Plant Methods

REVIEW

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CRISPR-Cas13a as a next-generation tool for rapid and precise plant RNA virus diagnostics

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Abstract

Plant viruses are among the most serious threats to global agriculture, causing significant yield losses and jeopardizing food security. Identifying these viruses is crucial to prevent widespread crop damage and ensure effective management. CRISPR-Cas13a, a subtype of the RNA-targeting Cas13 family, has emerged as a transformative tool in molecular diagnostics, specifically tailored to detect these plant RNA viruses with unparalleled precision. Unlike traditional methods such as ELISA and RT-PCR, which are often limited by sensitivity, equipment dependency, and long processing times, Cas13a offers exceptional specificity and attomolar-level sensitivity. Its RNA-guided collateral cleavage mechanism allows signal amplification, making it particularly suitable for field-deployable diagnostics. Recent advances in Cas13 engineering, including compact variants such as Cas13bt3 and Cas13Y, have further improved its delivery efficiency and minimized immune responses, enhancing its agricultural applications. Integration with amplification methods like LAMP and innovative biosensor platforms like graphene-based and electrochemical systems further enhances its diagnostic potential. While challenges remain, including off-target effects, reagent stability, and scalability, innovations in CRISPR RNA (crRNA) design, reagent encapsulation, and microfluidic technologies are actively addressing these barriers. CRISPR-Cas13a represents a cutting-edge solution for rapid, accurate, and accessible plant virus diagnostics, providing a powerful safeguard for crop yields and global food security.

Keywords CRISPR-Cas13a, Plant viruses, Molecular diagnostics, Field-deployable diagnostics, Biosensor platforms, Food security

Introduction

Plant viruses are a significant threat to global agriculture, causing severe crop losses and jeopardizing food security. Timely and accurately identifying these viruses is critical to implementing effective control measures and minimizing economic and agricultural damage. These pathogens are particularly challenging to manage due to their rapid mutation rates, extensive host ranges, and ease of transmission [1]. Traditional diagnostic methods, such as enzyme-linked immunosorbent assays (ELISA), real-time PCR, and next-generation sequencing (NGS), have been widely used to detect plant viruses. However, these methods often require complex infrastructure, significant expertise, and high costs, making them less accessible in resource-limited agricultural settings [2]. To address these limitations, innovative and efficient diagnostic approaches are urgently needed.

CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats) has emerged as a transformative technology in molecular biology and genetics, offering unprecedented precision in genome editing and diagnostics since its identification as a prokaryotic adaptive immune system [3]. Among CRISPR-associated proteins, Cas13a has gained attention for its RNA-targeting capabilities, enabling the detection and cleavage of specific RNA sequences with high specificity. This unique

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property makes Cas13a particularly suitable for detecting plant RNA viruses, which are among the most destructive pathogens in agriculture [4].

The mechanism of Cas13a is highly advantageous for diagnostics. Guided by RNA, it not only targets specific viral RNA but also triggers a collateral cleavage activity, amplifying detection signals. This capability allows the detection of viral RNA at extremely low concentrations, making it ideal for early diagnosis [5]. CRISPR-Cas13a has been integrated into diagnostic platforms such as SHERLOCK and HUDSON, which streamline RNA detection by combining isothermal amplification (e.g., RPA) with Cas13a's collateral cleavage activity [6]. These systems eliminate complex RNA extraction steps and enable field-deployable workflows, as demonstrated in rapid Tomato Brown Rugose Fruit Virus (ToBRFV) diagnostics [7].

CRISPR-Cas13a-based diagnostics address the limitations of traditional approaches by combining simplicity, speed, and affordability, particularly for field-deployable applications [8]. This review explores the potential of CRISPR-Cas13a in advancing plant virus diagnostics, focusing on its mechanisms, diagnostic platforms, and comparative advantages over conventional methods. By improving virus detection strategies, CRISPR-Cas13a contributes to sustainable agriculture and enhances global efforts to safeguard food security.

While DNA viruses (e.g., geminiviruses) are significant plant pathogens, this review focuses on RNA viruses due to their predominance (> 80% of plant viral species) and unique diagnostic challenges. CRISPR-Cas13a is uniquely positioned to address these challenges through direct RNA detection and amplification-free workflows, unlike DNA-targeting systems (e.g., Cas12/Cas14) requiring additional RNA virus analysis steps.

Types of virus detection methods and their comparison

Viruses, as obligate intracellular pathogens, infect a wide range of hosts, including plants, animals, and humans. Their genomes, particularly in RNA viruses, exhibit high mutation rates, leading to genetic diversity that complicates detection and management efforts. To address these challenges, various diagnostic techniques have been developed, each offering unique advantages and limitations based on sensitivity, speed, cost, and field applicability [9].

Traditional nucleic acid-based methods like PCR and RT-qPCR have long been the cornerstone of virus detection due to their high sensitivity and specificity. However, these methods are time-intensive, require advanced laboratory facilities, and demand skilled personnel, making them less practical for resource-limited or field settings [10]. Recent advancements in PCR technologies, such as quantitative PCR (qPCR) and digital PCR (dPCR), have significantly improved sensitivity and precision. qPCR now achieves detection limits as low as 1–10 copies/μL, with multiplex capabilities enabling simultaneous detection of multiple targets [11]. Digital PCR, with its absolute quantification and tolerance to inhibitors, has further enhanced reproducibility [12]. However, these methods remain reliant on thermocycling, specialized equipment, and trained personnel, limiting their field applicability. Additionally, they require RNA extraction and reverse transcription for RNA viruses, adding complexity and time [13, 14].

Alternatively, isothermal amplification methods, such as loop-mediated isothermal amplification (LAMP), offer faster results and require less complex equipment, enhancing their field applicability [15]. Immunological approaches, including ELISA and lateral flow immunoassays (LFIA), detect virus-specific proteins and complement nucleic acid-based techniques. ELISA provides robust sensitivity within 2–6 h and is suitable for basic laboratories. Modern ELISA platforms now incorporate nanomaterials and signal amplification strategies, achieving sensitivities of 0.1–10 ng/mL within 2–6 h [16]. Automated systems have reduced human error, and multiplex ELISA kits are available for high-throughput screening. Despite these improvements, ELISA still suffers from antibody cross-reactivity, inability to distinguish closely related viral strains, and dependence on stable protein epitopes, which may mutate rapidly in RNA viruses. LFIAs, meanwhile, deliver rapid results with minimal equipment, making them ideal for point-of-care testing, although they generally exhibit lower sensitivity compared to nucleic acid-based methods [17].

Recent LFIA innovations include gold nanoparticle-enhanced colorimetric signals and CRISPR-integrated strips [18]. These assays provide results in 15–30 min with minimal equipment, making them ideal for point-of-care use. However, their sensitivity (~ 0.1–10 ng/mL) remains inferior to nucleic acid-based methods, and they lack multiplexing capabilities for mixed infections [19].

