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ORIGINAL ARTICLE

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A novel and efficient method for identifying cotton leafroll dwarf virus infection in upland cotton (*Gossypium hirsutum*)

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Abstract

We report the development of a novel and more efficient, rapid, cost-effective and simple technique than current PCR-based identification methods for screening cotton (*Gossypium hirsutum*) plants for the presence of cotton leafroll dwarf virus (CLRDV). This protocol takes advantage of the PACE (PCR Allele Competitive Extension) system and uses PCR amplification of cDNA, coupled with sequence-specific fluorescent probes to differentiate between infected and uninfected cotton plants. This procedure has the potential for application in detection of other RNA viruses in a variety of other crops, by using primers specific for the RNA-dependent RNA polymerase (RdRP) gene and a widely conserved housekeeping gene in the host organism; in this case, the *G. hirsutum* polyubiquitin gene (GhUB).

KEYWORDS
assay development, cotton, cotton leafroll dwarf virus, KASP/PACE-PCR, viral detection, viral testing

1 | INTRODUCTION

Cultivated cotton (*Gossypium hirsutum* and *G. barbadense*) is the most produced natural fibre crop in the world. Cotton leafroll dwarf virus (CLRDV) is a viral pathogen that poses a serious threat to cotton production, specifically in Asia and South America (Distéfano et al., 2010), and more recently in the United State (Avelar et al., 2020). CLRDV is a causal agent of cotton blue disease and cotton leafroll dwarf disease, which results in the stunting and blistering of new leaves, as well as downward cupping of the leaves in susceptible cotton varieties (Cauquil & Vaissayre, 1971). It is a positive-sense, single-stranded RNA virus in the genus *Polerovirus* (family *Solemoviridae*), originating in South America and transmitted by aphids (Distéfano et al., 2010). Through piercing-sucking mouthparts, aphids simultaneously feed from the phloem and inject saliva into the plant, providing a rapid route to infect cotton (Ng & Perry, 2004). In addition, asexual reproduction in aphids can lead to large and rapid increases in infective vector populations, further adding to the efficiency of virus spread. Current methods for disease control include insecticide application and the destruction of weeds that may serve as offseason hosts to the virus and/or the vector.

Reverse transcription polymerase chain reaction (RT-PCR) is the prevailing method of CLRDV detection, but this technique is expensive and time-consuming and requires specialized equipment. With the discovery of a widespread infection of CLRDV in research fields at Texas A&M University during the summer of 2019 (Alabi et al., 2020), efforts were initiated to develop a more cost-effective, simple and efficient method to determine plant infection status. The new protocol involves the PACE (PCR Allele Competitive Extension) system, which uses sequence-specific amplification associated probes to assay for the presence of target nucleotide sequences.

PACE Assays (Pace & Probesure, 2020) utilize a primer mix, a master mix and a template (RNA, cDNA or DNA). Usually, there are two target-specific primers, each having a unique sequence that binds to the template for amplification by DNA polymerase, and one of two generic oligonucleotide tails that enable subsequent fluorescent reporting. These oligonucleotides are complementary to the oligos present in the mastermix that are associated with quenched fluorophores. As amplicons and mastermix oligos bind, the fluorophores are

unquenched that enables each de-quenched FAM or HEX fluorochrome to emit a signal. Primers, mastermix and sample are combined and undergo PCR using a standard thermocycler. Sequence-specific primers bind to the target nucleotide sequence and elongate, incorporating the tail oligonucleotides to each synthesized strand. As amplified oligonucleotides unquench increasing numbers of fluorophores in the mastermix, the fluorescent signals that are detected at the end of the PCR cycles also increase, similar to the KASP system described in Semagn et al. (2014). The FAM or HEX molecules are excited by light exposure in a PlateReader system, which then plots the fluorescence intensity for each of the two dyes (Figure 1).

By pairing primers specific to plant and virus genetic material, the plot output could be exploited to visually differentiate between infected and uninfected plants. The two groups (infected and uninfected plants) cluster distinctly from one another, simplifying classification between them. Using genes conserved amongst RNA viruses and plants for this type of assay would make it more widely usable for other crop species as well.

2 | RESULTS

First, primers that were capable of distinctly clustering uninfected versus infected plant materials were identified using the PACE system (Figure 2). The screened primers were designed to target a partial fragment of the CLRDV RdRP gene, based on AL674F/AL1407R primers published in Avelar et al. (2019), and the *G. hirsutum* housekeeping genes polyubiquitin gene and catalytic subunit of phosphatase 2A (Artico et al., 2010) (Table S1). It is important to note that these two genes are widely conserved across RNA viruses and the *Gossypium* genus, respectively. These primers were designed to only amplify either plant or viral sequences, and they correspond to a unique wavelength, such that upon fluorescent excitation and scanning, the uninfected samples would amplify along only one axis (either *x* or *y*, depending on which fluorophore was associated with the housekeeping primer), and the infected samples would amplify along both axes, because they contain both plant and viral cDNA.

