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Recombinase Polymerase Amplification and Their Application in Phytopathogen Detection

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***Abstract*— DNA identification method is indispensable for the detection of a plant pathogen. However, established techniques, though reliable, requires advanced equipment, and their application outside specialized laboratories is limited. Along with the advancement of molecular techniques, several isothermal amplification methods, including Recombinase Polymerase Amplification (RPA), has been developed in this study. In fact, RPA is a rapid and sensitive amplification method, operating optimally at 37-42℃ for 15 to 30 minutes with minimal sample preparation, and can amplify as low as 1-10 target copies. Furthermore, RPA has been a favourable method for the detection of plant pathogens due to its advantageous parameters. This review presents the current knowledge of RPA and its application in plant pathogen detection.**

***Keywords*— isothermal amplification; recombinase polymerase amplification (RPA); plant pathogens detection.**

I. INTRODUCTION

For decades, molecular diagnostic methods have enabled plant pathogen detection to become more rapid, specific, and sensitive. Since the discovery of polymerase chain reaction (PCR) in the 1980s, various development and adaptation have been reported, including quantitative PCR (qPCR). While perfect for specialized laboratories with skilled operators, there are still limitations to these methods, especially in resource-limited locations, as they require costly electrically powered equipment and strict thermal cycling conditions.

In recent years, there has been an increase in the advancement and adaptation of isothermal amplification methods [1]. As a result, several methods with different enzymatic mechanisms have been developed, including loop-mediated isothermal amplification [2], helicase-dependent amplification [3], rolling-circle amplification [4] and recombinase polymerase amplification [5].

Developed in 2000, loop-mediated isothermal amplification (LAMP) is a rapid and sensitive isothermal amplification method that operates between 60-70℃. It has equal sensitivity to the established method like PCR and can produce up to 109 copies of DNA within 30-60 minutes [1,6]. LAMP is compatible with various detection methods, including fluorescence, electrochemical, chemiluminescence,

colorimetric, and turbidimetric detection methods. However, LAMP products are not suitable for gene cloning and sequencing due to the mixture of DNA molecules of different sizes [2,7]. The limitation then is overcome by the addition of two additional steps of LAMP product digestion and primer extension [8]. LAMP reaction design is also complicated for the non-specialist as it requires a minimum of four target-specific primers that bind to six regions. The use of multiple primers also increases the risk primer-primer interaction [2,7].

Helicase-dependent amplification (HDA) is an alternative isothermal amplification method that is very similar to PCR except for the heat denaturation as it employs the use of DNA helicase to unwind double-stranded DNA (dsDNA) [7]. Originally operating at 37℃, it was modified to operate at 65℃ using thermostable helicase and DNA polymerase to increase the efficiency and specificity while simplifying the reagent component [1,3]. Similar to LAMP, HDA amplicons can be detected using various methods, including gel electrophoresis, fluorescence, electrochemical and lateral flow. Despite the simplicity of HDA, HDA reaction is prone to non-specific amplification, and thorough optimization and screening of primers are necessary to avoid false-positive and false-negative results [9].

Rolling-circle amplification (RCA) is a highly specific and sensitive isothermal amplification method developed in the 1990s. It requires small single-stranded circular DNA as a template which can be achieved using special oligonucleotide padlock probes (PLP) [1]. Employing the use of a single short primer, it produces long single-stranded DNA or RNA molecules with multiple repetitive units that correspond to the circular DNA template at 37℃ [4]. Like other nucleic acid amplification methods, RCA products can be hybridized to oligonucleotides with various functional groups for various detection strategies [7].

In fact, recombinase polymerase amplification (RPA), operating at low and constant temperature for a short incubation time, is a rapid isothermal amplification method. It has the sensitivity that equals established techniques like PCR and is tolerant towards PCR inhibitors and impurities. Aside from the conventional gel electrophoresis method, RPA has been successfully coupled with various detection strategies, including the real-time fluorescent method and lateral flow assay [10]. This review focuses on current knowledge of RPA regarding plant pathogen detection and its application.

