Genome-Informed Recombinase Polymerase Amplification Assay Coupled with a Lateral Flow Device for In-Field Detection of *Dickeya* Species

Gamze Boluk,¹ Shefali Dobhal,¹ Alex B. Crockford,² Michael Melzer,¹ Anne M. Alvarez,¹ and Mohammad Arif^{1†}

¹ Department of Plant and Environmental Protection Sciences, University of Hawaii at Manoa, Honolulu, HI 96822, U.S.A.

² Wisconsin Seed Potato Certification Laboratory, University of Wisconsin-Madison, Madison, WI 53706, U.S.A.

Abstract

Dickeya spp. cause blackleg and soft rot diseases of potato and several other plant species worldwide, resulting in high economic losses. Rapid detection and identification of the pathogen is essential for facilitating efficient disease management. Our aim in this research was to develop a rapid and field-deployable recombinase polymerase amplification (RPA) assay coupled with a lateral flow device (LFD) that will accurately detect *Dickeya* spp. in infected plant tissues without the need for DNA isolation. A unique genomic region (*mgIA/mgIC* genes) conserved among *Dickeya* spp. was used to design highly specific robust primers and probes for an RPA assay. Assay specificity was validated with 34 representative strains from all *Dickeya* spp. and 24 strains from other genera and species; no false positives or negatives were detected. An RPA assay targeting the internal

transcribed spacer region of the host genome was included to enhance the reliability and accuracy of the *Dickeya* assay. The detection limit of 1 fg was determined by both sensitivity and spiked sensitivity assays; no inhibitory effects were observed when 1 µl of host sap, macerated in Tris-EDTA buffer, was added to each reaction in the sensitivity tests. The developed RPA assay is rapid, highly accurate, sensitive, and fully field deployable. It has numerous applications in routine diagnostics, surveillance, biosecurity, and disease management.

Keywords: bacteria, blackleg and soft rot diseases, diagnostics, *Dickeya* spp., field deployable, recombinase polymerase amplification (RPA), RPA diagnostics, techniques

Dickeya spp. are Gram-negative, fermentative, and motile plant-pathogenic bacteria that produce extracellular pectinolytic enzymes which are imperative for the phytopathological process. Other key factors such as oxygen availability, temperature, and humidity also play a significant role in pathogenesis of *Dickeya* spp. (Perombelon 2002). Previously included in a diverse group of phytopathogens called *Erwinia chrysanthemi*, these soft-rotting and vascular phytopathogens were reclassified into six species of *Dickeya* (Samson et al. 2005) and now the genus includes eight species; namely, *Dickeya aquatica*, *D. chrysanthemi*, *D. dadantii*, *D. dianthicola*, *D. fangzhongdai*, *D. paradisiaca*, *D. solani*, and *D. zeae* (Brady et al. 2012; Parkinson et al. 2014; Tian et al. 2016; van der Wolf et al. 2014).

Dickeya spp. cause disease on a wide range of host plants, including potato (*Solanum tuberosum* L.) (Samson et al. 2005), which is a multibillion-dollar industry in the United States, valued at \$3.7 billion in 2018 by the National Potato Council. On potato, symptoms include blackleg, soft rot, and stem rot (Laurila et al. 2009; Ngadze et al. 2010; Perombelon 2002). In addition, wilting of leaves followed by discoloration of the vascular system are common symptoms. *Dickeya* spp. are widespread because potato plants are grown across Eastern and Northern Europe, Israel, and other parts of the world (Boluk and Arif 2019; Golanowska et al. 2016; Joutsjoki et al. 2005; Laurila et al. 2009; Palacio-Bielsa et al. 2006; Tsror et al. 2008). Apart from potato, other hosts include banana, tomato,

sugar beet, pineapple, onion, pear, maize, rice, and a variety of ornamental plants (Gardan et al. 2003; Ma et al. 2007; Palacio-Bielsa et al. 2006; Samson et al. 2005; Sueno et al. 2014; Tian et al. 2016). Pineapple heart rot, previously attributed to *E. chrysanthemi* (Kaneshiro et al. 2008), and stalk rot of corn differ somewhat in symptom expression but both diseases are now attributed to *D. zeae* (Kumar et al. 2017; Marrero et al. 2013; Sueno et al. 2014). Symptoms on pear include bleeding canker of tree trunks, which is caused by the recently defined species *D. fangzhongdai* (Tian et al. 2016).

In order to detect and identify *Dickeya* spp., several traditional diagnostic methods are used, including semiselective crystal-violet pectate medium, biochemical tests, and enzyme-linked immunosorbent assay (Samson et al. 2005; Singh et al. 2000), but these methods are often time consuming and laborious, or require a skilled technician. PCR and quantitative PCR (qPCR)-based methods are more sensitive; numerous methods have already been published for different *Dickeya* spp. However, these techniques require facilities that are adequately equipped to run such diagnostic tests (Czajkowski et al. 2015; Hommais et al. 2011; Humphris et al. 2015; Nassar et al. 1996; Pritchard et al. 2013, 2016). *Dickeya* spp. were characterized using multilocus sequence typing, amplified fragment length polymorphism, DNA-DNA hybridization (Avrova et al. 2002; Brady et al. 2012; Fujimoto et al. 2018; Marrero et al. 2013; Nassar et al. 1996; Slawiak et al. 2009; van der Wolf et al. 2014; Waleron et al. 2002), and other PCR-based methods, including end-point PCR, multiplex PCR, and real-time qPCR (Czajkowski et al. 2015; Dobhal et al. 2020). Among these methods, real-time qPCR has shown high sensitivity but cannot be performed in field.