The advent of CRISPR-Cas13 technology represents a paradigm shift in virus diagnostics. CRISPR-Cas13a, with its unique collateral RNA cleavage activity, enables ultra-sensitive detection within approximately 30 min. Unlike RT-qPCR, CRISPR-Cas13a diagnostics require minimal equipment and are cost-effective, making them highly suitable for field applications [20]. Platforms like SHERLOCK integrate CRISPR-Cas13 with isothermal amplification techniques such as LAMP or RPA, enabling rapid detection of plant viruses like ToBRFV and Potato Virus Y (PVY) in under an hour, even at minimal viral loads [15]. Also, CRISPR-Cas13a systems facilitate multiplex detection, a critical advantage for identifying

multiple pathogens simultaneously. Tools like CARMEN-Cas13 leverage Cas13a's collateral cleavage activity to detect numerous viral targets in a single assay, proving especially valuable for monitoring outbreaks or detecting viruses in agricultural settings. Additionally, innovations like heat-based sample preparation eliminate complex RNA extraction processes, simplifying diagnostics for resource-limited regions [6].

Despite their transformative potential, CRISPR-Cas13-based systems are not yet widely adopted in clinical diagnostics, where traditional methods like PCR and ELISA remain the gold standard due to their established reliability. However, ongoing optimization of CRISPR diagnostics is expected to bridge this gap, enabling broader adoption for both clinical and field applications. Table 1 provides a detailed comparison of these methods, illustrating how CRISPR technology complements traditional approaches by addressing their limitations and expanding the possibilities for rapid, cost-effective virus detection.

Major plant viruses and diagnostic requirements

Numerous RNA viruses cause devastating impacts on global agriculture, particularly due to their rapid spread and limited treatment options (Table 2). Several critical plant RNA viruses are known for their severe economic impact and the diagnostic requirements necessary to control their spread [25]. ToBRFV is a tobamovirus that infects tomatoes and peppers, leading to fruit discoloration, deformation, and significant yield losses. Since its emergence, it has rapidly spread across Europe, the Middle East, and North America, with economic losses estimated in the hundreds of millions of dollars globally [26]. PVY is one of the most damaging viruses in potato production, capable of causing up to 80% yield loss and reducing tuber quality. It also affects tobacco and tomato crops, with major implications for trade and seed certification [27]. Tobacco mosaic virus (TMV) is a well-known plant virus due to its stability and broad host range. Infections by TMV result in mosaic patterns and stunted growth in tomatoes and tobacco, and the virus remains highly persistent in the environment, making it difficult to eliminate [28]. Cucumber mosaic virus (CMV) infects over 1,200 plant species, including vegetables and ornamentals. It is transmitted by aphids and can lead to systemic infections that cause considerable losses in cucumbers, peppers, and melons [29].

Each of these viruses has specific diagnostic requirements based on factors such as required sensitivity, detection time, and preferred methods. ToBRFV typically requires detection sensitivity below 10 copies per microliter with a detection time of 30 to 60 min, for which CRISPR-Cas13a platforms like SHERLOCK and RT-PCR are commonly used. PVY requires approximately 100 copies per microliter sensitivity and can be

Table 1 Comparative analysis of traditional and CRISPR-based methods for virus detection

Detection method	Sensitivity (Limit of detection)	Time to result	Cost	Equipment needed	Applicability in field settings	References
ELISA	0.1–10 ng/mL	2–6 h	Low	Basic laboratory	Yes	[16, 21]
PCR	1–10 copies/µL	2–4 h	Medium	Advanced laboratory	Yes	[14]
LFIA	~ 0.1 to 10 ng/mL	15–30 min	Low	Minimal	Yes	[18]
RT-qPCR	1–10 copies/µL	1–2 h	Medium	Advanced laboratory	No	[11, 13]
LAMP	10–100 copies/µL	30–60 min	Medium	Moderate	Yes	[22]
RPA	10–100 copies/µL	~ 20 min	Medium	Moderate	Yes	[23, 24]
CRISPR-Cas13a	< 10 copies/µL	30–60 min	Medium	Minimal to Moderate	Yes	[8]
NGS	Single viral genome	Hours to Days	High	Advanced sequencing	No	[12]

Table 2 Key plant RNA viruses and their diagnostic requirements

Virus	Host plants	Economic impact	Required sensitivity	Detection time	Preferred detection methods
Tomato Brown Rugose Fruit Virus (ToBRFV)	Tomato, Pepper	Hundreds of millions of USD globally	< 10 copies/µL	30–60 min	CRISPR-Cas13a, RT-PCR
Potato Virus Y (PVY)	Potato, Tomato, Tobacco	Up to 80% yield loss	~ 100 copies/µL	30–60 min	LAMP, CRISPR-Cas13a
Tobacco Mosaic Virus (TMV)	Tobacco, Tomato	Persistent infection, yields losses	~ 10 copies/µL	~ 30 min	CRISPR-Cas13a, ELISA
Cucumber Mosaic Virus (CMV)	Cucumber, Melon, Pepper, Ornamentals	Systemic infections; wide host range	100–500 copies/µL	~ 60 min	RT-LAMP, CRISPR-Cas13a

detected within the same timeframe using LAMP or CRISPR-Cas13a methods. TMV, needing around 10 copies per microliter sensitivity, can be detected in about 30 min using CRISPR-Cas13a or ELISA. CMV, which requires sensitivity in the range of 100 to 500 copies per microliter, can be identified in about one hour using RT-LAMP or Cas13a-based tools [8]. These specifications guide the development of effective diagnostic platforms. CRISPR-Cas13a, particularly when combined with isothermal amplification methods, meets these demands by offering high sensitivity, speed, and portability suitable for field deployment. Table 2 summarizes key plant RNA viruses along with their economic impact and diagnostic requirements, supporting the need for rapid and sensitive field-based detection methods.

History of CRISPR

The discovery of CRISPR traces back to the late 1980 s when researchers identified unusual repetitive DNA sequences in bacteria. Francisco Mojica at the University of Alicante was among the first to recognize these sequences in *Haloferax mediterranei*, later coining the term "Clustered Regularly Interspaced Short Palindromic Repeats" (CRISPR) [30]. Earlier, Ishino et al. (1987) had documented similar sequences in Escherichia coli, though their function remained unclear. Over the next decade, comparative genomic studies revealed the widespread presence of CRISPR across diverse bacterial species, hinting at an evolutionary role in microbial defense [31]. A breakthrough came in 2002 when Jansen et al. discovered CRISPR-associated (cas) genes located near these repetitive sequences, suggesting a functional relationship between CRISPR arrays and Cas proteins [32]. The true significance of this system emerged in 2005 when scientists found that the spacer sequences within CRISPR arrays matched viral and plasmid DNA, indicating an adaptive immune mechanism [33]. This discovery fundamentally reshaped our understanding of bacterial defense, showing how CRISPR-Cas systems enable microbes to recognize and neutralize invading genetic elements.

CRISPR-Cas systems are classified into two major classes: Class 1, which relies on multi-protein effector complexes, and Class 2, which features single-effector proteins such as Cas9 [34]. Class 2 systems, particularly Cas9, revolutionized genome engineering after Doudna and Charpentier demonstrated its re-programmability for targeted DNA cleavage [35]. Researchers soon harnessed this precision to develop gene-editing tools, enabling transformative applications in medicine and agriculture. The impact of CRISPR technology is particularly evident in agriculture. For instance, CRISPR-Cas9 has been used to disrupt the OsSWEET14 susceptibility gene in rice (*Oryza sativa*), conferring resistance to *Xanthomonas oryzae*, the pathogen responsible for bacterial blight [36]. This approach reduces reliance on chemical pesticides, promoting sustainable farming practices. Similarly, CRISPR-edited wheat with enhanced drought tolerance exemplifies its potential to address climate-driven crop losses [37]. Building on the success of DNA-targeting Cas9, scientists identified RNA-targeting systems such as Cas13, which cleaves single-stranded RNA with high specificity [37]. The SHERLOCK platform, leveraging Cas13, has been adapted for agricultural use; for example, Zhang et al. developed a field-deployable assay to detect ToBRFV, enabling rapid containment of outbreaks [5]. Therefore, the advent of the Cas13 system has paved the way for groundbreaking advancements in precise gene recognition. This naturally leads us to explore the applications and structural classification of Cas13 proteins.

