Review Article

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CRISPR/Cas-based detection systems – emerging tools for plant pathology

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Abstract: Plant diseases pose a significant threat to global food security, causing substantial crop yield losses and economic damage. Traditional diagnostic methods often lack the speed, specificity, and sensitivity required for effective disease management. The emergence of CRISPR/ Cas-based detection systems offers a novel and highly efficient approach for identifying plant pathogens. This review explores the principles of CRISPR/Cas technology, its adoption for pathogen detection, and its advantages over conventional diagnostic techniques. Recent advancements in CRISPR-based detection tools, including SHERLOCK, DETECTR, and other Cas-mediated platforms, and their potential for rapid, field-deployable diagnostics have been discussed. As these technologies continue to evolve, they hold promise for transforming plant disease diagnostics and enhancing global agricultural resilience.

Keywords: Cas12a, Cas13a, SHERLOCK, DETECTR, isothermal amplification

1 Introduction

Plant diseases are considered one of the most important threats to crop production and thus to food supply in the modern world. Since the onset of agriculture 10,000–12,000

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years ago, the agro-ecosystems evolved toward high environmental homogeneity and low genetic diversity, which have driven the emergence and evolution of numerous crop pathogens and pests [1,2]. For the major crops, the global yield losses caused by pests and pathogens have been estimated in a range of 20-30%, depending on the crop and the international distribution of the production [3]. Plant diseases affect various aspects (components) of food safety both on local and global levels and may have disastrous consequences [4]; a notorious example of this is the case of the potato famine in Ireland (1845-1850) caused by Phytophthora infestans [5]. Thus, comprehensive disease control is a crucial task to maintain food safety in countries and regions of the world. The modern-day practices require a complex approach combining the utilization of crop genetic resources to increase their capability to resist diseases, direct eradication of the pathogens and their vectors, and the monitoring of the infection distribution within agricultural systems.

The crucial step in the monitoring of pathogens is their correct identification, as different infection agents require different approaches to disease management. To date, various methods with their advantages and limitations are used for plant pathogen diagnostics (Table 1). The oldest used methods of detection and diagnostics of plant pathogens are based on distinct visual features of different diseases, both on macro- and microscopic scales, as well as traditional microbiological approaches based on Koch's postulates, when applicable [6]. The visual examination method is simple and does not require special equipment, but it is not suitable for plants with latent infections, it depends on the subjective knowledge and experience of the investigator, and it is prone to errors [7-9]. The limitations of such traditional approaches have led to the development of a range of more precise laboratory-based methods, from serological assays to DNA markers. The serological methods, mainly based on enzyme-linked immunosorbent assay (ELISA), are widely used due to their low cost and good performance; however, they have limitations related to their specificity in different experimental contexts [6]. The DNA detection methods based on polymerase chain reaction (PCR) and sequencing became a standard in plant

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Table 1: Comparison of traditional and modern methods of plant disease diagnostics

Method	Advantages	Disadvantages
Visual examination of symptoms	Does not require expensive equipment and expendables	 Highly depends on the personal expertise of the researcher/practitioner Ambiguous symptoms of the broad groups of pathogens (bacteria, fungi, viruses, protists) Not suitable for latent infections
Microbiological methods (selective medium cultivation, inoculation experiments)	 Requires basic equipment Provides and maintains access to a viable pathogen 	 Not all pathogens can be grown in culture (e.g., viruses, obligate parasites) Limited accuracy Time and labor consuming
Serology assay (ELISA, immune blotting)	 Low cost High throughput Easy performance 	 Availability of specific antibodies, difficulty of developing a custom assay Low specificity Varying performance depending on material, experimental environment, etc.
PCR and sequencing	 High specificity on different levels of resolution, from species to races/pathotypes Wide range of available assays Easy to create custom assays using primer design tools and available genetic/genomic data on pathogens High reproducibility 	 Risks of cross-reactivity Requires expensive equipment, laboratory environment, and expendables High demand on time and labor May require highly proficient personnel for data interpretation and analysis
Isothermal amplification	 Requires basic laboratory equipment Portability High speed 	 Requires more expertise to design custom assays Potentially lower specificity comparing to PCR
CRISPR/Cas detection	 High specificity to the targets High sensitivity when coupled with amplification methods Quick results Can be adopted to simple environment 	 Lack of standardized assays Requires more expertise to design custom assays May have insufficient sensitivity without preliminary amplification step

pathogen diagnostics due to their exceptional sensibility and potential for customization [10–12]. However, despite continuous efforts to improve, this approach remains expensive, time, and labor consuming and requires a stationary laboratory environment. The demand for quick, portable, yet reliable technologies for rapid DNA- or RNA-based diagnostics has led to the development of methods of isothermal amplification: loop-mediated isothermal amplification (LAMP), recombinase polymerase amplification (RPA), helicase-dependent amplification, etc. [13]; however, these methods have their own limitations [14].