II. RPA MECHANISM

Developed in 2006 by Piepenburg et al., [11], Recombinase Polymerase Amplification (RPA) makes use of protein molecules involved in cellular DNA synthesis, recombination and repair. As described in Figure 1, RPA reaction begins when uvsX recombinase protein forms a recombinase-primer complex with oligonucleotide primers [11]. Unlike several amplification methods, an initial DNA denaturation step is not required for the primers to anneal to the complementary target sequence. The complex instead scans the double-stranded target DNA for homologous sequence and promotes strand exchange at cognate sites [12–14]. The resulting D-loop structure is stabilized by single-stranded DNA binding proteins (SSB), thus preventing primer ejection. As recombinase dissembles from the strand, strand displacing DNA polymerase gains access to the 3’ end of the oligonucleotide, resulting in primer elongation [11,15]. A continuous cycle of RPA leads to exponential amplification of target DNA and stops once the reagents and phosphocreatine pool depletes [16].

As RPA employs the use of enzymes instead of depending on the cyclic temperature to break and anneal the bonds between nucleotides, RPA reaction can take place at a low and constant temperature; 25 to 42℃. This consequently enables RPA reaction time to be as short as 15 to 30 minutes [5]. Furthermore, RPA primer design is also simple. Though it requires a longer primer length, 30 to 35 bases, the reaction is independent of primer melting temperature. Furthermore, several reports have reported on efficient amplification using normal PCR primers [17,18].

Besides, RPA reactions can also be coupled with probes for different detection strategies. Figure 2B describes a typical RPA reaction using an exo probe. The basic tetrahydrofuran (THF) residue is cleaved by exonuclease III (included in the kit) upon probe annealing to its target. Fluorophore and quencher are separated from each other, thus generating a fluorescent signal. In Figure 2C, nuclease formamide pyrimidine DNA glycosylates (fpg) cleaves the fpg probe at the dR group site, thus releasing the fluorophore from the probe to generate fluorescence. Figure 2A shows an RPA reaction coupled with a nfo probe for lateral flow detection. In A, THF residue is cleaved by Endonuclease IV (nfo) upon hybridization of nfo probe to target DNA, thus releasing polymerase extension blocking group at the 3’ end of the probe and triggering polymerase extension. Figure 2B shows the capture of both labels attached to RPA products by anti-labels fixed to lateral flow strip. The capture of both labels indicates a positive amplification reaction [19]. Further details on probe design are explained in the RPA detection method section.

III. APPLICATION IN PLANT PATHOGEN DETECTION

Since its introduction, Recombinase Polymerase Amplification (RPA) has been successfully used in various fields of studies; health science [20-21], agriculture [22-23], and food science [24-25]. There have been approximately seventy publications of RPA focusing on phytopathogen detection.

A. Primer

According to the manufacturer’s manual guideline, [19] RPA primers should be 30 to 35 bases long. Shorter primers will still function; however, they will have slower amplification kinetics. On the other hand, longer primers will increase the possibility of secondary structure formation that will lead to primer noises [19]. However, several studies have reported that normal polymerase chain reaction (PCR) primer length can be used efficiently [17,18]. As with PCR primers, sequences that promote secondary structures, hairpin and primer-primer interaction should be avoided when designing RPA primers. Though the melting point is not a critical point of RPA primer, GC content below 30% or above 70% is not recommended.