Classification of Cas13 proteins

The CRISPR-Cas13 family consists of four principal subtypes—Cas13a, Cas13b, Cas13c, and Cas13d—each with specific structural features that influence its RNA-targeting and cleavage behavior (Table 3). Cas13a, originally identified as C2c2, was the first functionally characterized subtype and is known for its strong collateral cleavage activity. This property has been widely utilized in diagnostic applications such as SHERLOCK, where its signal amplification capability enhances detection sensitivity [38]. In contrast, Cas13b has structural modifications that improve specificity and minimize off-target effects, making it well-suited for precise transcriptome editing [39]. Meanwhile, Cas13c, first identified in 2017 by Abudayyeh et al., was found to possess a locus and CRISPR RNA (crRNA) structure similar to Cas13a, with a protein length of approximately 1120 amino acids [40]. However, its RNA interference activity was observed to be weaker than that of Cas13a and Cas13b in human embryonic kidney (HEK) 293FT cells. This lower efficiency suggests that, while Cas13c retains fundamental RNA-targeting characteristics, its practical applications may be more limited compared to other Cas13 variants [41].

Cas13d, a notably compact variant, offers unique advantages for therapeutic delivery via size-limited vectors like adeno-associated viruses (AAVs), where packaging constraints impose strict transgene size limitations [42]. Recent advancements have led to the development of novel compact variants, such as Cas13bt3 and Cas13Y, which are approximately 800 amino acids in length—significantly smaller than traditional Cas13 subtypes that range from 967 to 1152 amino acids. Despite their reduced size, these variants

Table 3 Comparison of Cas13 subtypes based on structural features, applications, and performance metrics

Subtype CAS13	Structural features	Applications	Collateral cleavage efficiency	Off-target effects	Engineering potential	References
Cas13a	Large crRNA-binding pocket; robust RNase activity	SHERLOCK diagnostics; RNA silencing in plants and humans	High	Moderate; mitigated by crRNA redesign	Engineered for reduced off-target effects	[5]
Cas13b	Compact; unique structural fold	RNA knockdown; moderate diagnostics	Moderate	Lower than Cas13a; sequence-specific risks	Engineered for compact diagnostic tools	[50]
Cas13c	Distinct catalytic residues; less studied	Hypothetical RNA applications	Unknown	Unknown	Potential for RNA stability studies	[51]
Cas13d	Smallest subtype; ideal for compact systems	RNA knockdown; diagnostics	High	Reduced compared to Cas13a	Enhanced for compact RNA-based tools	[50]
Cas13x	Ultra-compact; emerging subtype	Diagnostic platforms require minimal space	Moderate	Presumed low based on structural studies	High adaptability for bioengineering	[41]
Cas13y	Combines features of Cas13a and Cas13b	Advanced diagnostics; RNA degradation studies	High	Moderate; higher than Cas13d	Potential for high-efficiency tools	[41]

maintain catalytic efficiency in RNA cleavage while offering greater versatility in delivery. This makes them particularly promising for therapeutic and agricultural applications, where efficient intracellular transport and minimal immune response are crucial factors. Structural studies confirm that these smaller variants retain the conserved HEPN (Higher Eukaryotes and Prokaryotes Nucleotide-binding) domains essential for RNA cleavage while achieving enhanced functional flexibility, allowing researchers to optimize their activity for diverse applications [43] (Table 2).

Structural analyses have also shed light on the conformational dynamics that Cas13 proteins undergo upon RNA binding. In Cas13a and Cas13d, for instance, the HEPN domains align more closely upon target recognition, forming an active catalytic site that facilitates both sequence-specific RNA degradation and collateral RNA cleavage [44]. Engineering these domains by modifying key residues or adjusting their alignment dynamics can significantly improve specificity while reducing unintended off-target effects, a key goal in the ongoing refinement of Cas13-based diagnostics and therapeutics. Beyond medical applications, Cas13-based technologies have shown considerable potential in plant biotechnology, particularly for viral diagnostics. The collateral cleavage activity of Cas13a has been adapted for fluorescence and lateral flow-based detection of economically significant plant viruses such as Tomato Spotted Wilt Virus With Cas13a in Tomatoes, enabling rapid, on-site diagnostics that supports timely disease management [45]. The compact nature of Cas13d makes it particularly useful for monitoring RNA expression in crops, providing valuable insights into plant stress responses and gene regulation under both biotic and abiotic stress conditions [46].

Further highlighting Cas13's utility in agricultural virology, studies on CasRx—a member of the Cas13 family—have demonstrated its superior efficiency in targeting RNA viruses under experimental conditions. For instance, research using the TRBO-GFP system in *Nicotiana benthamiana* found that CasRx outperformed both LwaCas13a and PspCas13b in reducing viral RNA levels when designed to target the replicase gene. This underscores the importance of optimized crRNA design and the strategic localization of Cas13 proteins to maximize functional efficacy. These findings emphasize the crucial role of structural optimization in enhancing Cas13-mediated RNA targeting, further broadening its potential applications [47]. The structural diversity and emerging variants of Cas13 provide a strong foundation for continued engineering and refinement. Building on these insights, further exploration of Cas13's structural diversity and molecular mechanisms provides a foundation for targeted engineering and refinement. Key aspects such as target recognition, HEPN domain dynamics, and strategies to enhance RNA cleavage efficiency play a crucial role in optimizing Cas13 for broader applications in virology, agriculture, and therapeutics [48].

Empirical validation of CRISPR-Cas13 diagnostics has been most robust for Cas13a and Cas13d. For instance, the SHERLOCK platform (Cas13a) achieves a false-positive rate of $\leq 1\%$ and false-negative rate of $\leq 3\%$ in detecting RNA viruses like Zika and SARS-CoV-2, with statistical significance ($p < 0.001$) [5, 6, 49]. Similarly, compact Cas13d systems exhibit a false-positive rate of $\leq 2\%$ in multiplexed assays [42]. For other subtypes (e.g.,

Cas13b, Cas13c), quantitative performance data remain limited, reflecting their niche applications in RNA knockdown rather than diagnostics. Future studies should expand validation metrics across all subtypes to enable direct comparisons.

Mechanism, structural dynamics, and engineering of Cas13

Cas13 proteins exhibit a bilobed architecture composed of two main domains: the Recognition (REC) lobe, responsible for binding crRNA, and the Nuclease (NUC) lobe, which houses the HEPN (Higher Eukaryotes and Prokaryotes Nucleotide-binding) domains necessary for RNA cleavage (Fig. 1). When Cas13 binds to its complementary RNA target via crRNA, the HEPN domains undergo significant conformational changes, forming an active catalytic site. This structural rearrangement allows Cas13 to precisely cleave its target RNA (cis-cleavage) while also triggering collateral cleavage (trans-cleavage) of non-target single-stranded RNAs (ssRNAs) in the surrounding environment. This collateral cleavage activity serves as the foundation for highly sensitive diagnostic platforms like SHERLOCK, which utilizes Cas13's indiscriminate RNA degradation to amplify detection signals, ensuring high specificity and sensitivity [5].

