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Review

CRISPR/Cas-Based Diagnostics in Agricultural Applications

Tanzena Tanny, Mohamed Sallam, Narshone Soda, Nam-Trung Nguyen, Mobashwer Alam, and Muhammad J. A. Shiddiky*

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ABSTRACT: Pests and disease-causing pathogens frequently impede agricultural production. An early and efficient diagnostic tool is crucial for effective disease management. Clustered regularly interspaced short palindromic repeats (CRISPR) and the CRISPR-associated protein (Cas) have recently been harnessed to develop diagnostic tools. The CRISPR/Cas system, composed of the Cas endonuclease and guide RNA, enables precise identification and cleavage of the target nucleic acids. The inherent sensitivity, high specificity, and rapid assay time of the CRISPR/Cas system make it an effective alternative for diagnosing plant pathogens and identifying genetically modified crops. Furthermore, its potential for multiplexing and suitability for point-of-care testing at the field level provide advantages over traditional diagnostic systems such as RT-PCR, LAMP, and NGS. In this review, we discuss the recent developments in CRISPR/Cas based diagnostics and their implications in various agricultural applications. We have also emphasized the major challenges with possible solutions and provided insights into future perspectives and potential applications of the CRISPR/Cas system in agriculture.

KEYWORDS: *CRISPR-Cas, pathogen diagnosis, GMO, nucleic acid detection, point-of-care testing, traditional strategies, plant protection, fast-track breeding*

1. INTRODUCTION

Diseases and invasive pathogens have a significant impact on agricultural production. Pests cause 20−40% of global yield loss each year. Plant diseases have a $220 billion global economic impact. Invasive pests are responsible for around $70 billion of this cost.[1] According to globally estimated data, the average global yield loss of five major crops (wheat, rice, maize, potato, and soybean) in food security hotspots is due to 137 disease-causing pathogens, and the loss is greater in highly populated food shortage regions and areas prone to pests and diseases.[2] The severity of the loss is contingent upon the timing of disease identification and the management method.[3,4] Early disease detection mitigates the loss by reducing the disease's spread and allowing for prompt efforts to eradicate the pathogens.[5] Utilizing disease-resistant or -tolerant high-yielding plant varieties is another strategy for reducing crop loss. Through conventional breeding, marker-assisted selection (MAS), and genetic transformation process, disease-resistant high yielding varieties are developed.[6−8] In molecular breeding programs, the use of polymerase chain reaction (PCR) and genome sequencing is required for continuous screening of genetic markers and target DNA, particularly for MAS and selection of transformed accessions.[7,9] Additionally, these technologies are commonly used in quarantine services for the detection of genetically modified (GM) organisms. However, these operations are arduous, time-consuming, and costly, and they require sophisticated equipment facilities and experienced technical management. A simple, rapid, on-field approach for detecting markers (SNPs, quantitative trait loci, linked genes) related to target traits can reduce the time and expense associated with these operations.

Visual detection of disease in the earliest stages of infection is challenging due to pathogens' microscopic shape and lengthy dormancy.[10] Additionally, many disease-causing agents exhibit identical symptoms, which makes the symptom based accurate diagnosis of disease extremely challenging. Immunoassays, PCR, and next generation sequencing based methodologies are widely used for detecting pathogens to address these issues.[10] These techniques are also laboratory based, time-consuming procedures that require specialists with expertise. Furthermore, a considerable amount of time is lost during the process of sample collection, transportation to the laboratory, and the delivery of the results to farmers.[11] Development and implementation of a simple, rapid, cost-effective on-site disease detection technology can reduce time and labor, accelerate disease management practices, and increase agricultural production.[12]

CRISPR (Clustered regularly interspaced short palindromic repeats) is a family of DNA sequences found in bacteria and plays an important role in the bacterial defense system with its associated Cas (CRISPR associate) proteins. Cas endonucleases recognize and cleave specific DNA targets complementary to CRISPR sequences termed as CRISPR/Cas technology. The precise nucleic acid detection and cleavage

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Table 1. Comparison of CRISPR/Cas and Existing Nucleic Acid Diagnostic Methods of Plant Pathogens

Detection method	Working principle	Advantages	Disadvantages	Ref
Polymerase chain reaction (PCR)	Amplification of DNA polymerase enzyme in artificial condition in thermal cycler	Mature and reliable technology	Time consuming, need thermal cycler and gel electrophoresis; effectiveness is subjected to DNA extraction, inhibitors, polymerase activity, expensive, lab-oriented	32,34,35
Real-time PCR (qPCR)	PCR amplification with fluorescent dye providing quantitative and real-time monitoring	Specific, sensitive, efficient, rapid, and universal; real time quantification and monitoring, most common technique	Time consuming, expensive, needs complex thermal cycler and specialized technical personnel, lab-oriented	34,35
FISH	Detection of DNA or RNA sequences in cells or tissues with fluorescently labeled DNA/RNA probes	Rapid, high sensitivity, specificity, and reproducibility	Autofluorescence, photobleaching, needs wide field epifluorescence or confocal laser scanning microscope, lab-oriented	10,34
Sequencing	Order of nucleotide bases of target DNA is determined with a sequencer	High reliability, high sensitivity, multiplexing	Specialized laboratories, expensive equipment, technical personnel, complex sample preparation and data processing time consuming	37
Nanopore sequencing/MiniON	DNA sequencing with a Fourth-generation portable sequencer	High-throughput results, easy detection of multiple pathogens, portable	High cost, not completely user-friendly	35,37
DNA microarray	A collection of known tiny DNA spots attached on a solid surface where pathogen DNA hybridizes	High throughput, sensitive, multiple detection	High-cost, low specificity, and accuracy, depends on PCR and imaging	5,33,43
LAMP	Amplification of DNA/RNA target in a single tube at 60−65 °C temperature with an isothermal polymerase and a set of four to six primers	Rapid, simple, isothermal condition, high sensitivity, high amplification, relatively cost-effective, in-tube visual detection, no denaturation needed; in-field detection	Complicated primer designing, complicated process for nonspecialist, many sizes of amplicons are generated, false positive	35,37,40
RPA	Amplification of DNA/RNA is done at 32−40 °C temperature	Simple, rapid, isothermal, body temperature, does not require an initial denaturation step	Sensitive to contaminants and off-target nucleic acid contamination long primers needed, specificity and sensitivity may vary	35,37
RCA	Amplification of DNA to form a long single stranded DNA or RNA using a circular DNA template and special polymerase at isothermal condition	Isothermal, high sensitivity and specificity	Expensive, the detection may be tricky	35,44
SDA	Isothermal amplification with restriction endonuclease mediated nicks of target and extension of nick site with polymerase by strand displacement	Rapid, isothermal condition	Inefficient for amplification of long transcripts	35,45
HDA	Isotheral amplification with helicase and polymerase enzyme	Rapid, isothermal condition, initial denaturation is not needed	Require extensive optimization	35,46
NASBA	Primer dependent transcription based continuous amplification of RNA in isothermal condition	Rapid, isothermal amplification	Expensive	35,47
CRISPR/Cas	Sequence-specific binding and cleavage of target sequence by Cas nuclease upon gRNA comparability, and signal generation through cis and trans cleavage of reporter molecules	Rapid, cost-effective, high sensitivity, high efficiency, single nucleotide specificity, robust, precision diagnostics, easily programmable and verily adaptable, multiplexing, simple design, POC application	Preamplification of target required, variable reproducibility	39,48−50

property of CRISPR/Cas technology has revolutionized the field of gene editing and has had a profound impact on medical science, biotechnology, and agriculture.[13−17] Its usefulness has expanded to include diagnostics and biosensors in addition to the broad fields of genome and chromatin alteration research.[13] As a precise, sensitive, and robust diagnostic technique, CRISPR technology has already demonstrated its effectiveness and dependability.[14,15] The discovery of indiscriminate cleavage activity on single-stranded RNA and DNA (ssRNA, ssDNA) by certain Cas proteins (Cas12, Cas13, and Cas14) has added new dimensions to nucleic acid diagnostics.[16−19] As a straightforward and natural adaptive immune mechanism, CRISPR has acquired community acceptance.[20] It is programmable and can be rapidly altered to the appearance of new diseases.[14] The CRISPR/Cas system is sequence-specific, and a two-step validation of the target, i.e., protospacer adjacent motif (PAM) recognition and single guide RNA (sgRNA) compatibility, enhances its specificity, sensitivity, and accuracy for target detection. In addition, the compatibility of CRISPR/Cas systems in creation of diverse signal transducing readouts, spanning from device-oriented fluorescent, electrochemical, and visual colorimetric readouts to lateral flow band on paper, has expanded various diagnostic choices.[14] It does not require complicated thermocyclers as the reaction temperature is 37 °C and requires a limited amount of reaction time. Furthermore, the reagents and reactions are easy to handle. These characteristics make CRISPR/Cas technology a potential and effective molecular tool to develop rapid, sensitive, and on-site diagnostic techniques.

The CRISPR/Cas system has been utilized for the identification of plant nucleic acids in various applications, such as the detection of genetically modified organisms (GMO),[21] differentiation and profiling of characteristics,[22] as well as the detection of plant pathogenic viruses,[23] bacteria,[24] fungi,[25] and insects,[26] through nucleic acid identification. Specific high sensitivity enzymatic reporter unlocking (SHERLOCK)[16] and DNA endonuclease targeted CRISPR trans reporter (DETECTR)[19] are two of the most well-known CRISPR based diagnostics tools in the healthcare sector that have been adopted in plant pathogen diagnosis.[24,27,28] Karmakar et al. provided a summary of the application of CRISPR technology for basic studies of host−pathogen

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interaction, virulence, and pathogen suppression in the field, as well as the CRISPR based methods for plant disease detection.[29] In another review, the current and potential applications of the CRISPR technology in plant pathology and disease management, including crop disease resistance, the research of host−pathogen interaction, and pathogen diagnostics, are described.[30] In their review on isothermal amplification based detection of plant viruses, Bhat et al. provided a summary of the integrated CRISPR/Cas system for on-site plant virus diagnostics.[31] Although the application of CRISPR technology for the detection of single nucleotide polymorphism (SNP) is still in its early stages, current advancement in the field clearly suggests that CRISPR technology can be utilized for the early diagnosis of plant diseases, the screening of germplasm for desired traits, and detection of genetically modified phenotypes using genetic markers or SNPs. Therefore, it is essential to have a thorough understanding of current CRISPR/Cas technologies for plant pathogens and trait diagnostics. The application of CRISPR diagnostics for plant genes and pathogen detection has been discussed partly in different reviews; however, a complete picture that summarizes all the advancements has not yet been published. In this review, an overview of the most used CRISPR/Cas based methodologies for plant nucleic acid and pathogen detection, highlighting the major technical challenges and limitations of existing techniques, is presented.

2. CURRENT MOLECULAR DIAGNOSTIC TOOLS FOR PLANT PATHOGENS

Various molecular diagnostic tools are currently used for plant pathogen diagnosis, including PCR, DNA microarray, DNA sequencing, and isothermal amplification techniques. PCR is the most commonly employed technique for pathogen diagnosis, where the target DNA is amplified in a thermal cycler and analyzed using gel electrophoresis systems.[32] Different types of PCR systems, such as real-time PCR, nested PCR, seminested PCR, multiplex PCR, droplet digital PCR, reverse transcriptase PCR, allele-specific PCR, PCR-RFLP, and AFLP, are utilized for pathogen diagnosis (refer to Table 1).[5,33−35] Real-time PCR, with its real-time, sensitive, and quantitative nature, is considered the gold standard nucleic acid amplification technique.[33] Although PCR based pathogen detection methods exhibit high accuracy and sensitivity, they are not ideal for point-of-care (POC) applications due to the requirement for complex thermal cyclers and time-consuming procedures.[31] Moreover, in some reports, CRISPR based detection has demonstrated higher sensitivity than PCR based detection.[36]

DNA microarrays (also known as DNA chips, gene chips, or biochips) offer multiplexing capability, with discrete microscopic spots of oligonucleotides specific to different pathogens immobilized on array surfaces, where labeled DNA is hybridized.[33] However, the reliance on PCR limits the field applicability of DNA microarray based detection, despite its improved sensitivity. DNA sequencing allows for precise identification of pathogens, but the multistep procedures, expensive instruments, expert handling, and complex bioinformatics analysis make it unaffordable for many resource-limited areas.[33,34,37] In terms of field applicability, speed, and cost-effectiveness, CRISPR/Cas technology outperforms PCR, sequencing, and microarray based detection approaches.[38,39]

For POC diagnostics, several isothermal amplification technologies have been developed, including loop-mediated isothermal amplification (LAMP), recombinase polymerase amplification (RPA), nucleic acid sequence based amplification (NASBA), helicase-dependent amplification (HDA), rolling circle amplification (RCA), and strand displacement amplification (SDA).[12,35] Among these, LAMP is particularly significant, as its set of primers enhances sensitivity, and in-tube colorimetric and fluorescent signals improve field applicability.[12,40] Isothermal amplification technologies do not require complex thermocyclers and can be integrated into simple hand-held devices, increasing their portability.[41] However, complex primer design, expensive reagents, and the need for instrumental product visualization introduce complexities in most of these methods compared to the CRISPR/Cas system.[35] A comparison of the advantages of CRISPR over other technologies is provided in Table 1.