The present review is focused on CRISPR/Cas-based detection, a relatively novel but rapidly growing in popularity tool for pathogen detection, in application to plant diseases. Since the first published results in 2017 [15], the method was extensively tested for human pathogens and, later, was widely adopted for plant pathology. The growing number of studies dedicated to the diagnostics

of plant pathogens indicates the increasing interest in this technology as a quick, portable, and reliable tool for plant pathology.

2 Diversity of bacterial CRISPR/Cas systems and their modifications

Since the discovery of CRISPR/Cas systems as an adaptive immune mechanism in bacteria and archaea [16], they have attracted attention both as a fundamental component of the evolution and ecology of prokaryotes [17] and as a promising tool for numerous practical applications [18]. The natural prokaryotic CRISPR/Cas systems have vast diversity. Based on available data on the composition of the systems and their evolutionary changes within various prokaryotic genomes, the current CRISPR/Cas classification

includes 2 classes, 6 types, and 33 subtypes [19]. Class I systems, including CRISPR/Cas types I, III, and IV, involve multi-component protein complexes (up to 11 in Archaeoglobus fulgidus), whereas class II systems, including types II, V [20], and VI [19], contain a single multi-domain Cas protein with modules for target and crRNA recognition and target cleavage [21]. Thus, due to simplicity, CRISPR/Cas class II systems have become preferable for molecular genetic research. The CRISPR/Cas9 system of this class became a widespread tool for genetic engineering.

The first system widely applied in genetic engineering was CRISPR-Cas9 (class 2, type II). It contains several conserved domains: REC1-REC3, HNH, and a RuvC-like domain, which ensure efficient nucleic acid detection and cleavage [22]. Cas9 introduces site-specific double-strand breaks at target sites. In synthetic CRISPR-Cas9 systems, crRNA and tracrRNA are combined into an artificial tetraloop, forming a single-guide RNA (sgRNA). The target is determined by a 98-nucleotide sequence, including a 20-nucleotide spacer located at the 5'-end of the sgRNA. The specificity of the editing system is defined by the spacer sequence used [23]. Protospacer recognition depends on the presence of a PAM sequence and absolute spacer homology. Precise cleavage at the target recognition site made Cas9 a popular tool in genetic engineering for gene editing via homologous or non-homologous repair of the affected regions [24,25]. The successful application of the CRISPR-Cas9 system from Streptococcus pyogenes has spurred interest in bacterial and archaeal genomes in search of simpler and more efficient tools.

Type V systems include Cas12 effector proteins, which functionally differ from type II systems. Cas12 proteins contain only a RuvC-like domain, responsible for DNA-targeting activity [19]. Cas12a offers several advantages over Cas9 in genetic engineering and pathogen detection. First, the SpCas9 protein is larger than Cas12a. Additionally, for its nuclease activity, Cas9 requires a chimeric molecule consisting of crRNA and tracrRNA - whereas CRISPR/Cas12a is more compact and requires only a single-guide RNA, simplifying cellular delivery. Second, Cas12a cleaves DNA differently than Cas9, which is crucial for transgenesis accuracy. While Cas9 creates "blunt ends" by cutting at a single site, Cas12a cleaves both DNA strands at a small distance apart (5-7 base pairs), forming "sticky ends." This feature enhances the precise integration of donor DNA fragments [26].

The Cas14 protein was discovered through metagenomic analysis of bacterial and archaeal databases. It is one of the smallest Cas proteins, ranging from 40 to 70 kDa, which is 400-700 amino acids smaller than other functional Cas proteins [27]. Functionally, Cas14 is more similar to type V proteins - its activity targets single-stranded DNA. However, unlike Cas9, Cas14 does not require a PAM sequence, which reduces constraints on protospacer selection. Cas14 contains an RuvC domain responsible for cleaving single-stranded DNA [27,28].