A key characteristic of Cas13 is its strong preference for single-stranded RNA (ssRNA) over double-stranded RNA (dsRNA), as it cannot unwind duplex structures. While this specificity enhances targeted RNA recognition, it also poses challenges when working with highly structured RNAs, such as viral genomes. To address this limitation, researchers have refined crRNA design strategies to specifically target single-stranded regions within RNA molecules. This optimization enhances targeting efficiency and minimizes off-target effects, making Cas13 highly suitable for precise transcriptome modulation and viral RNA detection [38, 44, 49]. In agricultural applications, this approach has significantly improved the detection of economically important plant pathogens, such as Tomato Spotted Wilt Virus [52]. Moreover, Cas13a's collateral cleavage activity has been successfully adapted for multiplexed detection systems, enabling the simultaneous identification of multiple RNA targets in complex infections. In *N. benthamiana*, for instance, CRISPR-Cas13a has been used to detect the TMV, Tobacco etch virus, and Potato virus X without requiring nucleic acid amplification [53]. By integrating this system with visual readouts like lateral flow strips, researchers have developed rapid, field-deployable diagnostic tools that provide results within 30 min, eliminating the need for laboratory-based procedures [54].

Beyond diagnostics, Cas13a is emerging as a powerful tool in therapeutic applications due to its programmability and high specificity. Unlike DNA-targeting CRISPR systems, Cas13a does not introduce permanent genetic modifications but instead regulates gene expression by selectively degrading RNA. This feature makes it a promising candidate for combating RNA viruses in both plants and humans. Notably, Cas13a has been utilized for the detection and degradation of SARS-CoV-2 RNA, leveraging its collateral cleavage activity to achieve rapid and reliable detection—an essential capability in pandemic response efforts [55].

To enhance Cas13a's performance in both diagnostic and therapeutic applications, researchers have

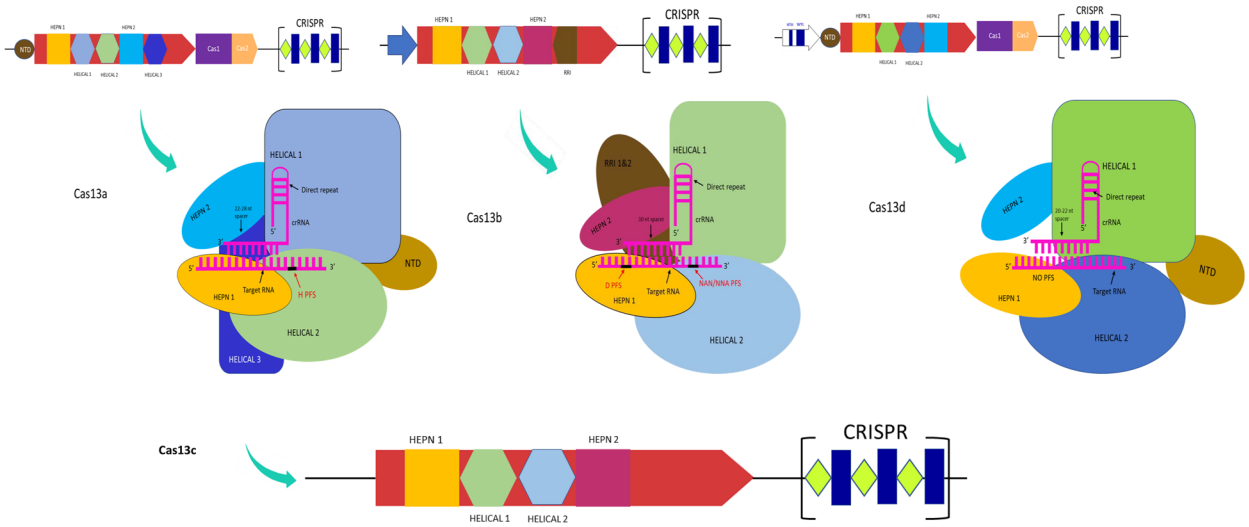

Fig. 1 Schematic representation of CRISPR loci and structure of Cas13a, Cas13b, and Cas13d. * The structural representation of subgroup C is not available; its linear form is depicted

introduced structural and functional modifications. Adjustments to the HEPN domains have been employed to fine-tune collateral cleavage activity, reducing off-target effects that could compromise cell viability in therapeutic contexts. Additionally, the development of compact Cas13 variants, such as Cas13bt3 (Cas13X.17), has addressed delivery challenges in therapeutic settings. These smaller variants maintain catalytic efficiency while being compatible with size-limited delivery systems, including nanoparticles and adeno-associated viruses. This advancement has proven particularly beneficial, where efficient intracellular transport is often hindered by the structural barriers imposed by cell walls [56]. Structural analyses further highlight Cas13's adaptability across diverse applications. Upon crRNA binding, the REC lobe undergoes a conformational shift, aligning the crRNA-RNA duplex within the catalytic HEPN domains, thereby enhancing specificity while preserving collateral cleavage efficiency. Mutational studies have also identified key amino acids within the HEPN domains that regulate activity, opening avenues for engineered Cas13 variants optimized for specific applications. For instance, reducing collateral cleavage through targeted HEPN mutations has improved therapeutic safety, while enhancing catalytic efficiency has increased diagnostic sensitivity [44]; [39].

CRISPR proteins compared: from Cas1 to Cas13 and beyond

The evolution of CRISPR proteins showcases an impressive journey of scientific innovation, driven by the pursuit of precision in genome engineering. Initially serving as bacterial immune defense mechanisms, CRISPR proteins have been transformed into powerful molecular tools, each with unique capabilities shaping the future of genetic research and therapeutic applications (Table 4). Among these, Cas13 has emerged as a groundbreaking RNA-targeting system, offering exceptional opportunities for diagnostics and gene regulation while advancing our understanding of molecular biology [57].

At the core of this system, Cas1 and Cas2 play crucial roles in acquiring and integrating new spacers into the CRISPR array, forming the foundation of the prokaryotic adaptive immune response. While essential

Table 4 Comparison of CRISPR-Cas subtypes based on applications and unique features

