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Onsite detection of plant viruses using isothermal amplification assays

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Summary

Plant diseases caused by viruses limit crop production and quality, resulting in significant losses. However, options for managing viruses are limited; for example, as systemic obligate parasites, they cannot be killed by chemicals. Sensitive, robust, affordable diagnostic assays are needed to detect the presence of viruses in plant materials such as seeds, vegetative parts, insect vectors, or alternative hosts, and then prevent or limit their introduction into the field by destroying infected plant materials or controlling insect hosts. Diagnostics based on biological and physical properties are not very sensitive and are time consuming, but assays based on viral proteins and nucleic acids are more specific, sensitive, and rapid. However, most such assays require laboratories with sophisticated equipment and technical skills. By contrast, isothermal-based assays such as loop-mediated isothermal amplification (LAMP) and recombinase polymerase amplification (RPA) are simple, easy to perform, reliable, specific, rapid, and do not require specialized equipment or skills. Isothermal amplification assays can be performed using lateral flow devices, making them suitable for onsite detection or testing in the field. To overcome non-specific amplification and cross-contamination issues, isothermal amplification assays can be coupled with CRISPR/Cas technology. Indeed, the collateral activity associated with some CRISPR/Cas systems has been successfully harnessed for visual detection of plant viruses. Here, we briefly describe traditional methods for detecting viruses, then examine the various isothermal assays that are being harnessed to detect viruses.

Introduction

Crops are susceptible to multiple viral pathogens that cause severe economic losses. Limiting these losses requires producers to identify the causal viruses. The oldest approach for detecting and diagnosing viruses that infect crops is examining their biological properties such as symptoms, host range, and transmission. In the early days of plant virology, symptoms were the primary means by which a viral disease was diagnosed and named, as evidenced by the names of many well-known plant viruses (Walkey, 1985). The symptoms produced in a range of test plants provide the first clues about the identity of a virus in the field or laboratory. For the grower, the nature and severity of disease symptoms determine the economic importance of a particular virus in terms of yield loss and reduced quality. When considering virus symptoms, it should be remembered that a virus is unlikely to cause just one symptom in an infected plant. Infection usually results in a range of symptoms, and a sequence of symptoms is frequently observed as the disease progresses.

Host range, where a virus can replicate in more than host, also provides key information about viruses, particularly emerging races of known pathogens. The sap transmission test, in which sap from an infected plant is used to inoculate a panel of indicator plants, is still the best means for routine diagnosis of many viruses, as this sensitive test provides essential information about the virus; including whether it is susceptible to specific host resistance genes. However, these tests may take several weeks and require panels of specific test plants. Moreover, not all plant viruses are sap transmissible, and it is often necessary to use natural virus vectors (insect vectors; aphids, beetles, thrips, whiteflies, leafhoppers; mites, nematode vectors, and fungal vectors). In addition, for viruses that cannot be transmitted by sap and vectors, the only means of confirmation may be grafting (Camarço et al., 1998; Hill, 1984).

Biological assays are very important for detecting and diagnosing plant viruses and are the only means to grow viruses. Such assays rely on indicator plants that shows specific symptoms when challenged with a specific virus. However, these assays are more time-consuming and labor-intensive than laboratory assays. Consistent, reproducible biological assay results require suitable vector-proof growth facilities and trained workers to produce ideal indicator plants. Also essential is familiarity with symptomatology. Infectivity assays are often needed to verify the results of laboratory tests, and inoculation of differential

hosts is an essential tool for identifying viruses at the species and strain levels (Chin et al., 2007; Davis et al., 2005; González et al., 2002; Roy et al., 1999). For example, biological assays are the only means to differentiate between papaya ringspot virus strains W and P. Viruses are also identified based on biophysical characteristics such as nucleic acid size, type, and sequence, protein composition and size, and particle shape (Stephanidis et al., 2007), as revealed by electron microscopy. However, electron microscopy is expensive, less sensitive, and cannot identify viruses to the species level.

The advent of ELISA in the 1970s improved virus detection, as this method was more sensitive than other methods performed at that time. With ELISA, results can be obtained within a few hours, and it can be used to test many samples. However, ELISA requires the production of virus-specific antisera. Attaching the antibody to an enzyme increases the sensitivity of detection of the antibody-antigen reaction and the resulting color reaction may be quantitatively measured. The sensitivity of ELISA is approximately 1–10 ng of virus per ml test sample. Several ELISA procedures have been developed (Clark and Bar-Joseph, 1984; Clark and Adams, 1977; Clark et al., 1986; Hampton et al., 1990; Mowat and Dawson, 1987). The major advantage of ELISA or other serological assays is that crude samples can be directly used in these assays, as no special procedure or equipment is required for test sample preparation.

Onsite detection of viruses using a serological assay is possible via lateral flow immunoassay (LFIA). Lateral flow or 'dipstick' methods are suitable for field use without the need for special equipment or technical skills. A lateral flow test typically incorporates a sample pad in close contact with a conjugate pad (Figure 1). This, in turn, is in contact with a membrane onto which test and control reagents have been immobilized. An absorbent pad wicks fluid away from the membrane. In these kits, virus coat protein-specific antibodies (capture antibodies) and species-specific antibodies are pre-immobilized onto membranes at the test and control lines, respectively. To perform the test, the sample is ground, and a few droplets of the extracted sap are loaded into the sample port of the device (conjugate pad), where it combines with virus-specific antibodies conjugated with colored latex beads or gold particles, forming a complex with the tagged antibodies. The bound antigen/antibody complex then moves by lateral flow through the membrane until it reaches the test line (where the virus-specific capture antibody is

immobilized). At the test line, the movement of the virus-specific antigen-antibody complex is arrested as it binds to the virus-specific capture antibody. As increasing amounts of antigen-antibody complex become captured at the test line, the line becomes visible as the colored latex or gold particles accumulate. The second capture line is the control line, comprising species-specific antibodies intended to arrest all unbound labeled antibodies that would also become visible to confirm that the assay has run correctly. LFIA is only suitable for detecting viruses that occur in high titer in the test samples. It may give false-negative results for test samples with low concentrations of virus. To overcome this issue, some new lateral flow assays (LFA) involve an initial amplification of the viral nucleic acid using labeled primers/probes, followed by end-point detection using lateral flow devices (Cassedy et al., 2021).

The next breakthrough in virus detection came with the invention of PCR in 1984. The method involves amplifying a specific region of the virus prior to its detection, thus increasing the assay's sensitivity many fold compared to ELISA or other serological assays. The important steps in PCR include extracting nucleic acids from the test plant, synthesizing two virus-specific primers, and setting up the PCR in a vial by adding extracted nucleic acids, primers, nucleotides, magnesium chloride, and *Taq* polymerase. The vial is then placed in the thermal cycler for denaturation, primer annealing, and extension using pre-determined settings. After the run, a positive reaction is identified by running the contents of the vial on a agarose gel. The presence of bands at the expected position indicates that the sample is positive for the virus (Henson and French, 1993).

