	x
Xanthomonas sp.	SHU202	Philippines	*O. sativa*,seed	C.M. Vera Cruz	-	-	-	-
Xanthomonas sp.	SHU222	Philippines	*O. sativa*,seed	C.M. Vera Cruz	-	-	-	-
Xanthomonas sp.	SHU268	Philippines	*O. sativa*,seed	C.M. Vera Cruz	-	-	-	-
Xanthomonas sp.	SHU303	Philippines	*O. sativa*,seed	C.M. Vera Cruz	-	-	x	-
Acidovorax avenae pv. *avenae*	NCPPB3112	Brazil	*Canna indica*	NCPPB[d]	-	-	-	x
A. avenae	BPJ4821	Philippines	*O. sativa*	C.M. Vera Cruz	-	-	x	x
A. a. pv. *citrulli*	94-21	USA	*Citrullus lanatus*	R. Walcott	-	-	-	-
Burkholderia andropogonis	3549	USA	*Saccharum officinarum*	L.E. Claflin	-	-	-	-
B. gladioli	O187	USA	*Allium cepa*	H.F. Schwartz	-	-	-	-
Curtobacterium flaccumfaciens	B473	USA	*Phaseolous* sp.	H.F. Schwartz	-	-	-	-
Escherichia coli	DH5α	USA	NA	Life	-	-	-	-

				Technologies				
Enterobacter sp.	O121	USA	*Allium cepa*	H.F. Schwartz	-	-	-	-
Enterobacter sp.	O174	USA	*Allium cepa*	H.F. Schwartz	-	-	-	-
Pseudomonas marginalis	ATCC10844	USA	*Lactuca sativa*	H.F. Schwartz	-	-	-	-
P. syringae pv. *syringae*	M72	USA	*Capsicum annuum*	H.F. Schwartz	-	-	-	-
P. syringae pv. *syringae*	M108	USA	*Solanum lycopersicum*	H.F. Schwartz	-	-	x	-
P. viridiflava	ATCC13223	USA	*Phaseolus coccineus*	H.F. Schwartz	-	-	x	-
Ralstonia solanacearum	K60	USA	*S. lycopersicum*	J.E. Leach	-	-	-	x
X. axonopodis pv. *vesicatoria*	85-10	USA	*Capsicum* sp.	A. Bogdanove	-	-	x	-
X. a. pv. *sojense*	4455	USA	*Glycine max*	L.E. Claflin	-	-	-	x
X. campestris pv. *campestris*	X1910	USA	*Brassica oleracea*	N. Dunlop	-	-	x	-
X. c. pv. carotae	NCPPB1422	New Zealand	*Daucus carota*	L.E. Claflin	-	-	-	x
X. translucens pv. cerealis	NCPPB1836	USA	*Secale cereale*	L.E. Claflin	-	-	-	-
Unknown	Ven	Venezuela	*O. sativa*,seed	V. Verdier	-	-	-	-

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589 [a]+ represents a positive amplification

590 [b]– represents absence of amplification

591 [c]x designates strain not tested

592 [d]National Collection of Plant Pathogenic Bacteria

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601 **Table 2.** LAMP primers for detection of *X. oryzae* pv. *oryzae* and *X. oryzae* pv. *oryzicola*

Target	**Primer**	**Sequence (5'-3')**
X. o. pv. *oryzae*	F3	CTTCAAGGCCAAGGACATC
PXO_00080[a]	B3	CACGATCTTGCAAGGGAT
	FIP	CGGTGCCGGACTGGATTTGCTAGGAATGAGC AATGCA
	BIP	GTAGTTGCCGACGGCTACCAGAAGCGTCCTC GTCTAA
	LoopF	TTTGAGGTCCCTTTCCACG
	LoopB	GTTTGTGCGCCGTCTATC
X. o. pv. *oryzicola*	F3	GGATCACAGTGATCGTGC
Xoryp_010100019045[b]	B3	CACTTATCGTCCAGTACGC
	FIP	CGATGCCGCCTTGATCGAGTTGTACTCCTACG ATGAGC
	BIP	ACCGAGTCGTTGCAGGTCTCTTGCGAAACAC AAGGAA
	LoopF	TTGTGACCACGCTGTCATT
	LoopB	TCGCCATCTCCAGTCCTAT
African *X. o.* pv. *oryzae*	F3	TATTGGGTGCTGCCGATGA
Hypothetical protein, draft sequence[c]	B3	GGCAACCTCACTTCCGTAAG
	FIP	ATGTAGCCATCATGCCCGCCTTTTCCCAGATT TGCGAGTCCTT
	BIP	GCGCTCTTCGGATGGTAGTGATTTTTGCCATG