Unlike Cas9, Cas12, and Cas14, Cas13 proteins of type VI have specificity for target recognition and cleavage of RNA instead of DNA. The HEPN domain in Cas13 is essential for RNA substrate binding and nuclease activity; like type V proteins, type VI proteins process their pre-crRNA [29]. Cas13a and Cas13b have a unique property: after specific target interaction, they exhibit non-specific activity against any RNA. Due to this. Cas 13 is considered a tool for genome interventions on the level of RNA, e.g., as an alternative for RNA interference for gene silencing or gene therapy [30,31].

3 Principles of CRISPR/Cas-based pathogen detection

The specific target binding by crRNAs laying in the basis of the function of CRISPR/Cas9 systems has raised an interest in their use for sequence recognition assays, as an alternative to traditional primers and probes. Such detection systems combine targeted recognition through specific binding of crRNA(sgRNA)/Cas9 complexes, followed by signal amplification, offering advantages over traditional methods like PCR in terms of speed, portability, and minimal equipment requirements. Earlier proposed assays were based on CRISPR/Cas9 and included diverse signal amplification methods. Different authors proposed a variety of signal detection approaches. Pardee et al. used crRNAs and Cas9 protein as part of paper-based biosensors for strain-specific detection of Zika virus [32]. SgRNA/Cas9 complex was proposed for use as a sequence-targeting agent in fluorescent in situ hybridization assay for detection of antibiotic-resistant Staphylococcus aureus [33]; the other study was focused on drug resistance in S. aureus, Acinetobacter baumannii, and Klebsiella pneumoniae [34]. Xu et al. proposed a method combining CRISPR/Cas9 target recognition with micro-bead-based ELISA and tested it for the detection of human papillomavirus [35]. Another approach based on luminescence from luciferin reporter and luciferase-linked Cas9 protein complex was tested for the detection of Mycobacterium tuberculosis [36]. An approach proposed by Huang et al. used specific target breaks made by Cas9 to trigger nicase-driven exponential circular amplification [37]. Other examples of using CRISPR/Cas9 for pathogen detection include Salmonella typhimurium [38], Orientia tsutsugamushi [39], Listeria monocytogenes, and

African swine fever virus [40]. However, the use of CRISPR/Cas9 systems for nucleic acid detection has not received as much attention as their applications as genome editing tools. Although the mentioned assays demonstrated higher accuracy compared to conventional PCR, they utilized diverse reporter/biosensor systems for signal detection and amplification, which were complicated and limited widespread adoption.

The nucleic acid detection systems based on proteins Cas12 and Cas13 have been considered more promising. The specific features of these proteins are the presence of trans-cleavage activity: Cas12 and Cas13 are capable of digesting any ssDNA or RNA, respectively, upon their activation by the duplex of the target and sgRNA [41-43], in contrast to Cas9, which cuts DNA exclusively at the binding site [44]. When used with reporter molecules, usually labeled DNA or RNA oligonucleotides, such collateral cleavage may amplify the detection signal to sufficient levels for visual or instrumental detection (Figure 1) [45]. The SHERLOCK (Specific High-sensitivity Enzymatic Reporter UnLOCKing) assay was the first method that defined the further development of CRISPR/Cas detection systems. The proposed method utilized RNA recognition and cleavage activity of Cas13a protein (previously known as C2c2; dual ribonuclease undergoing conformational change in response to target RNA recognition which leads to cisand trans-RNA cleavage activity [46]) coupled with RPA and reverse-transcription RPA (RT-PCR) and T7 in vitro transcription to detect DNA or RNA, correspondingly, of viral and bacterial pathogens both in culture and human samples [15]. The original protocol utilized RNA oligonucleotides labeled with a fluorescent dye and the quencher as a reporter; collateral cleavage activity of Cas13a protein led to the release of fluorescence detected and quantified

by conventional plate-reading or RT-PCR tools [47]; the modified assay, SHERLOCKv2, provided further development of the reporter system including use of colorimetric lateral flow cells and multiplex detection [48]. A similar approach involving the Cas12a protein was termed DNA endonuclease-targeted CRISPR trans reporter (DETECTR) [41]. Similar to Cas13a, Cas12a undergoes activation of trans-cleavage activity upon conformation changes caused by target recognition by sgRNA; the difference is that both targeting and cleavage substrates are ssDNA [49,50]. The absence of the required in vitro transcription step made this method a convenient alternative to CRISPR/Cas13based approaches; also, the ssDNA reporter molecules are more affordable and easier to use than RNA. As well as SHERLOCK, the combination with isothermal amplification of PCR increases sensitivity [51], and application of reverse transcription allows the use of the method also for RNA detection [52].