The field-deployable, loop-mediated isothermal amplification (LAMP) method is rapid and eliminates the requirement of expensive and sophisticated thermocycler (Dobhal et al. 2019; Larrea-Sarmiento et al. 2018; Notomi et al. 2000; Ocenar et al. 2019; Yasuhara-Bell et al. 2017). However, LAMP still relies on purified DNA and that is a challenging task to perform in field settings.

Recombinase polymerase amplification (RPA) is a comparatively new isothermal technique, which works at 37 to 42°C without the need of purified genomic DNA (Ahmed et al. 2018; Arif et al. 2016; Piepenburg et al. 2006). RPA now is being used to detect a multitude of pathogens that infect humans, animals, and plants. The RPA lateral

[†]Corresponding author: M. Arif; arif@hawaii.edu

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flow device (LFD) assay is rapid and can be done within 45 min from sample processing to detection (Ahmed et al. 2018). The amplified RPA product can be visualized on an LFD labeled with fluorescein isothiocyanate and biotin. This visual detection requires the modified oligonucleotides and probe used to amplify the target region.

The objective of this study was to develop a rapid RPA assay coupled with an LFD for specific and robust detection of *Dickeya* spp. in field settings without the need for purified DNA. The developed assay has tremendous applications in routine diagnostics, biosecurity, epidemiology, and disease management, and to effectively combat new disease outbreaks.

Materials and Methods

Source of bacterial strains and DNA extraction. In total, 58 strains were studied, including 34 strains of *Dickeya* spp. (Table 1) and 24 strains (Table 2) from closely related genera or species or other microorganisms found in the niche. These bacterial cultures were obtained from different hosts and geographical regions around the world. The strains were stored at -80°C and regrown on TZC (tetracycline chloride) medium (Dobhal et al. 2020; Larrea-Sarmiento et al. 2018). Plates were incubated at $26 \pm 2^{\circ}\text{C}$. A single colony was picked and multiplied to eliminate any possibility of contamination. The UltraClean Microbial DNA Isolation Kit (MO BIO, Carlsbad, CA, U.S.A.) was used to extract the genomic DNA from all pure strains. The concentration of extracted DNA was measured using a NanoDrop 2000 (Thermo Fisher Scientific Inc, Worcester, MA, U.S.A.); extracted DNA was stored in a freezer at -20°C . The identity of each strain was confirmed by amplifying either *dnaA* (chromosomal replication initiator protein) or 16S ribosomal RNA partial gene region (Dobhal et al. 2020).

RPA primers and probe design. The genomes of *D. dianthicola* (NZ_CP017638 and NZ_CM001840), *D. solani* (NZ_CP015137 and NZ_CP009460), *D. zeae* (NC_013592 and NZ_CP006929), *D. dadantii* (NZ_CP002038), *D. chrysanthemi* (NZ_CM001904), *D. fangzhongdai* (NZ_CP025003), and *D. paradisiaca* (NZ_CM001857) were retrieved from the NCBI GenBank genome database. The whole genomes were aligned using progressiveMauve plugged into Geneious 10.2.3 (Biomatters, Auckland, NZ), and used to evaluate primers and probe's in silico specificity (Dobhal et al. 2020). The *mglA/mgIC* genomic region was used to design specific RPA primers and a probe to detect all *Dickeya* spp. (Dobhal et al. 2020). The RPA sense and antisense primers and probe were designed manually (Table 3). The reverse primer DIC-RPAR1 was labeled with biotin at the 5' position and the probe was labeled with 6-carboxy-fluorescein (FAM) (Fig. 1). The RPA assay, designed using the host internal transcribed spacer (ITS) region, was also included in the validation (Ahmed et al. 2018) (Table 3).

The RPA reaction and analysis. The RPA reaction was performed using the TwistAmp Exo kit (TwistDX Limited, Maidenhead, U.K.) following the manufacturer's instructions. Briefly, RPA reactions were carried out in a total of 50 μl of reaction volume containing 29.5 μl of rehydration buffer, 0.6 μl (10 μM) of probe, 2.1 μl (10 μM) of each forward and reverse primer, 12.5 μl of nuclease-free water, 1 μl of DNA template or macerated plant sap (Ahmed et al. 2018), and 2.5 μl of magnesium acetate to activate the reaction. The reaction mixture was incubated at a fixed temperature of 42°C for 30 min. Each RPA run was performed with a positive control (target DNA) and a nontemplate control (nuclease-free water). After completion of the amplification reaction, 5 μl of RPA product was added to a mix of 500 μl of buffer (250 μl of

Table 1. Bacterial strains used an inclusivity panel to validate recombinase polymerase amplification (RPA) assay for specific detection of *Dickeya* spp.