Different variants of PCR, such as reverse-transcription PCR (RT-PCR), immunocapture PCR, nested PCR, multiplex PCR, real-time PCR, and so on have been developed (Bhat and Rao, 2020). PCR can also be used to detect multiple viruses in a sample using multiplex PCR. PCR is more sensitive than ELISA, as even a few copies of the viral nucleic acid present in the test sample can be amplified and detected. However, PCR requires sophisticated laboratory equipment and skilled personnel. PCR-based methods are cumbersome because they require the initial isolation of nucleic acids, followed by amplification of the target sequence and analysis of the products on an agarose gel.

By contrast, isothermal amplification can be performed at a single temperature without the need for thermal cyclers, and the results can be visualized as color changes without the need for agarose gel electrophoresis. These methods can be performed in a resource-poor laboratory, require less time, and produce comparable results to PCR-based methods. Of the several isothermal amplification assay methods available, loop-mediated isothermal amplification (LAMP) and recombinase polymerase amplification (RPA) are commonly used for the detection and diagnosis of infectious agents, including viruses.

Loop-mediated isothermal amplification (LAMP)

LAMP is an isothermal amplification assay that exploits the strand displacement activity of Bst DNA polymerase from *Bacillus stearothermophilus* for the efficient, robust amplification of any target nucleic acid using a minimum of four primers (F3, B3, FIP, and BIP) (Notomi et al., 2000). The sensitivity of the assay can be further increased by adding one or two additional primers (BL and FL) (Figure 2a) (Nagamine et al., 2002). Designing LAMP primers manually is possible but a bit complicated. Primers can be designed using the free online software package Primer Explorer version 5 (https://primerexplorer.jp/e/) or paid software (www.optigene.co.uk/lamp-designer /). Unlike PCR, the target nucleic acid is initially converted to a dumbbell-shaped structure that serves as a starting point for the cyclic phase of LAMP amplification. To convert the nucleic acid into a dumbbell-shaped structure, outward forward (F3), outward backward (B3), forward inner (FIP), and backward inner (BIP) primers are needed. FIP and BIP are long primers (~45 to 55 nucleotides) that contain both sense and antisense sequences. Readers can visit http://loopamp.eiken.co.jp/e/lamp/ for detailed guidelines for primer design.

The first step in the LAMP reaction is the annealing of a portion of the FIP to its complementary sequences (F2 region) in the target nucleic acid, resulting in the synthesis of a complementary strand. This is followed by the binding of primer F3 to its complementary region (F3c), leading to the displacement of the FIP-linked strand and producing a loop-structured end (Figure 2a). BIP then initiates the synthesis of another strand by binding to the complementary region (B2) on the FIP-linked strand. The synthesized strand is displaced upon B3-primed synthesis, leading to a dumbbell-shaped structure, which converts into a stem-loop structure by self-priming. This stem-loop

structure acts as the template for the cyclic amplification step of LAMP. The conversion of the target nucleic acid into a stem-loop structure is crucial for the success of LAMP. F3 and B3 are absent in the stem-loop structure, and hence these primers are no longer needed for the cyclic phase of LAMP amplification. The cyclic phase utilizes the stem-loop structure for repeated amplification cycles to produce many amplified strands of different lengths (Figure 2a). The sensitivity of the amplification can be increased using loop primers (LF and LB). LAMP reactions have been successfully coupled with a suitable reverse transcriptase (RT-LAMP) to transcribe a specific viral RNA sequence to DNA, which serves as a template for subsequent reactions. However, unlike standard RT, it is important to use a thermostable reverse transcriptase enzyme in RT-LAMP that can withstand temperatures up to 65 °C.

The standard LAMP reaction mix consists of isothermal buffer, $MgSO_4$, betaine, dNTPs, LAMP primers (F3, B3, BIP, FIP, LF and LB), Bst polymerase, thermostable reverse transcriptase (if performing RT-LAMP), and template nucleic acid. The LAMP assay does not require a highly purified nucleic acid template; in most cases, it works well with crude extract isolated from an infected plant. The LAMP reaction is incubated at 65 °C (may be optimized between 58 °C and 70 °C) for 30 to 60 minutes. The reaction can be stopped by placing the tube at 80 °C for 10 min to inactivate the enzyme (Bhat and Rao, 2020).

The results of LAMP can be visualized in many ways. The pyrophosphate molecule released during nucleotide synthesis in the LAMP reaction combines with magnesium ions to form magnesium pyrophosphate. Magnesium pyrophosphate is insoluble, and its accumulation turns the contents of the tube turbid (Figure 2b). Thus, the most straightforward way to visualize LAMP results is to observe the tube after the reaction with the naked eye for the presence of turbidity (Bhat et al., 2013; Fukuta et al., 2003a; Fukuta et al., 2003b; Mori et al., 2001; Nagamine et al., 2002; Nie, 2005; Notomi et al., 2000; Tomlinson et al., 2010). The addition of intercalating fluorescent dyes such as SYBR Green, ethidium bromide, or PicoGreen makes positive samples appear fluorescent under UV light (Almasi et al., 2013; Nie et al., 2012; Tomlinson et al., 2007). Similarly, the addition of calcein and $MnCl_2$ in the LAMP reaction results in green fluorescence under UV light (Figure 2c) (Mansour et al., 2015). Visual detection is also possible following the

addition of fluorexon dye, where a positive reaction is identified by a color change from light orange to green (Figure 2d). Finally, LAMP results can be visualized by agarose gel electrophoresis, which produces ladder-like bands when observed under a UV transilluminator. The turbidity of magnesium pyrophosphate, a byproduct of the reaction, can be detected in real-time with a real-time turbidimeter (Mori et al., 2001). Real-time detection is also possible by running the LAMP reaction on a real-time LAMP instrument and measuring the fluorescence emitted by SYBR Green or other dyes on a real-time basis). LAMP has been successfully used to detect many infectious agents of humans, animals, and plants. This technique has also been used to detect numerous plant viruses, as listed in Table S1.

LAMP combined with a lateral flow device for onsite detection

LAMP results can be visualized with a lateral flow device (LFD) when using labeled loop (FP and BP) or inner primers (FIP and BIP) at the 5′ end with biotin/digoxigenin (DIG)/Texas red or fluorescein isothiocyanate (FITC) (Figure 2e) (Peng et al., 2021; Tomlinson et al., 2010). The principle of immunochromatography using LFDs is that same as that described for LFIA. To detect LAMP products using an LFD, the labeled (biotin/DIG/FITC) LAMP product is diluted at the end of the reaction and applied to the sample port of the LFD. The LFD contains colored latex beads or gold nanoparticles conjugated with the appropriate antibodies produced in rabbit, such as anti-biotin/anti-DIG/anti-FITC, which bind to the LAMP product. This bound complex moves laterally through the membrane until it reaches the test line, where it binds to the label incorporated in the LAMP product, resulting in the formation of a visible colored line. No colored line will be visible for negative reactions in which only unincorporated primers are present. The control line will be visible when the colored latex combines with anti-rabbit antibody, which takes place within 15 min after the addition of the LAMP product. The presence of two colored lines indicates a positive reaction, while the presence of only a single control line indicates that the test sample is negative, and the absence of both colored lines indicates that the test failed. LFDs are also suitable for multiplex detection of different viruses simultaneously (Tomlinson et al., 2010; Tomlinson et al., 2013). Custom-made or commercial LFDs can be used to detect LAMP products. LAMP, followed by detection

using LFDs, has been successfully used to detect many pathogens, including cassava brown streak virus, citrus leaf blotch virus, tomato brown rugose fruit virus, and Ugandan cassava brown streak virus (Peng et al., 2021; Tomlinson et al., 2013) (Table S1).