Rapid and POC testing of causal agents of plant diseases is crucial for disease management and control.[12,32] In recent years, CRISPR/Cas technology has been widely employed for molecular diagnosis of diseases.[14,30,39] Its high sensitivity, specificity, speed, minimal equipment requirement, portability, adaptability, and versatility position the CRISPR/Cas system as an ideal for future molecular diagnostics for plant protection.[38,39,42]

3. CRISPR BASED ASSAY FOR PLANT DIAGNOSTICS

CRISPR-Cas is a natural immune system found in numerous archaea and bacteria.[51] CRISPR and its associated protein Cas confer sequence-directed protection against phage viruses in bacteria. Bacteria, when invaded by viruses, captures and incorporates small parts of the viral DNA in its own genomic DNA (CRISPR sequence), which is later used as a memory to generate RNA (gRNA) that can recognize viral DNA and chops them with Cas endonucleases to disable the viruses. The bacterial defense mechanism against invading genetic material is dependent on different Cas gene operons, spacers, and gRNAs. There are three major types (type I, II, and III) of CRISPR systems with various subtypes and chimeric variants.[52,53] The CRISPR-Cas system is divided into two classes, class 1 (types I, III, and IV) and class 2 (types II, V, and VI), based on the genes encoding the effector modules. Class 1 systems consist of CRISPR RNA (crRNA)-effector complexes with several subunits, whereas class 2 systems possess a single protein effector for defense.[54] Class 2 effectors, Cas 9 (type II), Cas12 (type V), and Cas13 (type VI) are the most frequently utilized CRISPR-Cas systems for nucleic acid targeting and genome editing.[53,55]

In general, Cas9 and Cas12 targets DNA and Cas13 targets RNA.[56] Nucleic acid identification and cleavage activity of the CRISPR Cas system is composed of a CRISPR associated endonuclease protein (Cas) and a gRNA. In CRISPR/Cas system, Cas nuclease is directed by gRNA toward the nucleic acid target which contains the compatible sequence of gRNA and cuts the target sequence after binding with the complementary sequence.[54,57] Target recognition depends on the presence of a Cas protein-specific protospacer adjacent motif (PAM) or protospacer flanking site (PFS) on the target sequence and sequence similarity of the target with gRNA. The protospacer adjacent motif in Cas9 and Cas12 is a two to six base pair DNA sequence (NGG, TTTN) positioned upstream or downstream of the gRNA-complementary target sequence.[58] Single nucleotide base PFS (A/T/C) is used by Cas13 for target RNA recognition, while certain Cas13 variants do not require PFS.[59] The Cas protein and gRNA complex

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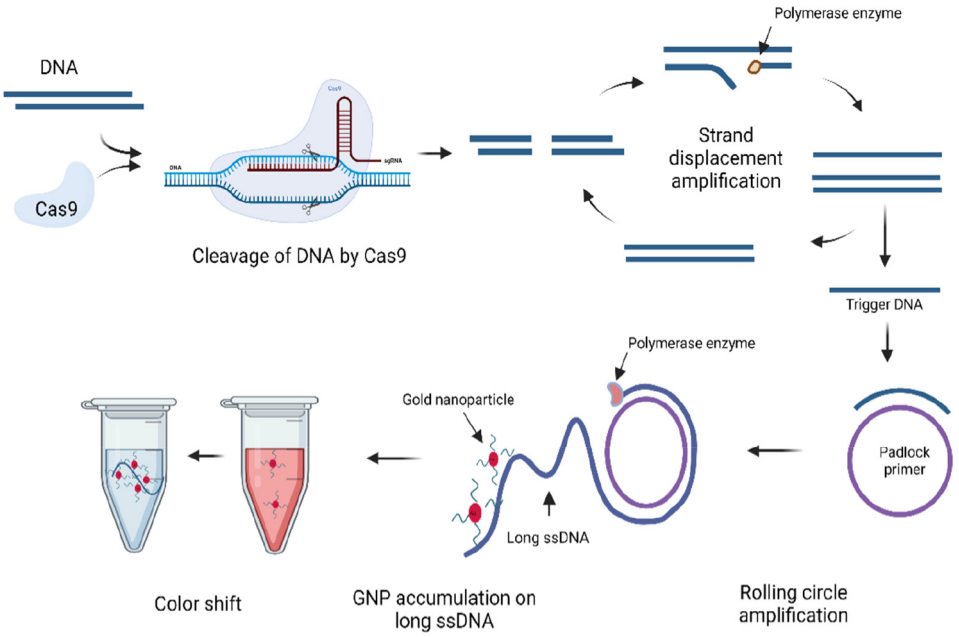

Figure 1. Schematics of CRISPR/Cas9-AuNP based detection of plant pathogens using Cas9 triggered isothermal amplification and colorimetric detection of targets.[68]

examine the double-stranded DNA (dsDNA) for the PAM site. Upon PAM recognition the complex unwinds the dsDNA,[60] and verifies the comparability of the target sequence with gRNA. The gRNA binds with the complementary target strand, displaces the nontarget strand, and generates an R-loop structure,[61] and in complementarity match, a double-stranded break (DSBs) of the target nucleic acid (DNA, RNA) occurred by Cas nuclease.[62] The Cas protein's cleavage of the target strand is known as the cis-cleavage activity. After cis cleavage, a complex comprising Cas protein, gRNA, and target sequence is generated in Cas12 and Cas13 systems and a second cleavage activity of nonspecific single stranded DNA/RNA is launched. This phenomenon is termed trans cleavage or collateral cleavage activity. The formed triplex complex (Cas/crRNA/Target) can cleave nontarget ssDNAs and ssRNAs without any sequence match.[19] This property of cis and trans cleavage has been utilized extensively in disease diagnosis systems.[17]

3.1. Cas9 in Plant Diagnostics. Cas9 endonuclease is the first discovered CRISPR protein that targets and cleaves dsDNA. It is a pioneer in genome engineering and CRISPR based biosensing technology. In the CRISPR/Cas9 system, the Cas9 protein is activated and led by a gRNA toward the target dsDNA. Cas9 binds to and unwinds the target dsDNA, and upon gRNA matching, a blunt double-stranded DNA break occurs three base pairs upstream of the PAM sequence.[60,63,64] The PAM sequence and gRNAs are important for target detection and the cleavage activity of the CRISPR/Cas9 system. The gRNA of Cas9 is composed of a crRNA that binds to the complementary sequence of the target DNA and a trans-activating CRISPR RNA (tracrRNA) that assists in binding to the Cas9 nuclease. These two RNAs (tracrRNA and crRNA) can be linearly organized into a single gRNA.[65] The Cas9 protein targets only dsDNA flanked by a Cas9-specific 3′ PAM sequence. The PAM requirement of the most commonly used and experimentally characterized Cas9 orthologue SpCas9a (Cas9 of *Streptococcus pyogenes*) is 5′-NGG-3′, while other Cas9 orthologues have different PAM requirements.[66] The cleavage is dependent on the nuclease domains HNH and RuvC, where HNH cleaves the complementary strand and RuvC cleaves[65] the noncomplementary strand.

Although the CRISPR/Cas9 system has been extensively utilized to develop plant resistance against viruses, bacteria, and fungi,[44] its application in the field of crop disease and plant pathogen detection is limited. Two types of Cas9 based detection platforms have been employed thus far for the detection of phytopathogens and plant nucleic acids. The first type is a visual colorimetric shift, where the target-specific cleavage activity of Cas9 serves as a trigger for isothermal amplification.[68] The resulting product is then used to induce the aggregation of AuNPs (gold nanoparticles) for signal transduction. In the second type, the precise binding capacity of Cas9 and dCas9 with the target (biotinylated or FAM-labeled) and labeled AuNPs is utilized to generate signals in lateral flow assays (LFAs). Examples of such assays include CRISPR/Cas9-mediated lateral flow nucleic acid assay (CASLFA)[67] and biotin-coupled specific CRISPR based assay for nucleic acid detection (Bio-SCAN).[71]

The target-specific cleavage activity of Cas9 was used as a trigger of isothermal amplification, and the product is used for signal transduction to develop a colorimetric method for the detection of plant pathogen *Phytophthora infestans*.[68] In the biosensing assay, target dsDNA of the pathogen is detected and broken by the Cas9/gRNA complex. The cleavage product contains the 3′●●●CGTTAC/NN●●●5′ sequence that is nicked with Nb.BsrDI endonuclease; Bst 3.0 DNA polymerase binds at the nicked site and extends the strand by displacing the downstream strand. Thus, from recurring cleavage and strand displacement amplifications, many ssDNAs are released which serve as the catalyst for rolling circle amplification (RCA) process along with a padlock probe. A long ssDNA with repeating hybridization sites is generated from the RCA process followed by strand displacement amplification. The subsequent long DNA hybridizes with the DNA strands of oligo-functionalized AuNPs and aggregates them, resulting in a noticeable shift from wine red to purple color (Figure 1). Two picomolar synthetic DNA can be detected visually through this

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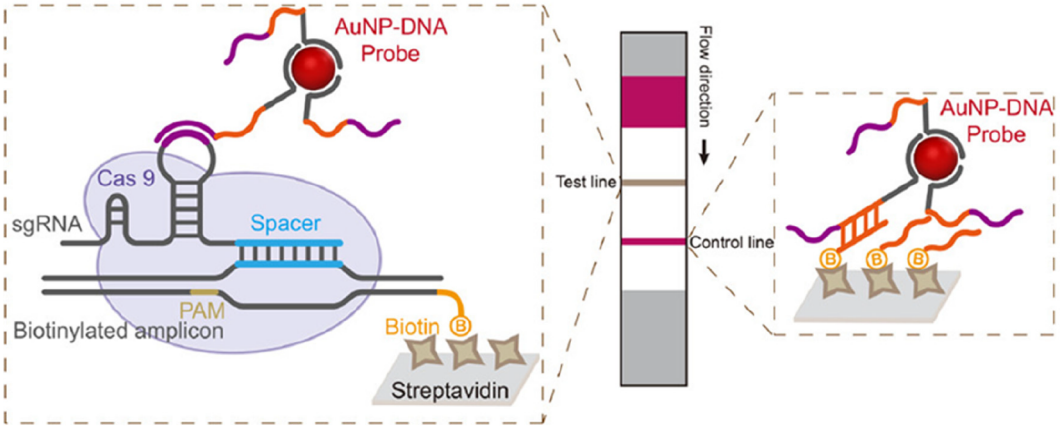

Figure 2. Schematics of the CRISPR/Cas9-mediated lateral flow nucleic acid assay (CASLFA). Reproduced with permission from ref 67. Copyright 2020 American Chemical Society.

Table 2. Cas9 Based Detection of Plant Pathogens and Transgenic Traits

Target plant/Pathogen	Nucleic acid extraction process	DNA amplification	Cas enzyme	Time	Limit of Detection	Detection Type	Assay name and ref
Phytophthora infestans	Genomic DNA extraction	SDA-RCA	Cas9		0.2 pm to 20 nm	Colorimetric	[68]
Transgenic rice, other targets - African swine fever virus (ASFV), *Listeria monocytogenes*	DNA extraction from rice powder	PCR, RPA	Cas9	1 h 40 min	100 copies, 0.01% of transgenic content	LFA	CASLFA[67]
Rust resistant in wheat cultivars	DNA/RNA extraction at lab	RPA, RT-RPA	dCas9	<1 h		LFA	Bio-SCAN[72]
Transgenic plant							
Mutant rice lines							
Puccinia striiformis f. sp. *Tritici*							
Magnaporthe oryzae Triticum fungus							
Tomato yellow leaf curl virus							
Tobacco mosaic virus							
Potato virus Y							
Pseudomonas bacteria							
Agrobacterium tumefaciens							

method, and it was successful in detecting *P. infestans* genomic DNA.[68] However, the detectability of the bacteria in plant extracts and the specificity of the method for detecting *P. infestans* in the presence of nonspecific pathogens or multiorganism environmental samples were not addressed.

In genetic research, the application of lateral flow tests is plagued by false positive results and lengthy, inefficient hybridization procedures.[69] Wang et al.[67] devised a CRISPR/Cas9-mediated lateral flow nucleic acid assay (CASLFA) technique to combat the problems. Within 1 h, the method can detect hundreds of copies of *Listeria monocytogenes*, transgenic rice (GMOs) and African swine fever virus genomic material. They harnessed biotinylated primers to amplify the targets, and Cas9/sgRNA (Cas9 single guide RNA) binds with the biotinylated targets. Cas9/sgRNA sequentially unwinds the dsDNA and releases the nontarget strand as a single strand upon locating the target with PAM.[70] They came up with a strategy to use this segment as the region to be hybridized[67] with a DNA probe connected to AuNP. They referred to it as the DNA unwinding hybridization assay. To promote universality and to utilize a common DNA probe, the sgRNA2 scaffold was rebuilt by inserting an extra sequence to form an expanded stem-loop structure. This stem loop served as the DNA probe's anchoring location.[67] The AuNP-DNA probes hybridize with either the unwound segment of the DNA or with the stem loop region of the sgRNA2 (Figure 2). As a result, a Cas9/sgRNA/biotinylated target/AuNP-DNA probe complex is formed. When the complex is run on LFA, the biotinylated portion of the target DNA is caught on the streptavidin line of the LFA strip, and gold buildup on the line generates a colorimetric band (Figure 2). In the absence of target, no biotinylated target is formed. As a result, no band is observed on the test line. The CRISPR/Cas9 enzymatic assay to generate the Cas9/sgRNA/biotinylated target/AuNP-DNA probe complex takes around 5 min, and LFA detection process takes only 3 min. The assay was able to detect 0.01% PCR amplified transgenic samples with the naked eye and grayscale analysis. In CASLFA, RPA was incorporated to reduce the time of target amplification to 20 min and temperature to 37 °C to remove the dependency on thermal cycler.

The LFA based Bio-SCAN method, which was created by Ali et al.,[71] for on-site detection of SARS-CoV-2, also utilized RPA for amplification and has been successfully applied by Sánchez et al.[72] for a variety of agricultural application. In this method, isothermal RPA amplification of target with FAM labeled primers was combined with dead biotinylated deactivated Cas9 (dCas9) protein to develop a biotinylated FAM labeled complex instead of target amplification with

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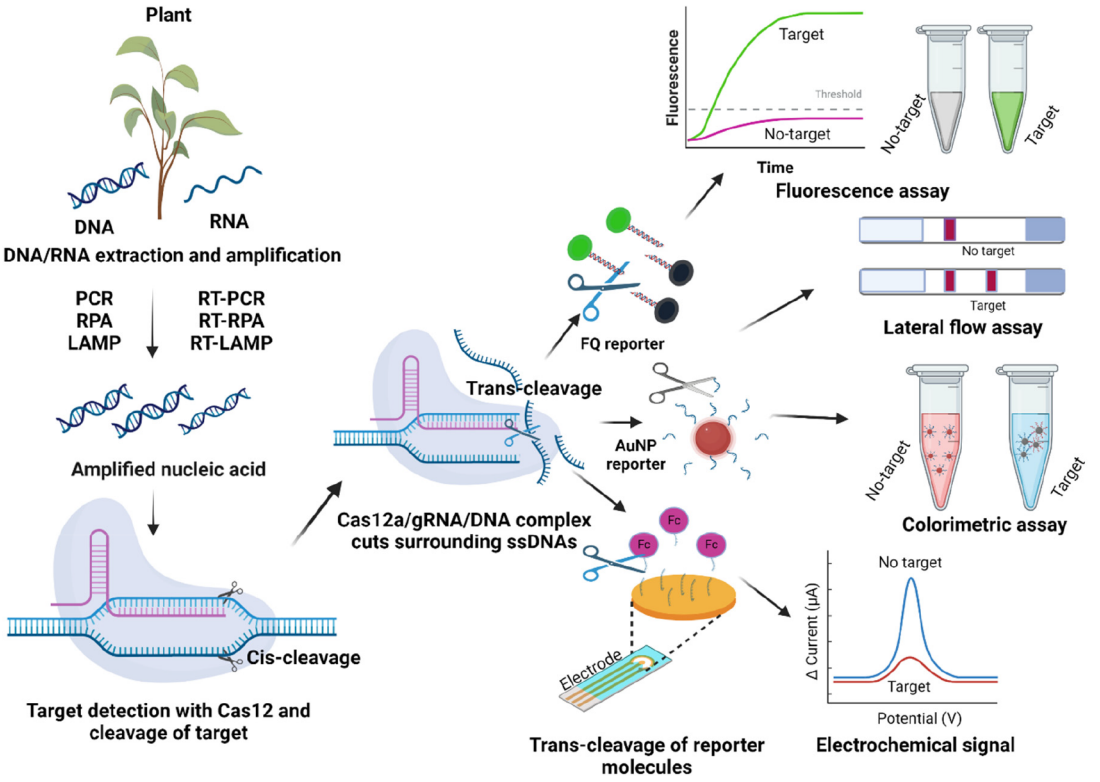

Figure 3. Schematics of the Colateral Clease-Coupled CRISPR/Cas12a biosensing method. Different types of signal transduction methods, such as fluorescent real-time, lateral flow assay, colorimetric detection, and electrochemical assay can be adapted with this method.

biotinylated primer. The complex hybridizes with AuNP reporters and gives visible bands on the streptavidin line of commercially available streptavidin−biotin based LFA strips. The reagents have a longer shelf life and can be applicable in resource limited field settings.[71,72] Bio-SCAN showed success in the detection of diverse phytopathogens, i.e., viruses, bacteria, and fungi (Table 2). Moreover, it demonstrated effectiveness in the detection of herbicide resistance alleles in rice plants and fungal resistance alleles in wheat germplasm.[72] Additionally, it was able to identify typical transgenic sequences (such as the *CaMV 35S* and *Ubi* promoters)[72] in total genomic DNA isolated from genetically modified crops (such as transgenic rice and tobacco). As a robust and easy on-site diagnostic platform, Bio-SCAN can facilitate plant protection, breeding, and genome engineering programs.