Isolate	Original ID	Organism	Location	Host	GenBank ^a	RPA ^b
A5415	CFBP2048	<i>Dickeya chrysanthemi</i>	United States	<i>Chrysanthemum morifolium</i>	MH453538	+
A5641	CFBP1270	<i>D. chrysanthemi</i>	Denmark	<i>Parthenium argentatum</i>	MH453539	+
A5576	PRI2120	<i>D. dadantii</i>	Comoros	<i>Perlagonium capitatum</i>	MK208948	+
A5642	CFBP3855	<i>D. dadantii</i>	France	<i>Saintpaulia</i> sp.	MH453542	+
A5643	CFBP6467	<i>D. dadantii</i>	Martinique, France	<i>Musa</i> sp.	MK208950	+
A6060	CFBP3698	<i>D. dadantii</i>	Cuba	<i>Musa</i> sp.	MK208956	+
A6061	CFBP1247	<i>D. dadantii</i>	United States	<i>Dieffenbachia picta</i>	MK208957	+
A5418	CFBP1200	<i>D. dianthicola</i>	United Kingdom	<i>Dianthus caryophyllus</i>	MK208961	+
A5566	PRI1363	<i>D. dianthicola</i>	The Netherlands	<i>Solanum tuberosum</i>	MK208962	+
A5567	PRI1370	<i>D. dianthicola</i>	The Netherlands	<i>S. tuberosum</i>	MK208963	+
A5569	PRI1372-B	<i>D. dianthicola</i>	The Netherlands	<i>S. tuberosum</i>	MK208965	+
A5573	PRI2114	<i>D. dianthicola</i>	United Kingdom	<i>D. caryophyllus</i>	MK208947	+
A5644	CFBP2015	<i>D. dianthicola</i>	France	<i>S. tuberosum</i>	MK208951	+
A5645	CFBP4155	<i>D. dianthicola</i>	The Netherlands	<i>Kalanchoe blossfeldiana</i> var. <i>Maes</i>	MK208952	+
A6058	CFBP1982	<i>D. dianthicola</i>	France	<i>Dahlia</i> sp.	MK208953	+
A6059	CFBP3706	<i>D. dianthicola</i>	Switzerland	<i>Cichorium intybus</i>	MK208955	+
PL22	GBp1A	<i>D. dianthicola</i>	Hawaii, U.S.A.	<i>S. tuberosum</i>	MK189269	+
PL23	GBp10B	<i>D. dianthicola</i>	Hawaii, U.S.A.	<i>S. tuberosum</i>	MK189270	+
PL24	GBp11A	<i>D. dianthicola</i>	Hawaii, U.S.A.	<i>S. tuberosum</i>	MK189271	+
PL25	GBp21C	<i>D. dianthicola</i>	Hawaii, U.S.A.	<i>S. tuberosum</i>	MK189268	+
A5579	PRI2127	<i>D. paradisiaca</i>	Columbia	<i>M. paradisiaca</i>	MK208943	+
A5420	CFBP4178	<i>D. paradisiaca</i>	Colombia	<i>M. paradisiaca</i>	MK208942	+
A5581	PRI2187	<i>D. solani</i>	Israel	<i>S. tuberosum</i>	MH453540	+
A5582	PRI2188	<i>D. solani</i>	Israel	<i>S. tuberosum</i>	MH453541	+
A5422	CFBP 2052	<i>D. zeae</i>	United States	<i>Zea mays</i>	MH453537	+
A6069	CFBP1277	<i>D. zeae</i>	United States	<i>Z. mays</i>	MK208960	+
A6056	3 leaf	<i>D. zeae</i>	Hawaii, U.S.A.	<i>Ananas comosus</i>	MH453535	+
A5269	1-6A	<i>D. zeae</i>	Hawaii, U.S.A.	<i>A. comosus</i>	MN045867	+
A5265	1-3A	<i>D. zeae</i>	Hawaii, U.S.A.	<i>A. comosus</i>	MK189273	+
A5423	CFBP6466	<i>D. zeae</i>	Martinique, France	<i>A. comosus</i>	MH453536	+
PL47	F4-3A2	<i>D. zeae</i>	Hawaii, U.S.A.	<i>Brassica oleracea</i> var. <i>sabellica</i>	MN045868	+
A5577	PRI2121	<i>D. zeae</i>	Malaysia	<i>A. comosus</i>	MK208949	+
A6066	CFBP1889	<i>D. zeae</i>	Malaysia	<i>A. comosus</i>	MK208958	+
A6067	CFBP1890	<i>D. zeae</i>	Malaysia	<i>A. comosus</i>	MK208959	+

^a GenBank accession number.

^b Positive amplification indicated by + symbol.

water + 250 µl of buffer; Milenia Biotec, Germany). An LFD was vertically inserted into the dilution mix and left for 5 min.

Determination of limit of detection and spiked assays. The genomic DNA of *D. dianthicola* (strain A5418) was 10-fold serially diluted from 1 ng to 1 fg to determine the detection limit. One microliter from each dilution was used for individual reactions. Two spiked assays were also performed to confirm any inhibitory effect by adding 1 µl of potato stem or tuber sap to each reaction containing 10-fold serially diluted genomic DNA. A nontemplate control was included in each assay to confirm the reliability of the assay.