Both original SHERLOCK and DETECTR protocols have been registered as trademarks by The Broad Institute, Inc., Cambridge, USA (USA registration number 6295441, Mar. 16, 2021) and Mammoth Biosciences, Inc., Brisbane, California, USA (USA registration number 6473033, Aug. 31 2021), respectively, and patented; main patents include US12037639B2 (USA), JP2023011606A (Japan), ES2927463T3 (Spain), EP4119663B1 (European Union), WO2018107129A1 (WIPO), AU2017371324B2 (Australia) for SHERLOCK and US20210102242A1, US11174470B2 (USA), EP3830301B1 (European Union) for DETECTR. The development of novel modifications of basic SHERLOCK/DETECTR assays continues to expand them for newer applications, improve their features, and avoid limitations. The protocol HOLMES (an one-HOur Low-cost Multipurpose highly Efficient System) was developed to allow quantitative detection of the target molecules [53,54]. As proof of concept, the use of

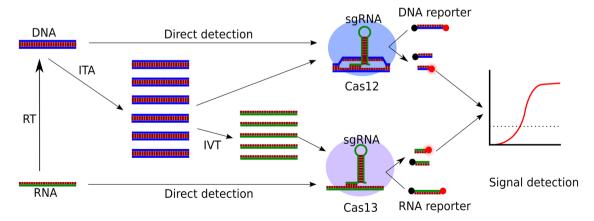


Figure 1: General principles of nucleic acid detection using CRISPR/Cas12-Cas13. RT – reverse transcription; ITA – isothermal amplification; IVT – *in vitro* transcription. Source: Created by the authors.

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Cas14 protein instead of Cas12a was proposed for nucleic acid detection with potentially higher fidelity [28].

Due to their versatility, accessibility, and customization potential, SHERLOCK and DETECTR-like methodologies became a standard for CRISRP/Cas-based detection of nucleic acid. A high speed of analysis and potential portability are usually considered the most important advantages of these tools, and the new protocols are developed to further improve these qualities.

Whereas the requirements for the basic procedure of CRISPR/Cas detection are limited by a thermostat and single tube reaction mixture, the methods proposed for the nucleic acid cleavage signal detection vary in their affordability (Figure 2). The most common approach described above utilizes fluorescent probes [48,53] and thus requires equipment for fluorescence detection, such as plate readers and real-time thermal cyclers; however, it was shown that the visual detection of fluorescence is also possible and provides qualitative test outcomes using only a UV light source [55]. An alternative approach is the colorimetric method utilizing

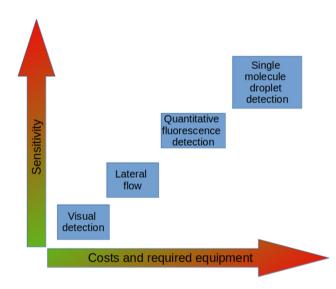


Figure 2: CRISPR/Cas signal detection methods ordered by their sensitivity and cost.

gold or iron nanoparticles as reporters [56-58]. In case of visual detection, similar to isothermal amplification methods (LAMP, RPA), which are usually used in conjunction with CRISPR/Cas, the methodology can be deployed using a minimal set of portable equipment like mini-centrifuges and solid-body thermostats [59]. Lateral flow elements [60] and biosensors [61] have been proposed to further increase the availability of express detection directly in the field. To exclude a step of isolation of the nucleic acids, a combination of SHERLOCK with the HUDSON (Heating Unextracted Diagnostic Samples to Obliterate Nucleases) method was proposed for direct detection of viruses in biological samples [62]. On the other hand, the implementation of new complicated techniques for precise signal detection paves the way for higher sensitivity and fidelity of nucleic acid detection [63]. Use of microchamber-based array was shown to increase sensitivity by excluding the amplification step and thus reducing possible amplification errors [64]. Similarly, the possibility of direct quantitative detection of RNA using a digital droplet platform without amplification was shown [65]. Thus, the CRISPR/Cas detection systems can be deployed at various scales, from express detection at the points of care (fields and gardens, in case of crop disease) to more complicated laboratory environments with different levels of precision and sensibility, depending on the goals. Therefore, the methodology of CRISPR/Cas detection has significant potential as both a rapid, portable diagnostic tool and a reliable and efficient tool for well-equipped laboratories.