The Bio-SCAN method is a more efficient and versatile tool for plant pathogen detection and GMO identification.[72] In contrast, the Cas9 based visual colorimetric assay[68] requires a more complex process involving several enzymes and cyclic probes, limiting its range of applications. Both CASLFA[67] and Bio-SCAN[72] methods have successfully identified a wide range of targets and have proven to be effective at the point-of-care. However, the Bio-SCAN method has several advantages over other techniques, including lower costs, increased stability, adaptability, and the ability to detect a broad range of elite plant alleles and phytopathogens such as viruses, bacteria, and fungi.[72] The use of commercial LFA strips, low-maintenance reagents, and redesigned primers and sgRNA have made the Bio-SCAN platform simple and adaptable for field application.[72] Although the design of special sgRNA2 is somewhat complex, CASLFA[67] surpasses Bio-SCAN and other LFA systems in terms of speed, sensitivity, and specificity.

3.2. Cas12 Diagnostics in Agriculture. Cas12 is a versatile single RNA-guided endonuclease that is smaller in size compared to Cas9. It belongs to class 2, type V CRISPR effectors and is capable of targeting and cleaving specific dsDNA.[57] It can trans-cleave neighboring nonspecific ssDNAs consecutively. It exhibits various subtypes, namely, Cas12a (formerly designated as Cpf1[73]), Cas12b, Cas12c, and Cas12f (previously known as Cas14).[55,74,75] The Cas12 enzyme requires an crRNA without tracrRNA and a T-rich PAM sequence (5′-TTTN-3′) upstream of the gRNA binding site on the dsDNA sequence for effective binding and cleavage of the dsDNA target. Unlike dsDNA targeting, ssDNA targeting and breakage are not limited by the presence of a PAM. The crRNA leads the Cas12 protein to the target dsDNA, and following PAM and crRNA match, a double-stranded staggered DNA break 18−23 base pairs downstream from the PAM region occurs.[57] Cas12 lacks the HNH domain, and only the RuvC nuclease domain is responsible for cleaving both strands of dsDNA in the presence of cations (Mg^{2+} and Ca^{2+}).[73,76,77] The cleavage of target dsDNA initiates collateral cleavage activity of surrounding nonspecific ssDNAs.[19,78] Apart from ssDNAs, Cas12a exhibits collateral cleavage of hairpin RNAs and ssRNAs longer than 11 bp only in the presence of a ssDNA target.[79] Trans cleavage of hairpin structures are quicker than single-stranded structures.[78,79]

The remarkable property of the Cas12a enzymes, which allows them to cleave ssDNA indiscriminately and nonspecifically upon target recognition, has found extensive use in plant disease diagnostics. This collateral cleavage activity is harnessed in CRISPR diagnostic systems to generate various types of signals, including fluorescent real-time, UV−visible, lateral flow, colorimetric, and electrochemical signals (Figure

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Table 3. Cas12 Based Detection of Plant Pathogens, Plant Species, and Transgenic Traits

Target plant/ Pathogen	DNA extraction process	DNA amplification	Cas enzyme	Time	Limit of detection	Detection Type	Assay name and ref
Fluorescent detection assays							
Fusarium graminearum, fungus	DNA Purification Kit	PCR	LbCas12a		1 fg/μL, 4 dpi	Plate reader	[84]
Tomato mosaic virus	RNA mini kit	RT-PCR	LbCas12a		15−30 ng of PCR products	Plate reader	[23]
Tomato brown rugose fruit virus							
Transgenic rice	Conventional SDS	PCR	LbCas12a		8×10^1 copies/ μL	UV gel system	[85]
Beet necrotic yellow vein virus	RNA extraction Kit	RT-RPA	Cas12a		0.1 pM synthetic; 0.1 ng (real sample)	Plate reader	DETECTR[86]
Fungicide-resistant *Puccinia striiformis*	Lab extraction	RPA	Cas12a	40 min	25 aM	Plate reader	iARMS[87]
Bactrocera zonata, *Ceratitis capitata* fruit fly	Total insect DNA, kit & Chelex 100	RPA	LbCas12a	1.5 h		Plate reader	[88]
GM corn Powder	DNA extraction kit	RCA	LbCas12a	30 min	45.0 pM.	Plate reader	isoCRISPR[89]
Potato virus X	RNA extraction kit	RT-RPA	LbCas12a	30 min	10 pM	Portable P51 fluorescence viewer	iSCAN-OP[90]
Tobacco mosaic virus					7−10 dpi		
Tomato yellow leaf curl virus	Conventional extraction	LAMP	LbCas12a	1 h	100 aM of synthetic dsDNA	Portable P51 fluorescence viewer	[91]
Tomato leaf curl New Delhi virus							
Erwinia amylovora Acidovorax citrulli bacteria	Simple DNA extraction,	RPA	LbCas12a-5M	40 min	40 CFU/reaction	Hand-held UV light	LbCas12a-5M[82]
Fungicide resistance in *Venturia carpophila*	Pericarp Sample boiled in buffer for 2 min	RPA		45 min	7.82×10^3 fg/ μL	Naked eye, UV flashlight/cell phone torch POC	One-pot RPA-CRISPR/ Cas12[92]
Maize chlorotic mottle virus	Extraction with trizol Reagent	RT-RAA	LbCas12a	45 min	2000 ng cDNAs diluted up to 10^{-5}	Naked eye under blue light POC	[93]
Xanthomonas oryzae pv *Oryzae* bacteria	DNA extraction under preheating and adding EDTA	RPA	Cas12a		3 dpi plant leaf, Xoo-DNA = 1×10^3 copies/μL, Xoo-cell = 2.5×10^3 cfu/mL	Visual LED transilluminator	RAC[24]
Phyllanthus amarus plant	Extraction kit	RPA	Cas12a	~2 h	0.8 fg accuracy degree of 90% for species authentication	Naked eye, LED transilluminator	Bar-cas12a[94]
Datura L.	Lab extraction	RPA	Cas12a	~1 h	single-copy 10 copies/μL	Microfluidic assay	[95]
GM soybean (Roundup Ready)	Tiangen Plant Genome Extraction Kit	LAMP	Cas12a	5 min	0.05% transgenic content	Naked eye, UV light	[97]
GM soybean powder	Tiangen Plant Genome Extraction Kit	PCR/LAMP	Cas12a		0.1% transgenic ingredients	Portable biosensor LED	Cas12a-PB[96]
Lateral flow assays							
Magnaporthe oryzae Triticum fungus	Lab extraction	RPA	Cas12a	30 min	0.001 μg/μL	LFA (PCRD)	DETECTR-NALFIA[25]
Candidatus phytoplasma trifolii bacteria	Extraction kit	RPA	Cas12a		1−100 aM	Plate reader, LFA	DETECTR[99]
Candidatus Liberibacter asiaticus bacteria	Extraction Kit	RPA	Cas12a	1.10 h	Attomole	Plate reader, LFA	DETECTR[98]
Pseudomonas syringae pv *Actinidiae* bacteria	Filter paper based DNA extraction method	RPA	Cas12c1	2.15 h	3.9 nM	Gel imager, LFA	Cas12c-DETECTOR[83]
Heterodera schachtii nematode	DNA Extraction Kit	RPA	Cas12a	<1 h	Single cysts and single female $s = 10^{-4}$, Single second-stage juvenile $s = 4^{-3}$, Genomic DNA = 0.001 ng template	Real time, PCR, LFA	[104]
Magnaporthe oryzae fungus, Bt rice	Filter paper based dipstick DNA extraction	RPA	LbCas12a	30 min		Plate reader LFD	[21]
Elsinoe fawcettii fungus	Infected leaf mashed in buffer	RPA	Cas12a	1 h	1 fg	LFA POC	[101]
Tomato brown rugose fruit virus	Rapid paper strip-mediated RNA extraction	RT-LAMP	LbCas12a	1 h	10^{-4} ng/rxn (RNA sample), 100 copies of transcript/rxn (Synthetic)	PCR, System, LFA	[102]
Maize chlorotic mottle virus	Crude RNA extracts alkaline-PEG buffer	RT-RAA	Cas12a	1 h	2.5 copies CP; 0.96 pg RNA	LFA	One-tube one-step RT-RAA/

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J. Agric. Food Chem. 2023, 71, 11765−11788

Table 3. continued

Target plant/ Pathogen	DNA extraction process	DNA amplification	Cas enzyme	Time	Limit of detection	Detection Type	Assay name and ref
Lateral flow assays							
							CRISPR-Cas12a[103]
Rice stripe virus Rice black-streaked dwarf virus Xanthomonas *oryzae* bacteria	Solid phage DNA/ RNA extraction kit	LAMP	FnCas12a	60 min	9 copies of DNA	Real-Time PCR LFA POC	Cas-PfLAMP[100]
Colorimetric assay							
Apple necrotic mosaic virus Apple stem pitting virus Apple stem grooving virus Apple chlorotic leaf spot virus Apple scar skin viroid	Modified alkaline polyethylene glycol (PEG) extraction method	RT-RPA	LbCas12a	1 h	250−2500 viral copies (Fluorescent), 1 fM (colorimetric)	Naked eye Field applicable	[114]
GM rice, miRNAs	Lab extraction	PCR, RPA	AsCas12a, Lbu-Cas13a	1 h	500fM miRNA	Naked eye AuNPs− Linker ssDNA	[106]
Grapevine red-blotch virus	Lab extraction	PCR	LbCas12a		10 aM to 1 pM, 2000−10000 dilutions of extracted DNA	Naked eye AuNPs− Linker ssDNA	[105]
Genetically modified - Bt-11 maize, CP4-EPSPS Soybean	Lab extraction	RAA	Cas12a	∼1 h	1 aM/0.1 wt % GM content	Naked eye portable, Gold nanorods	[107]
Electrochemical assay							
GM soybean and corn	Lab extraction	N/a	Cas12a	1 h	0.3 fmol/L ECL; 3 fmol/L FSV	Electrochemical Fe3O4@-AuNPs/DNA Fc & Ru	[108]
Collateral cleavage independent assays							
Mung bean yellow mosaic India virus *Ageratum enation* virus	Lab extraction	PCR, RT-PCR	LbaCas12a	15 min		Fluorescent Quantus Fluorometer blue light	CCI-CRISPR[109]

3). The trans-cleavage activity is utilized to cleave the ssDNA bond, releasing the reporter molecules, as depicted in Figure 3. In general, the fluorescent signal is generated by cleaving the ssDNA connection between the fluorophore and the quencher (FQ) reporter. This signal can be detected using a fluorescent reader or visually examined by illuminating with LED or blue light.[17] In lateral flow assays, visible bands are produced by cleaving the bonds between the FAM/FITC-biotin reporter (fluorescein phosphoramidite-FAM and fluorescein isothiocyanate-FITC) and trapping FAM-labeled AuNPs on the target line and biotin on the streptavidin line (control) (Figure 6B).[15,49] The cross-linker of AuNP particles is disrupted in colorimetric assays, leading to the aggregation of AuNPs and a visual color change.[17] It is worth noting that Cas12a detection methods developed without relying on the principle of trans-cleavage activity are rare.

The low concentration of target molecules in the analytes of interest poses a challenge to the sensitivity of CRISPR diagnosis. To address this, most CRISPR/Cas12a based detection systems incorporate a preamplification step before the enzymatic cleavage. This step increases the number of target DNA/RNA, enhancing the sensitivity and effectiveness of the method. Standard PCR or isothermal amplification techniques such as LAMP, RPA, RCA, or SDA are commonly employed for preamplification.[49] Major Cas12a based detection technologies, including DETECTR,[19] CDetection (Cas12b-mediated DNA detection[80]), and HOLMES (1 h low-cost multipurpose highly efficient system),[81] combine trans-cleavage activity with preamplification and various signal transduction processes. To increase the portability and field application of diagnostic assays, simple and rapid DNA/RNA extraction procedures from plant and pathogen samples, such as a filter paper dipstick, microneedle, extraction buffer, and the Chelex method, are employed. These methods enhance the convenience and practicality of the diagnostic assays,[21,24,82,83] making them suitable for field use.