Validation of RPA assay with naturally infected plant samples. Eight naturally infected potato plants that showed wilt and soft rot symptoms in the field (P1, P3, P4, P7, P8, P10, P21, and P22) were brought to the laboratory. The samples were disinfected using 0.6% sodium hypochlorite; plant tissue (1 cm²) was crushed in an Eppendorf tube using a small sterile pestle. A plant tissue kit (Wizard Genomic DNA Purification Kit; Promega Corp., Madison, WI, U.S.A.) was used to extract the genomic DNA following the manufacturer's protocol. Additionally, 50 mg of infected tissues were macerated for 1 min in 400 µl of Tris-EDTA (TE) (1×) buffer using a pestle. The macerated tissues were left for 10 min at room temperature to let the plant debris settle; 1 µl of the supernatant was used directly in the RPA reaction (Ahmed et al. 2018).

Confirmation of infection in potato samples. The genomic DNA of naturally infected potato tubers (P1, P3, P4, P7, P8, P10, P21, and P22) was amplified using the primer set of gene *dnaA* for *Dickeya* spp. (Dobhal et al. 2020). The PCR contained 10 µl of GoTaq Green Master Mix (Promega Corp.), 1 µl (5 mM) of each forward and reverse primer, 1 µl of template DNA, and 7 µl of Ultra-Pure DNase/RNase-Free distilled water (Thermo Fisher Scientific). The Dc-dnaA PCR conditions were as follows: initial denaturation at 94°C for 5 min; followed by 35 cycles of denaturation at 94°C for 20 s, annealing at 58°C for 1 min, and extension at 72°C for 1 min; final extension at 72°C for 2 min; and held at 12°C. The PCR was performed in a T100 Thermal cycler (Bio-Rad, Hercules, CA, U.S.A.). Agarose gel (1.5%) electrophoresis was used to separate PCR amplicons. The amplicons were visualized under UV light

in a gel documentation system (Bio-Rad Gel Doc-XR+; Bio-Rad). Amplified PCR products were cleaned by adding 2 µl of ExoSAP-IT (Affymetrix Inc, Santa Clara, CA, U.S.A.) in 5 µl of PCR product, and incubated at 37°C for 15 min followed by 80°C for another 15 min. Treated templates were sequenced using *dnaA*-F1 and *dnaA*-R1 sense and antisense primers. Sanger sequencing was performed at the GENEWIZ facility (Genewiz, La Jolla, CA, U.S.A.). The sequences of the sense and antisense strands of each strain were aligned and manually edited in order to rectify any sequencing error. Manually edited sequences were compared against the NCBI GenBank nucleotide and genome databases using the NCBI BLASTn tool. Geneious 10.2.3 was used for editing and alignment (Dobhal et al. 2020).

Validation of RPA assay with artificially infected plant samples. The potato stem tubers, sweet potato (*Ipomoea batatas* L. (Lam)) root tubers, and taro (*Colocasia esculenta* L. (Schott)) corms were surface sterilized by dipping them into a beaker containing 0.6% sodium hypochlorite for 3 min followed by three consecutive washes with sterilized water. Tubers were cut into thin slices using sterilized scalpel blades. Cut slices were placed in sterile petri dishes with filter paper containing 1 to 2 ml of sterile water to mimic a moist chamber. The potato, taro, and sweet potato slices were stab inoculated with 2 to 3 colonies of *D. zeae* (A6056) and *D. solani* (A5581) using a sterile toothpick. All slices were incubated for 24 h at 28°C. Tissues were macerated in TE buffer as described above. One microliter of the supernatant was used for the RPA assay. Two replicates of each infected samples of potato, taro, and sweet potato were tested along with the healthy controls (negative control).

RPA assay for plant control. Potato stem tubers were surface sterilized and cut into thin slices using sterilized scalpel blades. The slices were placed in sterile petri dishes the same as described above for the artificially infected plant process. The potato slices were stab-inoculated with *Pectobacterium wasabiae* (A1852), *P. carotovorum* subsp. *odoriferum* (A2686), *P. carotovorum* subsp. *brasiliensis* (A6152), and *P. carotovorum* subsp. *carotovorum* (A5278) using a sterile toothpick. All slices were incubated for 24 h at 28°C. The DNA was extracted using the Wizard Genomic DNA Purification Kit following the manufacturer's protocol.

Table 2. Bacterial strains used in exclusivity panel to recombinase polymerase amplification (RPA) assay for specific detection of *Dickeya* spp.