CRISPR subtype	Application	Unique features	References
Cas3	DNA degradation	Functions as a helicase-nuclease complex; suitable for large genomic deletions	[64]
Cas4	Spacer acquisition	Involved in CRISPR array spacer integration; limited direct applications	[65]
Cas5	crRNA processing	Essential for complex assembly in Type I systems	[66]
Cas6	crRNA maturation	Processes pre-crRNA into mature crRNA	[67]
Cas7	crRNA binding	Structural backbone of Type I CRISPR complexes	[68]
Cas8	Target DNA recognition	Binds PAM and initiates DNA unwinding	[69]
Cas9 (SpCas9)	Genome editing	Most widely used for DNA editing; high efficiency and specificity; requires PAM sequence (NGG)	[70]
Cas9 (SaCas9)	Genome editing	The smaller size compared to SpCas9; is useful for delivery in AAV vectors	[71]
Cas9 (StCas9)	Genome editing	Found in *Streptococcus thermophilus*; PAM sequence is NNAGAAW	[72]
Cas9 (SpCas9)	Genome editing	Most widely used for DNA editing; high efficiency and specificity; requires PAM sequence (NGG)	[70]
Cas10	Involved in the degradation of target RNA and DNA in the bacterial immune response	An enzyme with polynucleotidyl transferase activity that plays a role in the type III CRISPR-Cas system	[73]
Cas12a (Cpf1)	High-precision genome editing—creating cuts with sticky ends to facilitate the insertion of new sequences	DNA-directed endonuclease- Creates double-stranded nicks with sticky ends, Requires a crRNA for activity	[74]
Cas12b	Genome editing	Active at high temperatures; suitable for thermophilic conditions	[75]
Cas13a	Editing RNA and identifying viral RNAs	RNA-directed endonuclease- targeting and cleaving single-stranded RNA	[5]
Cas12f (Cas14)	In detecting target DNAs with diverse sequences	Small endonuclease with DNA cutting activity, recognition of target sequences without the need for a specific PAM sequence	[76]
Cas12j (CasΦ)	Genome editing, compact systems	Ultra-small Cas variant; effective for use in bacteria with minimal systems	[77]

for memory formation in bacterial defense, their direct application in genome editing remains limited due to incomplete understanding of their mechanisms [58]. In contrast, Cas13 has gained prominence as a highly adaptable RNA-targeting tool with direct applications in research and medicine. Unlike Cas1 and Cas2, Cas13 is specifically designed for RNA interference, making it an ideal candidate for precise RNA manipulation [38].

The introduction of Cas9 from *Streptococcus pyogenes* revolutionized genome editing by enabling targeted DNA cleavage and facilitating precise genetic modifications. This breakthrough significantly advanced biotechnology and medicine; however, concerns over off-target mutations and unintended genomic alterations have hindered its widespread adoption [3]. Unlike Cas9, Cas13 exclusively targets RNA, mitigating the risk of permanent genomic modifications and making it a superior choice for RNA-based applications, such as viral diagnostics. The development of the SHERLOCK system, which harnesses Cas13's enzymatic activity for high-sensitivity viral RNA detection, exemplifies its transformative potential in molecular diagnostics [59].

Beyond Cas13, other CRISPR proteins such as Cas12 and Cas14 have introduced further refinements. Cas12 facilitates precise DNA editing by generating staggered double-strand DNA breaks, making it a promising alternative to Cas9, particularly for high-fidelity genome modifications. However, like Cas9, Cas12 relies on host DNA repair mechanisms, which can limit its efficiency [60]. Cas13, on the other hand, bypasses this dependency by directly targeting RNA, enabling immediate and precise gene expression regulation. This unique ability has positioned Cas13 as an essential tool in pathogen detection and agricultural biotechnology, particularly in combating RNA viruses in crops. Researchers have successfully employed Cas13 to degrade viral RNA in infected plants, significantly reducing viral load and demonstrating its potential for developing virus-resistant crops [53]. Despite its advantages, Cas13 faces certain limitations. While off-target effects are less frequent compared to DNA-targeting CRISPR systems, further refinement is necessary to enhance its specificity. Additionally, Cas13's RNA-targeting nature restricts its use in genome editing applications requiring DNA modifications. These challenges underscore the need for continued innovation to expand Cas13's capabilities and precision [61]. Meanwhile, the search for even more versatile genome-editing tools has led to the discovery of Cas14, a compact protein capable of targeting single-stranded DNA (ssDNA) without requiring a PAM sequence. While Cas14's high specificity and small size make it an attractive candidate for high-fidelity genome editing, its potential remains under exploration, and it has yet to achieve Cas13's success in RNA-based diagnostics and gene regulation [62].

The progression from Cas1 and Cas2 to Cas9, Cas12, and Cas13 highlights the ongoing refinement of CRISPR technologies to enhance their precision and versatility. Each protein has introduced unique strengths while facing distinct challenges, shaping the trajectory of genetic research and biotechnological applications. Among these, Cas13 represents a significant leap forward, offering unparalleled opportunities in RNA diagnostics, gene regulation, and agricultural biotechnology. As research continues to refine and expand its capabilities, Cas13 is poised to play an increasingly vital role in advancing both fundamental science and applied biotechnology [63].

Engineering and future perspectives of Cas13a and advanced crRNA design

The RNA-cleavage activity of Cas13a, a CRISPR-associated enzyme, hinges on the precise design of its crRNA, which acts as a molecular guide to direct the enzyme to its target RNA. Central to this process are two critical crRNA regions: the seed domain (nucleotides 2–8) and the switch domain. The seed domain facilitates the initial "molecular handshake" with the target RNA, ensuring specificity, while the switch domain activates Cas13a's cutting ability upon binding. These elements collectively optimize Cas13a for diagnostic applications, as even minor crRNA design flaws can compromise accuracy. This interplay between crRNA structure and function underscores why its design is a cornerstone of effective diagnostics [78].

Advancements in computational tools have revolutionized crRNA design, enabling researchers to predict optimal sequences with high precision. Platforms like RNAfold and CRISPR can analyze RNA secondary structures to identify accessible regions, such as unpaired loops in viral genomes, which are ideal for Cas13a binding [79]. For instance, in combating the ToBRFV, computational predictions guided crRNA designs targeting viral loop regions, enhancing detection reliability while minimizing off-target effects [80]. Tools like Rosetta and molecular dynamics simulations further refine this process by modeling Cas13a-crRNA interactions, revealing how subtle structural tweaks can boost performance. These innovations exemplify how computational biology bridges the gap between theoretical design and practical application [81].

Despite its diagnostic promise, Cas13a's collateral RNA cleavage—useful for signal amplification in tools like SHERLOCK—poses challenges for therapeutic use. To address this, engineered Cas13a variants with reduced off-target activity have emerged. Modifications to the

crRNA spacer region fine-tune cleavage kinetics, while mutations in the HEPN domains, responsible for catalytic activity, enhance specificity. Researchers demonstrated that specific HEPN mutations could curtail unintended RNA degradation without sacrificing target cleavage efficiency, a breakthrough for safer applications. Such refinements highlight the delicate balance between harnessing Cas13a's power and mitigating its risks [42].

In plant virus diagnostics, Cas13a's ability to multiplex—detect multiple RNA targets simultaneously has proven transformative [53]. A notable example is the simultaneous detection of virus TMV, TEV, and PVX, enabling rapid on-site diagnosis and timely crop management. This capability is particularly vital in regions where mixed infections devastate yields, offering farmers a practical tool to curb losses. By turning complex lab processes into field-ready solutions, Cas13a empowers farmers to protect crop yields and food security, one RNA target at a time [82].

Integration of LAMP and RPA with CRISPR-Cas13 for enhanced plant virus detection

While LAMP enables robust and rapid detection, it has limitations, including potential non-specific amplification, a restriction on target sequence length, and uneven product structures, which limit its utility in downstream applications such as sequencing [83]. Additionally, its high sensitivity can lead to false positives due to aerosol contamination [84]. To enhance its diagnostic potential, researchers have integrated CRISPR-Cas12 and CRISPR-Cas13 with LAMP, improving specificity while maintaining rapid amplification. An example of the integration of LAMP with CRISPR-Cas12 is the development of a LAMP-Coupled CRISPR–Cas12a module for the rapid and sensitive detection of plant DNA viruses. This system utilizes the target-specific cleavage activity of Cas12a, combined with LAMP amplification, to achieve a highly specific and field-deployable diagnostic tool for plant pathogens such as tomato yellow leaf curl virus (TYLCV), demonstrating high sensitivity and reliability for early virus detection in plants [85]. Similarly, CRISPR-Cas13, which is highly effective for RNA virus detection, has been incorporated into RT-LAMP/Cas13 assays, demonstrating high sensitivity in detecting plant pathogens such as TMV, TEV, and PVX in field conditions [82].