For initial tests, known infected/RT-PCR positive ($n = 2$) and uninfected/RT-PCR negative ($n = 2$) cotton samples were used to assay primer effectiveness. Primers were designed using BatchPrimer3 v1.0 software (You et al., 2008), and screening assays were performed with thermocycler settings according to manufacturer recommendations. The best performing primer sets were assessed based on their ability to create distinct and separate clusters of infected versus uninfected samples along the proper axes (Figure 3). The primer sets that produced the tightest clusters were subjected to further testing on larger sample sizes, and eventually, the most efficient primer sets were chosen for all downstream analyses (Table S1). The results obtained with the most efficient primer pairs with nine uninfected and 12 infected samples are shown in Figure 4 (both corrected for cluster distinction and uncorrected).

To verify that the clusters resulted from differential amplification of cotton and CLRDV sequences, an assay that included plasmids carrying partial CLRDV RdRP sequence and devoid of plant RNA was ran. These provided a set of samples in which amplification would expectedly lead to signal along one axis only, namely, the axis opposite to the uninfected samples (Figure 5). These results indicated that the clusters emanated from target-specific amplification by the primer set.

Primer combinations and sequence-specific amplification assays were initially tested using a two-step RT-PCR method. The first step was first-strand cDNA synthesis, and the second step was PCR

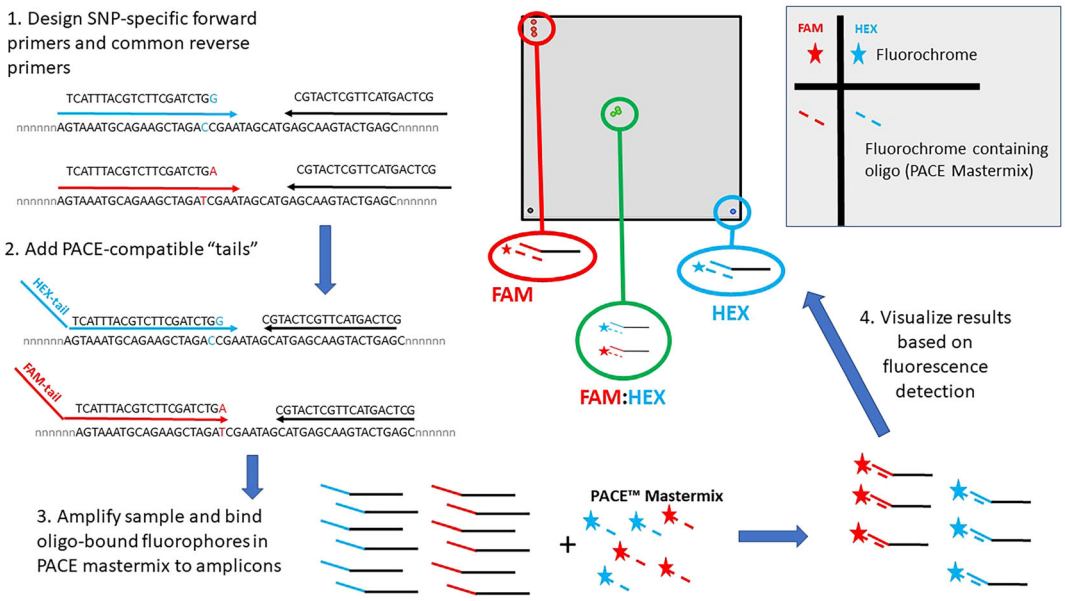

FIGURE 1 Step-by-step visual overview of canonical application of PCR allele competitive extension (PACE) assays for SNP (single nucleotide polymorphism) identification, often used in genotyping screens.