Compared to the conventional methods based on PCR, the CRISPR/Cas has several advantages (Table 2). CRISPR/Cas demonstrated sensitivity similar to or greatly surpassing PCR; e.g., for *Staphilococcus auresus*, the CRISPR/Cas system was able to detect 1.2 CFU whereas PCR detected 60 CFU [66]. Both methods have high specificity as they rely on specific oligonucleotide matching; however, CRISPR/Cas is more sensitive to the mismatches than PCR primers and thus allows precise identification of particular sequences and their variants [66,67]: even single mismatches in the target have been shown to reduce cleavage efficiency [56].

Table 2: Comparison of CRISPR/Cas detection and PCR

Feature	PCR	CRISPR/Cas-based detection
Sensitivity	High	Ultra-high (can surpass PCR/qPCR)
Specificity	High	High (can discriminate single nucleotides)
Speed	Hours	15–60 min
Equipment	Expensive, requires thermocycler	Simple, often isothermal
Cost	Higher	Potentially lower
Quantification	Excellent (qPCR)	Good
Practicality	Lab-based, skilled personnel	Point-of-care potential, user-friendly

This, as well as potentially low costs, demands for time and labor, and portability, as discussed above, makes CRISPR/Cas-based detection systems a tool of choice for many applications currently relying on PCR, including detection of pathogens of human [68], animals [69], and plants [70].

3.1 Use of CRISPR/Cas for detection of plant pathogens: current status and future perspectives

As described above, the key field of development and application of CRISPR/Cas-based pathogen detection and diagnostics is medicine. However, this technology has also been adopted for the demands of plant pathology and epidemiology. Similar to the primer- and probe-based method, the core of the methodology is versatile and can potentially be applied to any research object using custom-designed gRNA sequences. The early example is the use of CRISPR/ Cas9 coupled with LAMP and gold nanoparticles to detect Phytophthora infestans, the common pathogen of vegetable crops [56]. Tripathi et al. adopted the CRISPR/Cas9-nicase method [71] to detect banana bunchy top virus and banana streak virus in banana plants. However, the later studies focused on the systems based on Cas12 or Cas13 proteins, as their convenience and versatility have been proven by the use of systems like SHERLOCK and DETECTR for medical purposes. The cases of use of Cas13a protein with SHERL-OCK-like protocols for plant pathogen detection include identification of tomato spotted wilt virus [72] and fungus Verticillium dahliae in smoke tree [73]. Hak et al. proposed an express-protocol for direct detection of tomato brown rugose fruit virus (TBRFV) by CRISPR/Cas13a without steps of RNA isolation, RT and amplification, and in vitro transcription; although sensitivity of the assay was lower comparing methods combined with amplification, high portability and rapid execution have made it very promising as in field plant pathogen diagnostic tool [74].

The most used approach is the use of Cas12a protein, due to its compatibility with DNA and RNA targets (via reverse transcription) without the necessary step of *in vitro* transcription. A system for the direct detection of tomato mosaic virus (*Tobamovirus tomatotessellati*¹) and TBRFV (*Tobamovirus fructirugosum*) in tomato without a preliminary isothermal amplification step was developed and shown to differentially detect two viruses from 15 to

30 ng of PCR product [76]; another system for TBRFV has shown sensibility similar to conventional real-time PCR and has been recommended as suitable at the points-ofcare [77]. Another tomato viruses, tomato yellow leaf curl virus (Begomovirus coheni) and tomato leaf curl New Delhi virus (Begomovirus solanumdelhiense), have been successfully detected using Cas12a with LAMP [78]. Even in case of RNA viruses, the use of CRISPR/Cas12a combined with RT-RPA or RT-LAMP was considered preferable and tested for detection of beet necrotic yellow vein virus (Benyvirus necrobetae) in sugarbeet [79], tobacco mosaic virus (Tobamovirus tabaci), potato virus X (Potexvirus ecspotati), potato virus Y (Potyvirus yituberosi) in tobacco [80], maize chlorotic mottle virus (Machlomovirus zeae) in maize [81], apple necrotic mosaic virus (Ilarvirus ApNMV), apple stem pitting virus (Foveavirus mali), apple stem grooving virus (Capillovirus mali), apple chlorotic leaf spot virus (Trichovirus mali), apple scar skin viroid (Apscaviroid cicatricimali) in apple tree [82], citrus tristeza virus (Closterovirus tristezae) [83], and brassica yellows virus (Polerovirus TUYV) [84]. Although Cas13a could be used for direct detection of viral RNA, as shown by Hak et al. [74] and Marqués et al. [85], Cas12a detection combined with reverse transcription and isothermal amplification remains preferable due to higher sensitivity. Typical targets used for the detection of plant viruses include ORFs encoding coat (capsid) protein, as in the case of PCR-based or other sequence-specific assays.