3.2.1. Fluorescent Detection Assays. In Cas12 based fluorescence detection methods, the trans cleavage of FQ reporters produces a fluorescent signal in the presence of the target nucleic acid. In contrast, in the absence of the target, no signal is observed (Figure 3). The generated fluorescence signal can be quantitatively detected using a real-time PCR machine[104] and microplate reader,[84] or alternatively, at the end of the reaction using an LED[24] or fluorescence device[66] (Table 3). In addition to commercial devices, simpler approaches and portable devices have also been designed.[96]

Fluorescence Detection Assay with Sophisticated Lab Equipment. Cas12a enzymes trans-cleavage based fluorescent

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J. Agric. Food Chem. 2023, 71, 11765−11788

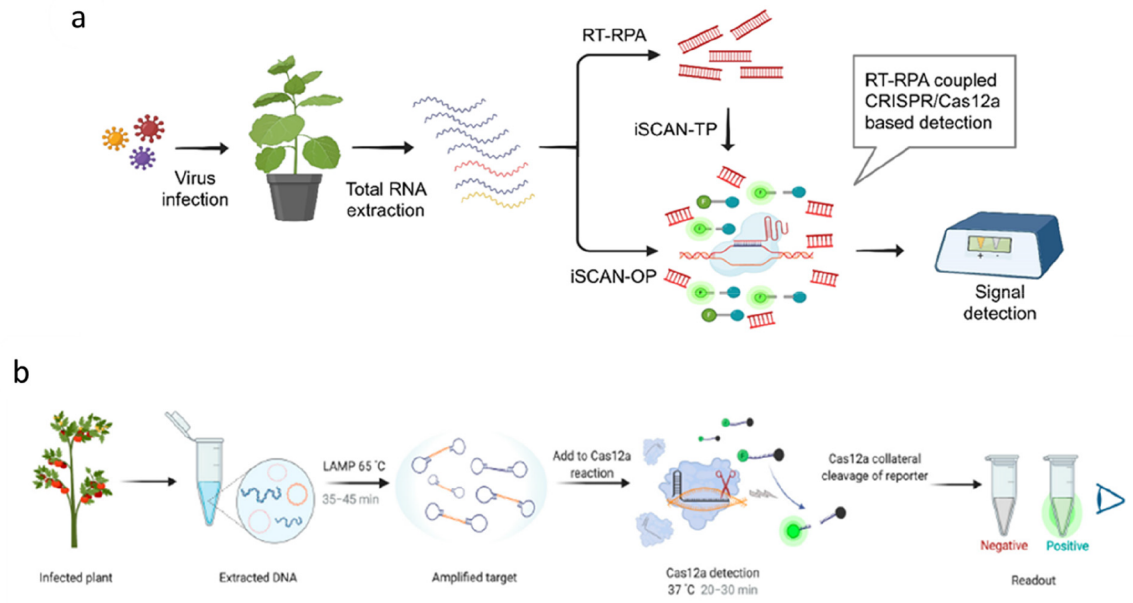

Figure 4. Field applicable CRISPR/Cas12a based detection methods. (a) iSCAN-OP method for the detection of plant RNA virus with the hand-held p51 fluorescent viewer device. Reproduced from ref 90. Copyright 2020 The Authors. (b) LAMP-coupled CRISPR/Cas12a based visible fluorescent assay for the detection of plant viruses. Virus DNA extracted from plants is amplified by LAMP followed by Cas12a trans cleavage of the HEX reporter molecule, generating a visual green-fluorescent signal by LED. Reproduced from ref 91. Copyright 2021 The Authors.

assay was used to detect wheat fungal disease fusarium head blight (FHB),[84] where PCR amplification of targeted gene fragments [internal transcribed spacer (ITS) and transcription elongation factor 1α (EF1α)] of the fungus was used for increasing the specificity. Using a fluorescent plate reader, the assay was able to detect 1 fg/μL of fungal DNA. The assay also detected the pathogens in plants only 4 days after infection and distinguishes it from other closely similar fungal infections. Alon et al. utilized similar techniques to detect RNA viruses like tomato brown rugose fruit virus (ToBRFV) and tomato mosaic virus (ToMV) from 15 to 30 ng of RT-PCR amplified targets. Using LbCas12a (Cas12a from *Lachnospiraceae bacterium*) and FAM reporter, the viruses was detected in greenhouse grown tomato plants containing both ToBRFV and ToMV.[23] Similar technique was also developed to identify rice transgenic lines and showed sensitivity for 8×10^1 copies/μL of DNA.[85]

Cas12 based diagnostic methods exploit isothermal amplification techniques, such as RPA and LAMP, for instrument-free and low-cost detection systems. In the DETECTR system, RPA-amplified nucleic acids are recognized by Cas12a and fluorescently detected through trans cleavage of the FQ reporter (Figure 3).[25] For instance, rhizomania caused by the beet necrotic yellow vein virus (BNYVV) in sugar beets was diagnosed using the DETECTR system coupled with the RT-RPA technique. Genomic RNA extracted from beet root at a concentration as low as 0.1 ng was successfully detected using a plate reader.[86] Similarly, the iARMS (isothermal amplification refractory mutation system) technique achieved a sensitivity of 25 aM using RPA amplification with a specific primer containing a single base mismatch at the 3′ end. This technique was used to detect fungicide resistance fungus carrying a mutation.[87] It was also employed for the differential detection of *Bactrocera zonata* and *Ceratitis capitata* aphids, two significant fruit pest species. To simplify the multistep DNA extraction procedure, Alon and his team utilized a Chelex based DNA extraction process to isolate DNA, and the entire Cas12a detection procedure (DNA extraction, RPA, and fluorescent signal detection) took only 1.5 h.[88]

Instead of the FQ reporter, utilizing a RCA primer as the substrate for trans cleavage, a label-free, isothermal, one-pot transgenic crop (corn) detection system was developed.[89] RCA primers hybridize with the G-quadriplex-anchored padlock probe and generate RCA amplicons. The RCA amplicons with G-quadriplex are thus identified by the G-quadriplex binding dye *N*-methyl mesoporphyrin IX (NMM), resulting in a strong fluorescent signal. In the presence of a target, the collateral cleavage of the RCA primer inhibits the production of RCA products and diminishes fluorescent signal. The assay successfully detected transgenic and nontransgenic corn from extracted genomic DNA with microplate reader and detection limit (LOD) was 45 pM for dsDNA target (Table 3).

Fluorescence Detection with Hand-Held/Portable Machines. Plate reader and RT-PCR machine limit the application of Cas12a mediated fluorescence system in laboratory. A commercially available portable p51 fluorescence viewer device was utilized to capture the fluorescent signal from fluorophores in the iSCAN-OP[90] (*in vitro* Specific CRISPR based Assay for Nucleic acids detection in One-Pot) method to increase field applicability (Figure 4a). In iSCAN-OP, three RNA viruses, potato virus X, potato virus Y, and tobacco mosaic virus, were diagnosed with picomolar sensitivity and specificity in a single step at a single temperature within 30 min. Using specially generated crRNA from infected plant samples, we determined the RT-RPA product of the viral coat protein coding sequence. The P51 fluorescence viewer was also useful for detection of tomato yellow leaf curl virus and tomato leaf curl New Delhi viruses using a LAMP-coupled CRISP-Cas12a fluorescent detection method (Figure 4b). In this method,[91] the released HEX fluorophore emits an intense fluorescent signal when a light-

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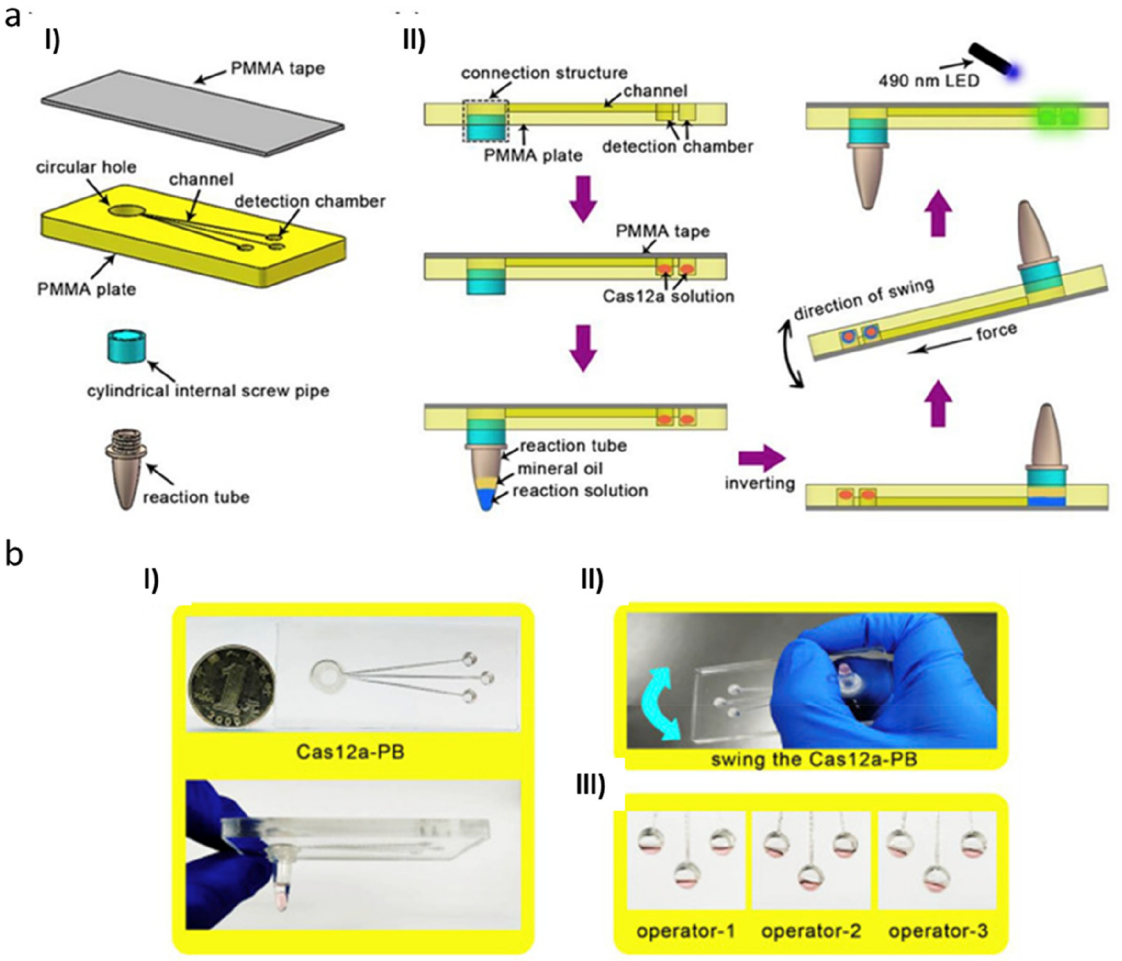

Figure 5. Schematics and actual form of CRISPR/Cas12a based portable biosensor (Cas12a-PB) method. (a) Components of Cas12a-PB (I) and schematics of the complete process (II). (b) The constructed form of the device and visual representation of the process. (I) the real image of Cas12a-PB picture. (II) A Cas12a-PB illustration with motion. (III) The liquid distribution in three detection chambers. Reproduced with permission from ref 96. Copyright 2020 Elsevier.

emitting diode (LED) shines on the molecule and is captured on P51 fluorescence viewer (Figure 4b). This approach generates fluorescent signals from extracted plant DNA samples (infected tomato and tobacco) within an hour. The in-tube visible fluorescent detection limit for synthesized dsDNA was 100 aM with no cross-reactivity with other prevalent Gemini viruses.

UV fluorescence torch was used by Jiao et al.[82] for developing a visual onsite detection technique for screening optimum quarantine pathogenic bacteria of horticultural crops, *Erwinia amylovora* and *Acidovorax citrulli*. They also characterized a newly designed LbCas12a variant (LbCas12a-5M) with a wider PAM (TNTN) preference and better trans-cleavage efficiency than the wild-type enzyme. They also combined a basic bacterial DNA extraction method with species-specific crRNA design and irradiated with UV fluorescence torch for visualization with the new LbCas12a-5 M cleavage to provide a straightforward, rapid (40 min) and visual detection of *A. citrulli* in a watermelon seed lot. This method screened the pathogens with a limit of detection (LOD) of 40 CFU (colony-forming unit) per reaction (Table 3). A mini UV torch was also utilized in the visual one-pot POC detection of fungicide resistance in *Venturia carpophila*.[92] The assay is capable of detecting 7.82×10^3 fg μL^{-1} copies of genomic DNA within 45 min. To conduct the experiment in a one-pot setup, CRISPR and RPA reagents were positioned on the side and bottom of the tube. This assay can be performed under field conditions with only a portable hot pot, as it involves rapid DNA extraction with a 2 min heat treatment in buffer, followed by visual observation of fluorescence using a UV flashlight or cell phone torchlight for detection. Another field-applicable CRISPR/Cas12a visual detection method for maize chlorotic mottle virus was reported by Duan et al.[93] The detection platform incorporates a simple trizol method for RNA extraction, isothermal RT-recombinase-aided amplification of the target, and visible green fluorescence emission under blue light. Naked-eye detection can be achieved within only 45 min without the need for any sophisticated equipment.

DETECTR with an LED transilluminator was able to detect the bacterial leaf blight disease pathogen *Xanthomonas oryzae* pv Oryzae (Xoo) in rice samples with the LOD of 1×10^3 copies/μL.[24] An LED transilluminator was also used with DETECTR to build a barcode coupled with the cas12a assay, "Bar-cas12a," for species authentication.[94] DNA barcodes are small sections of genes showing highly distinguishable species-specific sequences and are commonly employed for plant species authentication. "Bar-cas12a" was created to boost specificity and sensitivity, and it displayed 1000 times higher

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J. Agric. Food Chem. 2023, 71, 11765−11788

