Isothermal Amplification and Lateral-Flow Assay for Detecting Crown-Gall-Causing *Agrobacterium* spp.

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Accepted for publication 29 May 2017.

ABSTRACT

Agrobacterium is a genus of soilborne gram-negative bacteria. Members carrying oncogenic plasmids can cause crown gall disease, which has significant economic costs, especially for the orchard and nursery industries. Early and rapid detection of pathogenic *Agrobacterium* spp. is key to the management of crown gall disease. To this end, we designed oligonucleotide primers and probes to target *virD2* for use in a molecular diagnostic tool that relies on isothermal amplification and lateral-flow-based detection. The oligonucleotide tools were tested in the assay and evaluated for detection limit and specificity in detecting alleles of *virD2*. One set of

primers that successfully amplified *virD2* when used with an isothermal recombinase was selected. Both tested probes had detection limits in picogram amounts of DNA. Probe 1 could detect all tested pathogenic isolates that represented most of the diversity of *virD2*. Finally, the coupling of lateral-flow detection to the use of these oligonucleotide primers in isothermal amplification helped to reduce the onerousness of the process, and alleviated reliance on specialized tools necessary for molecular diagnostics. The assay is an advancement for the rapid molecular detection of pathogenic *Agrobacterium* spp.

Agrobacterium is a genus of bacteria that includes soilborne plant pathogens which can infect hundreds of different families of herbaceous and woody dicots, including ornamental trees, shrubs, fruit and nut trees, and grapevine (Otten et al. 2008). As the causative agent of crown gall disease, members of *Agrobacterium* cause malformations that manifest as tumors. These disfigurements in growth render some agricultural plants unsellable because of loss in aesthetic appeal. In young plants, infection by *Agrobacterium* spp. can compromise health due to decreased nutrient and water acquisition. In some species of plants, such as grapevine and rose, *Agrobacterium* infection can be systematic and persist without any overt expression of disease symptoms (Martí et al. 1999; Tarbah and Goodman 1987; Yakabe et al. 2012).

In a commercial setting, the pathogen can be introduced into a production setting via damaged galls from diseased plants and disseminated by irrigation or improperly sanitized tools. At present, there are just a few commercially available protectants and no curatives (Ryder and Jones 1991). In total, pathogenic *Agrobacterium* spp. cause an estimated 40% reduction in yield in the orchard and nursery industries, with annual losses in the millions of dollars (Kennedy and Alcorn 1980; Moore et al. 2001). The best course of action includes early detection and eradication of the diseased plants (Burr and Otten 1999).

Based on the more classical classification scheme, the *Agrobacterium* genus includes at least five recognized species that are distinguished based on phenotypic differences and host range. These include *Agrobacterium tumefaciens*, *A. rhizogenes*, *A. vitis*, *A. rubi*, and *A. larrymoorei*. However, regardless of phylogenetic classification, all pathogenic isolates require a tumor-inducing (Ti) plasmid to cause crown gall disease to plants (Christie and Gordon

2014). To infect plants, the bacteria require a wound, which releases attractants and provides ingress points. Perception of the attractant leads to the activation of *vir* genes on the plasmid, which execute a unique process of interkingdom gene transfer and genetic transformation of plants (Gelvin 2012). The *vir*-encoded protein products are necessary to process and translocate, via a type IV secretion system, T-DNA into plant cells. The T-DNA is a bordered segment of the plasmid that, upon transfer, leads to expression of genes that perturb plant hormone signaling and unregulated growth, manifested as crown galls, and cause the host to synthesize abundant levels of opines, nutrients for the infecting *Agrobacterium* spp.

Early detection is a critical component in managing *Agrobacterium*-caused diseases. Culturing *Agrobacterium* spp. from diseased tissues is inadequate for diagnosis, because nonpathogenic variants are widespread. Accepted methods include polymerase chain reaction (PCR) amplification of loci unique to the Ti plasmid directly from extracts of symptomatic plant tissue, or verifying the virulence of isolated bacteria. The *virD2* gene is the most conserved among pathogenic *Agrobacterium* spp. and was consequently advanced as a target for detection (Haas et al. 1995; Wang et al. 1990). *VirD2* nicks the T-DNA from the plasmid and pilots the resulting T-strand into the host nucleus (Herrera-Estrella et al. 1988, 1990; Young and Nester 1988). Due to the diversity of plasmid sequences, multiple sets of primer sequences have been designed, each with limited specificity (Bini et al. 2008; Haas et al. 1995; Johnson et al. 2013). For example, the primer sequences reported by Haas et al. (1995), though designed to what was identified, at the time, as the most conserved portion of the *virD2* gene, have limited use in detecting plasmids carried by *A. vitis* strains, commonly associated with infected grapevines (Bini et al. 2008).