Isolate	Original ID	Organism	Location ^a	Host	GenBank ^b	RPA ^c
A5150		<i>Enterobacter asburiae</i>	Hawaii	<i>Zingiber officinale</i>	MK182852	–
A1084	QR-6	<i>Erwinia amylovora</i>	California	<i>Pyrus</i> sp.	MK182851	–
		<i>Klebsiella</i> sp.	Hawaii	...	MK182844	–
A3131	ATCC13048	<i>Klebsiella aerogenes</i>	South Carolina	...	MK208954	–
A1867	F2c.papaya	<i>Pantoea</i> sp.	Hawaii	<i>Carica papaya</i>	MK182843	–
A6222	DP138	<i>Pantoea agglomerans</i>	Wisconsin	<i>Zea mays</i>	MH547382	–
A5513		<i>P. agglomerans</i>	Hawaii	Ornamental	MK182849	–
A5186	ATCC29267	<i>P. cypripedii</i>	California	<i>Cypripedium</i> sp.	MK182846	–
A5348	2d	<i>Pectobacterium aroidearum</i>	Hawaii	<i>Aglaonema</i> sp.	MK182847	–
A1850	IPM1260	<i>P. atrosepticum</i>	Colorado	<i>Solanum tuberosum</i>	MH453513	–
A6163	Eca31	<i>P. atrosepticum</i>	Wisconsin	<i>S. tuberosum</i>	NS	–
A6165	Ecb1	<i>P. betavascularum</i>	California	<i>Beta vulgaris</i>	MK250993	–
A6149	WPP5	<i>P. carotovorum</i> subsp. <i>brasiliensis</i>	Wisconsin	<i>S. tuberosum</i>	MH453522	–
A6152	WPP165	<i>P. carotovorum</i> subsp. <i>brasiliensis</i>	Wisconsin	<i>S. tuberosum</i>	MH453521	–
A5278	1-#21	<i>P. carotovorum</i> subsp. <i>carotovorum</i>	Hawaii	Irrigation water	MH453511	–
A5280	1-#31	<i>P. carotovorum</i> subsp. <i>carotovorum</i>	Hawaii	Irrigation water	MH453512	–
A5368	5X	<i>P. carotovorum</i> subsp. <i>carotovorum</i>	Hawaii	<i>Aglaonema</i> sp.	MH453510	–
A5350	5C	<i>P. carotovorum</i> subsp. <i>carotovorum</i>	Hawaii	<i>Aglaonema</i> sp.	MK208940	–
A2686	E43	<i>P. carotovorum</i> subsp. <i>odoriferum</i>	Hawaii	<i>Brassica oleracea</i> var. <i>capitata</i>	MH453519	–
A1852	M784	<i>P. parmanteri</i>	Colorado	<i>S. tuberosum</i>	MH453534	–
A1701	B94	<i>Xanthomonas euvesicatoria</i>	California	<i>S. lycopersicum</i>	MH492152	–
A3617	XV145	<i>X. vesicatoria</i>	South America	<i>S. lycopersicum</i>	MH492125	–
A2961	C58	<i>Agrobacterium tumefaciens</i>	New York	<i>Prunus avium</i>	NS	–
A3450	30	<i>Ralstonia solanacearum</i>	Trinidad and Tobago	<i>S. lycopersicum</i>	MK243481	–
...	...	Healthy plant	Hawaii	<i>S. tuberosum</i>	...	–

^a States in the United States (except for South America and Trinidad and Tobago).

^b GenBank accession number. NS = Sequencing quality was poor, not submitted to NCBI GenBank database.

^c Negative amplification indicated by – symbol.

IC-RPAF1 and IC-RPAR1 primers and IC-RPAP1 probe were used to detect host DNA, thereby enhancing the accuracy of the developed assays. The RPA primers and probe were designed to detect the genomic DNA of potato (Ahmed et al. 2018). Healthy potato tissues and samples infected with *Pectobacterium* spp. were assayed with *Dickeya* sp.- and host-specific primers and probes. The *D. dianthicola* (PL022) genomic DNA was used as a positive control; water was used as a nontemplate control. The inclusion of a plant control RPA assay is essential when detecting the pathogen from infected plant materials.

Results

Target unique genomic region, primer design, and in silico validation. The unique and conserved *mglA/mglC* genomic region used to design the primers and probe was selected after comparing genomes of *Dickeya* spp. and other closely related species, including *Pectobacterium* (Dobhal et al. 2020). The selected gene region was analyzed for specificity using NCBI databases. Manually designed long primers and probes were BLASTn against the NCBI GenBank database for in silico validation. BLASTn report showed high specificity—100%

match with only *Dickeya* sequences, and no sequence match occurred within any other genera, including *Pectobacterium*, *Pantoea*, or *Enterobacter*.

Specificity validation. The broad-range detection capability of the developed RPA assay was assessed with 34 strains of six different *Dickeya* spp., including *D. chrysanthemi*, *D. dadantii*, *D. dianthicola*, *D. paradisiaca*, *D. solani*, and *D. zeae*. In the exclusivity panel, 24 strains from seven other bacterial genera (*Agrobacterium tumefaciens*, *Enterobacter aerogenes*, *E. asburiae*, *E. cloacae*, *Erwinia* sp., *Erwinia amylovora*, *Klebsiella* sp., *P. atrosepticum*, *P. betavascularum*, *P. carotovorum* subsp. *brasiliensis*, *P. carotovorum* subsp. *carotovorum*, *P. carotovorum* subsp. *odoriferum*, *P. wasabiae*, *Ralstonia solanacearum*, *Xanthomonas euvesicatoria*, and *X. vesicatoria*) were included to confirm high specificity. No false positives or false negatives were detected. The positive results were only obtained with *Dickeya* spp. (Fig. 2A) and no positive amplification was observed with any strains of the exclusivity panel (Fig. 2B). The RPA assay was also negative when tested against the healthy plant DNA. Results appearing after 10 min were not considered. The primers and probe were able to detect only *Dickeya* spp., indicating that the

Table 3. List of primers and probes used to develop the recombinase polymerase amplification (RPA) lateral-flow strip assay for *Dickeya* spp.