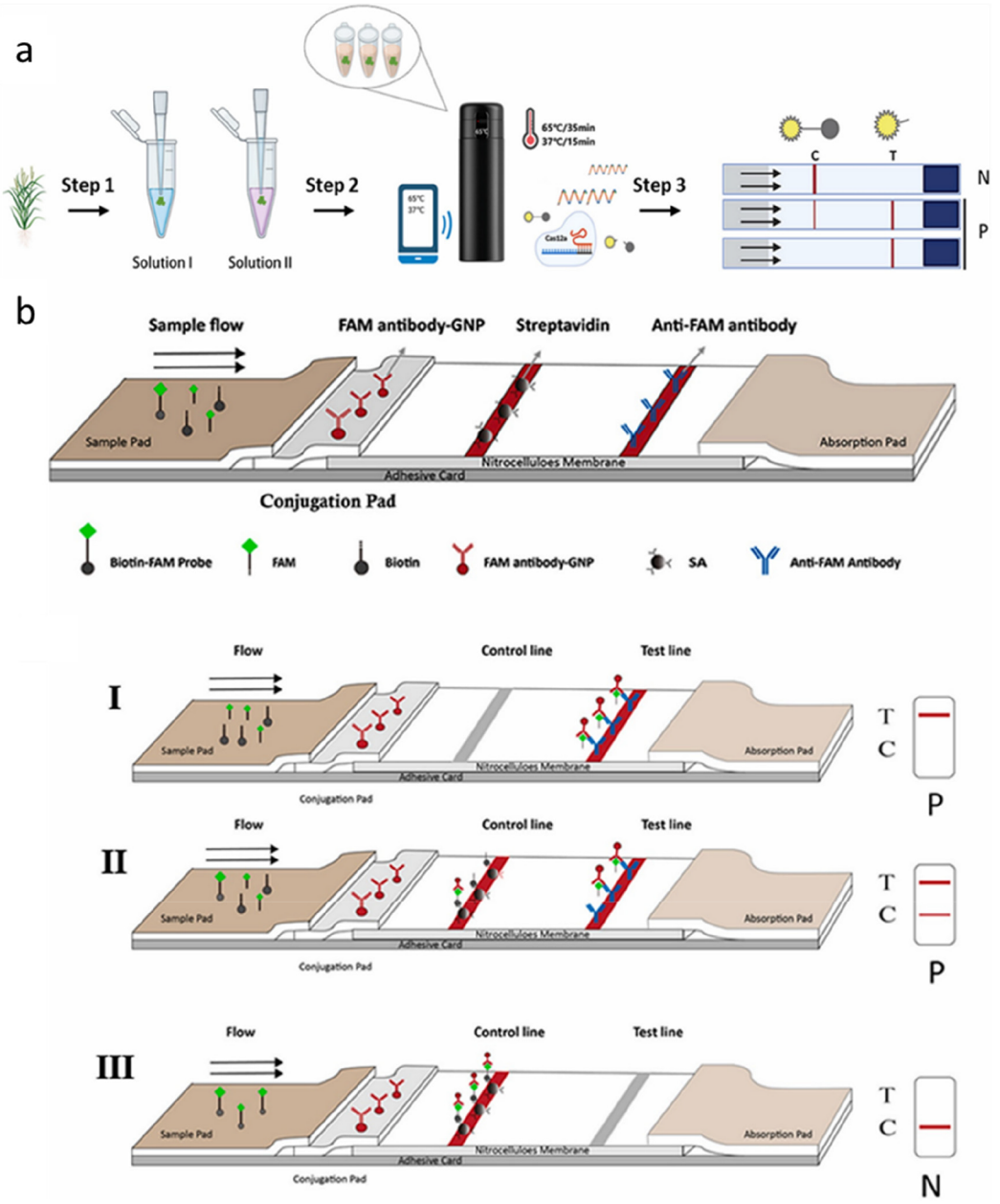

Figure 6. Visual, field applicable, and real-time CRISPR/Cas detection using lateral flow assay. (a) Schematic illustration of the Cas-PfLAMP system. DNA extraction (Step 1), amplification (Step 2), and CRISPR/Cas experiment using a transportable thermal flask (Step 3), followed by the paper based lateral flow detection. (b) Illustration of the lateral flow assay. Reproduced with permission from ref 100. Copyright 2022 Elsevier.

sensitivity to agarose gel electrophoresis with a 0.8 fg LOD and a 90% degree of accuracy for *P. amarus* species authentication. Recently, a Cas12a microfluidic fluorescence system was proposed for the identification of plant DNA barcodes. This system demonstrated remarkable sensitivity and speed in distinguishing different species of *Datura L.* with high accuracy.[95] To integrate the DETECTR assay into portable fluorescence detection equipment, a centrifugal microfluidic platform/chip was utilized. This innovative setup enables the identification of single-copy DNA mini barcodes. The microfluidic chip itself is designed to accommodate several reaction wells, ranging from 2 to 16, allowing for the simultaneous detection of multiple samples. This multiplexing capability significantly enhances the efficiency of the species identification. Notably, the platform exhibited an impressive 100% consistency with morphological species identification, indicating its strong potential for DNA barcoding in field applications. In terms of detection time, the bar-cas12a assay requires approximately 2 h to detect a single species, while the microfluidic assay can analyze multiple samples in around 1 h. Both systems have demonstrated superior performance compared to traditional species authentication methods, such as PCR, high-resolution melting, and sequencing. Additionally, they reduce the dependency on sophisticated instruments, making on-site detection more feasible and practical. The advancements achieved by these innovative fluorescence based systems offer promising prospects for reliable and efficient species authentication in various contexts.

Fluorescence Based Portable Biosensor. For contamination-free Cas12a based fluorescent assay, a special reaction

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vessel with seals were made by Wu et al.[97] This special reaction tube was used in a LAMP-coupled Cas12a based visual UV detection method for the detection of the CaMV35S promoter in genetically modified (GM) soybean (Roundup Ready). The vessel tube contains LAMP reagents, and at the end of LAMP amplification CRISPR reagents are added on the seal of the tube and a lid with needle disrupts the sealing. With a short centrifugation, the CRISPR reagents are mixed with the amplified products. The products could be visibly identified under UV light (254 nm) in the reaction vessel after they were kept at 37 °C for 5 min. This vessel was utilized and refined to build a CRISPR/Cas12a based portable biosensor (Cas12a-PB)[96] capable of detecting the CaMV35S promoter and the lectin gene in GM soybean powder. The biosensor was composed of polymethyl methacrylate (PMMA) and PMMA tape, and it contained three detection chambers that had channels connecting them to the reaction vessel (Figure 5a,b). The detection chambers contain preloaded CRISPR/Cas12a reagents and FQ reporters (Figure 5bIII), while LAMP amplification is done in the reaction vessel (Figure 5bI). The amplified LAMP products are transferred to the detection chamber through the connecting channel by swinging the Cas12a-PB in the opposite direction (Figure 5aII,bII). With the aid of a 3D printing detection instrument with a mobile phone, the CRISPR reaction can be seen as it occurs in the detection chamber. The positive samples produced a green signal under 490 nm LED illumination, while the negative samples produced a black signal. The instrument is powered with mobile technology and contains a LED light (490 nm), filter (520 nm), card slots, and a display slot. The biosensor can distinguish between GM soybean and GM maize powders and non-GM peanut and rice powders with a detection threshold of 0.1% transgenic soybean components.[96]

3.2.2. Lateral Flow Assays. Lateral flow assay (LFA) produces visual results without the need for an instrument. In the LFA assay, FAM antibody labeled AuNPs conjugate with the FAM-biotin reporter and aggregate on the streptavidin control line in the absence of target. In the presence of target, cleavage of FAM-biotin reporter releases the AuNPs which are captured on anti-FAM test line and generate visual bands (Figure 6b). Lateral flow assays and fluorescent assays are incorporated in Cas12a based detection to enhance visual detection and field applicability.

A nucleic acid lateral flow immunoassay (NALFIA) was designed for precisely and sensitively screening wheat blast pathogen in the seedling stage.[25] The method was able to identify the pathogen only 2 days after inoculation, whereas PCR needed 4 days. With a specific gRNA intended to target a conserved region of *Magnaporthe oryzae Triticum* (MoT), the assay can detect DNA concentrations as low as 1 ng/μL (isolated genomic DNA) (Table 3). The RPA amplification and LFA process took only 30 min and cost $6 as commercial PCRD strips were used. It was predicted that in large scale production the cost will be reduced to $4 USD. However, in the assay, a conventional genomic DNA isolation procedure was used, which made it less field applicable.

Attomolar sensitivity was attained with a similar assay for the detection of citrus greening disease and potato purple top disease bacteria *Candidatus Liberibacter asiaticus* and *Candidatus Phytoplasma trifoli*.[98,99] Wang and Zhong introduced the 5′-TG PAM based Cas12c-DETECTOR platform, which has the potential to recognize single nucleotide polymorphism (SNP). With this technique two of the three sgRNAs designed to target the *HopZ5* gene (a particular *vir* gene of Psa) of *Pseudomonas syringae* (Psa) bacteria, causing bacterial canker disease in kiwifruit, was successfully detected. The Cas12c-DETECTOR was also combined with lateral flow stripe for visual paper based detection and excited with blue light for visual fluorescent detection.[83]

Zhang et al.[21] reported a field applicable lateral flow assay with a RPA-Cas12a based fluorescent assay to identify the rice blast fungus *Magnaporthe oryzae* and the *Cry1C* gene of genetically modified (GM) bt-rice (Figure 3). To simplify the procedure, they used paper strips (paper dipsticks) to extract the genomic DNA from the rice leaf, and RPA amplification of the target is performed at body temperature. These steps were followed by a paper based LFA with an FAM-biotin reporter to make the detection system visible and inexpensive. The method successfully detects the bt-rice and rice blast pathogen in 30 min without any instrument The developed process is user-friendly and field applicable, as body temperature is used for target amplification and only papers were used for DNA extraction and LFA detection. Moreover, the procedure is also economic, as per test only cost $3.5.

Using a similar on-site diagnosis platform, pathogen *Elsinoe fawcettii* of citrus scab disease was detected from 1 fg of isolated genomic DNA within 1 h (Table 3). Cross-reactivity with other nonscab fungi was absent. The assay demonstrated a positive response in detecting fungal DNA in tissue lysate obtained by simply pressing the infected leaves in different buffers (TE buffer, PEG buffer, and GE3 buffer).[101] Bernabé-Orts et al. utilized identical RT-LAMP technologies at the laboratory and field levels to detect ToBRFV virus in infected tomato plant samples. Amplification of the movement protein (MP) gene followed by column-mediated RNA extraction and LbCas12a trans cleavage of the reporter yielded sensitivity comparable to RT-PCR with no cross-reactivity to nearby viruses.[102]

Zhu et al.[100] applied PAM (TTT)-containing LAMP primers to eliminate the dependency of Cas12 enzyme on PAM and thus has developed a distinct PAM-free LAMP-assisted and CRISPR-Cas12a cleavage (Cas-PfLAMP)-mediated on-spot visual detection tool for rice pathogens. This platform was able to identify two RNA viruses (rice stripe virus and rice black-streaked dwarf virus) and a bacterium (*Xanthomonas oryzae*) from field-collected rice leaf samples. With the solid phage nucleic acid extraction method, LAMP amplification, and portable lateral flow strip test, the detection platform was stable, quick, visible, on-site, and sensitive to the rapid detection of several rice pathogens. The entire process could be carried out on the field using a smart thermos cup, a portable device, and a lateral flow strip (Figure 6a). The test is sensitive to 3 to 9 copies of DNA, and the total analysis time is 60 min. 2.5 copies (CP gene) of maize chlorotic mottle viruses were detected within an hour with one-tube one-step reverse transcriptase recombinase aided (RT-RAA)/CRISPR-Cas12a lateral flow assay developed by Fan and Zhang et al.[103] The assay is field applicable as it uses alkaline-PEG buffer for RNA extraction in 10 min and requires only a portable metal incubator for amplification. The total process took place in a single tube and in one step, and we were able to detect the virus in 0.96 pg of infected plant RNA.

3.2.3. Colorimetric Assays. Over the years, AuNPs have been widely used for naked-eye detection due to their intrinsic surface plasmon resonance (SPR) properties, simplicity, and cost-effectiveness.[110−112] The aggregation and dispersion of

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J. Agric. Food Chem. 2023, 71, 11765−11788

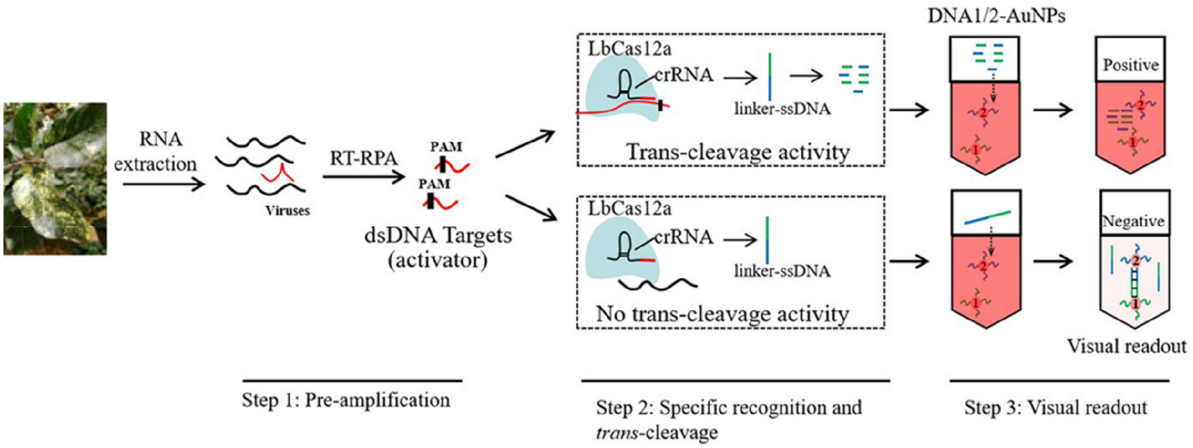

Figure 7. Flowchart of the RT-RPA-coupled CRISPR/Cas12a based colorimatric test for plant virus detection. Virus RNA isolated from plants is amplified by RT-RPA, followed by Cas12a transcleavage of linker DNA, producing a colorimetric pink signal via plasmonic AuNP solution. Reproduced with permission from ref 114. Copyright 2020 The Authors.

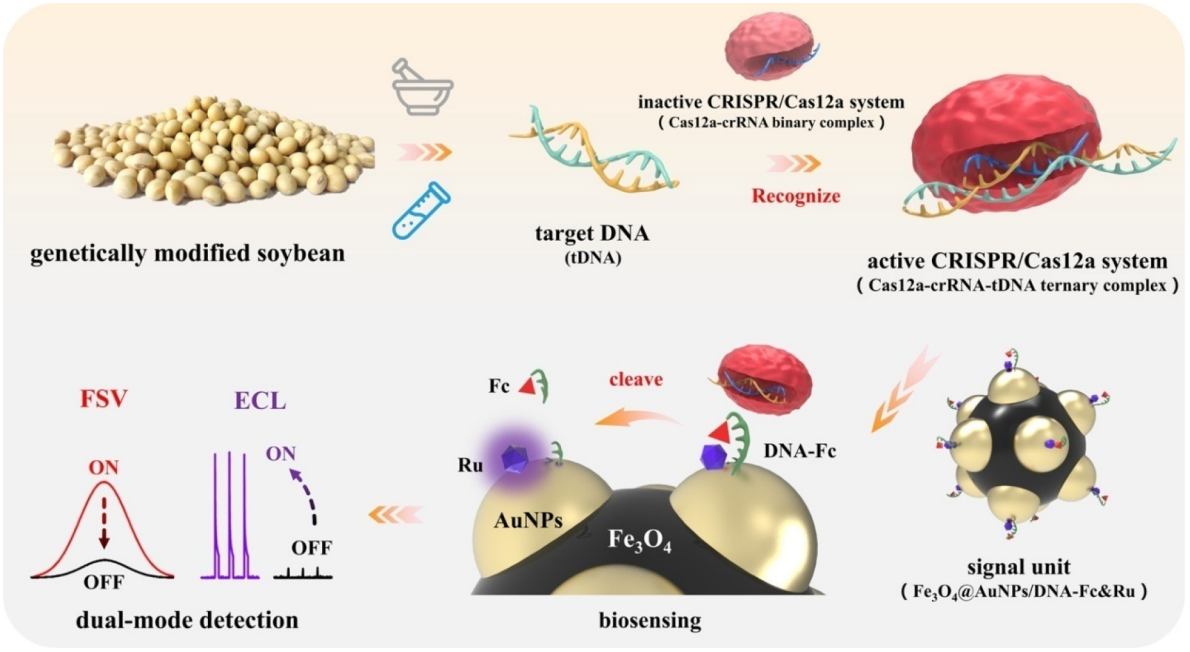

Figure 8. Schematics of a CRISPR/Cas12a mediated electrochemical biosensor for genetically modified plant detection. Reproduced with permission from ref 108. Copyright 2021 American Chemical Society.