Though PCR is widely accepted and used by diagnostic labs, the method nevertheless requires expertise and specialized equipment and can be time consuming, depending on the number of samples. Loop-mediated isothermal amplification (LAMP) eliminates reliance on equipment for thermocycling or visualizing amplified products and can produce results in 30 min (Notomi et al. 2000). Nonetheless, LAMP requires the ability to maintain a constant high

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*The e-Xtra logo stands for “electronic extra” and indicates that one supplementary figure is published online.

temperature and primers that anneal to six different regions of the target gene sequence, making it difficult to design LAMP for use against diverse alleles of a targeted gene.

Recombinase polymerase amplification (RPA) is an isothermal method that works at a constant low temperature using stable reaction components that do not require refrigeration (Piepenburg et al. 2006). This method has been used for on-site detection of a variety of pathogens that affect human, animal, and plant health (Hansen et al. 2016; Lau et al. 2017; Londoño et al. 2016; Moore and Jaykus 2017; Wang et al. 2016). RPA relies on a recombinase protein to mediate primer invasion of the target DNA, and continuous amplification takes place via strand-displacement synthesis. Additionally, modified oligonucleotides used in RPA can be coupled with a modified probe to allow for visual detection on a disposable lateral-flow strip. The lateral-flow strip has two antibodies to immobilize and detect the amplicon, which is dually labeled with fluorescein isothiocyanate and biotin. Results can be visualized in as little as 30 min.

Our goal was to develop a specific molecular detection assay to accelerate diagnostics of pathogenic *Agrobacterium* spp. primarily associated with hosts other than grapevine. We describe the application of RPA and a lateral-flow-based method for detecting pathogenic, crown-gall-causing *Agrobacterium* isolates. Primers and probes were designed to detect multiple alleles of *virD2* and, thus, are broadly applicable for detecting most pathogenic *Agrobacterium* spp. As expected, these new oligonucleotide tools accurately distinguished between a plasmid-carrying and a plasmid-lacking strain and, furthermore, detected all tested variants of *virD2*. We additionally demonstrate that the detection limit of RPA is 3.5 pg of DNA extracted from cultured bacteria. Finally, we highlight the rapidity of the method. DNA extracted from gall tissues and cultured bacteria can be added directly to the RPA reaction and *virD2* can be detected via the lateral-flow strip, dramatically reducing cost, time, and effort required for detection.

MATERIALS AND METHODS

Agrobacterium isolates. *Agrobacterium* spp. used in this study are described in Table 1. Bacteria were maintained on solid MGY (mannitol-glutamic acid-yeast) medium at 28°C or grown overnight at 28°C in MGY medium with shaking at 200 rpm (Keane et al. 1970).

Induction of crown galls on *Nicotiana benthamiana*. Crown galls were induced using the method previously described (Anand et al. 2007). Overnight cultures were pelleted and bacterial cells were washed with sterile water. The bacteria were resuspended to a concentration of 10⁹ CFU/ml in liquid *Agrobacterium* induction media (1/10 Murashige-Skoog basal salts; 2-*N*-morpholino-ethanesulfonic acid, pH 5.8, at 0.5 mg/ml; and 1% glucose) supplemented with acetosyringone (150 µg/ml) and placed at room temperature (24°C) for 14 to 16 h. The induced cultures were washed with sterile water and resuspended in 0.9% NaCl. Galls were induced on 4- to 6-week-old *Nicotiana benthamiana* by wounding the stems with a 22-gauge needle dipped in bacterial suspension. For a mock-inoculated control, plants were similarly wounded using a needle dipped in induction medium. Plants were maintained in a growth chamber with a daily cycle of 10 h of light and 14 h of darkness at 25°C.