Primer name	Sequence ^a	L (nt) ^b	GC (%)	BLASTn ^c		
				QC (%)	E-value	ID (%)
DIC-RPA-F	5'-GCATTGTCGAAACCAAGAACCACGACGAGAACGA-3'	34	50.00	100	6e-08	100
DIC-RPA-R	5'-Biosg/ATGCCGCTGTCTTTTCAGCCAGGTGAGCGCACTT-3'	33	57.60	100	2e-07	100
DIC-RPA-LP	5'-FAM/TAGCGTCGTTACACCTTTAATGATGCAAGG/Internal dSpacer/CTGTTACCATGAAAGCTA/Spacer-3'	49	42.86	100	6e-05	100
IC-RPAF1 ^d	5'-AACACAAAC GACTCTCGGCAACGGATATCTCG-3'	32	50.00	...	...	...
IC-RPAR1 ^d	5'-Biosg/ATGGCTTCGGGCGCAACTTGCGTTCAAAGACT-3'	32	53.10	...	...	...
IC-LP ^d	5'-FAM/TGAAGAACGTAGCGAAATGCGATACCTTGGT/Internal dSpacer/TGAATTGCAGAAATCCCGTGA/Spacer-3'	50	44.00	...	...	...

^a FAM = 6-carboxy-fluorescein.

^b Length in nucleotides (nt).

^c QC = query cover and ID = percent identity.

^d Ahmed et al. (2018).

Fig. 1. A, Schematic representation of the recombinase polymerase amplification (RPA) principle and mechanism. The nfo probe is 48 to 52 nucleotides long, modified at the 5' end with an antigenic 6-carboxy-fluorescein (FAM) label, and blocked at the 3' end. The tetrahydrofuran (THF) residue for the detection by nfo nuclease is placed at 30 nucleotides from the 5' end. When the probe forms the double-stranded DNA, the THF is cleaved by nfo nuclease. After the cleavage, the probe with a free 3' end is formed that functions as a primer for an extension by the polymerase. For the genomic amplicon to be detected in sandwich assay format, a reverse primer is labeled at the 5' end (biotin). The genomic amplicons have two labels at both ends. **B,** Schematic representation of the RPA lateral flow device principle and mechanism. The HybriDetect dipstick, which is a ready-to-use universal dipstick, contained anti-FAM with the gold particle in the sample pad, anti-biotin in the detection line, and anti-rabbit antibody in the control line. Sample and the specific solution were added to the sample pad. In the incubation time, the genomic amplicon with FAM and biotin labels bind to the gold nanoparticles, allowing the detection.

Fig. 2. Specificity validation of the recombinase polymerase amplification (RPA) for *Dickeya* spp. **A**, Inclusivity panel; 14 representative strains of *Dickeya* spp. Lane PC = positive control, *Dickeya dianthicola* (PL22), lane 1 = *D. chrysanthemi* (A5415), lane 2 = *D. chrysanthemi* (A5641), lane 3 = *D. dadantii* (A6066), lane 4 = *D. dadantii* (A6060), lane 5 = *D. dianthicola* (A5566), lane 6 = *D. dianthicola* (A5567), lane 7 = *D. dianthicola* (PL023), lane 8 = *D. dianthicola* (PL025), lane 9 = *D. paradisiaca* (A5420), lane 10 = *D. paradisiaca* (A5579), lane 11 = *D. solani* (A5581), lane 12 = *D. solani* (A5582), lane 13 = *D. zeae* (A5423), lane 14 = *D. zeae* (PL47), and lane NTC = nontemplate control (water). **B**, Exclusivity panel; 14 close relative representative bacterial strains. Lane PC = positive control, *D. dianthicola* (PL22), lane 1 = *Agrobacterium tumefaciens* (A2961), lane 2 = *Enterobacter aerogenes* (A3131), lane 3 = *Erwinia amylovora* (A1084), lane 4 = *Erwinia* sp. (A5350), lane 5 = *Klebsiella aerogenes* (A3131), lane 6 = *Pectobacterium atrosepticum* (A6163), lane 7 = *P. carotovorum* subsp. *brasiliensis* (A6149), lane 8 = *P. carotovorum* subsp. *carotovorum* (A5368), lane 9 = *P. carotovorum* subsp. *odoriferum* (A2686), lane 10 = *P. wasabiae* (A1852), lane 11 = *Pantoea agglomerans* (A5513), lane 12 = *P. ananatis* (A6222), lane 13 = *Ralstonia solanacearum* (A3450), lane 14 = *Xanthomonas euvesicatoria* (A1701), and lane NTC = nontemplate control (water).