AuNPs exhibit distinguishable optical properties. The dispersed colloidal AuNP solution appears red/pink in color, while the aggregated state results in a blue/colorless color due to a shift in the absorption peak to longer wavelengths.[113] In visual colorimetric experiments, DNA-labeled AuNPs, linker DNA, and the trans-cleavage activity of the LbCas12a enzyme are utilized to induce a change in the state of the AuNPs. Jiao et al.[114] reported a Cas12a-mediated AuNP-associated visual assay for identifying five distinct apple RNA viruses (Table 3), where the ssDNA linker was used for aggregation of DNA functionalized AuNPs. In the presence of an intact linker, ssDNA cross-links DNA-AuNP particles, producing a colorless solution. However, in the presence of viral DNA, Cas12a trans-cleaves the ssDNA linkers, which keeps the steady state condition of the AuNPs in the solution and thus produce red color (Figure 7). Fresh crude samples were prepared with a simple alkaline polyethylene glycol (PEG) extraction method using a hand-held tissue homogenizer at room temperature, and the procedure was validated using 52 samples obtained from commercial orchards. The RT-RPA-CRISPR/Cas12a platform could detect viruses in fresh leaf crude samples in an hour or less, with a detection limit of 250−2500 viral copies (apple stem pitting virus, apple stem grooving virus, and others). Similar to this, plasmonic AuNPs were used to visually identify grapevine red-blotch virus from PCR-amplified products.[105] A similar colorimetric detection platform has demonstrated universal application by successfully identifying various pathogens such as bacteria, viruses, miRNAs, and transgenic rice.[106]

Qian et al.[107] developed a naked eye multicolour colorimetric Cas12a technique with gold nanorods (GNRs) for the detection of GMOs. Instead of AuNPs and ssDNA linkers, the color shift of GNRs generated by the invertase-glucose oxidase cascade reaction and the Fenton reaction was

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J. Agric. Food Chem. 2023, 71, 11765−11788

utilized as signal readout. In the presence of an RPA amplified target in the system, invertase linked to functional magnetic beads with a ssDNA linker is released by ssDNA cleavage. Free invertase concentration is proportional to the amount of active Cas12a. Sucrose in the solution is converted to glucose by invertase, which is then oxidized by glucose oxidase to create hydrogen peroxide. The intermediate hydroxyl (−OH) molecules are produced by inducing a Fenton reaction (with Fe^{2+} in an acidic environment) to produce hydroxyl (OH) molecules. The OH etching of the GNRs causes color alterations or blue shifts in the localized surface plasmon resonance (LSPR) peak, which can be visualized with the naked eye. The method can detect between 2.24 and 24.08% GM content in actual maize samples.

3.2.4. Electrochemical Assays. Ge et al. developed a PCR-free electrochemical biosensor for the detection of genetically modified soybean.[108] They immobilized ruthenium (Ru)- and ferrocene (Fc)-containing ssDNA (DNA-Fc) probes on AuNP coated iron(II,III) oxide nanoparticles (Fe_3O_4NPs) and used them as a signal unit. The composite bionanomaterial Fe3O4@AuNPs/DNA-Fc&Ru was adsorbed on the sensing layer of the polished magnetic glassy carbon electrode to achieve voltammetric and electrochemiluminescence responses (Figure 8). The ssDNA cleavage releases the electroactive Fc from the bionanomaterial Fe3O4@AuNPs/DNA-Fc&Ru surface, attenuating the voltammetric current, whereas the free-attached Ru on the surface of the signal unit enhances the ECL signal when the bionanomaterial dispersion is dropped and measured on the magnetic carbon electrode. Using the dual detection mode, the biosensor produced a detection sensitivity of 0.3 fmol/μL.[108]

A universal CRISPR-Cas12a (cpf1) based electrochemical biosensor (E-CRISPR) was reported for the detection of viral nucleic acids with picomolar sensitivity.[115] In this assay, a gold electrode with an immobilized methylene blue (MB)-ssDNA reporter on the surface is used as the signal detection unit. The presence of MB on the electrode surface leads to a strong electrochemical signal. However, in the presence of target DNA, Cas12a cleaves the ssDNA and detaches the MB from the gold surface, resulting in a significant reduction in the number of square wave voltammetry (SWV) signals. The researchers also designed an aptamer based cascade with E-CRISPR, which enabled the detection of specific proteins. A ssDNA aptamer is used as a recognition unit for the protein of interest, serving as both the target for Cas12a and the recognizing unit. When the protein is present, the ssDNA aptamers bind to the protein, reducing the available target for Cas12a. Consequently, a high electrochemical signal is not obtained. This on-chip electrochemical biosensing platform holds significant potential for the detection of plant pathogenic nucleic acids and protein aptamers.

3.2.5. Collateral Cleavage Independent Assays. The majority of CRISPR/Cas12a diagnostic procedures include collateral cleavage. However, Srivastava et al. reported a collateral cleavage independent CRISPR/Cas12a based detection method (CCI-CRISPR) to detect two significant begomo viruses, Mung bean yellow mosaic India virus (MYMIV) and *Ageratum enation* virus (AEA), from soybean field samples (Table 3). They targeted the DNA-A and DNA-B subcomponents of MYMIV and AT-rich promoter region of the alpha satellite subcomponent of AEA virus. The crRNAs are designed to target sequences with and without PAM regions. Cas12a binds with both regions (with and without PAM) but cleaves only the dsDNA with the PAM region. QuantiFluor dsDNA dye produces a fluorescent signal when it attaches to free dsDNA binding sites. They claimed that Cas12a-induced cleavage in the presence of PAM, enhances the availability of the free binding sites, hence amplifying the signal. In contrast, cleavage frees LbCas12a in the absence of PAM, resulting in a decrease in binding sites and fluorescence signal. The detection occurred, with an increase and decrease in fluorescent signal relative to the control dsDNA.[109] The CCI-CRISPR system is cost-effective due to the lower cost of the applied dsDNA dye compared to fluorescent quencher (FQ) reporters. This cost advantage makes it a potential alternative method for pathogen diagnosis.

CRISPR/Cas12 is the most widely used method for detecting the genomic contents of plant viruses (Table 5), bacteria, fungi, and nematodes. It has also been applied to GMO detection and species authentication. Each of the developed techniques has its own uniqueness. For point-of-care (POC) applications, several techniques have been implemented with Cas12a assays, including (i) simple and rapid nucleic acid extraction, (ii) isothermal amplification at body temperature or using portable hot pots, and (iii) visual screening with LFD, AuNPs, portable fluorescent devices, or UV devices.

The fastest field-applicable Cas12a assay took 30 min to detect transgenic rice and blast fungus with picomolar sensitivity using LFD stripes[21] from plant samples to signal detection. RAA-mediated[103] and LAMP-mediated[100] DETECTR-LFA detection platforms require almost 1 h in the field, but they have high sensitivity, detecting 2.5−9 copies/μL, respectively. Attomolar sensitivity was achieved with DETECTR-LFA detection of *Candidatus* bacteria in the lab.[99,98] Among the POC applicable fluorescence detection systems, iSCAN-OP[90] requires the least amount of time. In 30 min, this contamination-free one-pot assay provides in-tube detection of RNA viruses. The hand-held fluorescence viewer is expensive, so a more affordable UV torch system was used for POC detection of *V. carpophila*[92] to develop another portable one-pot assay, which takes a total of 45 min in the field. However, the sensitivity of this method was 7.82×10^3 fg μL^{-1} copies of genomic DNA, while the sensitivity of iSCAN-OP was 10 pM. Due to differences in metrics (pM, CFU, ng/μL, copies, etc.), comparing techniques is difficult.

The Cas12a colorimetric assay with gold nanorods showed significant sensitivity of the 1 aM DNA target with RPA amplification, compared to Cas12a-mediated fluorescent reporter assays.[107] This assay can detect 0.1% genomic content in BT-maize with the naked eye. Meanwhile, 0.05% of the genetic content of GM soybean powders can be detected with the naked eye under UV light using the Cas12a fluorescent assay conducted in a designed reaction vessel.[97] Individual CRISPR reactions in the vessel take only 5 min. Additionally, the AuNP-mediated colorimetric assay can detect 250−2500 copies of apple viral DNA[114] with RT-RPA and 10 aM to 1 pM of grapevine viral DNA with PCR amplification.[105] Electrochemical biosensors (EB)[108] provide high sensitivity (0.3 fmol/L LOD) and accuracy (90−112% recoveries) in GMO detection directly from extracted samples without any target amplification (e.g., PCR, isothermal amplification) step. They also show prospects in non-nucleic acid target detection and cost-effective POC detection.[115,116]

The DNA targeting and collateral cleavage activity of Cas12 enzymes, as well as their versatility in adapting to advanced

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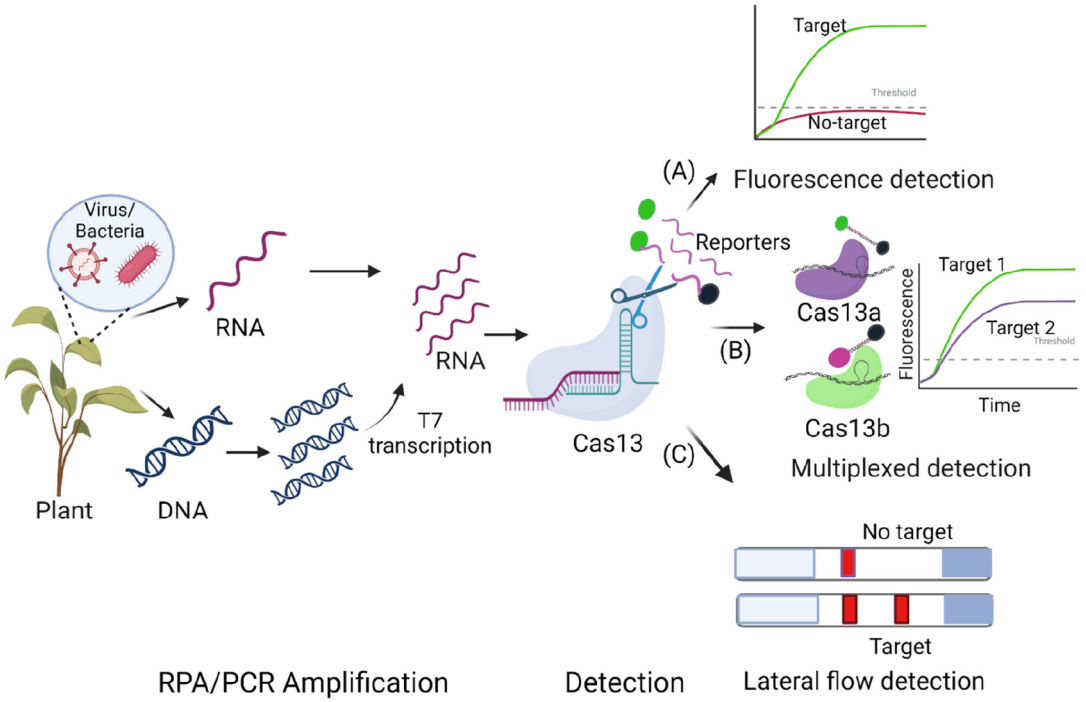

Figure 9. Schematics of a gene detection approach using CRISPR/Cas13 (SHERLOCK) that combines DNA/RNA extraction and isothermal amplification. Different modes of signal transductions, such as, fluorescent real-time,[36,122] multiplexed readouts (e.g., detection of two genes employing two-color fluorescence with Cas13a and Cas13b[27]), and lateral flow detection can be used in this biosensing approch.[27,121]

amplification and signal readout systems, make them a potential next-generation diagnostic tool.[17] CRISPR/Cas12 has shown promising advances in the nucleic acid based detection of plant pathogens and species authentication. With modification and improvement, several techniques are applicable for simpler field-level diagnosis.[82,92,100] By adopting specially designed ssDNA aptamers as targets for cleavage, non-nucleic acid targets of pathogenesis (such as virulence proteins, effector molecules, and smaller molecules)[17,49,115,117] can be explored to expand plant disease diagnostics.

3.3. Cas13 in Plant Diagnostics. Cas13 is a recently discovered Cas endonuclease that possesses RNase activity. It belongs to type II, class VI CRISPR/Cas effectors and is capable of targeting and cleaving ssRNAs both *in vitro* and *in vivo*.[118] Similar to Cas12, Cas13 is guided by a single crRNA to a complementary target sequence and exhibits cis and trans cleavage activity.[17,119] In the CRISPR/Cas13 system, the precursor crRNA (pre-crRNA) is converted into mature crRNA, which carries a 28−30 nucleotide spacer sequence.[120] Cas13a is guided by the crRNA to target a complementary ssRNA and cleave it upon recognition. Effective cleavage activity is achieved with crRNAs containing at least 18 bp, and the seed region of the crRNA plays a crucial role in binding. Cas13a activity is dependent on a single PFS for target sequence detection. Cas13's cleavage activity is greater on targets with PFS 3′ A, C, or U than on those with PFS 3′ G.[119] In contrast, in different orthologues like LshCas13a and LwaCas13a (Cas13a from *Leptotrichia shahii* and *Leptotrichia wadeii*), the PFS preference may vary.[59,120] Cas13a's cleavage activity is guided by two higher eukaryotes and prokaryotes nucleotide-binding endo-RNase domains (HEPN)[119] that break RNA beyond the crRNA-ssRNA duplex.