Extraction of genomic DNA. *Agrobacterium* DNA was extracted using the DNeasy Blood and Tissue Kit per the manufacturer's instructions (Qiagen). Galls or mock-inoculated plant tissues were surface sterilized for 1 min in 95% ethanol, washed three times with sterile water, and cut finely with a sterile razor blade. Cut tissues were then transferred to a lysing matrix tube (Lysing Matrix A; MP Biomedicals) with 400 µl of cetyltrimethylammonium bromide buffer (100 mM Tris-Cl, pH 8.0; 20 mM EDTA, pH 8.0; 1.4 M NaCl; 2% [wt/vol] cetyltrimethylammonium bromide; and 1% polyvinyl pyrrolidone 40,000). The tube containing the plant tissue was homogenized at 6.0 m/s for 40 s on a Fast-Prep 24 (MP Biomedicals). The lysing matrix tube was then incubated in a heat block at 65°C for 10 min. The DNeasy Blood and Tissue Kit was used to extract DNA from the lysates (Qiagen).

A NanoDrop ND-1000 spectrophotometer (Thermo Scientific) was used to determine the quality and quantity of the extracted genomic DNA. Genomic DNA was diluted in sterile deionized water to a concentration of 35 ng/µl and serially diluted 10-fold from 35 ng/µl to 3.5 fg/µl.

Genome sequencing, assembly, and analyses. Total genomic DNA was used to prepare Nextera XT libraries, and the resulting multiplexed libraries were sequenced on an Illumina HiSeq 3000 to generate 150-mer paired-end sequencing reads (Center for Genome Research and Biocomputing [CGRB], Oregon State University). FastQC was used to assess sequencing reads for quality (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc/>). BBduk v.35.82, with the parameters “ktrim = rk = 23 mink = 9 hdist = 1 minlength = 100 tpe tbo”, was used to remove adapter sequences from the reads. SPAdes v. 3.1.1, with the parameters “-careful -k 21,33,55,77,99”, was used to correct errors and de novo assemble the reads (<https://sourceforge.net/projects/bbmap/>) (Bankevich et al. 2012). Blobtools was used to assess assemblies and, if necessary, guide elimination of contigs likely derived from contaminating bacteria (blobtools v0.9.19.6; <https://zenodo.org/record/439101#.WXeqMITyph>) (Kumar et al. 2013). Prokka was used to annotate the genome sequences (Seemann 2014).

The *virD2* sequence from *A. tumefaciens* C58 was used as a query in a BLASTN (version 2.5.0) search against the National Center for Biotechnology Information nr database, subset to *Rhizobium/Agrobacterium* (taxid:227290), using the autoMLSA package with relaxed settings (minimum coverage = 45%) (Davis et al. 2016). In total, 38 sequences were identified. The *virD2* sequence was also extracted from the genome sequence of *A. vitis* 80/94. MAFFT (version 6.864b) with default settings was used to align 39 total *virD2* sequences (Kato and Standley 2013). The *virD2* tree was generated using RAXML (version 8.1.17) and visualized in FigTree (version 1.4.3) (Stamatakis 2014). CLC Sequence Viewer (Qiagen) was used to align the *virD2* sequences and to identify the most conserved sequence regions.

PCR and RPA assays. Primers and probes were designed according to the instructions of the manufacturer (TwistDx Limited). Primer and probe sequences are provided in Table 2. The bottom-strand primer was modified to include a 5' biotin tag and probes included a 5' FAM (6-fluorescein amidite) group, a dSpacer, and a 3' C3-spacer.

Standard PCR consisted of 0.4 mM each primer, 2.5 mM dNTP, 1× ThermoPol buffer, 0.5 U of Taq DNA Polymerase (New England Biolabs), and 1.0 µl of genomic DNA template (of varying concentration) in a final volume of 25 µl. Cycling parameters were a

TABLE 1. *Agrobacterium* isolates used in this study^a

Isolate name	Accession number	Species	Opine type	Host	Reference
LMG215	NBZF00000000	<i>Agrobacterium tumefaciens</i>	n/a	Hop	This work
C58	GCA_000092025.1	<i>A. tumefaciens</i>	Nopaline	Cherry	Wood et al. 2001; Goodner et al. 2001
B131/95	GCA_001692155.1	<i>A. rhizogenes</i>	Nopaline	P/A root	Davis et al. 2016
N2/73	GCA_001692195.1	<i>A. tumefaciens</i>	Nopaline	Raspberry	Davis et al. 2016
B140/95	GCA_001692245.1	<i>A. tumefaciens</i>	Octopine/agropine	P/A root	Davis et al. 2016
15-1187-1-2a	GCA_001692265.1	<i>A. tumefaciens</i>	Nopaline/chrysopine	Yarrow	Davis et al. 2016
V80/94	NBZE00000000	<i>A. vitis</i>	Vitopine/nopaline	Grape	This work

^a Abbreviations: n/a = not available, Hop = common hop, and P/A root = peach/almond rootstock.