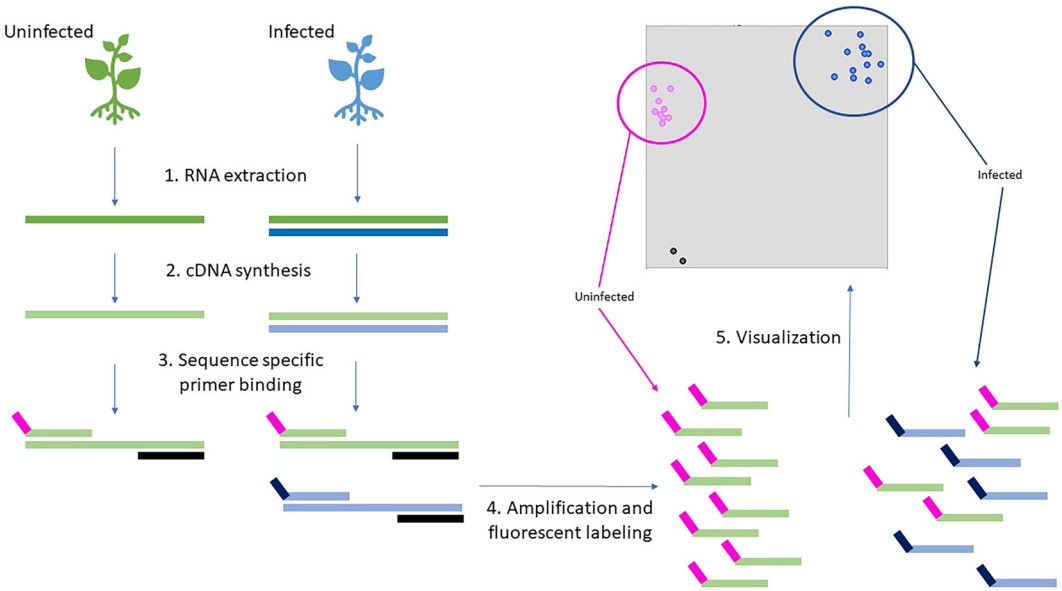

FIGURE 2 Conceptual layout of PCR allele competitive extension (PACE) system that would allow for cotton leafroll dwarf virus infection status detection in cotton. (1) Dark green lines represent plant RNA, whereas dark blue lines represent viral RNA. Both RNA templates are present in total RNA extracts from infected individuals. (2) The plant cDNA (light green lines) and viral cDNA (light blue line) were synthesized from the RNA template in the previous step. (3) Primers specific for plant (pink) and viral (navy blue) gene targets bind to the respective cDNA templates and start the amplification and fluorescent tagging process. (4) During the amplification phase, more and more fluorescently labelled DNA molecules are synthesized. (5) Visualization of fluorescence levels with a plate reader enables classification of relative strength of plant- and virus-specific signals, for example, where pink dots are uninfected samples, and blue dots are infected samples.

FIGURE 3 An example of three primer that clearly clustered infected (blue) and uninfected (pink) controls along appropriate axes (*y*-axis for uninfected plant tissue, and along both axes for infected plant tissue). Black dots represent no template controls (NTCs).

amplification using the PACE system to identify infected and uninfected clusters of samples.

This two-step method was used to screen 42 plants from two glasshouses for CLRDV infection. Some of these glasshouse cotton plants were brought in from the field where the virus was known to be present; these plants, however, had not been previously tested for CLRDV. Other samples collected from plants in proximity to plants brought in from the field were tested for CLRDV in this screen as well to determine if any transmission took place within the glasshouses. As depicted in Figure 6, the assay was successfully discerned between these unknown samples via visual scoring as infected (31) or uninfected (11). This shows the robustness of the assay for differentiating between infected and uninfected samples even when the number of samples in each group is highly variable. For example, in Figure 6, where there are many more infected than uninfected plants.

After establishing the PACE-PCR method for detection, the workflow was streamlined by developing a one-step method that was sufficiently robust and accurate for distinguishing between infected and uninfected tissue. The PACE-RT One Step RT-PCR Kit (3CR Bioscience) was used with thermocycler settings according to manufacturer recommendations. Repeatability was used to assess robustness; separate aliquots from each of the tested samples were included in each

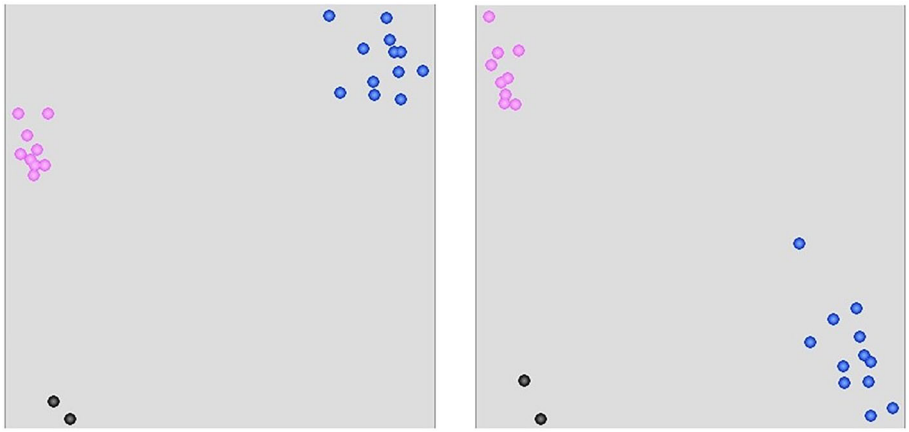

FIGURE 4 Two-step PCR allele competitive extension (PACE) assay results with the optimal working primer set. Nine uninfected (pink) and 12 infected samples (blue) were assayed in this experiment and both uncorrected (left) and Y-factor corrected (right) images are displayed. Adjustments for corrected image are normalization = 1.0, Y-factor correction = 1.0, X-factor correction = 0.22. This correction was made to elaborate visual distinction between infected and uninfected clusters and does not change the amplification status of any samples. Black dots represent no template controls (NTCs).

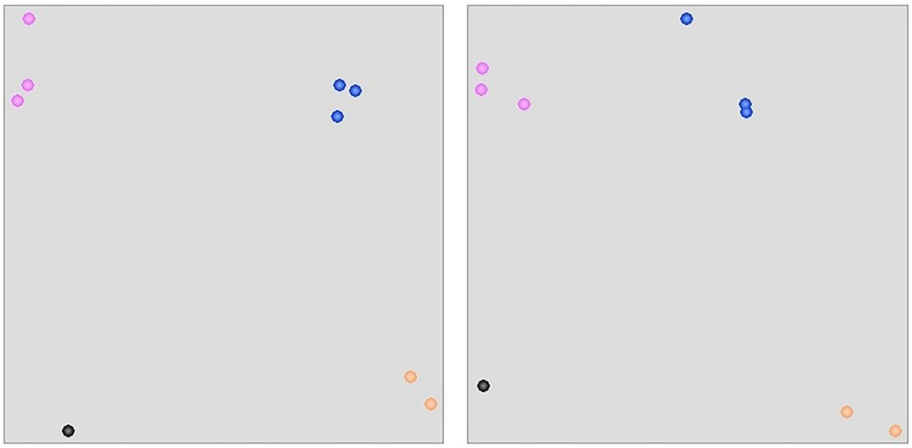

FIGURE 5 Two-step PCR allele competitive extension (PACE) assay results with three infected (blue), three uninfected (pink), two plasmid (orange), and one no template control (black) samples. Plasmid samples containing only viral sequences were expected to amplify along the *x*-axis, uninfected samples containing only plant sequences were expected to amplify only along the *y*-axis, and infected samples containing both types of sequences were expected to amplify along both axes. The results were each replicated once to confirm clustering vis-à-vis left and right plots.