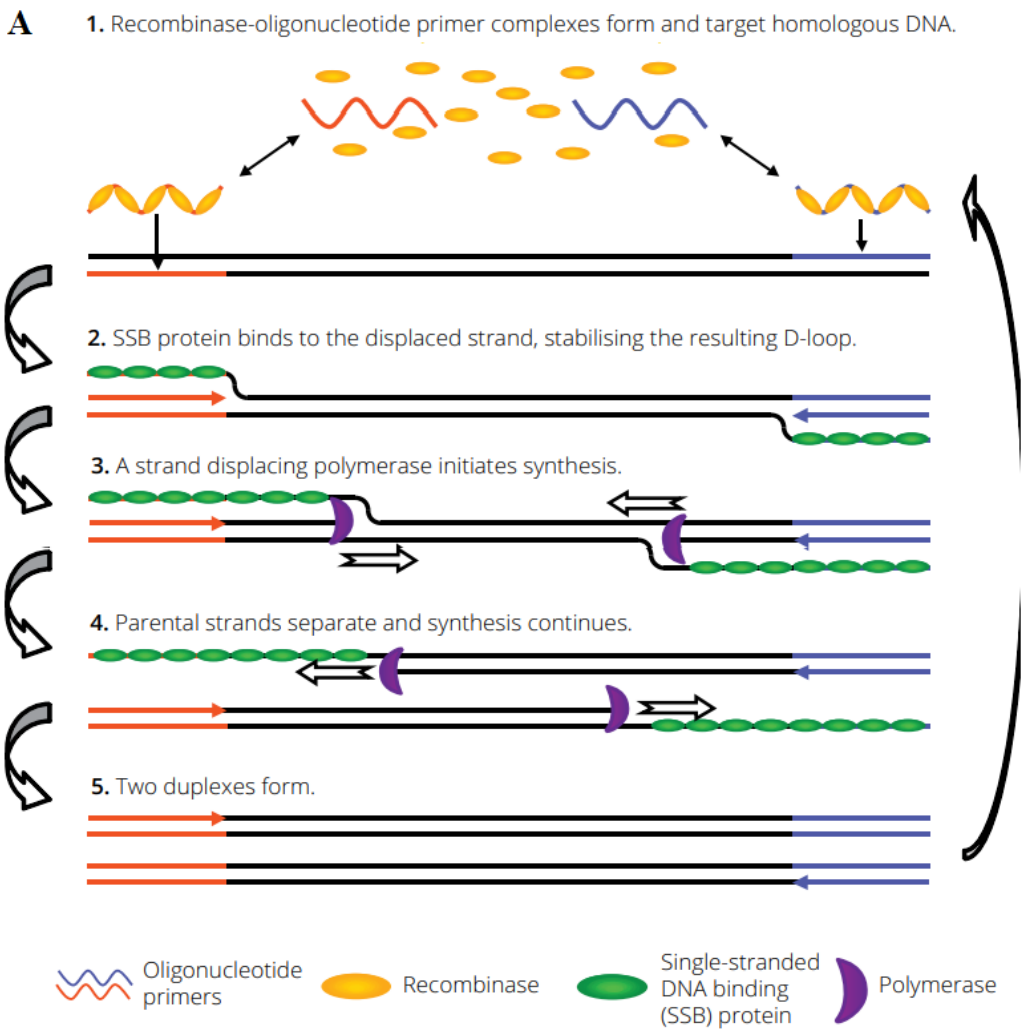

Fig.1. Schematic diagram of RPA reaction mechanism. Adapted from TwistAmp® instruction manual [19].

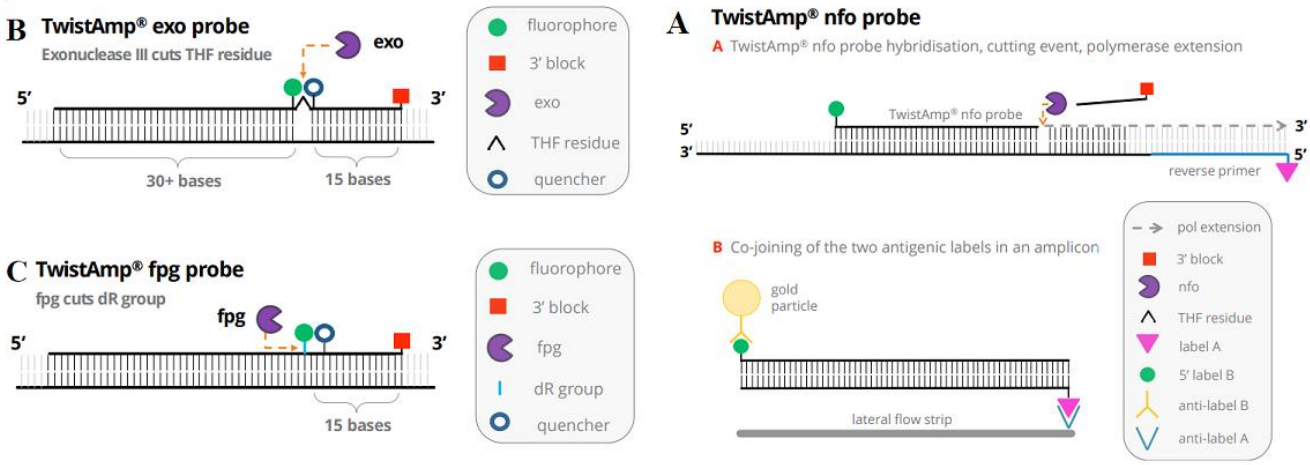

Fig.2. Schematic diagram of RPA reaction mechanism. A) nfo probe, B) exo probe and C) fpg probe. THF = tetrahydrofuran, dR = deoxyribose. Adapted from TwistAmp® instruction manual [19].