10-min denaturation at 94°C; followed by 30 cycles of 30 s at 94°C, 30 s at 55°C, and 90 s at 72°C; followed by 10 min at 72°C. Products were resolved on a 2.0% 1× Tris-acetate EDTA (TAE) agarose gel, stained with ethidium bromide, and visualized under UV light.

RPA was optimized following the manufacturer's instructions (TwistAmp Basic; TwistDx Limited). Reactions consisted of 0.48 µM each primer, 29.5 µl of rehydration buffer, 12.2 µl of water, and 1.0 µl of genomic DNA (of varying concentration). A volume of 2.5 µl of 280 mM magnesium acetate (MgAc) was added to initiate the reaction, which was subsequently incubated at temperatures ranging from 20 to 40°C. Reaction times of 1, 10, 20, 30, and 40 min were tested. The RPA products were purified using the QIAquick PCR purification kit (Qiagen), resolved on a 2.0% 1× TAE agarose gel, stained with ethidium bromide, and visualized under UV light.

Products from PCR and RPA were sequenced on an ABI 3730 DNA Analyzer (CGRB, Oregon State University). The PCR products were prepared using previously described methods (Creason et al. 2014). The RPA products were purified using the QIAquick PCR purification kit (Qiagen).

For RPA coupled to lateral-flow detection, reactions consisted of 0.42 µM top-strand primer, 0.42 µM biotin-labeled bottom-strand primer, 0.12 µM probe, 29.5 µl of rehydration buffer, 12.2 µl of water, and 1.0 µl of genomic DNA (of varying concentration). Reactions were vortexed briefly and then added to a freeze-dried pellet provided by the manufacturer (TwistAmp nfo; TwistDx Limited). The reaction was initiated by addition of 2.5 µl of 280 mM MgAc. Reactions were incubated at 37°C for 30 min. The dual-labeled amplicon was visualized using a lateral-flow dipstick (Milenia Biotec GmbH). The product (1 µl) was diluted in 49 µl of 1.0× phosphate-buffered saline with Tween (PBST) and 10 µl was applied to the base of the dipstick. The lateral-flow strip was subsequently submerged in 100 µl of 1.0× PBST and incubated at room temperature until appearance of the positive control band, typically within 2 min.

RESULTS

Design of oligonucleotides and selection of isolates for testing. The *virD2* gene is the most conserved gene of oncogenic plasmids and was previously developed for use in discriminating pathogenic *Agrobacterium* spp., making it a good marker for molecular diagnostics (Haas et al. 1995). To improve upon the use of *virD2* in discriminating more of the known pathogenic genotypes from nonpathogenic bacteria, we first generated a maximum-likelihood phylogenetic tree based on all publically available *virD2* sequences (Fig. 1). This set of sequences was augmented by the inclusion of the *virD2* sequence extracted from the genome sequence of *A. vitis* 80/94, determined herein. The 39 *virD2* sequences from the Ti plasmids formed five distinct clades and those from the root-inducing (Ri) plasmids, which are traditionally associated with hairy root disease, formed separate clades. The *virD2* sequence from *A. vitis* 80/94 grouped with other vitopine-catabolizing *A. vitis* strains of clade IV. We confirmed that the 39 *virD2* sequences accurately represented the diversity of the gene by comparing them to another approximately 130 *virD2* sequences (A. J. Weisberg and J. H. Chang, unpublished data).

As previously described, the *virD2* genes have a conserved region in the 5' end of the sequence that has been used as a target for amplification (Supplementary Figure S1) (Bini et al. 2008; Haas et al. 1995; Johnson et al. 2013). Because of the demonstrable utility of this region, we based our design of oligonucleotide reagents on the 5' end of *virD2*. We targeted three sequences within the first 500 bp of the 5' region and designed primer sequences to meet the requirements for use in RPA assays (Fig. 2A). The lateral-flow detection method requires an oligonucleotide probe that anneals internal to the amplification primers. To this end, we targeted two regions internal to the sequences for designing primers (Fig. 2A). These regions, however, were not as conserved in one clade of *A. vitis* Ti plasmids (Fig. 2B). Nonetheless, our focus is on isolates found on hosts other than grapevine. Therefore, we designed probe

Fig. 1. *virD2* genes of oncogenic tumor-inducing (Ti) plasmids of *Agrobacterium* spp. form distinct clades. Maximum-likelihood phylogenetic tree of *virD2* sequences from diverse *Agrobacterium* spp. In total, 39 sequences were identified using BLASTN, aligned, and used as input for tree generation. Isolates are distinguished based on host type (woody, herbaceous, grapevine, or lab strain) and opine catabolism. Clades of Ti plasmids are labeled. Isolates used for testing are boxed.