of five separate assay runs. In each case, the infected and uninfected samples were consistently separated into two clusters along the expected axes (Figure 7). This one-step protocol significantly reduced the amount of time required to complete virus testing for each plant from ~4 to ~2.5 h and brought the cost of screening isolated RNA down to 0.16 USD per sample from 3.53 USD, that is, about a 20-fold cost savings for consumables. This efficient, rapid, cost-effective and simple testing method is likely to become useful to test for cotton viral infections and RNA virus presence in other crop species as well.

A one-way analysis of variance of the X-coordinate location against the infection status differs significantly ($\alpha = .05$, *p*-value < .0001) between infected and uninfected controls in the one-step reaction, and differences between uninfected and NTC samples were insignificant (Figure 8). When the same analysis was performed with the Y coordinates, there were only significant differences between the isolated controls (both infected and uninfected) and the NTC samples. This indicated that there was significant amplification along the *y*-axis in the case of both types of samples, which is to be expected as both samples contain plant cDNA. The Y coordinates analysis shows that the assay amplified the cDNA samples successfully without any indication of random amplification as shown in the NTC samples differing significantly from others in the Y-direction. The analysis of the X coordinates revealed that, although the visual plots for the one-step assay are not as tightly clustered as those for the two-step assay, the infected and uninfected individuals can be easily identified based solely on their amplification in the X-direction.

3 | DISCUSSION

This newly developed PACE assay makes the identification of CLRDV infection more efficient, rapid, cost-effective and simpler than current assays with equally robust and reproducible results. By taking advantage of the PACE system's ability to create distinct clusters based on specific primer-based amplification and fluorophore excitation, a method of visual detection with straightforward analysis was developed. Simplicity is critical, especially for a disease that has proven itself to be sporadically asymptomatic (Huseth, 2019).

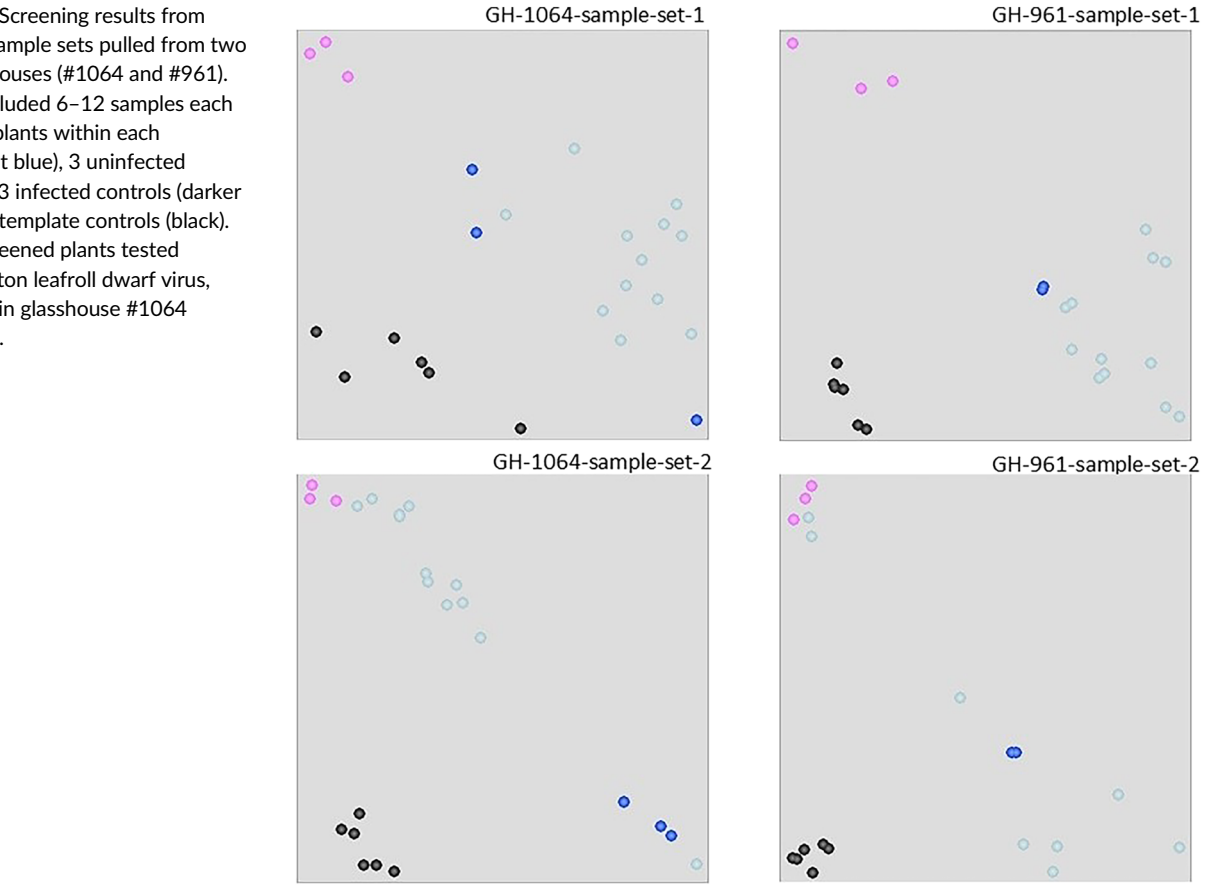

FIGURE 6 Screening results from four different sample sets pulled from two different glasshouses (#1064 and #961). Each screen included 6–12 samples each from different plants within each glasshouse (light blue), 3 uninfected controls (pink), 3 infected controls (darker blue), and 6 no template controls (black). Most of the screened plants tested positive for cotton leafroll dwarf virus, although some in glasshouse #1064 tested negative.