B. Temperature and Time

The application of RPA is significantly more prominent in field-based or point-of-care settings because of its low and constant reaction temperature and rapid incubation time. The temperature and time in most published reports are optimized for temperatures between 37 and 42℃ with an incubation time of 15 to 30 minutes [26–28]. However, RPA has also been reported to have successful amplification reactions at temperatures as low as 25℃, though at a longer incubation time [29,30]. On the other hand, when the reaction temperature is as high as 42℃, 1 to 5 minutes reaction time have been observed to be sufficient for reliable detection [31-32].

C. Template Quality

PCR/RT-PCR require a high-quality nucleic acid template due to their intolerant towards inhibitors. In contrast, RPA proves to be more efficient or beneficial compared to PCR due to its high tolerance towards contaminants. Indeed, RPA can be carried out using impure template DNA or spiked DNA template in crude plant extract [26,33] and minimally processed infected plant samples. Few milligrams of the tissue were ground with extraction buffer. The supernatant was directly used for RPA [33–35]. The majority of the reports using crude extract as the template had used General Extraction Buffer (GEB3 or GEB2) as the extraction buffer [36-37], followed by 0.5M NaOH [38]. Other alternatives include Tris-EDTA (TE) buffer [33,39], CTAB-Tris buffer [10], Tris-HCl [40] and phosphate-buffered saline [41]. There were also reports on the direct use of plant sap without sample preparation for RPA. The stem of the sample was cut, and the sap was squeezed out using pliers into sterile capillary tubes before it was directly used as the template. However, the detectable fluorescent signal of the impure sample could only be observed at 2X speed of assays with pure extracted DNA [42].

D. Sensitivity

In terms of sensitivity, RPA has been reported to have equal sensitivity to PCR; 1 pg/uL of a pure RNA transcript of tomato chlorotic dwarf viroid, 10 copies of total DNA of Candidatus Phytoplasma mali and 3 pg/uL of total RNA of cucumber mosaic virus [10,37,43]. However, the sensitivity varies slightly depending on sample purity and detection method. The pure nucleic acid sample was reported to have a faster detection rate at 3 to 5 minutes compared to crude DNA extract at 6 to 16 minutes when measuring detectable fluorescent signal [42,44], while the minimum limit of detection reported for the pure nucleic acid sample and impure DNA extract is comparable; 10^{-3} to 10^{-4} dilution [45]. Detection by real-time RPA using fluorescent was also reported to be more sensitive than the end-point detection method using lateral flow. Real-time RPA detected 1 to 10 gene copies, whereas lateral flow RPA detected between 10 to 100 copies of DNA [42].

E. Detection Method

Generally, RPA products can be detected in real-time during the amplification reaction and at the end-point, following amplification reaction, depending on the reagents used. For real-time detection, the most common method is by using an exo probe, a specific probe for TwistAmp exo kit reaction [10,43,35].

Exo probe is a 46 to 52 bases oligonucleotide, homologous to target DNA with dT-fluorophore and dT-quencher flanking the 5’ end and 3’ end of tetrahydrofuran residue (dSpacer) and a 3’ end-block to prevent probe elongation. Upon probe annealing to target DNA, exonuclease III included in the TwistAmp Exo kit recognizes and cleaves the probe at the dSpacer site, producing two fragments and separating the fluorophore from the quencher. The generation of fluorescent enables the RPA amplicon to be observed in real-time. However, degradation of DNA because of the exonuclease makes it unsuitable to be detected using gel electrophoresis [19,46].

Fpg probe, on the other hand, is an approximately 35 bases oligonucleotide. The probe has a 3’ end block to prevent probe elongation, a quencher at the 5’ end and a fluorophore that is attached to an abasic nucleotide via a C-O-C linker called dR-group. Upon hybridization of the probe to target DNA, nuclease formamide pyrimidine DNA glycosylates (fpg), available in TwistAmp fpg kit, cleaves the probe at the dR site, releasing the fluorophore to generate fluorescence. As opposed to the exo kit, the amplicons can be detected both in real-time using fluorescent and at the end-point using gel electrophoresis as the final yield of the amplicon is not reduced [19,46]. Table I summarizes RPA assay using the fluorescent detection method.

Besides probe, RPA reactions can also be monitored using DNA intercalating fluorophores such as SYBR Green I [23] and SYTO9 [47]. However, these dyes are non-specific and cannot distinguish target from non-specific amplicons, thus giving rise to false-positive results [47].