TABLE 2. Oligonucleotides used in this study

Name	Sequence (5' to 3')	Source
Haas_A	ATGCCCGATCGAGCTCAAGT	Haas et al. 1995
Haas_C	TCGTCTGGCTGACTTTCGTCATAA	Haas et al. 1995
VirD2_f1	GCAGCCGAGATGTTTGGG	This work
VirD2_f2	GCCAGACGAGGAAAGGC	This work
VirD2_r1	GGCGTCGTAATTCAGTTGGG	This work
VirD2_r1_Biotin	/5Biosg/GGCGTCGTAATTCAGTTGGG	This work
VirD2_r2	GCGATCGATGTGGAAGGC	This work
Probe 1	/56-FAM/ATGCCCGATCGAGCTCAAGTATTCGCG/idSp/TTGTGCCRGAGGTGG/3SpC3/	This work
Probe 2	/56-FAM/TTATGACGAAAGTCAGCCAGACGAGGAAAG/idSp/CAACAGGAGTTGACCACCC/3SpC3/	This work

1 to anneal to the 5' end of the amplified region and target the same region as primer Haas_A (Haas et al. 1995). To be more inclusive, probe 1 included degenerate sites. Probe 2 was designed to anneal central to the amplified region and is a derivative of Haas_C (Haas et al. 1995).

The phylogenetic tree was also used to guide the selection of a diverse set of isolates that represent each of the *virD2* clades for use in testing and optimizing the RPA assays (Fig. 1). Isolates representing multiple *Agrobacterium* spp. as well as different plasmid-encoded opine metabolism types were chosen from our collection. Host type from which isolates were obtained (herbaceous, woody, and grapevine) was also used as a selection criteria. Although not a direct target of our assay design, one grapevine isolate was included to test whether the use of the primers and probes could be extended. We also included in subsequent tests LMG215, a nonpathogenic isolate collected from *Humulus lupulus* (the common hop). We generated a draft genome sequence of LMG215 and identified it as a member of the *A. tumefaciens* species. Based on molecular markers, LMG215 clusters most closely to reference isolate *A. tumefaciens* strain B6 and, thus, is a member of genomospecies 4 (data not shown) (Costechareyre et al. 2010). Moreover, the de novo assembled genome sequence does not include a Ti or Ri plasmid. The absence of an oncogenic plasmid was verified by the failure of any sequencing reads from LMG215 in

aligning to any reference Ti or Ri plasmid sequence. LMG215 is used hereafter as a negative control in testing the specificity of molecular diagnostic tools.

Testing primers and probes. Six combinations of primers were tested using the RPA basic kit and purified genomic DNA from *A. tumefaciens* C58 at 35 ng/μl as a template, and compared with their efficacy in standard PCR. In RPA, four of the tested combinations yielded products of expected sizes (Fig. 2C). Sequence analysis of the products showed them to be identical to the targeted sequences of *virD2*. In standard PCR, all six pairs successfully amplified a product of expected size and were identical in sequence to their targeted region of *virD2* (Fig. 2D). The difference in results between RPA and PCR was not surprising and is consistent with the need to test primers in RPA basic, prior to advancing to use in RPA nfo. Additionally, regardless of the primer pair tested, no products were observed in reactions that lacked DNA or had genomic DNA from LMG215, the plasmid-lacking isolate (LMG215 data not shown). The primer pairs are specific to *virD2*-encoding and, thus, pathogenic *Agrobacterium* bacteria. Because primers Haas_A and VirD2_r1 best met recommendations of the manufacturer, all subsequent tests relied on the use of this pair of primers. VirD2_r1 was modified with the addition of a 5' biotin tag for lateral-flow analysis and is subsequently referred to as VirD2_r1_Biotin.