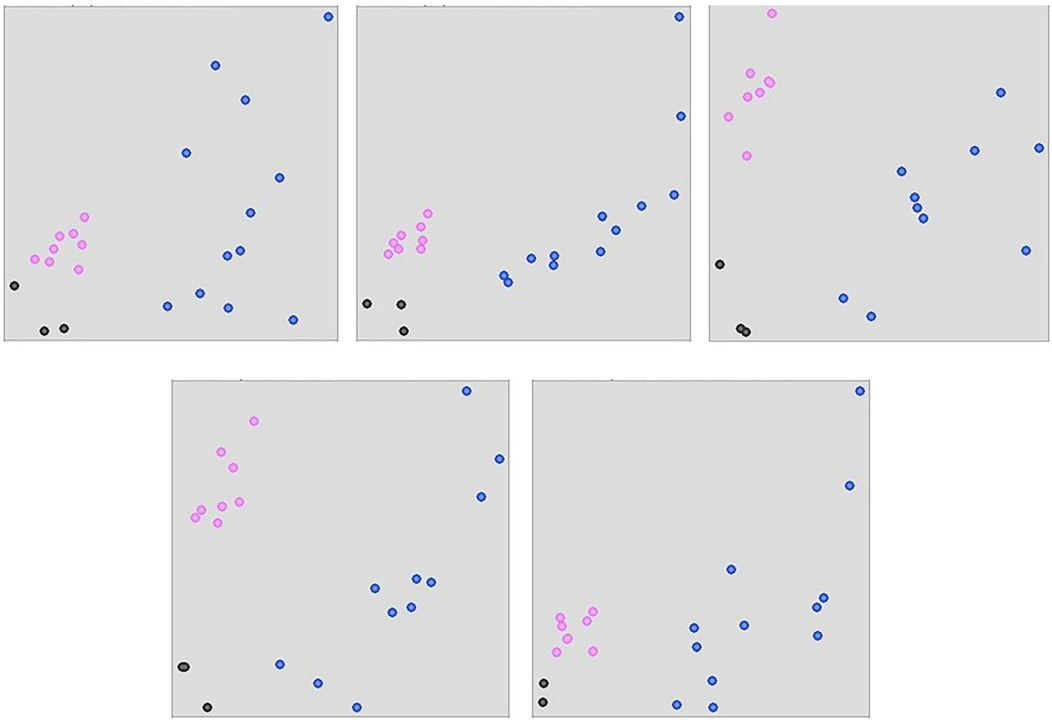

FIGURE 7 Five replicated trials using one-step reverse transcription PCR allele competitive extension (PACE) assay for CLRDV detection. The assay was performed with RNA samples from 7–8 uninfected (pink) and 11–12 infected (blue) cotton plants. KlusterCaller adjustment settings for visualization are normalization = 1.0, Y-factor correction = 1.0, X-factor correction = 0.22–0.26.