The majority of the reports describing the end-point detection of RPA products rely on lateral flow assay using TwistAmp nfo kit and nfo probe. Nfo probe is a 46 to 52 base nucleotide with an antigenic label at the 5’ end, polymerase extension blocking group at the 3’ end and an internal abasic nucleotide substituted with tetrahydrofuran residue called dSpacer. The antigenic label is usually carboxyfluoroscein group (FAM), Alexa fluor488 or digoxigenin. Annealing of nfo probe to target DNA is activated by the hybridization of both forward and reverse primers to template DNA. Upon annealing of the probe to the target DNA, nfo nuclease cleaves the dSpacer region, thus turning the probe into amplification primer, running equidirectional to one of the primers. Aside from the probe, the labelling of the opposing amplification primer at the 5’ end with an antigenic group, i.e., biotin, is required. RPA products produced will have both labels, making them detectable in a sandwich assay format [5]. RPA assays using the lateral flow detection method is summarized in Table II.

Aside from lateral flow, other end-point detection method includes agarose gel electrophoresis [34,45]. Gel electrophoresis is widely used for product visualization in the laboratory due to its simplicity. However, RPA products purification is usually necessary to avoid smeared bands on the gel, which is caused by excess proteins and crowding agents in the reagents. Several methods have been reported to be good purification steps for RPA products, namely PCR product purification kit, an additional heating step at 65℃ or 95℃ for 10 minutes and high-speed centrifugation for 3 to 5 minutes to suspend the protein [28]. Table III summarizes RPA assays using the gel electrophoresis detection method.

TABLE I: RPA METHODS WITH FLUORESCENT DETECTION.

Target	**Template**	**Time (min)**	**Temp (℃)**	**Kit/Reagent**	**Sensitivity**	**Ref**
Cucurbit yellow stunting disorder virus	Total RNA	30	40	AmplifyRP XRT	100fg/uL	[41]
Candidatus liberbacter	1) Crude extracted with 0.5M NaOH 2) Total DNA	10	37	TwistAmp Basic/ SYBR Green I	1) 50-fold dilution 2) 0.1ng/uL	[23]
Yam mosaic virus	Total RNA	30	37	TwistAmp exo	14pg/uL	[48]
Potato virus Y	1) Total RNA 2) Crude extracted with GEB3	20	39	AmplifyRPA Acceler8	1) 0.01ng/uL 2) $1X10^{-2}$ dilution	[49]
Phytophthora infestans	1) gDNA 2) gDNA spiked with healthy crude extract	30	39	TwistAmp exo	1) 50fg/uL 2) 50fg/uL	[40]
Macrophomina phaseolina	gDNA	30	39	TwistAmp exo	200 fg	[36]
Fusarium oxysporum	gDNA	30	39	TwistAmp exo	1 pg	[35]
Phytophthora species	Total DNA	30	39	TwistAmp exo	1 pg	[50]
Leptosphaeria maculans	1. gDNA 2. plasmid DNA	30	39	TwistAmp exo	1. 21.6 pg 2. 75.4 fg	[51]
Phytophthora species	1) gDNA 2) gDNA spiked with crude plant extract	30	39	TwistAmp exo	3.3 pg/uL	[26]
Fusarium oxysporum	gDNA	30	37	TwistAmp Basic/SYTO9	0.1 ng	[47]
Xanthomonas species	gDNA	25-30	41	TwistAmp exo	10^{6} -10^{7} CFU/ml	[52]
Candidatus phytoplasma oryzae	Plasmid DNA	20	39	TwistAmp exo	1-10 copies/uL	[42]
Potyviruses	Crude extracted with alkaline-PEG	30	37	TwistAmp exo-RT	$1X10^{-3}$ dilution	[44]
Rose rosette virus	1) RNA transcript 2) viral RNA	20	42	TwistAmp exo-RT	1 fg/uL	[53]
Cucumber mosaic virus	1) Total RNA 2) Crude extracted with GEB3	25	40	TwistAmp exo-RT	1) 3pg/uL 2) $1X10^{-5}$ dilution	[43]
Candidatus phytoplasma mali	Total DNA	20	42	TwistAmp exo Lateral flow/nfo kit	10 copies	[10]