The efficacy of the probes was tested using the RPA nfo assay, and included primers Haas_A and VirD2_r1_Biotin and genomic DNA from *A. tumefaciens* C58 at 35 ng/μl. We optimized probe detection by testing combinations of duration of reaction (time) and temperature, which were varied but kept within manufacturer-recommended ranges. Temperature had a strong effect (Fig. 3). Reactions with either probe reliably amplified a product at 40°C in as little as 10 min, albeit with low signal intensity. In contrast, neither of the two probes yielded detectable products when the lowest temperature was used (20°C). Extending the time for amplification could mitigate some temperature effects. With incubation of 20 min at 37°C, both reactions yielded a prominent and visible product. Reactions that included probe 2 successfully amplified a product at a temperature as low as 30°C. Extending reaction times to 30 min allowed for reactions to work at a temperature as low as 25°C when using probe 2. In contrast, reactions with probe 1 were not successful at temperatures lower than 37°C, regardless of the duration of the reaction. Based on these comparisons, probe 2 appeared to be more robust than probe 1. In subsequent tests, a reaction condition of 37°C for 30 min was used.

Testing the detection limit of RPA. We determined the detection limit of lateral-flow strip-based detection of RPA.

Fig. 2. Oligonucleotide primers and probes were designed to the 5' region of *virD2*. **A**, Schematic of the 5' approximately 500 nucleotides of *virD2* and relative positions of designed oligonucleotide primers. **B**, Alignment of *virD2* sequences from tested *Agrobacterium* spp. with primer and probe sequences designed for use in recombinase polymerase amplification (RPA) coupled to lateral-flow detection. Shading indicates percent identity of the consensus. Inverted triangles indicate position of abasic sites in probes 1 and 2. **C**, Image of ethidium-bromide-stained gel with amplicons derived from the RPA basic assay (combinations indicated). **D**, Image of ethidium-bromide-stained gel with amplicons derived from standard polymerase chain reaction (PCR). For both RPA and standard PCR, in total, 35 ng of DNA from *Agrobacterium tumefaciens* C58 was used as a template (C58). Water and DNA from LMG215 (data not shown) were used as negative controls. Sizes of the amplified products are listed.

Fig. 3. Time and temperature were optimized for using recombinase polymerase amplification (RPA) nfo to detect *virD2* of *Agrobacterium tumefaciens* C58. Two probes were independently tested in RPA nfo using primers Haas_A and VirD2_r1_Biotin and 35 ng of genomic DNA from *A. tumefaciens* C58. Reactions were run at the indicated times (top) and temperatures (bottom) and assayed using lateral-flow strips. The “test” band indicates trapping of amplicons dually labeled with FAM (6-fluorescein amidite) and biotin, which are complexed with gold-labeled anti-FAM antibodies and captured via anti-biotin antibodies. Gold particles that are not complexed with the amplicon are captured by anti-rabbit antibodies at the “control” line, which serves as a control for flow of the strip. Reactions with sterile water were included for each probe and temperature tested and assessed for 40 min. Results were repeated three times with similar results.

Genomic DNA from *A. tumefaciens* C58 was serially 10-fold diluted and tested using primer sets Haas_A + VirD2_r1_Biotin with probe 1 or probe 2 (Fig. 4A). Results were compared with that of a standard end-point PCR of 35 cycles. RPA with either oligonucleotide set reliably detected a product from as little as 3.5 pg of input DNA. A product can be detected from as little as 3.5 fg of starting DNA but results were difficult to discern and more difficult to repeat (data not shown). In contrast, the use of end-point PCR could only yield a faintly detectable product if the amount of DNA was more than 35 pg; results were more reliable with 350 pg of input DNA. These data indicate that lateral-flow strip detection of the RPA assay, using either probe, has a detection limit 10 to 100× lower than that of traditional PCR methods at detecting *virD2* from a genomic DNA template.

Using aliquots of DNA from the same starting stock of *A. tumefaciens* C58 DNA, the total assay time of the RPA method was compared with that required for traditional PCR detection. The RPA method coupled with lateral-flow detection could detect *virD2* in as little as 30 min, whereas the traditional method, including thermocycling and resolving products via gel electrophoresis, took upwards of 3 h. This represents a substantial savings in time and cost for labor, especially if there is a large number of samples.