Recombinase polymerase amplification (RPA)

RPA technology was developed by Piepenburg et al. in 2006 (Piepenburg et al., 2006) and commercialized by TwistDx (www.twistdx.co.uk). This technique has been used to detect various pathogens, including fungi, bacteria, and viruses that infect animals, humans, and plants. RPA is an isothermal procedure that requires a single temperature for target amplification and an enzyme to separate the strands of double-stranded (ds)DNA to achieve primer binding at the target region on the template (Figure 3a). At the beginning of the RPA reaction, in the presence of ATP and a crowding agent (high molecular weight polyethylene glycol), the recombinase protein integrates with the primers to form a recombinase-primer complex (Zhang et al., 2014). This complex identifies the complementary sequence on the template and allows the primer to anneal after the separation of the dsDNA strands by the recombinase. After the primer annealing, the recombinase separates from the complex, leaving the 3′ end available to the DNA polymerase to extend the chain. The separated DNA strands are then stabilized by single-stranded binding protein (SSB) as the DNA polymerase extends the chain, forming a new dsDNA that acts as a template for further amplification.

Although the manufacturer recommends using primers 30–35 nucleotides long, some recent reports describe successful RPA using standard PCR primers (Lobato and O'Sullivan, 2018). Primers with long tracts of guanines and cytidines at the 3′ end and guanines at the 5′ end are not recommended (www.twistdx.uk.com). Similarly, primers with a GC content of > 70% or < 30% are not recommended. The ideal amplicon length is ~100–200 bases. RPA is generally carried out at 37 °C to 42 °C for 15–30 min (Piepenburg et al., 2006). The template for RPA can be dsDNA, single-stranded ssDNA, or cDNA. In addition, primer design is simple, and there is no need to consider the annealing temperature (Boyle et al., 2014). RPA assays can be performed on crude samples (does not require a highly purified template) and are less prone to contamination than conventional

PCR assays (Rojas et al., 2017). RPA can be performed in a simple incubator, dry bath, or at room temperature without the need for a thermocycler. Finally, RPA reagents are supplied in lyophilized form without the need for a freezer for transport and storage. These features make RPA suitable for use onsite or in a laboratory with minimum facilities. RPA can be combined with a lateral flow assay, making it ideally suited for the onsite detection of viruses in only 15–30 min. Another advantage of RPA is its suitability for multiplexing, depending on the sequence of the target pathogen, target amplicon size, and primer design (Kersting et al., 2014). As primers can compete for recombinase proteins, the ratios and concentrations of primers must be optimized for multiplex RPA assays (Lau, 2016). In most cases, RPA is highly specific, with 100% specificity for the target sequence.

RPA is performed using the basic kit available from TwistDx. Primers (forward and reverse), template, and rehydration buffer are added to the freeze-dried reaction pellets, followed by mixing. The addition of magnesium acetate will immediately initiate the RPA reaction. The tube is placed in an incubator at a suitable temperature (optimum 37 to 42 °C). The tube is removed from the incubator after 4 min, inverted vigorously, and placed back in the incubator for 20 to 40 min. The results of RPA can be visualized by agarose gel electrophoresis. To avoid smeared bands, the RPA product can be purified to remove proteins and crowding agent prior to gel electrophoresis. Colorimetric detection of the RPA product is possible if either primers or dNTPs labeled with biotin are used in the reaction (Lobato and O'Sullivan, 2018). After the reaction, the addition of streptavidin-HRP conjugate, followed by the substrate (tetramethylbenzidine and hydrogen peroxide), results in a change in color whose intensity depends on the amplicon concentration.

RPA can also be performed using an RNA template (RNA viruses) by adding a compatible reverse transcriptase enzyme directly into the RPA reaction mix in a one-step format called RT-RPA. Alternatively, the RNA can be converted into cDNA separately and a portion of the cDNA can be used as a template for RPA (Aman et al., 2020a; Aman et al., 2020b; Aman et al., 2022). RPA has been successfully used to detect several infectious agents, including viruses. Plant viruses that have been detected using the RPA assay are listed in Table 1. The sensitivity of detection by RPA can be enhanced by including probes in the reaction for real-time detection via fluorescence or endpoint detection in lateral flow assays (Daher et al., 2016).

Real-time detection of RPA products based on fluorescence

RPA products can also be viewed and quantified in real-time with a fluorimeter using fluorescent probes. This assay is performed using a TwistAmp Exo kit or TwistAmp fpg kit (Daher et al., 2016; Powell et al., 2018; Stringer et al., 2018). The TwistAmp Exo kit uses a specially designed probe called the Exo probe, which consists of a complementary 46–52 nucleotide sequence internal to both primers. The Exo probe contains a polymerase extension blocking group at the 3′ end and an internal abasic nucleotide analog (the tetrahydrofuran residue THF, which replaces a conventional nucleotide, also called dSpacer) flanked by a dT-fluorophore and a matching dT-quencher group (Figure 3b) (Lobato and O'Sullivan, 2018). During the RPA reaction, primers generate targets for annealing of the probe. When the probe is annealed to the template strand, the THF residue is cleaved by the exonuclease, separating the fluorophore and quencher, thus generating a fluorescent signal that can be monitored with a fluorimeter. The amount of signal produced is directly proportional to the concentration of amplicons.

Like the Exo probe, the fpg probe in the fpg kit also contains a complementary sequence internal to both primers that is approximately 35 nucleotides long (Powell et al., 2018). This probe is modified at the 5′ end with a quencher group and contains a fluorophore on an abasic nucleotide analog 4 to 5 nucleotides downstream of the quencher. The fluorophore is attached to the ribose group of the abasic nucleotide through a C-O-C linker termed the dR group. During the RPA reaction, primers generate targets for annealing of the probe. When the probe is annealed to the template strand, the fpg cleaves the probe at the dR position, thus separating the fluorophore and quencher, thereby generating a fluorescent signal that can be monitored with a fluorimeter. The amount of signal produced is directly proportional to the concentration of the amplicon. These approaches were used to develop detection assays for cucumber mosaic virus infecting banana (Srivastava et al., 2019), cucurbit yellow stunting disorder virus infecting watermelon and squash (Kalischuk et al., 2020), plum pox virus infecting stone fruits (Zhang et al., 2014), potato virus Y (Cassedy et al., 2022), rose rosette virus (Babu et al., 2017b), yam mosaic virus, and yam mild mosaic virus (Silva et al., 2018) (Table 1).

Instead of a specific probe, real-time detection is also possible using intercalating dyes in the RPA reaction, such as SYBR Green or Eva Green (Lobato and O'Sullivan, 2018). However, these dyes cannot discriminate RPA amplicons from non-specific amplified dsDNA products or primer-dimers.