TABLE II: RPA METHODS WITH LATERAL FLOW DETECTION

Target	Template	Time (min)	Temp (°C)	Detection method	Sensitivity	Ref
Hop stunt viroid	1) Total RNA 2) RNA transcripts	20	39	AmplifyRP Acceler8	1) 10 ng/uL 2) $2X10^{9}$ copies	[54]
Tomato chlorotic dwarf viroid	1) RNA transcript 2) Crude extracted with GEB3	15	39	AmplifyRP Acceler8	1) 1pg 2) 25-fold dilution	[37]
Phytophthora infestans	gDNA	20	39	TwistAmp nfo	500fg/uL	[29]
Little cherry virus 2	1) Total RNA 2) Crude extracted GEB3	15	39	AmplifyRP Acceler8	1) 0.1 ng/uL 2) $1X10^{-2}$ dilution	[55]
Candidatus phytoplasma pruni	1) Purified PCR amplicon diluted in water 2) Purified PCR amplicon diluted in a crude extract	20	39	AmplifyRP Acceler8	1) 5.0-0.05 ag 2) 0.5 ag	[56]
Milkvetch dwarf virus	1) Viral vector 2) Crude extract 200mg leaf + 2ml plant lysis buffer -homogenize mesh bag-supernatant	30	37	TwistAmp basic/microsphere test strip	1) 10^{1} copies/uL 2) $1X10^{-2}$ dilution	[57]
Phytophthora cactorum	gDNA	30	39	TwistAmp nfo	100 fg/uL	[58]
Clavibacter & C. nebraskensis	1) gDNA 2) gDNA spiked with plant extract	30	39	TwistAmp nfo	1) 10 pg/uL (clavibacter) and 100 fg/uL (C. nebraskensis) 2) 10 pg/uL (clavibacter) and 1 pg/uL (C. nebraskensis)	[33]
Pectobacterium	1) gDNA 2) gDNA spiked with host gDNA	30	37	TwistAmp exo	10 fg/uL	[39]
Potato spindle tuber viroid	1) RNA transcripts 2) Total RNA	30	37	TwistAmp nfo	1) 10^{6} copies 2) $1X10^{-7}$ dilution	[59]
Phytophthora sojae	gDNA	20	39	TwistAmp nfo	0.01 ng/uL	[60]
Candidatus liberibacter asiaticus	Total DNA	20-30	38	TwistAmp nfo	1 pg/uL	[27]
Dickeya species	1) gDNA 2) gDNA spiked with host sap	30	42	TwistAmp exo	1 fg/uL	[61]
Phytophthora hibernalis	gDNA	20	38	TwistAmp nfo	0.1 ng/uL	[62]
Candidatus phytoplasma oryzae	Plasmid DNA	20	39	TwistAmp nfo kit	10-100 copies/uL	[42]
Verticillium dahliae	1) gDNA 2) soil gDNA	20	37	TwistAmp nfo	1) 10 pg/uL 2) 10^{3} spores/g soil	[63]
Dickeya species	1) plasmid DNA 2) culture with plant extract	30	39	TwistAmp basic	1) 50 copies/mL extract 2) 250-700 CFU/mL extract	[64]
Phytophthora capsici	gDNA	20	40	TwistAmp nfo	10 pg/uL	[65]
Verticillium alfalfa	1) gDNA 2) total DNA	20	37	TwistAmp nfo	1) 800 fg/uL 2) 3.25 pg	[38]
Rice black-streaked dwarf virus	cDNA	20	37	TwistAmp nfo/reverse transcriptase	$1X10^{-1}$ dilution	[66]
Candidatus phytoplasma mali	Total DNA	20	42	TwistAmp nfo	10 copies	[10]

TABLE III: RPA METHODS WITH GEL ELECTROPHORESIS DETECTION.