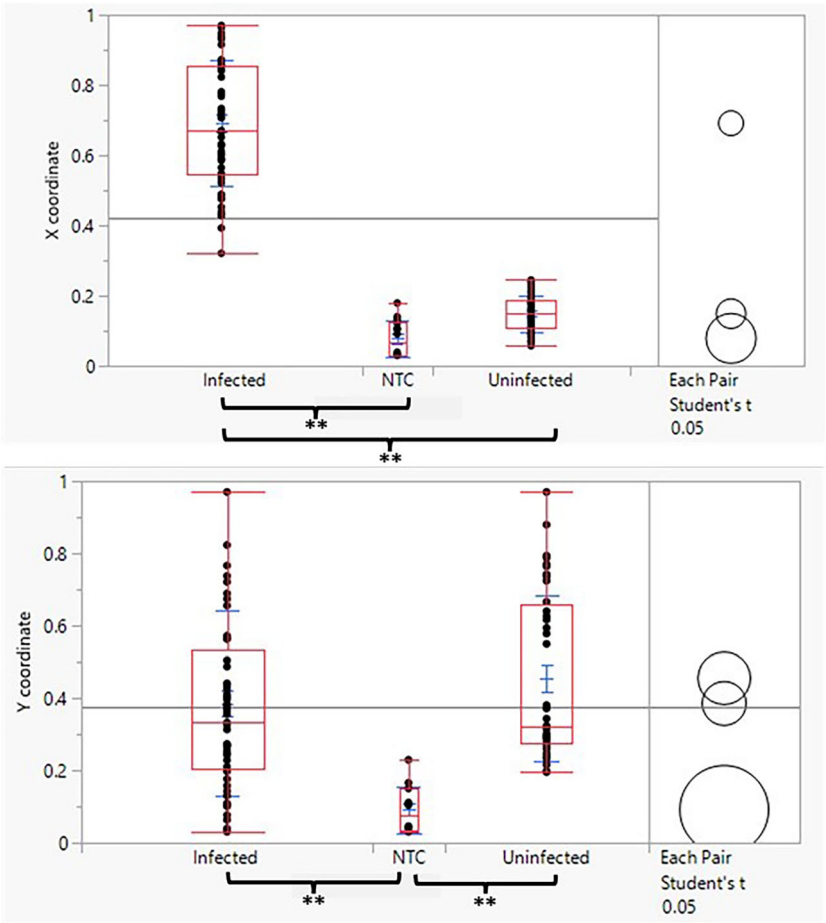

FIGURE 8 One-step reverse transcription PCR allele competitive extension (PACE) assay reaction statistics over the course of all five replicates with the same set of known controls. Significantly different groups are denoted with asterisks. Note specifically that there are significant differences in *x*-axis amplification between infected and uninfected individuals, as well as infected individuals and NTCs. And for *y*-axis amplification, only the NTC samples were significantly different from the other two groups.

Inconsistency in the presence and type of symptoms has made CLRDV infections in the southern United States challenging to diagnose in the field and thus difficult to contain. The assay reported in this study was developed to address this challenge and enhance the identification of CLRDV infection in cultivated cotton.

Both two-step and one-step methodologies differentiate infected and uninfected cotton plants efficiently and effectively. Although the two-step assay takes longer (1.5 h longer) and is also more expensive (~3.37 USD more per sample) than the one-step option, the clusters displayed for scoring are tighter and easier to identify (Figure 9). The one-step assay is faster and cheaper but produces clusters that are not as tight as those in the two-step. Although still possible to distinguish visually, one-step results can be confirmed through statistical analysis (Figure 8).

Assay accuracy was evaluated through several rounds of testing with controls that were known to be infected or uninfected with CLRDV. A plasmid cloned with the partial CLRDV-RdRp gene was also able to show that the primers amplify along the expected axes based on their target sequences and corresponding fluorescent tags. The two-step assay was also tested in a glasshouse screen that allowed determination of the infection statuses of otherwise untested plants.

During the glasshouse experiments, some differences were observed between glasshouse levels of infection, despite all screened glasshouses containing plants from the field where infection was first identified. Two possibilities may explain this finding: Some plants that were brought in were not infected, and therefore, there was no infection found in the glasshouses in which those plants reside. Alternatively, different insect population levels within each glasshouse may be the cause of this difference in infection rate, because aphids are the known vector for CLRDV. Being that several individual cotton plants 'resident' to glasshouses (having not come in contact with the field) were identified as CLRDV-positive, it is possible that within-glasshouse transmission of CLRDV resulted in varying numbers of infected and uninfected individuals in each glasshouse screen.

This method of viral infection detection is not limited to applications in cotton. Other crop plants threatened by RNA viral infections detrimental to overall production could benefit from screening using this assay, including those such as turnip yellows virus and chickpea stunt virus, to name two with highly similar RdRP sequences to CLRDV. And although these two viruses share sequence similarity with the RdRp gene we target in our assay, we are confident in our case that we detected CLRDV due to typical symptoms of the disease identified in the field and that at present, there are no reports of either of the other viruses mentioned here infecting cotton. This assay is also likely amenable to other regions of the RdRP gene, other viral genes altogether, as well as DNA virus detection.

Being that the RdRP is a highly conserved gene with an essential function in viral replication (Hengxia & Peng, 2019), thus