Detection of RPA products via lateral flow assay

To increase the sensitivity and facilitate the instrument-free detection of RPA products, a lateral flow assay (LFA) can be used, allowing the results to be visualized extremely rapidly. This assay is performed using a TwistAmp nfo kit containing two primers (a conventional forward primer and a reverse primer labeled at its 5′ end, usually with biotin), one probe, and the enzyme nfo (endonuclease IV). The probe should have a complementary sequence internal to both primers with lengths varying from 46 to 52 nucleotides. The probe is modified at the 5′ end with an antigenic label (usually a carboxyfluorescein group (FAM), or Alexa Fluor 488 or digoxigenin), a polymerase extension blocking group at the 3′ end, and an internal abasic nucleotide analog (the tetrahydrofuran residue THF, which replaces the conventional nucleotide, also called dSpacer) placed at least 30 nucleotides from the 5′ end and 15 nucleotides from the 3′ end (Figure 3c) (Lobato and O'Sullivan, 2018).

During the RPA reaction, primers generate targets for annealing of the probe. When the probe is annealed to the template strand, the THF residue is cleaved by the nfo enzyme, leaving a 3′ hydroxyl group at the 3′ end of the probe, thus converting the probe into a primer that can extend the chain. RPA performed in this manner produces amplicons with two antigenic labels (FAM and biotin) on either side and can be processed via a lateral flow assay to visualize the results. The diluted (1:10 or 1:100) products of RPA are loaded onto the sample pad of the lateral flow strip, and the sample pad region of the strip is placed into PBST buffer. The labeled amplicons bind to gold-labeled FITC-specific antibodies and move through the capillarity. The amplicons bound with gold particles become immobilized when they combine with biotin ligand molecules at the test line and generate a red or blue line. Gold particles that have not been captured move further to reach the control lines, where they become immobilized by species-specific antibodies to produce a red or blue line. Thus, the development of colored test and control lines indicate a positive

reaction, while the development of only the control line indicates that the sample is negative for the test virus.

By combining RPA with a lateral flow assay, results can be obtained in less than 1 h with a sensitivity of detection ranging from 1 to 10 DNA copies (Lobato and O'Sullivan, 2018). The method is also suitable for detecting multiple viruses if different antigenic labels are used for different viruses. RPA-LFD assays have been developed to detect milk vetch dwarf virus, little cherry virus 2, plum pox virus, potato virus Y, rice black-streaked dwarf virus, and tomato spotted wilt virus (Cao et al., 2020; Cassedy et al., 2022; Lee et al., 2021; Mekuria et al., 2014; Zhang et al., 2014). Similar assays have also been developed for the detection of viroids such as hop stunt viroid, potato spindle tuber viroid, and tomato chlorotic dwarf viroid (Hammond and Zhang, 2016; Ivanov et al., 2020; Kappagantu et al., 2017) (Table 1). Further improvements of LFA were reported by several researchers (Cordray and Richards-Kortum, 2015; Crannell et al., 2014; Jauset-Rubio et al., 2016; Rohrman et al., 2012).

CRISPR/Cas for onsite detection

CRISPR/Cas (clustered regularly interspaced short palindromic repeats/CRISPR-associated) is a naturally occurring adaptive immune system used by prokaryotes such as bacteria and archaea to avoid invading viruses and plasmids (Doudna and Charpentier, 2014; Jinek et al., 2012). When a virus infects a bacterium for the first time, it incorporates a small portion of the viral sequence into the host's genome. Later, when the same bacterium is infected by the same virus for the second time, it transcribes the incorporated viral sequences into CRISPR RNA. CRISPR RNA forms a complex with tracrRNA and CRISPR-associated (Cas) protein, which binds to the complementary sequence in the invading viral DNA/RNA and generates double-strand breaks (DSBs), thereby destroying the virus. The CRISPR/Cas system can be introduced into a eukaryotic cell to cut its DNA at the desired site using single guide RNA (sgRNA) containing a complementary sequence to the targeted genomic region. Upon introduction of sgRNA and Cas9 in the nucleus of the target organism, the sgRNA binds to and guides Cas9 to the target sequence to generate a site-specific DSB. The DSB is then repaired by non-homologous end joining (NHEJ) or

homology-directed DNA repair (HDR) if a donor DNA molecule is provided (Jinek et al., 2012).

CRISPR/Cas is widely used as a genome-editing tool due to its target specificity and the ease of targeting any gene simply by varying the gRNA sequence. Due to their ease of target-dependent programmability, CRISPR/Cas systems have also been used for virus interference in various organisms including plants (Ali et al., 2015a; Ali et al., 2015b; Ali et al., 2018; Aman et al., 2018a; Aman et al., 2018b; Aman et al., 2020a; Mahas et al., 2019; Mahas and Mahfouz, 2018; Mahas et al., 2018). Following the discovery of increasing numbers of Cas proteins with different activities, CRISPR/Cas systems have been used to diagnose infectious organisms, including viruses. CRISPR/Cas is also employed for the detection of microRNAs (miRNAs), single nucleotide polymorphisms (SNPs), and DNA methylation (Kim et al., 2021b).

Among the various Cas proteins, Cas3 and Cas9 cleave dsDNA; Cas12 and Cas14 cut either dsDNA or ssDNA; and Cas13 cleaves ssRNA (Abudayyeh et al., 2016; Aman et al., 2018a; Aman et al., 2018b; Aman et al., 2020a; Harrington et al., 2018; Kim et al., 2021b; Zetsche et al., 2015). To enable the specific binding of gRNA-Cas protein complexes and cleavage, most Cas proteins (Cas3, Cas9, and Cas12) require a tri/tetranucleotide sequence next to the gRNA binding region known as the protospacer adjacent motif (PAM). The Cas13 recognition site next to the crRNA consists of a single nucleotide and is called the protospacer recognition site (PFS). By contrast, Cas14 cleaves ssDNA in a PAM-independent manner and dsDNA in a PAM-dependent manner (Kim et al., 2021b). Upon binding and cleaving of the specific target nucleic acid, certain Cas proteins (Cas3, Cas12, Cas13, and Cas14) show collateral, non-specific activities against ssRNA (Cas13a) or ssDNA (Cas 12a and Cas14a) (Abudayyeh et al., 2016; Aman et al., 2020a; Chen et al., 2018; Harrington et al., 2018). This collateral (trans-cleavage) activity occurs multiple times for a single target bound by the CRISPR-gRNA complex.

This non-specific cleavage has been exploited for diagnostic purpose (Abudayyeh et al., 2016; Aman et al., 2020a; Aman et al., 2020b; Aman et al., 2022; Chen et al., 2018; Chen et al., 2021), leading to the development of a Cas13-based detection assay for RNA viruses known as SHERLOCK (Specific high-sensitivity enzymatic reporter unlocking) (Gootenberg et al., 2018; Gootenberg et al., 2017). In this method, the RNA template is

converted into cDNA via reverse transcription. The cDNA is then subjected to RPA, and the RPA-amplified DNA molecules are subjected to *in vitro* transcription to obtain RNA templates that serve as the substrate for specific cleavage by the Cas13a-gRNA complex. Following target recognition and cleavage, the non-specific trans-cleavage nuclease activity of Cas13a becomes activated, and this enzyme cleaves the labeled ssRNA probe (labeled with a quencher and reporter). Once cleaved, the released reporter generates a fluorescent signal that can easily be quantified or detected under UV light. Different reporters that are compatible with LFA devices have also been used to facilitate signal detection at point-of-care (Figure 4) (Gootenberg et al., 2018).