Target	Template	Time (min)	Temp (°C)	Kit/Reagent	Sensitivity	Ref
Chili veinal mottle virus	Total RNA	20	38	TwistAmp Basic RT	10fg/uL	[67]
Cucumber green mottle mosaic virus	Total RNA	15	37	TwistAmp Basic RT	0.5pg/uL	[68]
Banana bunchy top virus	1) Total DNA 2) Crude extracted using 0.5M NaOH	30	37	TwistAmp Basic	1) 0.01ng/uL 2) 1X 10^{-6} dilution	[45]
Cucumber green mottle mosaic virus	cDNA	30	38	TwistAmp Basic RT/Reverse Transcriptase	1X10^{-6} µg	[69]
Peach latent mosaic virus	Total RNA	5	42	TwistAmp Basic RT	100fg/uL	[32]
Apple stem grooving virus	Total RNA	1	42	TwistAmp Basic RT	4.7ng/uL	[70]
Maize chlorotic mottle virus	cDNA	30	38	TwistAmp Basic/Reverse Transcriptase	2.3X10^{-6} µg/uL	[71]
Barley yellow dwarf virus	Total RNA	5	42	TwistAmp Basic RT	50fg/uL	[31]
Tomato yellow leaf curly virus	1) Plasmid DNA 2) Total DNA 3) Crude extracted with 0.5M NaOH	39	37	TwistAmp Basic	1) 9.6pg 2) 1X10^{-6} dilution 3) 1X 10^{-3} dilution	[28]
Piper yellow mottle virus	1) Total DNA 2) Crude extracted with 0.5M NaOH 3) cDNA	40	39-42	TwistAmp Basic	1)1X10^{-5} dilution 2) 1X10^{-3} dilution 3) 1X10^{-5} dilution	[34]
Rose rosette virus	RNA transcript	20	42	TwistAmp Basic RT	1fg	[72]
Potato virus Y	cDNA	20	25-42	TwistAmp Basic/reverse transcriptase	2pg	[30]

IV. ALTERNATIVE DETECTION METHOD

Aside from lateral flow, fluorescence and gel electrophoresis detection, alternative detection strategies have been reported [22,73,74] and are summarized in Table IV.

Several studies have reported Recombinase Polymerase Amplification (RPA) detection using the colorimetric method. In a report by Wang and Yang, [73] they exploited the optical property of gold nanoparticles (AuNP) to produce visual detection of RPA amplicon. Complementary thiol-labelled oligonucleotides conjugated to AuNP was used to hybridize to RPA amplicons in a high-temperature setting at 95°C, where the RPA amplicon fragments denatured into single-stranded DNA. As the colour variation of the AuNP probe is affected by the growth, size and shape of AuNP, the assay can be considered as semi-quantitative, where the colour varies from light pink in a negative control reaction to rich blue-violet and navy-blue colours in a high template concentration reaction [75]. The detection assay enabled detection of tomato yellow leaf curl virus (TYLCV) using the naked eye, without any equipment [73].

In another report, the study combined the use of real-time RPA (RT-RPA) for target pre-amplification, LbCas12a/crRNA for sequence recognition and AuNP probe for visual detection of multiple RNA viruses in apple. The indiscriminative non-specific single-stranded DNA trans-cleavage activity of Cas12a/crRNA, which is activated upon recognition of target DNA, was employed to cleave linker-ssDNA and thus prevent AuNP aggregation and colour changes. Compared to the usually reported red to magenta to blue colour changes of AuNP aggregation, the study was based on the detection of red (AuNP dispersion) to colourless (linker-ssDNA-induced AuNP aggregation) colour change [75-76].

In fact, RPA detection using an electrochemical biosensor has also been reported. The detection strategy involved hybridization of RPA amplicons containing biotin on one end and 10 nucleotides barcode on another end, with AuNP-DNA probe and streptavidin-coated magnetic beads, which then the AuNP was separated, transferred to the working electrode, activated, and analyzed with differential pulse voltammetry (DPV) response. As the amount of AuNP separated is proportional to the amount of amplified target DNA, positive RPA reaction was monitored from the DPV signal [77]. An alternative approach is based on solid-phase RPA and electrochemical impedance spectroscopy (EIS). RPA master mix was first added onto AuNP-modified screen-printed carbon electrode (SPCE), followed by target DNA.

Following incubation, the reaction was characterized by IES [22].

Surface-enhanced Raman scattering (SERS) has also been exploited for the detection of plant pathogens in multiplex RPA reactions. Double tagged RPA amplicon with biotin and nucleotide barcode was hybridized with streptavidin magnetic beads and SERS nanotags containing AuNP probe and Raman reporters - each pathogen with a different reporter. The beads were separated, washed, and used for SERS detection on a portable Raman microscope. Raman reporter's specific scattering patterns (upon laser excitation) enabled detection and differentiation of each pathogen in the reaction [78].

TABLE IV: RPA WITH ALTERNATIVE DETECTION METHOD.