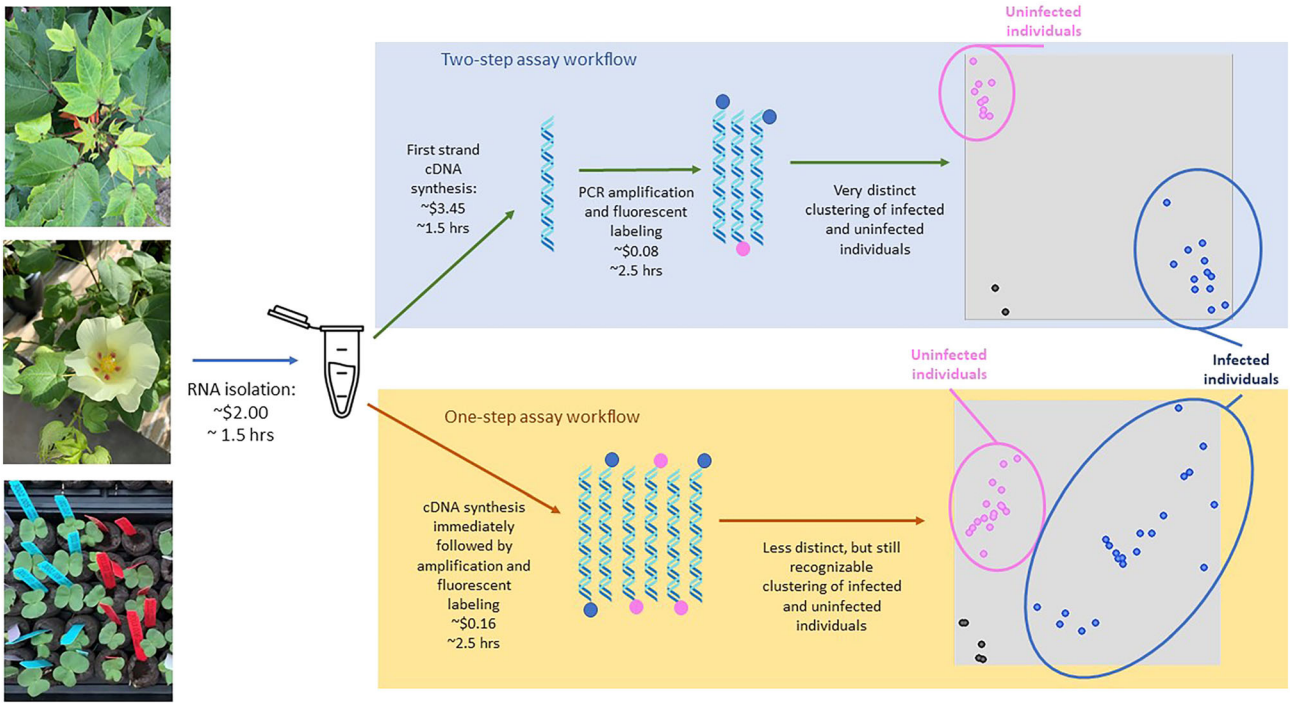

FIGURE 9 Comparison between time and financial costs of one- versus two-step PCR allele competitive extension (PACE) assays described in this report.

infection success, it is a very good target for this assay. It's inter-virus sequence conservation and importance to infection both imply that mutations in this gene are less likely to occur at a level that would affect the efficiency of this assay. This also provides our assay, as it is currently designed, with specificity to detection of RNA viruses. Thus, the primers designed in this study for PACE assay detection of CLRDV would potentially detect all currently known genetic variants of the virus (Ramos-Sobrinho et al., 2021). Preliminary data (not shown) using imperfect sequence matches to RdRP-targeting primers used in this assay show that these primers are tolerant to few base pair changes (<5), but not to more than 10 changes. The mutation rates of RNA viruses are typically high (Sanjuán et al., 2010), but by targeting more highly conserved genes using primers that are tolerant to minimal mismatching, this assay should remain useful to breeders and researchers alike in the coming future of this virus.

4 | EXPERIMENTAL PROCEDURES

4.1 | Plant material

Infected controls used in this study were the same as those tested positive for CLRDV by RT-PCR, followed by Sanger sequencing confirmation, by Alabi et al. (2020). Original identification of CLRDV in the field was deduced by typical symptoms of this specific disease, including leaf distortion, upward leaf cupping, shortened internodes and dwarfing. These plants were taken into the glasshouse post-field season and maintained under normal growth conditions and periodically cut back to promote new growth for RNA extraction. One young leaf and a quarter of a mature leaf were collected from the same plant for RNA isolation. Uninfected controls are lab-grown TM-1 x 3-79 F1 total RNA, extracted from half of a fully expanded (~2-week-old) cotyledon per sample.

4.2 | RNA extraction

Total RNA was extracted from each sample using the OPS Synergy™ 2.0 Plant DNA Extraction Kit (OPS Diagnostics, Lebanon, NJ, USA), with some modifications. The 500 µL of homogenization buffer was supplemented with 7 µL of DTT before grinding samples and the RNase A treatment was omitted. The RNA was eluted with 50 µL Molecular Biology Grade Water and quantified using a DeNovix DS-11 Spectrophotometer (DeNovix Inc., Wilmington, DE, USA) with concentrations normally ranging from 600 to 1200 ng/µL and 260/280 nm absorbance ratios between 1.93 and 2.05. The RNA samples were stored at −80°C when not in use.

4.3 | Two-step: cDNA synthesis

Complementary DNA (cDNA) was synthesized using the igScript™ First- Strand cDNA Synthesis Kit (Intact Genomics Inc., St. Louis, MO, USA) under conditions specified by the manufacturer. A 1 µL aliquot of the stock RNA per sample was used for cDNA synthesis.

4.4 | Two-step: PACE assay amplification

PACE assays were performed according to the manufacturer's recommendations. Briefly, the reaction mixture consisted of 1 μL of the stock first-strand cDNA, 3 μL nuclease-free H_2O, and 4 μL PACE Assay Mix including primers. These were ran in 96- or 384-well plates, depending on number of samples and controls in each experiment.

4.5 | One-step PACE-RT assay

The PACE-RT One Step RT-PCR Kit (3CR Bioscience) was used for this experiment, and sample preparation and thermocycler settings were performed according to manufacturer recommendations. This experiment works best when performed in a 384-well plate to maintain temperature consistency across the small reaction volume. Reaction mix should also be made fresh for every experiment.

4.6 | Data analysis using fluorescent plate reader visualization software

Each reaction plate was scanned using a PHERAstarPlus Fluorescent Plate Reader (BMG LabTech, Cary, NC, USA). Visual analysis was performed using KlusterCaller (LGC Biosearch Technologies, Teddington, Middlesex, UK). It is important to denote empty wells in the program, or else, results will be difficult to interpret. If the NTC samples amplify in any direction, the plate was likely contaminated while it was being loaded, and the results are unreliable.