Another detection assay called DETECTR (DNA endonuclease targeted CRISPR trans-reporter) was subsequently developed for the detection of human papilloma virus (Chen et al., 2018). In this method, the target region is initially amplified using RPA to increase the assay's sensitivity. The resulting dsDNA is used as the target for Cas12a-gRNA-mediated cleavage. A ssDNA probe labeled with a reporter and quencher molecules is used to monitor the collateral (trans-cleavage) activity of Cas12a. The other Cas protein exploited for diagnosis is Cas14, which does not require a PAM sequence for specific cleavage of the target ssDNA and is small (nearly half the size of Cas12a). Cas14-DETECTR is useful for detecting ssDNA pathogens and SNPs (Aquino-Jarquin, 2019; Harrington et al., 2018). Subsequently, Cas12a-based HOLMES (one-hour low-cost multipurpose highly efficient system) and a Cas3-based assay named CONAN (Cas3 operated nucleic acid detection) were developed. CONAN is similar to DETECTR, except that it uses multiple Cas proteins to achieve higher sensitivity (Figure 4) (Li et al., 2018; Yoshimi et al., 2020). The sensitivity of CRISPR-based diagnostic assays is comparable to that of PCR-based assays. However, the current CRISPR-based diagnostic assays require prior amplification of the target nucleic acid molecule by RPA, LAMP, or PCR, adding to the time and cost of the assays (Dong et al., 2018; Gootenberg et al., 2017). To overcome this problem, non-amplification-based CRISPR/Cas assays are currently being developed (Aman et al., 2020a; Bruch et al., 2019; Hajian et al., 2019; Kim et al., 2021b).

CRISPR/Cas-based onsite detection of plant DNA viruses

LAMP followed by CRISPR–Cas12a-based detection using a real-time fluorescence assay was used to detect tomato yellow leaf curl virus and tomato leaf curl New Delhi virus (Mahas et al., 2021) (Table S1). This assay takes less than an hour and successfully detects both viruses in plants with high sensitivity and specificity. The assay provides easy-to-interpret visual readouts using a simple, low-cost fluorescence visualizer, making it suitable for point-of-use applications. Similarly, Bernabé-Orts et al., successfully detected tomato brown rugose fruit virus using RT-LAMP followed by CRISPR–Cas12a-based detection using both real-time fluorescence detection and calorimetrically using LFD (Figure 5) (Bernabé-Orts et al.). Furthermore, Alon et al., performed differential detection of tomato brown rugose fruit virus and tomato mosaic virus infecting tomatoes using a similar approach, except that they used RT-PCR instead of RT-LAMP or RT-RPA for the initial conversion of viral RNA into dsDNA to act as a substrate for Cas12a (Alon et al., 2021).

CRISPR/Cas-based onsite detection of plant RNA viruses

Recently, Aman et al., developed a one-pot detection assay named iSCAN one-pot (iSCAN-OP), a simple, quick, efficient RT-RPA method coupled with a CRISPR/Cas12a-based one-step detection assay to detect potato virus X, potato virus Y, and tobacco mosaic virus (Table 1) (Aman et al., 2020b). In this method, viral RNA is initially converted into dsDNA via RT-RPA, allowing it to act as a substrate for Cas12a cis activity (Figure 5). The targeting of the dsDNA by Cas12a elicits its collateral action, which in turn cuts the ssDNA reporter molecules and releases the signal combined the iSCAN-OP assay with a commercially available P51 fluorescence viewer device to enable rapid, inexpensive, in-field diagnosis of viruses (Aman et al., 2020b; Chen et al., 2018). This assay can be performed at a single temperature within 30 min and provides a robust system for plant virus detection.

RT-RPA followed by CRISPR/Cas12a-based detection using real-time fluorescence was also used to detect beet necrotic yellow vein virus (BNYVV) (Ramachandran et al., 2021). Jiao et al., performed CRISPR/Cas12a-based detection of viruses infecting apples, such as apple necrotic mosaic virus, apple stem pitting virus, apple stem grooving virus, apple chlorotic leaf spot virus, and apple scar skin viroid (Jiao et al., 2021) (Table 1). Each virus/viroid was detected directly from crude leaf extracts after

simultaneous multiplex RT-RPA with high specificity. The results could be identified with the naked eye using oligonucleotide-conjugated gold nanoparticles and LFDs. The sensitivity of the CRISPR/Cas12a-RT-RPA platform is equivalent to that of RT-qPCR, with detection limits ranging from 250 to 2500 copies of virus. This method is simple and quick, requiring less than an hour from sample collection to the visualization of results.

Concluding Remarks

Plant diseases impose serious limitations on the cultivation of many crops in all growing regions due to a lack of rapid and sensitive methods for proper diagnosis. The key factors in any efficient disease management program are the reliable identification of pathogens and an understanding of their natural dissemination mechanisms. Immunoassay and nucleic acid-based techniques are currently the most used diagnostic methods in plant pathology. Among these techniques, PCR, and its modifications as well as various ELISA formats are the most popular. With the development of isothermal assays such as LAMP and RPA and their coupling with CRISPR/Cas systems, the detection of viruses and other pathogens has become far easier, as these assays do not require laboratory setup or specific technical skills. These tests can be performed onsite using crude extracts from plants as a template. The assays are more sensitive and require less time compared to conventional PCR assays, and the results can easily be interpreted visually.

These assays are useful for detecting viruses in seeds, vegetative propagules, weed hosts, and vectors that harbor viruses so that appropriate measures can be taken to contain the spread of the viruses within the field/locality. These assays can be used for screening germplasm accessions of crops for resistance against viruses and would also serve as a tool to study epidemiology and predict the outbreak of the disease.

Both isothermal assays discussed here are suitable for onsite detection of plant viruses and other infectious agents in resource-poor settings without the need for any special technical skills. Results of both assays can be visualized in real-time or endpoint detection using a lateral flow device. Further, among the two assays, the RPA assay is more suited for onsite detection as it can be performed using a pair of primers, lyophilized reagents (that can be stored at room temperature) and crude extracts from the test plant at low temperature (37-42 C), in a short time (5-20 min) and amenable for multiplexing.

However, RPA is affected by the initial concentration of the target template, concentration of magnesium acetate, mixing step, and the need for a purification step if results are viewed through agarose gel electrophoresis. On the contrary, LAMP is performed using 4-6 primers at higher temperatures (58-68 C) for a duration of up to 1 hr and is not easily amenable for multiplexing. Further, like RPA, LAMP is also affected by the initial concentration of the template, concentrations of magnesium sulfate, betaine, temperature, and time. CRISPR-based diagnostic assays are very specific, sensitive, and suitable for onsite detection either by real-time through fluorescence or endpoint through a lateral flow device. Besides its utility in the detection of infectious agents, it is used in the detection of miRNA, DNA methylation, and non-nucleic acids such as proteins. The current CRISPR-based diagnostic assays require prior amplification of the target nucleic acid molecule by RPA, LAMP, or PCR, adding to the time and cost of the assays. A small beginning has been made on the development of non-amplification-based CRISPR/Cas assay but more research efforts are needed in this direction.