In Cas13 based diagnostics, the collateral cleavage of ssRNA quenched fluorescence reporter is employed for signal transduction upon the recognition of isothermally amplified target ssRNAs.[16,27,28] Signal transduction can be achieved through fluorescence or lateral flow assays. The RNA-targeting CRISPR/Cas13a system, with its collateral cleavage activity of nonspecific RNAs, holds tremendous diagnostic potential for plant RNA viruses and valuable plant characteristics. The most widely used Cas13 based CRISPR detection system is SHERLOCK.[16] SHERLOCK operates on a similar principle to the DETECTR technology, with the difference being that the target molecules and transducing reporter molecules are ssRNAs.[27,28] In the SHERLOCK system, the target dsDNA/ssRNA is amplified using RPA/*RT*-RPA, transcribed into ssRNA using T7 RNA polymerase, and then detected through Cas13a's trans cleavage of the ssRNA linked to the fluorescent reporter molecule.[16] Gootenberg et al. adapted the SHERLOCK method for quantifying soybean glyphosate resistant genes and detecting multiple plant genes in a single reaction from a mixture of soybean crude extracts within 15 min.[27] They integrated a simple genomic DNA extraction method from ground soybean seeds with isothermal amplification (RPA) and fluorescent and lateral flow detection methods to construct a colorimetric, multiplex, portable detection system (Figure 9). They targeted the glyphosate resistance gene *CP4 EPSPS* (5-enolpyruvylshikimate 3-phosphate synthase from *Agrobacterium sp.* strain CP4) of glyphosate-resistant (GR) soybeans for GMO detection and housekeeping gene lectin (*LE1*) as a control for detection. The SHERLOCK method was able to discern 20% change in *CP4 EPSPS* gene levels.

For multiplex detection, Gootenberg et al.[27] employed two sets of enzyme reaction mixtures (Cas enzyme with gRNA specific for distinct targets) in the same reaction, along with two separate reporters, to generate two signals with different spectra in the presence of two different target RNAs (Figure 9B). In this setup, the LwaCas13a enzyme mix cleaves the

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Table 4. Cas13, Cas14, and Cas12/13 Based Detection of Plant Pathogens and Transgenic Crops

Target plant/Pathogen	Target and NA extraction process	DNA amplification	Cas enzyme	Time	LOD	Detection type	Assay name and ref
			Cas13				
Glyphosate resistant soybean	Simple gDNA extraction	RPA	PsmCas13b; LwaCas13a	30 min		Fluorescent two-color signal/Fluorescent reader Multiplex, colorimetric, Visual LFA	SHERLOCK[27]
Tomato Spotted Wilt Virus	RNA extraction kit	RPA	LwCas13a	20 min	2260 copies/μL	Fluorescent plate reader	[36]
Tobacco mosaic virus, Tobacco etch virus, Potato virus X (PVX)	Crude DNA extracts (lysis for 6 min)	No amplification	Cas13a/d	30 min		Fluorescence RT-PCR	[122]
			Cas12 and Cas13				
Tobacco mosaic virus, Tobacco etch virus, Potato virus X	Leaf crude sample, silica gel spin columns, lysis solution without RNA extraction	RT-PCR RT-RPA	LbCas12a LbCas13a RfCas13d	30 min		Fluorescent RT-PCR machine Multiplex LFA	[122]
Fusarium graminearum, Fusarium verticillioides -fungus, Rice black-streaked dwarf virus	Crude DNA extracts (lysis for 6 min)	RPA, RT-RPA	RfxCas13d, AsCas12a, LbCas12a, LwaCas13a	Single detection-26 min, Multiplex-30 min	10^9 aM without RPA, 1 aM with RPA	Fluorescence visual, UV flashlight	[121]
			Cas14				
Banana ripening miRNA	From banana fruit Plant miRNA kit	RCA	Cas14a/Cas12f		1.839 pM	Fluorescent RT-qPCR	[22]

poly-U reporter with the HEX fluorophore in the presence of the *EPSPS* gene, while the PsmCas13b enzyme mix cleaves the poly-A reporter with FAM in the presence of the *LE1* gene (Figure 9B). Consequently, two distinct fluorescence signals are generated from the HEX and FAM fluorophores simultaneously in the same reaction, revealing the presence of two different target molecules.

For making the system field applicable, a visible portable lateral flow assay with collateral cleavage of FAM- and biotin-functionalized RNA was utilized (Figure 9C). In the presence of the target, the ssRNA reporters are breached and free antibodies are captured on the test line, while in the absence of the target the RNA reporters on streptavidin lines (1st line) adsorbed anti-FAM antibodies labeled with AuNPs and no bands are visible in the test line. The RPA amplification from crude soybean extract to the lateral flow signal of resistant seeds with LwaCas13a took about 30 min.[27]

As described earlier, Cas13a has high potential in the diagnosis of plant RNA viruses. A similar fluorescence assay was utilized for rapid diagnosis of destructive tomato spotted wilt RNA virus from infected tomato leaf samples (TSWV).[36] The conserved region of the virus's N gene (nucleocapsid gene) was targeted for RPA amplification using cDNA synthesized from pure RNA and the assay took 30 min for RPA and 20 min for fluorescent detection (Table 4). The assay[36] successfully detected traces of the virus in infected plant leaf samples and its transmitting vector, the western flower thrips (*Frankliniella occidentalis*) aphid, with a detection limit 10 times greater than traditional RT-PCR (2,260 copies/L). However, this assay is not field applicable as it requires a qPCR machine for cDNA synthesis and a plate reader for fluorescence signal measurement. In comparison, the SHERLOCK method is easier to apply in the field for TSWV detection as it utilizes rapid DNA extraction and does not require complex instrumentation.[27] It is also faster and capable of multiplex detection. The SHERLOCK technology shows potential in various agricultural applications, from trait detection to rapid and multiplex pathogen diagnosis.

3.4. Combination of Cas12 and Cas13 Based Detection and Multiplexing. Incorporation of both Cas12 and Cas13 enzymes in biosensing assays was utilized for multiplexing and universal detection of DNA and RNA of plant pathogens. Duan et al.[121] harnessed the targeting and cleaving capabilities of LwCas13a's ssRNA and LbCas12a's ssDNA and combined them for developing a rapid field deployable RPA-CRISPR/Cas system for multiplexed detection of plant pathogens (Figures 3 and 9). The DNA of *Fusarium graminearum* (FG) and *Fusarium verticillioides* (FV) fungus and RNA of rice black-streaked dwarf virus were diagnosed in extracted crude plant samples using fluorescent and lateral flow assays (Table 4). For multiplex reaction, FAM labeled ssDNA and HEX labeled ssRNA reporters were used with LbCas12a and LwCas13a in the same reaction to detect FV and FG together. In the presence of target, LbCas12a preferentially cleaves ssDNA reporters and LwCas13a cleaves ssRNA reporters, which generates two types of fluorescence signal with different wavelengths when excited with a portable fluorescence flashlight (i.e., LUYOR-3415RG).[121] This developed technology is similar to the improved SHERLOCK platform reported by Gootenberg et al.[27] (Figure 9B). Most importantly, they advanced the system by using filter glasses, which made it visual. For visual observation of FAM, a 488 nm excitation filter was utilized, while a 520 nm filter was used for HEX, with yellow and red glasses, respectively. Furthermore, to make the system field-applicable, a portable solar generator and dry bath incubator were employed for DNA crude extract preparation and RPA amplification.[121] With this method a single target was detected in 30 min, while a multiplex sample required 60 min.

Sánchez-Vicente et al.[122] used target specific gRNA to distinctly detect TMV, PVX, and tobacco etch virus from a plant sample infected with multiple plant viruses (Table 5). Like the previous method, the assay induces fluorescence signal and lateral flow band in the presence of viral nucleic acid. The difference is that they performed CRISPR experiments in separate tubes with crRNA specific for each virus following a multiplex amplification of targets with three different primer

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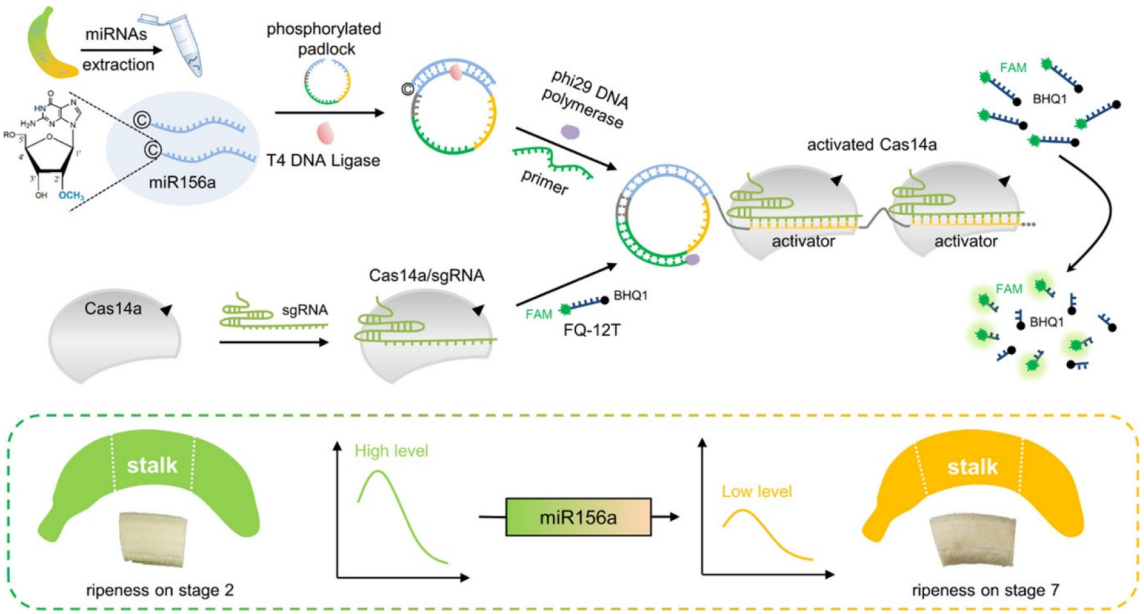

Figure 10. Fluorescent signal evaluation of the banana ripening process using the Cas14R assay for the identification of miR156a. Reproduced with permission from ref 22. Copyright 2021 American Chemical Society.

pairs specific to each virus. In comparison to a plant infected with a single virus, the multiple virus-infected plant produced a higher signal. In addition, a lateral flow readout for visual purposes was generated by dipping the lateral flow strip in a CRISPR/Cas12a combination. In the end, a field-applicable CRISPR/Cas12a system was established by extracting plant total nucleic acid in 5 min with alkaline lysis solution, RT-RPA amplification in 30 min, and fluorescence and biotin labeled ssDNA probe for lateral flow readout.[122] They also utilized CRISPR-Cas13a/d based method for amplification free detection of the viruses with fluorescent signal which can be detected by fluorescent reader. It can also be detected visually as the fluorophores emit green light when excited with blue light.

3.5. Cas14 Based Detection System. Cas14, also known as Cas12f, is smaller in size (40−60 kDa) compared to other Cas proteins, consisting of only 400−700 amino acids in length. It targets ssDNA without requiring a PAM for cleavage.[74] Specifically, it cuts ssDNA targets outside the crRNA/DNA target heteroduplex and does not exhibit cleavage activity for dsDNA. Cas14 also possesses trans-cleavage activity of nonspecific ssDNAs, similar to other Cas12 enzymes. It exhibits a preference for longer lengths of ssDNA reporters (FQ) for trans cleavage. The presence of secondary structure reduces its activation, and cleavage is inhibited in the presence of a 2-base pair mismatch in the middle portion of the ssDNA reporter. Due to its smaller structure and sensitivity to single or double base mismatches in the middle position of gRNA, Cas14a has been utilized for mutation detection and shows strong potential in the diagnosis of plant SNP.[74,143]

The small structure and single base sensitivity of the Cas14a enzyme have been utilized for targeting plant microRNA (miRNA) profiling.[22] miRNAs are often challenging to quantify but are crucial for plant biological activities. Chen et al. proposed a CRISPR/Cas14a trans cleavage coupled with a RCA (Cas14R) system for the detection of plant miRNA without the need for reverse transcription and demethylation. This system was effectively employed to profile miRNA156a involved in banana ripening. For miRNA detection, a padlock probe containing miR156a and Cas14A recognition sequences, as well as an RCA primer, was designed (Figure 10). When miR156a hybridizes with the padlock probe, T4 ligase ligates the 3′ and 5′ ends of the probe, forming a cyclic probe. The cyclic probe initiates an RCA reaction with the polymerase enzyme, resulting in a long RCA product comprising a repeating target sequence for gRNAs. Recognition of the RCA products by Cas14a activates the trans cleavage of the FQ reporter, thereby transducing a fluorescence signal.[22]

4. MAJOR CHALLENGES ASSOCIATED WITH CURRENT CRISPR BASED TECHNOLOGIES AND HOW TO OVERCOME THESE ISSUES

The main challenge of the CRISPR/Cas system lies in the low sensitivity and kinetics of Cas enzymes, which correspond to the low abundance of pathogens present in the samples. The viral load or bacterial load is extremely low at infected plant samples, and the CRISPR system requires a particular amount of target sequence to recognize or obtain an identifiable signal (low sensitivity challenge).[16,19,77] Therefore, most CRISPR/Cas systems employ a target amplification approach. Polymerase chain reaction (PCR, RT-PCR), quantitative PCR, or isothermal amplification (RPA, RT-RPA, or LAMP) are utilized as a preamplification step.[14] Dependency on instrumentation hinders the portability and field application of the CRISPR diagnostics. Isothermal amplification is mostly used to bypass the need of instrumental incorporation and reduce time and increase field[16,27,28] applicability of the systems.