Fig. 3. Determination of detection limit of *Dickeya* spp.-specific recombinase polymerase amplification (RPA) assay. **A**, Sensitivity assay performed with 10-fold serially diluted genomic DNA of *Dickeya dianthicola* (A5418). Lane 1 = 10 ng, lane 2 = 1 ng, lane 3 = 100 pg, lane 4 = 10 pg, lane 5 = 1 pg, lane 6 = 100 fg, lane 7 = 10 fg, lane 8 = 1 fg, and lane NTC = nontemplate control (water). **B**, Spiked assay; host stem sap was added to RPA reaction containing 10-fold serially diluted *D. dianthicola* genomic DNA. Lane 1 = 10 ng, lane 2 = 1 ng, lane 3 = 100 pg, lane 4 = 10 pg, lane 5 = 1 pg, lane 6 = 100 fg, lane 7 = 10 fg, lane 8 = 1 fg, and lane NTC = nontemplate control (water). **C**, Spiked assay; host potato tuber sap was added to RPA reaction containing 10-fold serially diluted *D. dianthicola* genomic DNA. Lane 1 = 10 ng, lane 2 = 1 ng, lane 3 = 100 pg, lane 4 = 10 pg, lane 5 = 1 pg, lane 6 = 100 fg, lane 7 = 10 fg, lane 8 = 1 fg, and lane NTC = nontemplate control (water).

developed RPA assay is accurate and highly specific for the detection of *Dickeya* spp.

Determination of detection limit. A 10-fold serial dilution of *D. dianthicola* (A5418) genomic DNA from 1 ng to 1 fg was used to evaluate the detection limit. In addition, two spiked assays were performed by adding 1 µl of healthy potato leaf or potato tuber sap to the RPA reaction containing 10-fold serially diluted genomic DNA to confirm the inhibitory effect of the host sap. The sensitivity assay with purified *D. dianthicola* genomic DNA (Fig. 3A) and the two spiked sensitivity assays (Fig. 3B and C) unanimously detected 1 fg (approximately 1 genome copy or 1 CFU) of *D. dianthicola* genomic DNA. The sensitivity results indicated that the developed RPA assay is highly sensitive and has no inhibitory effect of host sap.

Detection from naturally and artificially infected plant samples. The developed *Dickeya*-specific RPA was evaluated using naturally and artificially infected plant materials. Three experiments were performed: (i) the genomic DNA was isolated from naturally infected plant materials and used for RPA detection (Fig. 4A), (ii) sap from the infected plant material was used to perform the RPA (Fig. 4B), and (iii) the sap from artificially inoculated potato, sweet potato, and taro were used for RPA detection (Fig. 5). No differences in the results were obtained when RPA assays were performed using purified genomic DNA and infected plant tissue sap. The identity of each naturally infected plant sample was confirmed by isolating the bacteria followed by sequencing; sequencing confirmed that all plant tissues were infected with *D. dianthicola*. The *Dickeya* sp. was accurately detected when *Dickeya*-specific RPA was performed with sap of different artificially infected host tissues (potato, sweet potato, and taro); 100% accurate results were obtained (Fig. 5). All three experiments indicated that the *Dickeya* spp. were accurately identified regardless of DNA quality (DNA or plant sap) and sample type (different hosts). No amplification was observed with the negative controls or healthy potato.

Host target detection. An RPA assay designed to detect the potato and other solanaceous plant species was used to cross-validate the possibility of getting false negatives (Ahmed et al. 2018). The potato stem tubers were artificially inoculated with different *Pectobacterium* spp. Both *Dickeya*-specific and host-specific RPA assays were performed (Fig. 6). The host-specific RPA showed positive results with all infected samples, while all results were negative with *Dickeya*-specific RPA (Fig. 6). *Dickeya*-specific RPA only showed positive amplification with the positive control (*D. dianthicola*). The inclusion of a host-specific RPA when detecting from infected plant materials will enhance the reliability of the results.

Fig. 4. Detection of *Dickeya* spp. from naturally infected potato plant samples using recombinase polymerase amplification assay. **A**, Detection of *Dickeya dianthicola* from purified genomic DNA. Lane PC = positive control *D. dianthicola* (PL22), lane 1 = P1, lane 2 = P3, lane 3 = P4, lane 4 = P7, lane 5 = P8, lane 6 = P10, lane 7 = P21, lane 8 = P22, and lane NTC = nontemplate control (water). **B**, Detection of *D. dianthicola* from infected plant sap. Lane PC = positive control *D. dianthicola* (PL22), lane 1 = P1, lane 2 = P3, lane 3 = P4, lane 4 = P7, lane 5 = P8, lane 6 = P10, lane 7 = P21, lane 8 = P22, and lane NTC = nontemplate control (water).

Discussion

Soft rot diseases have a significant economic impact on many crops, including potato, pineapple, corn, banana, and ornamentals. Detection and differentiation of pathogens such as *Dickeya* and *Pectobacterium* spp. that cause soft rot is challenging due to genetic heterogeneity and production of nearly identical symptoms. We have developed a sensitive, rapid, and specific RPA assay to specifically detect *Dickeya* spp. The developed rapid assay was effectively demonstrated in-field, which is imperative for time-sensitive commodities. Additionally, this assay unanimously distinguishes the strains from the genera *Dickeya* and *Pectobacterium*.