Future efforts on the onsite detection should focus on the development of affordable, cost-effective and robust assays. RPA and LAMP based technologies coupled with CRISPR enzymes are revolutionizing pathogen diagnostics. Future improvements should focus on 1) building an inexpensive and portable device for in-field deployment 2) quick extraction methods to bypass the need for nucleic acid purification 3) multiplexing, to establish and optimize the detection of multiple pathogens simultaneously 4) to develop an artificial intelligence (AI) application to be able to share the data locally and globally which would aid in the better decision making process to manage the outbreak of diseases. With improvements and technology transfer to a simple yet affordable device, CRISPR-based diagnostic technologies has the ability to revolutionize onsite pathogen diagnostics.

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Table 1. Recombinase polymerase amplification (RPA) and reverse transcription (RT)-RPA based assays used for the detection of different plant viruses

Virus name (*Genus; Family*)	Target gene	Host	Detection method	Time (min)	Temperature (° C)	Sensitivity	Reference
DNA viruses							
banana bunchy top virus (*Babuvirus; Nanoviridae*)	replicase initiator protein	Banana	AGE	30	37	10 times more than PCR	(Kapoor et al., 2017)
bean golden yellow mosaic virus (BGYMV) (*Begomoviurs; Geminivirdae*)	C1 region	Bean	AGE	30	37	Equal to ELISA	(Londoño et al., 2016)
citrus yellow mosaic virus (CYMV) (*Badnavirus; Caulimoviridae*)	ORF3	Citrus	AGE	30	37	10 times less than PCR	(Kumar et al., 2018)
milk vetch dwarf virus (MDV) (*Nanovirus; Nanoviridae*)	CP	Cowpea	AGE, LFD	30	37	10^1 copies of MDV	(Cao et al., 2020)
piper yellow mottle virus (PYMoV) (*Badnavirus; Caulimoviridae*)	ORF 2	Black pepper	AGE	40	37	10 times more than PCR	(Mohandas and Bhat, 2020)
tomato mottle virus (ToMoV) (*Begomoviurs; Geminivirdae*)	C1 region	Tomato	AGE	30	37	Equal to ELISA	(Londoño et al., 2016)
tomato yellow leaf curl virus (TYLCV) (*Begomoviurs; Geminivirdae*)	C1 region	Tomato, tobacco, bean	AGE	30	37	Equal to ELISA	(Londoño et al., 2016)
RNA viruses							

Virus		Host	Method			Sensitivity	Reference
apple chlorotic leaf spot virus (ACLSV) (*Trichovirus; Betaflexiviridae*)	-	Apple	CRISPR/Cas 12a with oligonucleotide-conjugated gold nanoparticle	20	37	RNA transcripts of 0.01– 1 fM (25 viral copies) sensitivity equal to RT-qPCR	(Jiao et al., 2021)
apple necrotic mosaic virus (ApNMV) (*Ilarvirus; Bromoviridae*)	-	Apple	CRISPR/Cas 12a with oligonucleotide-conjugated gold nanoparticle	20	37	RNA transcripts of 0.01– 1 fM (25 viral copies) sensitivity equal to RT-qPCR	(Jiao et al., 2021)
apple stem grooving virus (ASGV) (*Capillovirus; Betaflexiviridae*)	CP	Apple and pear	AGE	1	42	10 times less than RT-PCR; Total RNA diluted up to 4.7 ng/µl	(Kim et al., 2018)
	-	Apple	CRISPR/Cas 12a with oligonucleotide-conjugated gold nanoparticle	20	37	RNA transcripts of 0.01–1 fM (25 viral copies) sensitivity equal to RT-qPCR	(Jiao et al., 2021)
apple stem pitting virus (ASPV) (*Foveavirus; Betaflexiviridae*)	CP	Pear	Capillary gel electrophoresis	4	42	1000-fold higher than RT-PCR; 1 fg/µL of RNA	(Kim et al., 2019)

Virus	Gene	Host	Method			Sensitivity	Reference
		Apple	CRISPR/Cas 12a with oligonucleotide-conjugated gold nanoparticle	20	37	RNA transcripts of 0.01–1 fM (25 viral copies) sensitivity equal to RT-qPCR	(Jiao et al., 2021)
barley yellow dwarf virus (BYDV) (*Luteovirus; Luteoviridae*)	CP	Oat	AGE	5	42	100 times more than RT-PCR; 50 fg/μl RNA	(Kim et al., 2020)
beet necrotic yellow vein virus (BNYVV) (*Benyvirus; Benyviridae*)	RNA-1	Sugarbeet	CRISPR-Cas12a Reporter Assay with fluorescence signal	60	42	0.1 pM of Target DNA	(Ramachandran et al., 2021)
chilli veinal mottle virus (ChiVMV) (*Potyvirus; Potyviridae*)	CP	Tobacco	AGE	20	38	10-fold more sensitive than RT-PCR; 10 fg RNA	(Jiao et al., 2020)
citrus concave gum-associated virus (CCGaV) (*Coguvirus; Phenuiviridae*)	RNA-1	Apple	AGE	30	38	10-fold more sensitive than RT-PCR	(Liu et al., 2021)
cucumber green mottle mosaic virus (CGMMV) (*Tobamovirus; Virgaviridae*)	CP	Watermelon	AGE	30	38	10-fold more sensitive than RT-PCR; 1.0×10^{-6} μg RNA	(Jiao et al., 2019b)
cucumber mosaic virus (CMV) (*Cucumovirus; Bromoviridae*)	CP	Banana	Real-time visual fluorescence (Exo probe)	25	40	3 pg/μl of RNA; up to 10^{-5} dilution of the	(Srivastava et al., 2019)

						crude leaf extract	
cucurbit yellow stunting disorder virus (CYSDV) (*Crinivirus; Closteroviridae*)	CP	Watermelon, squash	Real-time visual fluorescence (Exo probe)	30	40	2.5 pg purified total RNA	(Kalischuk et al., 2020)
ginger chlorotic fleck-associated virus 1 (GCFaV-1) (*Tombusviridae*)	CP	Ginger	AGE	50	39	100-fold more sensitive than RT-PCR	(Naveen and Bhat, 2020)
ginger chlorotic fleck-associated virus 2 (GCFaV-2) (*Ampleovirus; Closteroviridae*)	CP	Ginger	AGE	30	39	1000-fold more sensitive than RT-PCR	(Naveen and Bhat, 2020)
little cherry virus 2 (LChV2) (*Ampelovirus; Closterovridae*)	CP	Sweet cherry, mealybug	LFD	15	39	More than RT-PCR	(Mekuria et al., 2014)
maize chlorotic mottle virus (MCMV) (*Machlomovirus; Tombusviridae*)	CP	Maize	AGE	30	38	10-fold more than or less than RT-PCR	(Gao et al., 2021; Jiao et al., 2019a)
onion yellow dwarf virus (OYDV) (*Potyvirus; Potyviridae*)	CP	Onion	AGE	25	42	10-fold more than RT-PCR	(Kumar et al., 2021b)
plum pox virus (PPV) (*Potyvirus; Potyviridae*)	CP	Apricot, cherry, peach, plum	LFD, fluorescence (AmplifyRP)	15	39	More sensitive than Immunostrip	(Zhang et al., 2014)
	CP	Potato	AGE	30	39	100 times more than RT-PCR	(Kumar et al., 2021a)