The process of preamplification increases sensitivity. However, it also increases the complexity of designing primers and probes as well as the cost of associated enzymes and reagents. In addition, they provide an elevated level of pathogen nucleic acid amplification in the sample, which might result in cross-contamination and false positive results. A one-pot test could reduce the likelihood of cross-contamination.[123] Detection from unprocessed samples (without

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Table 5. Application of CRISPR/Cas Based Diagnostics in the Detection of Plant Viruses

Target plant/Pathogen	Enzyme	Time	Limit of detection	Detection type	Ref
			Cas9		
Tomato yellow leaf curl virus Tobacco mosaic virus Potato virus Y	dCas9	<1 h		LFA	72
			Cas13		
Tomato Spotted Wilt Virus	LwCas13a	20 min	2260 copies/μL	Fluorescent real time	36
			Cas12a		
Grapevine red-blotch virus	LbCas12a		10 aM-1 pM, 2× 10^3−1× 10^5 dilutions of extracted DNA	Colorimetric AuNPs	105
Potato virus X Potato virus Y Tobacco mosaic virus	LbCas12a	30 min	10 pM	P51 fluorescence viewer, multiplex	90
Tomato brown rugose fruit virus	LbCas12a	1 h	10^{-4} ng/rxn (RNA sample), 100 copies of transcript/rxn (Synthetic)	Fluorescent LFA (band)	102
Tomato brown rugose fruit virus Tomato mosaic virus	LbCas12a		15−30 ng of PCR products	Fluorescence Plate-reader	23
Tomato yellow leaf curl virus Tomato leaf curl New Delhi virus	LbCas12a	1 h	100 aM of synthetic dsDNA	P51 fluorescence viewer	91
Beet necrotic yellow vein virus	Cas12a		0.1 pM (synthetic) 0.1 ng (real sample)	Fluorescent	86
Apple necrotic mosaic virus Apple stem pitting virus Apple stem grooving virus Apple chlorotic leaf spot virus Apple scar skin viroid	LbCas12a	1 h	250−2500 viral copies (Fluorescent), 1 fM (colorimetric)	Colorimetric	114
Mung bean yellow mosaic India virus	LbaCas12a	15 min		Fluorescent	109
Ageratum enation virus				Fluorometer blue light	
Maize chlorotic mottle virus	LbCas12a	45 min	2000 ng of total RNA cDNAs diluted up to 10^{-5}	Naked eye under blue light	93
Rice stripe virus Rice black-streaked dwarf virus	FnCas12a	50 min	9 copies of DNA	Fluorescent real time, Visual LFA (band), on-spot	100
			Cas 12 and Cas13		
Tobacco mosaic virus, Tobacco etch virus, Potato virus X	LbCas12a LbCas13a	30 min		Fluorescent real time and end point, Multiplex	122
	RfCas13d			Visual LFA band	
Rice black-streaked dwarf virus	LbCas12a, LwaCas13a	Single-26 min, Multiplex-56 min	10^9 aM without RPA, 1 aM with RPA	Fluorescent visual, Flashlight with red and yellow filter glass LFA, multiplex	121

preamplification) can be a viable method, but it has limits (humic substances, polysaccharides, phenolic compounds, and enzymes).[5,12,25] The most effective approach to enhance sensitivity is to identify or engineer highly active Cas enzymes with increased collateral cleavage activity and/or a higher turnover rate in cleaving ssDNA/RNA. This would enable the detection of trace amounts of the target without the need for preamplification and generate detectable signals. The multiplex-crRNA technique is another method for enhancing the sensitivity of the reaction without preamplification. Multiple crRNAs targeting distinct areas of the target dsDNA within the same CRISPR/Cas12a system can enhance sensitivity. This approach is 64 times more sensitive and can detect up to 1 pM of the sample without the need for preamplification.[125]

A further constraint of the system is the dependence of the key Cas proteins (Cas9, LbCas12a, and Cas13a) on certain PAM and PFS sequences.[126] This limits the system's applicability to a broader variety of target sequences without PAM/PFS. To circumvent this constraint, the CRISPR/Cas system is merged with target amplification using PAM-containing primers[100] or the inclusion of an extra PAMmer to begin the cleavage[127] Additionally, other Cas orthologues with varying PAM/PFS preferences or shorter PAM or PFS can be investigated and utilized. Cas12c with 5′-TG, a version of Cas12, has been used for the detection of bacterial canker disease.[83]

For CRISPR detection, the off-target effect or cleavage of an undesired target is still a formidable constraint.[77] Designing sgRNA devoid of off-target activities is a challenging obstacle. To create specific sgRNA, in-depth knowledge of the entire genome sequence of the target, host, and related organisms and a suitable database is necessary. Targeting conserved regions of the target sequence is crucial for the construction of efficient gRNAs. To generate sgRNA for genome editing and gene targeting, numerous gRNA creation tools and web based applications are available.[128,129] Most techniques focus on decreasing the off-target effect of gRNA, and libraries are available for Cas9/sgRNA design for genome editing,[130,131] whereas tools for constructing gRNA for diagnostic purposes are limited.

Another limitation is the sensitivity and mismatch tolerance of gRNA. The CRISPR system relies on the similarity between

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the sgRNA and the target. In certain instances, Cas proteins exhibit cleavage activity even with 2 to 3 bp mismatches in crRNA. Different Cas proteins behave differently when varied mismatches are present.[132] Cas12a can tolerate 2−3 mismatches further away from the PAM sequence than it can in the 5′ proximal region of PAM.[133] In the instance of Cas12f, mismatches in the middle area of crRNA are more detrimental to cleavage activity than mismatches in other regions[74] The cleavage activity is dependent on the position of mismatches and the degree of sequence similarity between crRNA and the target. Consequently, the potential for false-positive results due to off-target sequence capture is a significant issue that can impact the accuracy of identification.

The activity of Cas12a endonuclease depends on crRNA. Cas12 shows varied cleavage activity for various crRNAs.[134] Designing a highly active crRNA is therefore essential for DNA detection assays. Zhang et al. discovered that crRNA has a substantial impact on cleavage efficiency. Among the 14 crRNAs examined for the LFA detection assay, only three were found to be active, although all of them performed well in the fluorescent detection assay. They also discovered crRNAs that can degrade dsDNA completely.[21]

In CRISPR based diagnostic tests, liquid reagents are utilized to make the process simple and versatile. However, reagents such as gRNA, Cas protein, and cleavage reporter must be stored at a low temperature in a freezer. This raises the need for equipment use and electricity consumption. A reasonable approach to tackle this issue could be lyophilization of the components.[135] The stability and sensitivity of the components must be extensively checked. In CRISPR diagnosis, sample preparation requires a distinct procedure involving the grinding of plant tissue, the application of a buffer, the utilization of kits, and incubation at a higher temperature. For point-of-care applications at home and in the field, simple sample preparation with an efficient detection efficiency is required. Incorporating simple and quick DNA extraction techniques can reduce procedure time and complexity.[27] It is possible to exploit the filter paper dipstick method,[136] heat lyophilization, and other simple DNA isolation techniques.

A CRISPR detection investigation comprises multiple phases, including sample extraction, target amplification, CRISPR reaction, and signal transduction. Separate procedures lengthen the process and increase the risk of contamination. The issue can be resolved by including reagents to reduce the number of steps and creating reaction containers to allow experiments to be conducted in a single pot. It is also possible to perform each step on a single device.[96]

5. UNIVERSAL APPLICATION

CRISPR technology can be used to detect all kinds of phytopathogens including viruses, bacteria, fungi, and insects. It can target RNA, DNA, microRNA, protein, tiny molecules, and aptamers.[56] Diverse types of pathogens or diseases can be detected by a single CRISPR system by targeting nucleic acids from different samples. By altering the gRNA, multiple targets can be recognized in a single system.[72] No changes to proteins or experimental settings are necessary in the CRISPR system for target detection. The only adjustments required are the design of highly active, target-specific gRNAs and the application of the appropriate isothermal amplification technique (such as RPA, RT-RPA, LAMP, or RT-LAMP) for RNA and DNA targets. By implementing this strategy, five distinct apple RNA viruses and viroids were successfully identified by simply modifying the gRNA sequences.[114]

In addition, a single CRISPR diagnostic platform can be utilized for detecting different kinds of pathogens, including viruses, bacteria, fungi, and insects, as well as plant nucleic acids of GMOs and specific traits.[72] For instance, rice blast pathogens and bt-rice genes were efficiently detected using a single method and highly active crRNAs. DNA and RNA can also be targeted on the same platform by switching the isothermal amplification method from RPA, LAMP to RT-RPA, RT-LAMP.[28,31] Moreover, DNA and RNA targets can be identified in a single reaction by combining Cas12a and Cas13a with distinct transducing signals.[122] Additionally, distinct orthologues of Cas proteins can perform multiplexing. In the same manner, distinct types, and variations of Cas proteins (LwaCas13a, LbCas12a, AsCas12a, RfxCas13d),[18,121] in conjunction with distinct transducing signals were employed for multiplexing.

The rigorous identification of Cas9/Cas12a for recognizing polymorphic sequences was broadened by a newly discovered CRISPR technique that employs gRNAs with universal bases. Using a single gRNA, this method enables the system to target many human SNPs or evolutionary variants of a single gene. Consequently, it allows flexibility in the detection of naturally occurring polymorphisms or variants without complexity.[137] Pathogens exhibit a significant degree of genetic variety.[138] It complicates the diagnostic procedure when using the CRISPR/Cas technology. The gRNA developed for one variant of a pathogen may be incapable of targeting other variations. However, the developed technology minimizes the complexity and extends the universality of the CRISPR diagnostic system.

Sequence-specific targeting of nucleic acids in accordance with gRNA sequence similarity and PAM sequences can be used to diagnose all types of plant diseases. The unique ability of Cas12 and Cas13 proteins to cleave ssDNA is being exploited as a diagnostic concept for nucleic acid. CRISPR/Cas technology is utilized for the detection of the zika virus, dengue virus, human papillomavirus, and the newly created fast COVID test kits. SHERLOCK,[16] DETECTR,[19] HOLMES,[139] and HUDSON (heating unextracted diagnostic samples to obliterate nucleases)[124] are all well-established, collateral cleavage based CRISPR based diagnostics tools.[28,140] These technologies can diagnose plant diseases, detect important traits and SNPs, and serve as biosensors for GMOs. The SHERLOCK method has been used to genotype and quantify soybean traits.[27] The DETECTR system has been utilized for plant pathogen and genetically modified crop diagnostics.[21,24,99]

The uses of the developed CRISPR technologies are not restricted to a single organism or pathogen. Applicability ranges from the diagnosis of pathogens in clinical samples[147] to animal and plant samples[17] and even the testing of food-borne pathogens[141] for toxicity. Single platform technology is appropriate for the selective and sensitive detection of diverse targets (DNA, RNA, miRNA, protein, small molecule, aptamer) from distinct pathogens (viruses, bacteria, fungi, insects) of human and plant diseases.[83,142] Consequently, a single CRISPR/Cas technology has demonstrated enormous cross-field application potential. Detection of polygenic traits and SNPs is a complex procedures. Rapid SNP detection can be useful for the marker-assisted selection of economically important traits. Improvement of CDetection,[80] Cas12c-

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DETECTOR,[83] or application of high-fidelity detection property of miniature Cas14[74,143] enzyme can be applied for rapid, multiplex detection of SNPs and polygenic traits. The CRISPR/Cas technology can be a next-generation tool for biomarker and nucleotide variant detection to increase the efficiency of plant and animal breeding program.

6. FUTURE PERSPECTIVES

CRISPR/Cas technology, with its diverse range of Cas enzymes such as Cas12, Cas13, and Cas14 has opened up new possibilities in disease detection.[13−18] These tools hold great promise for future disease detection and management due to their sensitive and specific detection capabilities, rapid adaptation to emerging pathogens, and compatibility with various signal readout platforms. Effective pathogen identification is crucial for disease management in plants. CRISPR based diagnostics have already demonstrated their efficacy as point-of-care, field-deployable devices for diseases like COVID-19.[123,144−147] Similar techniques can be applied to diagnose plant diseases, offering enormous potential. By the utilization of CRISPR/Cas detection methods, the time and cost associated with transporting samples to laboratories for extensive investigations can be significantly reduced.

CRISPR technology also holds immense potential in marker-assisted breeding techniques for detecting single nucleotide polymorphisms (SNPs). Traditional methods such as sequencing and PCR based SNP selection are expensive and time-consuming.[34,35,37] However, methods like SHERLOCK can effectively distinguish between SNPs in human genotyping and cell-free DNA (cfDNA).[16,18] The CDetection method has also shown sensitivity to single-base pair variations when using optimized guide RNAs (tgRNA).[80] Leveraging the natural accuracy, sensitivity, and specificity of CRISPR based nucleic acid detection systems,[15] we can target disease-related genes of pathogens,[105] detect genetically modified content in foods and crops,[96,97] identify SNP variants,[83,86] and analyze genes associated with economically important traits[72] in crops.

To fully utilize the potential of CRISPR based diagnostics for disease diagnosis and accelerated breeding, it is crucial to address the current challenges of multiplex detection and portability. Multiplex detection methods that can simultaneously identify multiple pathogens or genetic variations are highly desirable in disease management. By developing robust and reliable multiplex detection assays, we can streamline the diagnostic process, allowing for comprehensive pathogen profiling and informed decision-making. On the other hand, the portability of diagnostic devices is a critical factor, especially in resource-limited settings or areas with limited access to centralized laboratories. Designing portable devices that enable on-site testing can revolutionize disease diagnosis and breeding programs. By integrating CRISPR based detection systems into portable platforms, such as hand-held devices or portable kits, we can bring diagnostics closer to the point of need. This not only saves time and resources but also empowers farmers, plant breeders, and field workers with the ability to quickly detect and manage diseases in real-time.

The major challenges associated with the multiplex detection and portability involve integrating multiple steps of the CRISPR system into a single hand-held or portable device. These steps comprise the isolation, purification, cleavage, recognition, and detection of target nucleic acids in a semiautomated or fully automated manner. Addressing these challenges requires a multidisciplinary effort involving researchers from engineering, plant genetics and plant pathology, and analytical chemistry disciplines. The development of a hand-held or portable device for multiplexed analysis introduces additional complexities, particularly in integrating multistep processes for detecting multiple analytes within a single device. However, we believe that leveraging knowledge from advanced engineering and current biomedical diagnostic platforms would be beneficial in advancing this aspect of CRISPR diagnostics research, ultimately leading to the realization of fully integrated portable devices for CRISPR diagnostics. This advancement would unlock the full potential of CRISPR diagnostics, revolutionizing crop improvement and disease management strategies.

AUTHOR INFORMATION

Corresponding Author

Muhammad J. A. Shiddiky − *School of Environment and Science (ESC) and Queensland Micro and Nanotechnology Centre (QMNC), Griffith University, Nathan, QLD 4111, Australia; Rural Health Research Institute, Charles Sturt University, Orange, NSW 2800, Australia;* orcid.org/0000-0003-4526-4109; Email: mshiddiky@csu.edu.au

Authors

Tanzena Tanny − *School of Environment and Science (ESC) and Queensland Micro and Nanotechnology Centre (QMNC), Griffith University, Nathan, QLD 4111, Australia*

Mohamed Sallam − *School of Environment and Science (ESC) and Queensland Micro and Nanotechnology Centre (QMNC), Griffith University, Nathan, QLD 4111, Australia*

Narshone Soda − *Queensland Micro and Nanotechnology Centre (QMNC), Griffith University, Nathan, QLD 4111, Australia*

Nam-Trung Nguyen − *Queensland Micro and Nanotechnology Centre (QMNC), Griffith University, Nathan, QLD 4111, Australia*

Mobashwer Alam − *Queensland Alliance for Agriculture & Food Innovation, The University of Queensland, Nambour, QLD 4560, Australia*

Complete contact information is available at: https://pubs.acs.org/10.1021/acs.jafc.3c00913

Notes

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ABBREVIATIONS:

POC = point of care; gRNA = guide RNA; LFA = lateral flow assay; LFD = lateral flow device; LbCas12a = Cas12a from *Lachnospiraceae bacterium*; LbCas13a = Cas13a from *Leptotrichia buccalis*; Rfcas13d/RfxCas13d = Cas13d from *Ruminococcus flavefaciens*; FnCas12a = Cas12a from *Francisella novicida*; AsCas12a = Cas12a from *Acidaminococcus* sp; dpi = day after post inoculation; hr = hour; min = minute; gDNA = genomic DNA,

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