Molecular methods such as multiplex PCR (Diallo et al. 2009) and real-time qPCR (Dobhal et al. 2020; Laurila et al. 2009; van der Wolf et al. 2013) are available for the detection of *Dickeya* spp. However, these tools are relatively time consuming and currently have limited applications in quarantine and confirmatory diagnostics. A LAMP assay was also developed using *mgIC* gene by Yasuhara-Bell et al. (2017); however, the LAMP assay has a few limitations, including a higher temperature requirement (65°C) and the design of the complex primers. Moreover, the reported LAMP assay showed a detection limit of only 5 pg compared with the RPA sensitivity of 1 fg (approximately 1 genome copy). Additionally, *Dickeya*-specific LAMP required a qPCR or other device to quantify the fluorescence, which limited its adaptation in unequipped or low-budget labs. In both this and a previous study (Ahmed et al. 2018), RPA showed no adverse effect of plant inhibitors on detection sensitivity; this represents a major advance over the other molecular methods, including a field-deployable LAMP assay. Compared with LAMP and other molecular methods, RPA is more sensitive, tolerant toward inhibitors, and easier to visualize, and the tubes can be incubated in the palm of one's hand (Ahmed et al. 2018; Arif et al. 2016). The RPA does not require expensive instruments. In previous studies using LAMP for detection of *X. euvesicatoria* (Larrea-Sarmiento et al. 2018), the inclusion of host DNA reduced assay sensitivity but RPA was not adversely affected by adding the host sap.

Based on in silico analyses, it was predicted that the primers and probe would detect all species within the genus *Dickeya*. The

Fig. 5. Detection of *Dickeya* spp. from infected potato, sweet potato, and taro samples. Left lane PC = positive control/*Dickeya dianthicola* (PL22), lane 1 = *D. zeae* (A6056), lane 2 = *D. solani* (A5581), and lane NTC = nontemplate control (water). Middle lane PC = positive control/*D. dianthicola* (PL22), lane 1 = *D. zeae* (A6056), lane 2 = *D. solani* (A5581), and lane NTC = nontemplate control (water). Right lane PC = positive control/*D. dianthicola* (PL22), lane 1 = *D. zeae* (A6056), lane 2 = *D. solani* (A5581), and lane NTC = nontemplate control (water).

Fig. 6. Host-specific recombinase polymerase amplification (RPA) to validate the developed *Dickeya*-specific RPA. **A**, Infected potato slices with different *Pectobacterium* spp. **B**, Detection using *Dickeya*-specific recombinase polymerase amplification (RPA). Lane PC = positive control/*Dickeya dianthicola* (PL022), lane H = healthy potato, lane 1 = *Pectobacterium wasabiae* (A1852), lane 2 = *P. carotovorum* subsp. *odoriferum* (A2686), lane 3 = *P. carotovorum* subsp. *carotovorum* (A5278), lane 4 = *P. carotovorum* subsp. *brasiliensis* (A6149), and lane NTC = nontemplate control (water). **C**, Detection of host target using host-specific RPA assay. Lane H = healthy potato, lane 1 = *P. wasabiae* (A1852), lane 2 = *P. carotovorum* subsp. *odoriferum* (A2686), lane 3 = *P. carotovorum* subsp. *carotovorum* (A5278), lane 4 = *P. carotovorum* subsp. *brasiliensis* (A6149), and lane NTC = nontemplate control (water). No false negatives or false positives were observed.

analysis showed 100% identity only with *Dickeya* strains, which was expected because the selected genome region was unique to *Dickeya* spp. It has already been proven that a unique target genome sequence is peremptory for the development of a robust and highly specific assay (Dobhal et al. 2020; Larrea-Sarmiento et al. 2018; Ouyang et al. 2013). The specificity validation using the strains included in inclusivity and exclusivity panels indicated that the developed RPA is highly specific and only detects the strains from *Dickeya* spp.; no amplification or cross-reactivity was observed when tested against *Pectobacterium*, a closely related genus. No discrepancy was observed when RPA was validated using the naturally and artificially infected plant samples (Figs. 4 and 5). When different hosts (potato, taro, and sweet potato) were artificially inoculated with *Dickeya* strains and sap exuding after the infection was used to perform the *Dickeya*-specific RPA assay, all infected samples were detected with 100% accuracy. The procedure can be completed within 45 mins from sample preparation to final detection on LFD (Fig. 5), and this is remarkably rapid compared with the qPCR and LAMP assays (Dobhal et al. 2019; Ocenar et al. 2019; Ouyang et al. 2013).

The coupling of an LFD with RPA facilitates the application of this technique for on-site detection. LFD and lyophilized RPA reagents do not require refrigeration for transport. Inclusion of an RPA assay targeting the host ITS region enhanced the reliability of the results—it eliminated the uncertainty of obtaining false negatives, which is a critical aspect of a reliable diagnostic assay (Fig. 6).

In summary, we used a unique target genome region within *Dickeya* spp. to design a highly specific, robust, and rapid RPA assay for on-site detection of devastating soft rot and blackleg pathogens in *Dickeya* spp. The developed assay is user friendly, does not require DNA isolation, and has no effect of plant inhibitors. The assay has

applications in routine diagnostics, epidemiological studies, disease management, and biosecurity.

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