potato virus X (PVX) (*Potexvirus; Alphaflexiviridae*)	CP	*Nicotiana benthamiana*	CRISPR/Cas 12a-induced fluorescence	30	37	Picomolar range (femtomolar range with longer reaction times)	(Aman et al., 2020b)
potato virus Y (PVY) (*Potyvirus; Potyviridae*)	CP	Potato	AGE, LFD, fluorescence	20	25-40	Equal to RT-PCR	(Babujee et al., 2019; Cassedy et al., 2022; Wang et al., 2020)
	CP	*Nicotiana benthamiana*	CRISPR/Cas 12a-induced fluorescence	30	37	Picomolar range (femtomolar range with longer reaction times)	(Aman et al., 2020b)
rice black-streaked dwarf virus (RBSDV) (*Fijivirus; Reoviridae*)	P10	Rice	LFD	20	37	Equal to RT-PCR	(Zhao et al., 2019)
rice yellow mottle virus (RYMV) (*Sobemovirus; Solemoviridae*)	ORF 2	Rice	AGE	05	41	Equal to RT-PCR	(Juma et al., 2021)
rose rosette virus (RRV) (*Emaravirus; Fimoviridae*)	RNA 3	Rose	AGE, fluorescence	20	42	1 fg/μl of viral transcript	(Babu et al., 2017a; Babu et al., 2017b)
sugarcane mosaic virus (SCMV) (*Potyvirus; Potyviridae*)	CP	Maize	AGE	30	38	10-fold more than or less than RT-PCR	(Gao et al., 2021)
sugarcane streak mosaic virus (SCSMV) (*Poacevirus; Potyviridae*)	CP	Sugarcane	AGE	05	38	100-fold higher than RT-PCR	(Feng et al., 2018b)
sugarcane yellow leaf virus (SCYLV) (*Polerovirus; Solemoviridae*)	CP	Sugarcane	AGE	10-20	27-39	10 times lower than RT-PCR	(Feng et al., 2018a)

tobacco mosaic virus (TMV) (*Tobamovirus; Virgaviridae)*	CP	*Nicotiana benthamiana*	CRISPR/Cas 12a-induced fluorescence	30	37	Picomolar range (femtomolar range with longer reaction times)	(Aman et al., 2020b)
tomato spotted wilt virus (TSWV) (*Orthospovirus; Tospoviridae)*	CP	Pepper	LFD	10	38	Equal to RT-PCR	(Lee et al., 2021)
yam mild mosaic virus (YMMV) (*Potyvirus; Potyviridae)*	CP	Yam	Fluorescence	30	37	1×10^{-3} dilution of crude extract	(Silva et al., 2018)
yam mosaic virus (YMV) (*Potyvirus; Potyviridae)*	CP	Yam	Fluorescence	30	37	1×10^{-3} dilution of crude extract	(Silva et al., 2018)
Viroids							
apple scar skin viroid (ASSVd) (*Apscaviroid; (Pospiviroidae)*	Full	Apple	AGE	10	42	10 times more than RT-PCR; Total RNA diluted up to 20 pg/µl	(Kim et al., 2021a)
	Full	Apple	CRISPR/Cas 12a with oligonucleotide-conjugated gold nanoparticle	20	37	RNA transcripts of 0.01–1 fM (25 viral copies) sensitivity equal to RT-qPCR	(Jiao et al., 2021)
hop stunt viroid (HSVd) (*Hostuviroid; Pospiviroidae)*	Full	Hops	LFD	20	39	2×10^9 copies of HSVd trimeric transcript (less than RT-PCR)	(Kappagantu et al., 2017)

peach latent mosaic viroid (PLMVd) (*Pelamoviroid; Avsunviroidae*)	Full	Peach	AGE	05	42	1000-fold more than RT-PCR.	(Lee et al., 2020)
potato spindle tuber viroid (PSTVd) (*Pospiviroid; Pospiviroidae*)	Full	Potato	LFD	30	39	10^6 copies of *in vitro* transcribed PSTVd RNA	(Ivanov et al., 2020)
tomato apical stunt viroid (TASVd) (*Pospiviroid; Pospiviroidae*)	Full	Tomato	Fluorometer	20	39	27 to 81- fold dilution of crude extract	(Kovalskaya and Hammond, 2022)
tomato chlorotic dwarf viroid (TCDVd) (*Pospiviroid; Pospiviroidae*)	Full	Tomato	LFD	19	39	1 pg pure RNA. Sensitivity equal to RT-PCR	(Hammond and Zhang, 2016)

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Figure legends

Figure 1: Schematic diagram of the lateral flow immune assay (LFIA).

(A) Illustration of a lateral flow strip. The sample is applied to the sample pad and transported to the conjugate pad via an absorbent pad to form an antigen-antibody complex. The complex moves via lateral flow, where virus-specific antibodies capture it at the test line. The band's intensity at the test line indicates the amount of test antigen. The band at the control line confirms that the assay was done correctly.

(B) Representative lateral flow immune assay. The sample sap is added to the sample well (S) and moves along the lateral flow strip via capillary action. Two bands indicate a positive test, while a single control band indicates a negative test. No band or only a band at the test line indicates an invalid result.

Figure 2: Amplification via Loop-mediated isothermal amplification (LAMP) and various detection methods.

(A) Diagram of LAMP primers located on the target sequence and the amplification process. The FIP primer binds to its complementary sequence and begins amplifying the first strand, followed by binding and amplification of the second strand by the F3 primer. The following steps involve the amplification of the FIP-amplified strand by the B3 primer. Finally, FIP and BIP continue the LAMP cycle, resulting in dumbbell-shaped amplicons. A full stepwise description of the individual steps is provided in the figure.

(B) Magnesium pyrophosphate-based visual detection of the LAMP product. During LAMP, a large amount of pyrophosphate ion is produced as a by-product, which reacts with magnesium provided in the reaction mixture. The resulting product, magnesium pyrophosphate, forms a white precipitate that allows easy visual detection. The turbidity of the final LAMP reaction confirms the presence or absence of the targeted nucleic acid.

(C) Calcein-based fluorescence detection of the LAMP product. The reaction of the by-product pyrophosphate with magnesium or calcein-manganese aids in the visualization of the LAMP product by producing a precipitate or emitting bright green fluorescence under UV light. Calcein-based fluorescence is enhanced by the presence of magnesium in the reaction mixture.

(D) Fluorexon-based visual detection of the LAMP product. Upon completion of the LAMP reaction, fluorexon-MnCl turns from orange to green. The resulting green fluorescence is visualized with the naked eye under UV light.

(E) Lateral-flow based detection of the LAMP product. To visualize LAMP with LFA, the target nucleic acid (RNA/DNA) is amplified via RT-LAMP or LAMP with DIG, biotin, or FITC-labeled primers. The labeled LAMP product is diluted and applied to the lateral flow strip. After 5 to 15 minutes of incubation, the appearance of bands on the test and control lines indicates the presence of the target nucleic acid.

Figure 3: Recombinase polymerase amplification (RPA)-based amplification and its detection methods.

(A) Schematic diagram of the RPA assay. An ideal RPA assay consists of forward and reverse primers, a recombinase protein that helps bind primers to the target nucleic acid, ssDNA-binding proteins to stabilize the ssDNA, and a polymerase to amplify the primer-bound strands. The stepwise amplification process is shown in the figure.