Although not necessary for clear two-step result visualization, for the one-step assay, it is strongly recommended to correct for a lack of *x*-axis control using the visualization options in KlusterCaller™. In this study, one-step assays were visualized with an X-factor correction of 0.22–0.26, and a Y-factor correction of 1.0. Changing this setting does not alter the amplification status of the samples, but it only changes the view of the plot on the screen to account for empty space along the *x*-axis because there should not be any points that fall exclusively along that axis (Figure 5).

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CONFLICT OF INTEREST

Co-authors Nisha Jain and John E. Holme are employees of 3CR Bioscience Ltd. All other authors declare that they have no conflicts of interest.

AUTHOR CONTRIBUTIONS

SMT and BLC performed experiments and data collection and analysis in this study. RNV designed the experiment originally and created the initial primers used to start the study. OJA and DMS provided advice regarding viral and plant biology, as well as tools that were integral to the success of this project. JEH and NJ provided materials to perform experiments and troubleshooting advise for said materials.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author, SMT, upon reasonable request.

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REFERENCES

Alabi, O. J., Isakeit, T., Vaughn, R., Stelly, D., Conner, K. N., Gaytán, B. C., & Brown, J. K. (2020). First report of cotton leafroll dwarf virus infecting upland cotton (*Gossypium hirsutum*) in Texas. *Plant Disease*, *104*(3), 998. https://doi.org/10.1094/pdis-09-19-2008-pdn

Artico, S., Nardeli, S. M., Brilhante, O., Grossi-de-Sa, M. F., & Alves-Ferreira, M. (2010). Identification and evaluation of new reference genes in *Gossypium hirsutum* for accurate normalization of real-time quantitative RT-PCR data. *BMC Plant Biology*, *10*, 49. https://doi.org/10.1186/1471-2229-10-49

Avelar, S., Ramos-Sobrinho, R., Conner, K., Nichols, R. L., Lawrence, K., & Brown, J. K. (2020). Characterization of the complete genome and P0 protein for a previously unreported genotype of cotton leafroll dwarf virus, an introduced polerovirus in the USA. *Plant Disease*, *104*, 780–786. https://doi.org/10.1094/PDIS-06-19-1316-RE

Avelar, S., Schrimsher, D. W., Lawrence, K., & Brown, J. K. (2019). First report of cotton leafroll dwarf virus associated with cotton blue disease symptoms in Alabama. *Plant Disease*, *103*(3), 592–592. https://doi.org/10.1094/PDIS-09-18-1550-PDN

Cauquil, J., & Vaissayre, M. (1971). La maladie bleue du cotonnier en Afrique: transmission de cotonnier à cotonnier par *Aphis gossypii* Glover. *Coton et Fibres Tropicales*, *26*, 463–466.

Distéfano, A. J., Bonacic Kresic, I., & Hopp, H. E. (2010). The complete genome sequence of a virus associated with cotton blue disease, cotton leafroll dwarf virus, confirms that it is a new member of the genus Polerovirus. *Archives of Virology*, *155*(11), 1849–1854. https://doi.org/10.1007/s00705-010-0764-3 Epub 2010 Jul 31. PMID: 20677026.

Hengxia, J., & Peng, G. (2019). A structure-function diversity survey of the RNA-dependent RNA polymerases from the positive-strand RNA viruses. *Frontiers in Microbiology*, *10*, 1945. https://doi.org/10.3389/fmicb.2019.01945

Huseth, A., (2019). Detection of cotton leafroll dwarf virus (CLRDV) in North Carolina. NC State Extension News. Retrieved November 3, 2021, from https://cotton.ces.ncsu.edu/2019/12/detection-of-cotton-leafroll-dwarf-virus-clrdv-in-north-carolina/

Ng, J. C. K., & Perry, K. L. (2004). Transmission of plant viruses by aphid vectors. *Molecular Plant Pathology*, *5*, 505–511. https://doi.org/10.1111/j.1364-3703.2004.00240.x

Pace & Probesure. - Kasp™ & Taqman® master mix alternatives at unbeatable prices. 3CR Bioscience. (2020, January 22). Retrieved November 1, 2021, from https://3crbio.com/products/information/

Ramos-Sobrinho, R., Adegbola, R. O., Lawrence, K., Schrimsher, D. W., Isakeit, T., Alabi, O. J., & Brown, J. K. (2021). Cotton leafroll dwarf virus US genomes comprise divergent subpopulations and harbor extensive variability. *Viruses*, *13*(11), 2230. https://doi.org/10.3390/v13112230

Sanjuán, R., Nebot, M. R., Chirico, N., Mansky, L. M., & Belshaw, R. (2010). Viral mutation rates. *Journal of Virology*, *84*(19), 9733–9748. https://doi.org/10.1128/JVI.00694-10

Semagn, K., Babu, R., Hearne, S., & Olsen, M. (2014). Single nucleotide polymorphism genotyping using Kompetitive allele specific PCR (KASP): Overview of the technology and its application in crop improvement. *Molecular Breeding*, *33*, 1–14. https://doi.org/10.1007/s11032-013-9917-x

You, F. M., Huo, N., Gu, Y. Q., Luo, M.-c., Ma, Y., Hane, D., Lazo, G. R., Dvorak, J., & Anderson, O. D. (2008). BatchPrimer3: A high throughput web application for PCR and sequencing primer designi. *BMC Bioinformatics*, *9*, 253. https://doi.org/10.1186/1471-2105-9-253

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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