While LAMP has proven effective, RPA has emerged as a strong alternative, offering faster amplification (within 20 min) at a lower temperature (37–42 °C) [23]. Unlike LAMP, which requires four primers, RPA operates with three core proteins, making it simpler while maintaining high efficiency [24]. This lower temperature requirement allows RPA to be used in resource-limited settings, making it particularly advantageous for field-based diagnostics. However, both LAMP and RPA benefit greatly from integration with CRISPR-Cas13, as its RNA-targeting and collateral cleavage activity significantly enhance detection sensitivity and specificity. Upon binding to target RNA, Cas13 activates a trans-cleavage reaction, degrading nearby reporter molecules, which produces a fluorescent or lateral flow-based signal, enabling visual confirmation of viral presence [86] (Fig. 2). The SHERLOCK platform, initially designed for human viruses [5], has been successfully adapted for plant RNA viruses like ToBRFV and TMV, achieving single-base resolution without thermocycling [87].

This technology enables real-time, field-based diagnostics, allowing farmers to identify viral infections before widespread crop damage occurs. Further refinements in

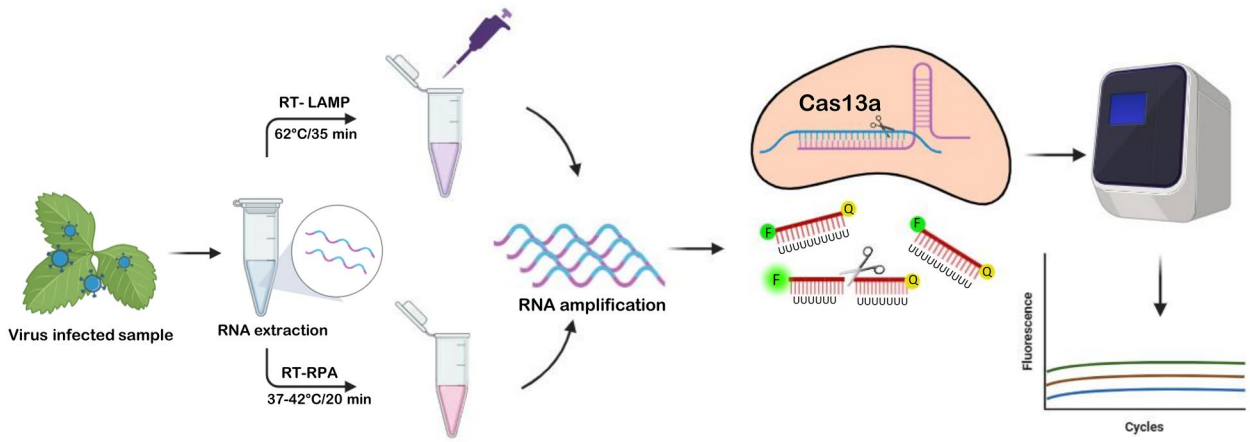

Fig. 2 Schematic representation of CRISPR-Cas13-based detection methods integrated with isothermal amplification techniques: **A** Loop-mediated isothermal amplification (LAMP) **B** Recombinase polymerase amplification (RPA)

CRISPR-Cas13-based diagnostics have led to the development of SHINE, an upgraded version of SHERLOCK that integrates reverse-transcription RPA (RT-RPA) and Cas13 cleavage into a single-step reaction [88]. This streamlined system minimizes contamination risks, accelerates detection to under 15 min, and maintains high specificity, making it a powerful tool for rapid virus identification in field settings [89]. SHINE has already demonstrated its effectiveness in detecting plant viruses, even in complex environments where laboratory access is limited [90].

While LAMP provides a fast and simple amplification method, the integration of RPA with CRISPR-Cas13 offers greater sensitivity and versatility, particularly for detecting RNA viruses in plants [82]. These advancements in molecular diagnostics are transforming agricultural disease management by enabling rapid, on-site virus detection, preventing the spread of infections, and ensuring sustainable crop production. By leveraging the unique strengths of LAMP, RPA, and CRISPR-Cas13, researchers are equipping farmers with powerful diagnostic tools that are reshaping plant health surveillance and global food security strategies [91].

Economic considerations of CRISPR-Cas13a diagnostics

The widespread adoption of CRISPR-Cas13a diagnostics for plant viruses depends not only on its technical merits but also on its economic feasibility compared to conventional detection methods. When evaluating diagnostic tools, researchers and practitioners must consider both the direct costs of implementation and the long-term economic benefits of early and accurate virus detection.

Traditional diagnostic methods such as RT-qPCR and ELISA have established infrastructure and protocols, but they often require significant financial investment. A standard RT-qPCR test typically costs between 15 and 25 (USD) per sample when accounting for reagents, equipment maintenance, and labor [11]. ELISA tests are somewhat less expensive at 2 to 10 (USD) per test but suffer from lower sensitivity and specificity [17]. These methods also demand specialized laboratory equipment and trained personnel, creating barriers for resource-limited agricultural settings.

In contrast, CRISPR-Cas13a diagnostics offer a more cost-effective solution for many applications. The SHERLOCK platform, for example, can be performed for approximately 0.50 to 1 (USD) per test when using lyophilized reagents [6]. This dramatic cost reduction stems from several factors: the elimination of expensive thermocycling equipment, simplified sample preparation protocols, and the potential for multiplexed detection of multiple pathogens in a single reaction. Field-deployable formats using lateral flow strips further reduce costs by removing the need for fluorescent readers or other specialized detection equipment [45].

The economic advantages of CRISPR-Cas13a extend beyond per-test costs. Early detection of plant viruses enabled by this technology can prevent substantial agricultural losses. For instance, rapid identification of Potato Virus Y (PVY) infections could save an estimated $200 million annually in global potato production by enabling timely intervention [8]. Similarly, containment of ToBRFV outbreaks through CRISPR-based surveillance has preserved millions of dollars in tomato exports [92]. These examples demonstrate how the slightly higher initial investment in CRISPR development can yield significant long-term returns through crop preservation.

However, some economic challenges remain for widespread CRISPR-Cas13a implementation. The development of specific crRNA guides for novel viral strains requires initial research investment, though this cost decreases as shared crRNA libraries become established. Reagent stability in field conditions may necessitate additional formulation expenses, though recent advances in lyophilization and encapsulation are addressing these concerns [93]. Additionally, regulatory approval processes for CRISPR-based diagnostics may incur costs that vary by region.

As technology matures, the cost gap between CRISPR diagnostics and traditional methods continues to narrow. The scalability of CRISPR systems, combined with their potential for decentralized testing, positions them as economically viable solutions for global agricultural monitoring. Future developments in reagent production and automation are expected to further reduce costs, making CRISPR-Cas13a diagnostics increasingly accessible to farmers and agricultural workers worldwide.