CRISPR/Cas12a assay got extensively used for the diagnostics of fungal diseases in plants. The list of the successfully tested pathogens includes, but not limited by Alternaria solani in potato [57], Sporisorium scitamineum and Colletotrichum falcatum in sugar cane [86], Diaporthe helianthi in sunflower [87], Sclerotium rolfsii in cassava [88], Verticillium dahliae [89], Venturia carpophila in peach [90], Fusarium circinatum in pines [91], F. asiaticum in rice [92], F. graminearum in wheat [93], Leptosphaeria maculans in Brassica [94], Magnaporthe grisea (syn. Magnapothe oryzae) in rice [95], Diaporthe aspalathi and D. caulivora in soybean [96], Elsinoë fawcettii [97] and Alternaria spp. [98] in Citrus, etc. Systems for the detection of oomycetes Phytophthora ramorum [99], P. sojae [100], and P. cambivora [101], deleterious pathogens of wild range of plant species. The typical target loci used for the identification of fungal species are 16S rRNA gene [86], ITS [87,88,94], Cal gene, EF1-α [87], GAPDH [89], CYP51C [92], etc. The bacterial pathogens for which CRISPR/Cas-based diagnostic systems have been proposed include Acidovorax citrulli [102], Xylella fastidiosa [103], Xanthomonas albilineans [86], Candidatus Liberibacter asiaticus [104], and Candidatus Phytoplasma trifolii [105]; ITS and 16S rRNA gene sequences were used as a target to identify bacterial species.

¹ Hereafter, the binary species names of viruses are according to the ICTV database [75].

The listed studies report detection sensibility from attogram to nanogram amounts of the target, depending on whether isothermal pre-amplification has been used or not. With such sensibility comparable or surpassing conventional PCR and real-time PCR-based assays [106], and taking into account costs, time, and labor demands, interest in the application of CRISPR/Cas-based detection systems in plant pathology is growing, as shown by the available publications. As the existing studies show, the prevailing approach is the use of CRISPR/Cas12a in conjunction with isothermal amplification methods (LAMP, RPA). In this direction, this technology may be considered an upgrade of isothermal amplification, allowing it to overcome its limitations (like lower specificity compared to PCR) at a low cost of additional manipulations and time. On the other hand, CRISPR/Cas detection protocols can be further developed as an independent, amplification-free tool. For plant viruses, which are predominantly RNAbased, a direct detection with CRISPR/Cas13 without steps of reverse transcription and amplification can be a potential game-changer, significantly increasing speed and portability of the assay. However, due to higher sensitivity, amplification-coupled protocols remain prevailing. The accumulated data lead to the development of common protocols, e.g., for the detection of viruses [107], which will make the wider adoption of the technology to crops and their pathogens easier. The applicability range of the CRISPR/Cas assays varies from express systems for rapid in field and as part of a sophisticated laboratory environment, with the corresponding scaling of sensitivity and fidelity of the detection. Thus, CRISPR/Cas detection systems are considered a promising alternative to PCR-based methods, both as is and as an extension of isothermal amplification assays.

4 Conclusion

The pathogen detection methods based on CRISPR/Cas have proven themselves a promising and reliable alternative to traditional PCR-based approaches. The main features include high specificity and sensibility, rapid preparation and result reading, versatility in various research and practical cases, and, of particular importance, low demand for equipment and potentially high portability. As was shown by numerous studies on human pathogen tests, CRISPR/Cas systems proved their viability and so pose interest not only for medicine but also for plant pathology. The portability and accessibility of the technology make it suitable for the express testing of infected plants in the field. The available research data indeed show the growing interest in CRISPR/Cas detection for a wide range of plant pathogens, and so development and improvement of the technology is a promising field of study and practical use in plant pathology and epidemiology.

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