(B) Exonuclease-based detection of the RPA product. A 46-52bp long Exo probe flanked by a quencher and fluorophore binds to the amplified product. Exo probe contains a THF (tetrahydrofuran) residue known as dSpacer, which is cleaved by the exonuclease, thus releasing the fluorophore from the quencher. The observed fluorescent signal indicates the presence of the target nucleic acid.

(C) Endonuclease-based detection of the RPA product. A 46-52 bp long oligonucleotide probe labeled with FAM or Alexa fluor binds to the target strand. The annealed probe is cleaved by the nfo enzyme, freeing the 3′-OH group of the probe, and is used as a primer in the subsequent reactions. The resulting amplicons

are produced with FAM and biotin using a biotin-labeled reverse primer. The final RPA product is applied to the LFA strip, and the results are visualized after the appearance of matching lanes.

Figure 4: CRISPR-based diagnostic platforms.

A schematic diagram of the different CRISPR-based diagnostic platforms for nucleic acid detection. SHERLOCK, Cas12-DETECTR, Cas12-HOLMES, and Cas14-DETECTR make use of a single effector, while CONAN uses a multi-component Cas3 system for nucleic acid detection with their respective gRNAs. In the SHERLOCK system, the target is amplified with a forward primer appended with a T7 promoter sequence. The amplified product is reverse transcribed and used as a template for Cas13. In the Cas14-DETECTR system, the template is amplified with a phosphorothioate (PT)-containing primer to protect one strand from T7 exonuclease activity. The PT-protected strand is then recognized by Cas14. CONAN is based on a multi-component system. After target recognition, the highly specific collateral activity of these proteins is activated, causing them to cleave the corresponding reporter. Due to this activity, all of these systems have been effectively harnessed for efficient, robust, precise CRISPR-based nucleic acid platforms.

Figure 5: Onsite CRISPR-based detection of plant viruses.

A schematic flow diagram of nucleic acid detection using CRISPR/Cas-based biosensing technologies. The extracted nucleic acid from plants infected with either RNA or DNA viruses is amplified via RT-RPA/RT-LAMP (for an RNA template) or RPA/LAMP (for a DNA template) using target-specific primers. The amplified product is subjected to CRISPR/Cas-based detection, and the signal readout can be detected via real-time PCR or visually using different reporter systems. FAM/biotin and FAM or HEX reporters are primarily used for LFA and visual detection (under UV or LED light), respectively. All figures were created with BioRender.

Author contributions

MM conceived the idea. A.I Bhat, RA and MM wrote the paper.

Conflict of interest

All authors declare that they have no conflict of interest.

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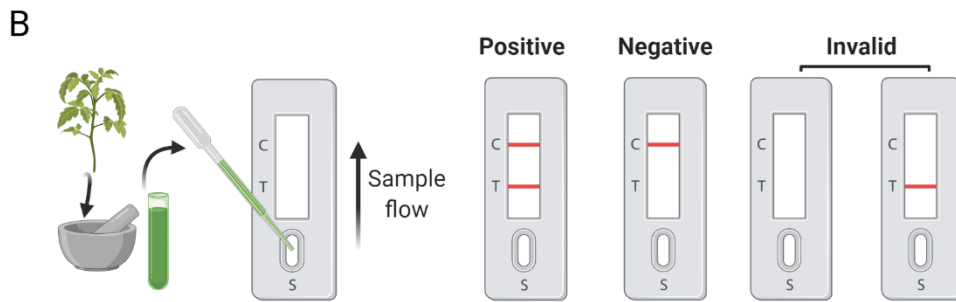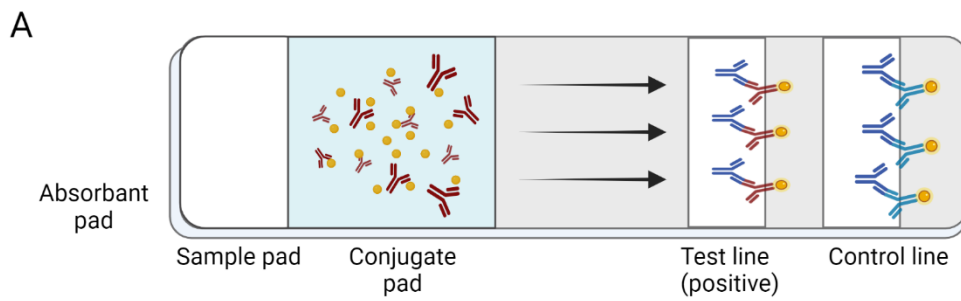
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Figure 1

A

Absorbant pad

Sample pad   Conjugate pad

Test line (positive)   Control line

B

Sample flow

Positive   Negative   Invalid

Sample loading and incubation

Add drop of sample in sample well (S). Capillary action moves sample across lateral flow test.

Interpreting results

Positive: one strip each in C well and T well
Negative: one strip in C well

Figure 2

A — LAMP primers on the target sequence

F3c F2c F1c B1 B2 B3
F3 F2 F1 B1c B2c B3c

1. **Forward inner primer (F1c-F2)** binds to F2c region and starts complementary polimerization

2. **F3** binds tp F3c and displaces **F1c-F2** strand and initiates a new polimerization cycle

3. **Backward inner primer (B2-B1c)** binds displaced strand and starts polymerization

4. **B3** displaces **B2-B1c** strand and initiates a new polimerization cycle

5. A **F1c-F2** primer binds the dumbbell structure and synthesizes a complementary strand

6. A **B2-B1c** primer binds the newly synthesized strand and continues the LAMP cycle

7. Additional **loop primers** can be added to increase the shape complexity of the amplicons

8. The cycle produces millions of loop complexes that can be detected using a specific assay

LAMP products (Loops and concatemers)

B — 65 °C, 15-30min, Before LAMP, After LAMP
Magnesium phyrophosphate based visual detection

C — 65 °C, 15-30min, UV, Before LAMP, After LAMP
Calcein and MnCl$_2$ based visual detection

D — 65 °C, 15-30min, UV, Before LAMP, After LAMP
Fluorexon based visual detection

E — RNA / DNA
RT_LAMP / LAMP products with DIG / Biotin or FITC labelled primers
Diluted LAMP product, Sample flow
Positive, Negative
Result

Figure 3

A

RPA/RT-RPA starting genetic material

① Recombinase primer complex formation

② Primer annealing and SSB protein binding

③ Polymerase-based amplification

④ Amplified product

|||| Primers
● ssDNA binding proteins (SSB)
● Recombinase
● Polymerase
∿ ssDNA
∿ RNA

B

dT-F   dT-Q   → dSpacer / dR group
● block group
Exonuclease
F

Amplification
Fluorescence
Threshold
No amplification
Copies per reaction (Ct)

C

nfo endonuclease   → dSpacer
F   ● block group

F
B
F
B

Positive   Negative
C   C
T   T
S   S

Readout

Figure 4

Figure 5

Plant infected with DNA virus → Extract DNA → Viral DNA

Plant infected with RNA virus → Extract RNA → Viral RNA

RPA / RT-RPA LAMP / RT-LAMP

Isothermal amplified product

CRISPR / Cas-based cis and trans cleavage

+ive   -ive
Visual onsite detection

Amplification
Fluorescence
Threshold
No amplification
Copies per reaction (Ct)
Real-time detection