Target	Time (min)	Temp (°C)	Kit/reagent/equipment	Detection method	Sensitivity	Ref
Tomato yellow leaf curl virus	30	37	TwistAmp basic/AuNP probe	Colorimetric	10-50 nM	[73]
Apple RNA viroid	20	37	TwistAmp basic/Cas12a/AuNP probe	Colorimetric	250-2500 copies/reaction	[74]
Pseudomonas syringae	20	37	TwistAmp basic/AuNP probe-streptavidin beads/DPV	Electrochemical response	214 pM	[77]
Citrus tristeza virus	60	25±3	TwistAmp basic/AuNP-modified SPCE/EIS	Electrochemical response	1 pg/uL	[22]
Fusarium oxysporum, Pseudomonas syringae, Botrytis cinerea	20	37	TwistAmp basic/SERS nanotag-streptavidin beads/Raman microscope	Spectroscopy	2.32 copies	[78]

V. ADVANTAGES AND DISADVANTAGES OF THE RPA METHOD

Owing to the technology of recombinase protein in aiding the DNA amplification process, RPA assay does not require high temperature and long incubation time for DNA amplification. Optimally operating at a single temperature between 37 and 42°C without the initial denaturation step, Recombinase Polymerase Amplification (RPA) requires less energy for its reaction and is suitable for the non-instrumented amplification reaction. RPA also has equal sensitivity to the polymerase chain reaction (PCR), having a successful amplification reaction even when the target copies and concentration are low. In addition, RPA can amplify both DNA and RNA from various kinds of organisms, even in the presence of PCR inhibitors or crude extracts. The method has also been successfully used to amplify DNA/RNA from plant sap without sample preparation. RPA also has a simple primer design. Though RPA requires a longer primer length than PCR, thorough consideration of primer annealing temperature is unnecessary, and a general annealing temperature between 50 and 100 is adequate. In terms of detection methods, RPA can be manipulated for various types of detection methods both in real-time and post-amplification process, which makes it suitable for both laboratory settings with trained staff and resource-limited settings with non-expert users [79].

However, there are several limitations to RPA. First, RPA is only suitable for amplification of small DNA fragments of not more than 500 bp and is not suitable for full-length gene study and whole-genome sequencing. Second, RPA amplicons usually need to be purified before detection and visualization using the gel electrophoresis method. Third, contrary to the real-time qPCR method, which is cycle-based, RPA reaction is time and kinetic based. The kinetic energy of RPA depends on target concentration, temperature, and mixing step. Therefore, lengthy optimization is sometimes required for an efficient RPA reaction.

VI. FUTURE APPLICATION OF RPA

Ever since the discovery and report of Recombinase Polymerase Amplification (RPA) in 2006, there has been a rapid increase in RPA applications and publications. The majority of the reports have focused on its optimization to increase efficiency while extending its application to the point-of-care (POC) setting. As a result, it is expected that there will be an exponential growth of RPA usage in the POC setup. Considering the advantages of RPA, RPA shows a high potential for complete integration with POC setting for early disease detection. However, more improvements are needed to simplify the reaction step and process for the use of non-experts and to minimize reagent and instrumentation for better integration to the POC setting. For example, it employs the use of a simple extraction buffer for nucleic acid extraction and on-chip fixation of amplification reagents to simplify the reaction steps. Nevertheless, RPA is a relatively new isothermal amplification method compared to loop-mediated isothermal amplification (LAMP) and helicase-dependent amplification (HDA). Given its advantages, RPA has a great capacity to be used in the field for early warning of plant pathogens and consequently help in the disease management process.

VII. CONCLUSION

Established molecular diagnostic methods like polymerase chain reaction (PCR) provide high specificity and sensitivity for pathogen detection. While ideal for resourceful laboratories, it is costly and requires a constant power supply for the strict thermal conditions. Recombinase Polymerase Amplification (RPA) is a remarkable isothermal nucleic acid amplification method that is capable of amplifying target nucleic acids at a

low and constant temperature of 37 to 42℃ for a short time, 15 to 30 minutes. With a sensitivity that equals established methods like PCR, amplifying as low as 1 to 10 target copies and good tolerance against PCR inhibitors, RPA is suitable to be employed in POC setting with limited resources and expertise for an early warning against phytopathogen attack. Its ease of reaction mechanism and reagents compared to other isothermal methods coupled with its versatility to be manipulated for various detection strategies like fluorescent, lateral flow, colorimetric, and electrochemistry will also allow further improvements to be made. Given that the technology is relatively new, despite its disadvantages, it is expected that more advancements will be made for POC detection of plant pathogens.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this paper.

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