Optimizing CRISPR-Cas13 sensitivity via nanoparticle

Gold nanoparticles (AuNPs) have emerged as a crucial component in CRISPR-Cas13-based biosensors due to their unique optical, electronic, and biocompatible properties. By amplifying detection signals and enabling multiple detection modes, gold nanostructures such as spherical nanoparticles significantly enhance the performance of these biosensors [19, 94]. For instance, the incorporation of AuNPs into optical biosensors allows for enhanced fluorescence quenching, colorimetric shifts, and plasmonic resonance signals upon target recognition (Fig. 3A). Such features have been utilized to develop highly sensitive diagnostic platforms for detecting RNA viruses. These systems offer rapid detection with minimal sample preparation [95]. In this context, an innovative gene detection method using colorimetric changes has been developed by integrating CRISPR/Cas12a/13a technology with gold nanoparticles. Unlike

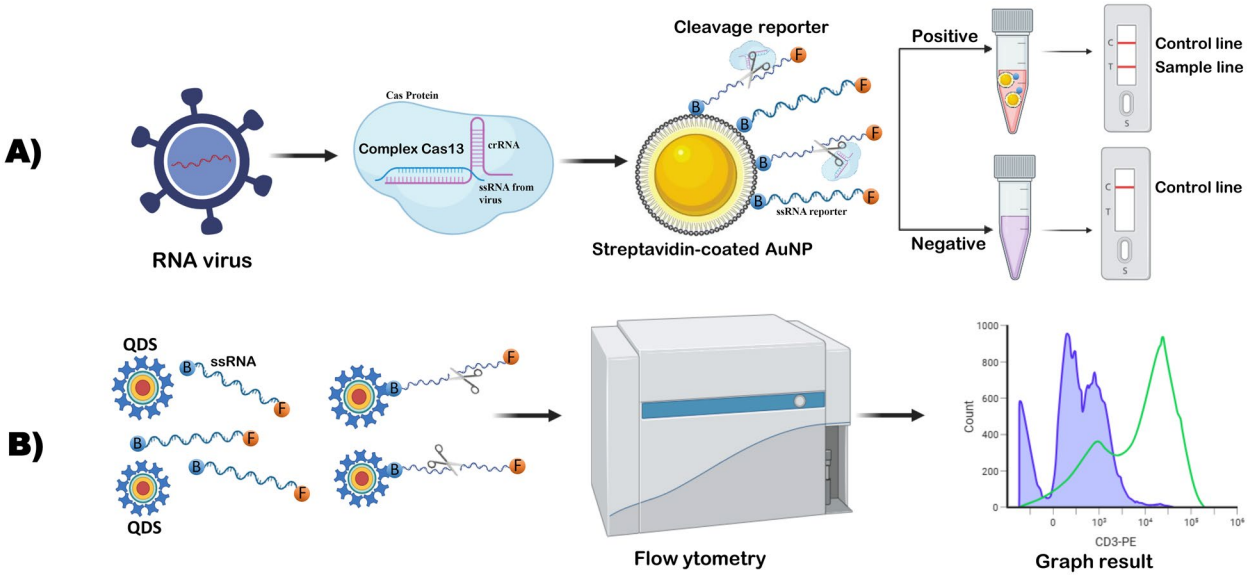

Fig. 3 Schematic representation of CRISPR-Cas13-based RNA virus detection using nanomaterial-assisted platforms. **A** Detection workflow using CRISPR-Cas13a coupled with streptavidin-coated gold nanoparticles (AuNPs) for lateral flow assay (LFA). Upon recognition of viral RNA, the Cas13-crRNA complex binds and cleaves the reporter RNA labeled with biotin (B) and fluorophore (F). The cleavage of reporters disrupts their binding to the AuNPs, altering the LFA signal. A positive sample shows two lines (control and test), indicating target RNA presence, while a negative sample shows only the control line. **B** Quantum dot (QDS)-based fluorescence assay integrated with CRISPR-Cas13a and flow cytometry. The Cas13a complex cleaves ssRNA reporters linked between biotin and fluorophore tags, disrupting their association with quantum dots. This cleavage results in detectable shifts in fluorescence intensity, which are measured via flow cytometry and represented in histogram plots. The graph shows fluorescence signal distribution between positive and negative samples, allowing quantitative assessment of viral RNA presence

traditional AuNPs-based systems, this new approach eliminates the need for high-temperature steps, simplifies the process, and improves accuracy by leveraging CRISPR's precise, room-temperature targeting. The platform's versatility was demonstrated by successfully detecting genetically modified rice. Moreover, the combination of CRISPR-Cas13a with gold nanoparticles has led to the development of an advanced molecular detection tool [96]. This system integrates CRISPR's accuracy in identifying RNA sequences with AuNPs' ability to generate clear electrical signals in solid-state nanopores—tiny channels that "count" molecules by measuring disruptions in electric current. Such an approach holds great potential for revolutionizing diagnostics with high precision and sensitivity. With these advancements, gold nanoparticles continue to play a pivotal role in the development of CRISPR-based biosensors, significantly enhancing modern diagnostic techniques [97]. Quantum dots, another promising nanomaterial, are widely used as fluorescent labels in CRISPR-Cas13 biosensors due to their unique optical properties, including high brightness, photostability, and tunable emission spectra. These labels enable accurate detection of target molecules by providing strong and detectable signals. (Fig. 3B) For instance, in a study focusing on SARS-CoV-2 detection, quantum dots were integrated into a fluorescence immunochromatography system combined with isothermal amplification and CRISPR-Cas13a. This approach allowed for rapid and highly sensitive detection of viral RNA, showcasing the ability of quantum dots to enhance signal amplification and improve diagnostic accuracy [98].

Optimizing CRISPR-Cas13 sensitivity via electrochemical integration

Graphene, a material with exceptional electrical conductivity, large surface area, and robust mechanical properties, has also been extensively employed in biosensor design. Its ability to interact with nucleic acids through π-π stacking interactions makes it a promising candidate for CRISPR-based diagnostics. When integrated with CRISPR-Cas13 systems, graphene-based materials can amplify signal outputs by serving as efficient transducers for detecting target RNA molecules (Fig. 4). For example, Li et al. utilized a graphene field-effect transistor (GFET) combined with CRISPR-Cas13a for the amplification-free detection of SARS-CoV-2 and respiratory syncytial virus (RSV) RNA [99, 100]. Similarly, Yu et al. demonstrated the use of solution-gated graphene transistors (SGGT) modified with CRISPR-Cas13a for the rapid and unamplified detection of SARS-CoV-2 RNA [101, 102]. Additionally, Sun et al. reported high-intensity vector signal