		CTTGTTTTGTGCA
Asian *X. o.* pv. *oryzae* PXO_03925[a]	F3	GGTGGTCAGCGCATCGA
	B3	ACTGCTGCTGTTCCAACG
	FIP	ATGCTGACGCGCAGCTGCTTTTAGCCCAGATGCCGCAC
	BIP	TGACGGCAATGAATACCCCGCTTTTGACCGACTGGCGTGCT

[a]Design based on *X. oryzae* pv. *oryzae* PXO99A (PRJNA28127) genome sequence

[b]Design based on *X. oryzae* pv. *oryzicola* BLS256 (PRJNA54411) genome sequence

[c]Design based on *X. oryzae* pv. *oryzae* NAI8 (PRJNA228925) and BAI3 (Verdier et al, unpublished) draft genome sequences

Table 3. LAMP detection of *X. oryzae* pv. *oyrzae* and *X. oryzae* pv. *oryzicola* using pathovar-specific assays in seedlots (200 seeds) inoculated with one seed carrying 10^4 CFU of *X. oryzae* pv. *oryzae* (*Xoo* PXO99) or *X. oryzae* pv. *oryzicola* (*Xoc* BLS256) (0.5% contamination). Each sample was analyzed in triplicate against each primer set where presence of an amplification curve at least two out of three runs was interpreted as a positive result.

Samples	Amplification from seed with pathovar-specific assays			
	Pathovar-specific *X. oryzae* pv. *oryzicola* LAMP assay		Pathovar-specific *X. oryzae* pv. *oryzae* LAMP assay	
	Xoc BLS256	*Xoo* PXO99	*Xoo* PXO99	*Xoc* BLS256
1	3/3	0/3	2/3	2/3
2	3/3	0/3	3/3	0/3
3	3/3	0/3	3/3	0/3
4	3/3	0/3	3/3	0/3
5	3/3	0/3	3/3	2/3
6	3/3	0/3	2/3	0/3
7	3/3	0/3	1/3	1/3
8	3/3	0/3	3/3	0/3
9	3/3	0/3	0/3	1/3
10	3/3	0/3	2/3	0/3
11	3/3	0/3	3/3	0/3
12	3/3	0/3	3/3	2/3
13	3/3	0/3	3/3	0/3
14	3/3	0/3	3/3	3/3
15	3/3	0/3	3/3	0/3
16	3/3	0/3	3/3	0/3
17	3/3	0/3	3/3	0/3
18	3/3	0/3	3/3	0/3
19	3/3	0/3	3/3	0/3
20	3/3	0/3	3/3	0/3
21	3/3	0/3	3/3	0/3
22	3/3	1/3	2/3	0/3
23	3/3	0/3	2/3	0/3
24	3/3	0/3	3/3	3/3
25	3/3	0/3	3/3	0/3
26	3/3	1/3	3/3	0/3

27	3/3	0/3	2/3	1/3
28	3/3	1/3	3/3	0/3
29	3/3	0/3	2/3	0/3
30	3/3	0/3	3/3	0/3
BPJ4821[a]	0/9	0/9	0/9	0/9
SHU199[a]	0/9	0/9	0/9	0/9
Water (NTC)	0/9	1/9	0/9	0/9
Total positives[b]	30	0	28	5
Total negatives[b]	0	30	2	25
Sensitivity	100.00		93.33	
Specificity	100.00		83.33	

[a]Genomic DNA from bacteria isolated from seeds (strains BPJ4829 and SHU199) were included as negative controls and are referenced in Table 1.

[b]Total positives and negatives refer to inoculated seed preparations, negative and no template controls were not included in this sum or for sensitivity and specificity calculations.

638 **Table 4**. LAMP detection of *X. oryzae* pv. *oyrzae* and *X. oryzae* pv. *oryzicola* in leaf tissue
639 inoculated by syringe infiltration 72 hpi. Three independently inoculated leaves were analyzed at
640 least twice.

Strain[a]	**Number of positives/total samples amplified with primer set:**			
	Pathovar-specific *X. oryzae* pv. *oryzae*	Pathovar-specific *X. oryzae* pv. *oryzicola*	African lineage *X. oryzae* pv. *oryzae*	Asian lineage *X. oryzae* pv. *oryzae*
PXO99[A]	3/3	0/3	0/3	3/3
MAI1	3/3	0/3	3/3	0/3
BLS256	0/3	3/3	0/3	0/3
Uninoculated leaf	0/3	0/3	0/3	0/3
No template[b]	-	-	-	-

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642 [a]Strains are referenced in Table 1.

643 [b]No template controls contained water and were included in each reaction. A "–" represents the
644 absence of any amplification signal.

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