We next tested whether RPA could be used to detect *virD2* directly from liquid culture-grown bacterial cells of *A. tumefaciens* C58. Using serial dilutions of the bacterial suspension, we found that RPA was successful in yielding a product from as few as 10,000 untreated bacterial cells (Fig. 4B). We next asked whether the process could be expedited even further. Single colonies of bacteria were selected from an agar plate and either resuspended in 100 µl of water or added directly in the RPA reaction. For the former treatment, 1.0 µl of the resuspended bacteria was either immediately transferred to the RPA reaction or transferred after 30 min of boiling. A positive product was detected in each of the three cases (data not shown). The ability to confirm the presence of *virD2* directly from a bacterial colony represents an additional savings in time and costs associated with labor.

Testing the sensitivity of RPA. We tested the RPA nfo assay on a panel of *Agrobacterium* isolates that were selected based on criteria described above (Table 1). Both probes were tested on DNA extracted either from liquid culture-grown bacteria or galls

Fig. 4. Recombinase polymerase amplification (RPA) nfo probes have good limits of detection for *virD2* of *Agrobacterium tumefaciens* C58. **A**, Genomic DNA was resuspended at 35 ng/µl and serially diluted 10-fold. A volume of 1.0 µl from each concentration was tested; the dilutions are indicated. The genomic DNA was also amplified using standard end-point polymerase chain reaction of 35 cycles. Products were visualized on a 1% Tris-acetate EDTA agarose gel. The size of the products is indicated. **B**, Bacteria were grown overnight, concentrated to 1×10^{10} CFU/ml, and diluted 10-fold; the number of CFU, quantified based on optical density at 600 nm, are indicated. For both, the two probes were independently tested in RPA nfo, with primers Haas_A and VirD2_r1_Biotin. Reactions were done for 30 min at 37°C and assayed using lateral-flow strips. The “test” band indicates trapping of amplicons dually labeled with FAM (6-fluorescein amidite) and biotin, which are complexed with gold-labeled anti-FAM antibodies and captured via anti-biotin antibodies. Gold particles that are not complexed with the amplicon are captured by anti-rabbit antibodies at the “control” line, which serves as a control for flow of the strip. Results were repeated three times with similar results.

artificially induced on *N. benthamiana* (Fig. 5). For assays using DNA from artificially induced galls, the negative controls were DNA from plants that were mock inoculated or inoculated with nonpathogenic isolate LMG215. Concentration of DNA from galls varied from 110 to 450 ng/µl, and 1 and 5 µl of each DNA sample was tested. Neither of the oligonucleotide sets yielded a detectable product in reactions that included DNA from isolate LMG215 or from mock-inoculated plants. These results confirm the specificity not only of the amplification primers but also of the oligonucleotide probes. RPA with probe 1 showed far greater applicability and yielded products from all the tested pathogenic isolates, either from bacterial or gall DNA extracts, including *A. vitis* 80/94 (Fig. 5). All gall DNA extracts worked, regardless of volume or starting concentration. Despite showing superior robustness in previous optimization experiments, probe 2 proved less suitable in this application, being able to detect only three of the six tested *Agrobacterium* spp., with results being consistent between the volumes tested (Fig. 5). Each of these three detected isolates were isolated from woody hosts, carry plasmids that belong to clade V, and encode for nopaline metabolism (Fig. 1). The difference in efficacy was not surprising, considering the design of the two probes (Table 2).

DISCUSSION

Crown gall continues to be a significant problem for the nursery and fruit and tree nut industries. Here, we present a facile, rapid, and specific molecular diagnostic tool for detecting diverse phytopathogenic *Agrobacterium* spp.

One of our goals was to develop a molecular diagnostic tool that is generalizable and sufficient for detecting most, if not all, of the *Agrobacterium* spp. and Ti plasmid variants commonly encountered on plants other than grapevine. Representative *virD2* alleles, including approximately 130 generated in a separate study

Fig. 5. Recombinase polymerase amplification (RPA) nfo probes vary in their ability to detect *virD2* alleles of *Agrobacterium* spp. The two probes were independently tested in RPA nfo, using primers Haas_A and VirD2_r1_Biotin. In total, 35 ng of DNA extracted from culture-grown isolates of tumor-inducing (Ti) plasmid-carrying *Agrobacterium* spp. (C) or from infected galls (G) was tested. DNA from the Ti plasmid-negative strain LMG215 and mock-inoculated plants (M) were included as negative controls. Reactions were done for 30 min at 37°C and assayed using lateral-flow strips. The “test” band indicates trapping of amplicons dually labeled with FAM (6-fluorescein amidite) and biotin, which are complexed with gold-labeled anti-FAM antibodies and captured via anti-biotin antibodies. Gold particles that are not complexed with the amplicon are captured by anti-rabbit antibodies at the “control” line, which serves as a control for flow of the strip. Results were repeated twice with similar results.