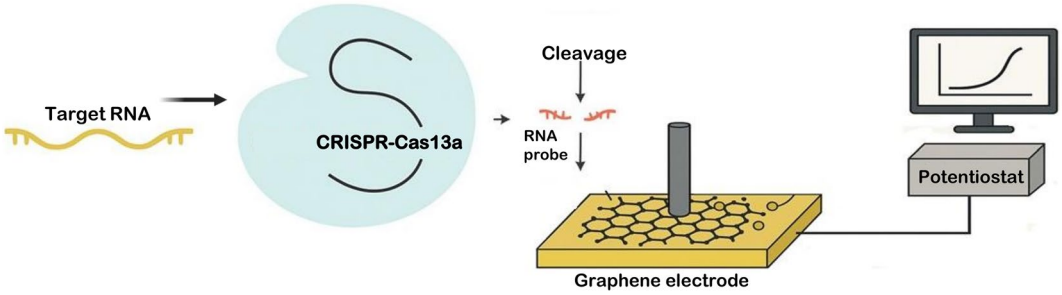

Fig. 4 Integration of CRISPR-Cas13 with electrochemical biosensing platforms for RNA virus detection. This schematic illustrates a graphene-based electrochemical detection system coupled with CRISPR-Cas13a for sensitive and label-free RNA virus diagnostics. Upon recognition of the target viral RNA by the Cas13a-crRNA complex, collateral cleavage of single-stranded RNA reporters is triggered. These cleavage events induce detectable changes in the electrochemical signal, as measured by devices such as graphene field-effect transistors (GFETs) or solution-gated graphene transistors (SGGTs). The high conductivity and surface area of graphene enhances the sensitivity and responsiveness of the system, enabling rapid and amplification-free detection. This platform offers potential for point-of-care applications in plant virus diagnostics

detection of SARS-CoV-2 RNA using stabilized graphene field-effect transistors (GFET) coupled with CRISPR-Cas13a [103, 104]. Despite these advancements, the use of graphene-integrated CRISPR-Cas13 systems in plant diagnostics remains unexplored.

Electrochemical methods have gained attention as powerful tools for enhancing the sensitivity and accuracy of CRISPR-Cas13-based biosensors. These methods utilize the high sensitivity of electrochemical systems and the RNA-targeting capabilities of Cas13, particularly its trans-cleavage activity, to amplify detection signals without requiring nucleic acid amplification [105]. The successful integration of electrochemical techniques with CRISPR-Cas13 has been demonstrated in various studies. For instance, Ma et al., developed an electrochemical biosensor incorporating CRISPR-Cas13, achieving high sensitivity in pathogen detection [106]. Similarly, Zhuang et al. reported the effective use of electrochemical nucleic acid sensors combined with CRISPR-Cas systems for real-time RNA analysis [107]. Additionally, Chen et al., applied a CRISPR-Cas13a-powered electrochemical biosensor for detecting SARS-CoV-2 mutations, highlighting its potential for precise and rapid diagnostics. These electrochemical approaches not only enhance the sensitivity of CRISPR-based biosensors but also simplify their design, making them suitable for point-of-care applications [108].

The integration of nanoparticles such as gold, quantum dots, and graphene, along with advanced electrochemical techniques, has significantly expanded the capabilities of CRISPR-Cas13-based biosensors (Fig. 3B). These innovations offer unparalleled sensitivity, rapid detection, and portability, making them ideal for diverse applications, including clinical diagnostics and agricultural pathogen detection [109]. As research in this field continues to evolve, these technologies are expected to play a crucial role in addressing global challenges in health and agriculture.

Advances in CRISPR-Cas13a-based strategies for plant virus detection

The effectiveness of CRISPR-Cas13a-based diagnostics is heavily dependent on RNA sample quality, as RNA degradation can significantly impair detection accuracy. The HUDSON method [6] stabilizes RNA in unextracted samples, enabling CRISPR-Cas13a diagnostics in field settings. This integration bypasses laboratory-grade RNA extraction, as demonstrated in resource-limited agricultural environments [109].

Ensuring high specificity in CRISPR-Cas13a diagnostics requires optimized crRNA design. Computational tools play a crucial role in identifying target RNA sequences while minimizing off-target effects. Platforms such as CaSilico provide bioinformatics-driven solutions for predicting target sites and optimizing crRNA functionality, enhancing precision across applications including plant virus detection. Studies have demonstrated that targeting conserved viral RNA regions with well-designed crRNAs significantly improves diagnostic accuracy, particularly in distinguishing mixed infections. Additionally, expanding crRNA libraries to cover diverse viral strains holds promise for increasing detection versatility [110].

The integration of portable detection devices with CRISPR-Cas13a systems has further expanded the potential for real-time virus diagnostics in agricultural settings. Researchers have explored the use of portable fluorescence readers and lateral flow strips for rapid and accurate pathogen detection without requiring sophisticated laboratory equipment. These innovations facilitate timely intervention in plant disease management [91]. Researchers highlighted the utility of portable detection

platforms coupled with cas13a in monitoring a variety of viruses in crops such as tomato, cucumber, and turnip, enabling more effective disease control strategies [53]. Another breakthrough in this field is the development of multiplexed diagnostic platforms, such as CARMEN-Cas13, which allow for the simultaneous detection of multiple viruses. This capability is particularly useful for identifying co-infections in crops. Although the application of CARMEN-Cas13 has not yet been demonstrated in plants, it has shown great potential in detecting multiple human-associated viruses simultaneously, managing the detection of 169 viruses through 4500 crRNA constructs [111].

Beyond enhancing detection capabilities, researchers have prioritized the stabilization of Cas13 reagents for effective use in diverse agricultural environments. Lyophilization has emerged as a reliable approach to maintaining Cas13 activity at room temperature, enabling deployment in tropical regions where cold storage is impractical [6]. Additionally, encapsulation techniques further protect Cas13 reagents from environmental fluctuations, ensuring consistent diagnostic performance in field conditions [93]. These advancements collectively contribute to the development of field-deployable CRISPR-Cas13 diagnostics, facilitating rapid and reliable viral detection in remote agricultural settings.

Conclusion

CRISPR-Cas13a technology has transformed plant virus detection by addressing the limitations of traditional diagnostic methods. With its exceptional sensitivity, specificity, and flexibility, this system has emerged as a crucial tool for identifying plant RNA viruses, particularly in resource-limited settings and agricultural environments. The integration of Cas13a with amplification techniques such as LAMP and advanced biosensor platforms has significantly narrowed the gap between laboratory research and practical agricultural applications. This combination enables rapid, field-deployable diagnostics, which are essential for early detection and timely intervention in viral outbreaks. Recent advancements have further enhanced the efficiency of Cas13a, making it suitable for diverse environmental conditions [82, 86] Additionally, improvements in crRNA design, reagent stabilization, and the development of smaller, more efficient Cas13 variants have greatly enhanced the system's accuracy, durability, and transduction efficiency, reinforcing its role in virus management [112]. However, challenges remain, including nonspecific effects, reagent stability across varying climatic conditions, and scalability for high-throughput detection. Addressing these limitations requires further research focused on enhancing RNA stability, developing lyophilization techniques, and incorporating microfluidic systems to improve the method's real-world applicability [93]. Expanding crRNA libraries to target emerging plant pathogens, advancing multiplexing capabilities, and refining Cas13a delivery methods could further broaden the scope of this technology. Continued innovation and optimization will ensure that this system becomes an indispensable tool for plant health management and agricultural sustainability.

Author contributions

M.K., conceptualization, investigation, writing original draft. A.G., supervision, writing, review & editing. A.N., supervision, review & editing. M.R., writing, review & editing. A.T., editing.

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Data availability

No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate

The research reported here did not involve experimentation with human participants or animals. Therefore, there was no need for their consent to participate.

Consent for publication

The research does not contain any person's data in any form, and all authors have consent for publication. There were no human participants so there is no need for participants to consent to publish.

Competing interests

The authors declare no competing interests.

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