(A. J. Weisberg and J. H. Chang, *unpublished data*), were used to guide the design of oligonucleotide tools. Primers Haas_A and VirD2_r1 showed the greatest applicability and could detect all the isolates tested (Fig. 5). Based on *in silico* analyses, it is predicted that the use of these two primers could be extended to detecting Ri plasmids as well as isolates with Ti plasmids that belong to clade I, which are currently not available in our collection (Fig. 1). The design of the probes was more challenging. For probe 1, we introduced two degenerate sites, which likely contributed to increasing the ability to detect a wide range of Ti plasmids. However, based on the high number of polymorphic sites in clade I of the *A. vitis* Ti plasmid, these pathogenic variants may not be detectable with this probe. Probe 2 could detect from lower amounts of input DNA but had lower utility and could only detect plasmids of clade V.

In addition to the applicability of this method for detecting most *Agrobacterium* spp., RPA offers advantages in being exceptionally rapid, with a good limit of detection. We could reliably detect *virD2* from as little as 3.5 pg of DNA. This level is comparable with that of quantitative PCR, without the need for specialized equipment (Johnson et al. 2013). Moreover, the DNA extraction or culturing steps could be circumvented and bacteria or DNA from gall tissue could be added directly to reactions. As few as 10,000 cells are necessary. For tests using DNA from gall tissues, we did not attempt to normalize the amount used because of the challenges in determining the fraction that is derived from the bacteria. Nonetheless, despite the likelihood that only a small fraction of the DNA was derived from *Agrobacterium* cells, RPA coupled to lateral flow could adequately detect *virD2*. Using DNA from bacteria and in direct comparison with the traditionally used process for PCR-based diagnostics, we estimate that the method described in this study saved 2 h and 40 min, providing a savings in time, effort, and cost.

However, there were some challenges in applying RPA and additional difficulties that have yet to be overcome. The design of oligonucleotide tools is restricted by the need to target three conserved regions that are within an amplifiable distance and, thus, knowledge of the genetic variation of the targeted sequence is required. Interestingly, we found that the buffer that was provided for use with the lateral-flow strips was unusable, because it consistently led to false positives when negative control RPA reactions were applied (data not shown). Using 1× PBST instead of the provided buffer solved this issue. Specific to *Agrobacterium* spp., there are documented cases in which plants showing symptoms of crown gall fail to yield culturable pathogenic *Agrobacterium* bacteria (Bélanger et al. 1995). It is possible that commonly used semiselective media are insufficient for supporting growth of all taxa of *Agrobacterium*. It is also likely that galls are devoid of or occupied by few plasmid-carrying bacteria, because opportunistic bacteria may have subsequently colonized, outcompeted, and displaced founder pathogenic genotypes. Finally, despite the depth of our sequencing effort, we can never exclude the possibility that yet-to-be discovered Ti plasmids with more divergent *virD2* sequences exist and evade currently used detection methods.

In summary, we analyzed a large dataset of diverse *virD2* sequences to design oligonucleotides for use in a coupled RPA and lateral-flow assay to detect pathogenic *Agrobacterium* spp. associated with plants other than grapevine. Assay conditions were optimized and we used isolates that represent most of the genetic diversity to demonstrate the developed tools to be efficacious with all parameters examined. The RPA method shows promise for use in the diagnostic laboratory setting and represents a significant step toward providing onsite detection methods that can be used by nonexperts and completed without specialized equipment.

ACKNOWLEDGMENTS

We thank H. Lederhos for her assistance and the members of the CGRB for their assistance with whole-genome and DNA sequencing. This work

was supported by the United States Department of Agriculture (USDA) National Institute of Food and Agriculture (NIFA) award 2014-51181-22384 to J. H. Chang and M. L. Putnam. E. A. Savory was supported by USDA-NIFA postdoctoral fellowship 2013-67012-21139. A. J. Weisberg is supported by USDA-NIFA postdoctoral fellowship 2017-67012-26126. Finally, we thank the Department of Botany and Plant Pathology for its support of S. L. Fuller and the computing infrastructure. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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