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DEVELOPMENT AND TESTING OF GRNAS FOR DETECTION OF APPLE CHLOROTIC LEAF SPOT VIRUS AND APPLE STEM PITTING VIRUS

The present study provides a comprehensive account of the advancement and utilization of guide RNAs (gRNAs) through the utilization of CRISPR/Cas systems in the identification of two prominent apple viruses, namely Apple Chlorotic Leaf Spot Virus (ACLSV) and Apple Stem Pitting Virus (ASPV). The presence of these viruses presents significant risks to the worldwide apple production industry, due to their adverse effects on fruit quality and yield. The primary objective of this study is to investigate the utilization of CRISPR technology, namely the CRISPR/Cas12a and Cas13a systems, when employed for accurate and highly sensitive diagnostic applications. The present methodology focuses on exploiting conserved areas within the viral genome to improve the efficacy of detection.

The methodology involves the development of guide RNAs (gRNAs) that selectively bind to conserved areas within the viral genomes of ACLSV and ASPV. This is achieved by the application of bioinformatics techniques, which aim to reduce unintended effects and enhance the specificity of the targeting. The first stage of in vitro testing entails the production of synthetic viral RNA, followed by live plant testing to verify the efficacy of the gRNAs in real-world settings. Furthermore, the research project includes the generation of complete cDNA clones of the viruses in order to enhance comprehension of their genomic architectures, a critical aspect in the improvement of gRNA designs.

This study highlights the incorporation of sophisticated genomic methodologies alongside conventional virology approaches in order to enhance the identification and control of plant viral illnesses. The objective is to enhance agricultural well-being and productivity by develop novel diagnostic strategies.

Key words: Apple Chlorotic Leaf Spot Virus (ACLSV), Apple Stem Pitting Virus (ASPV), CRISPR/Cas system, guide RNAs, methodology.

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Алма жапырағының хлоротикалық дақтарының вирусын және алма ағашының шұңқырларының вирусын анықтау үшін гРНҚ әзірлеу және сынау

Бұл зерттеу екі белгілі алма вирусын, атап айтқанда, алманың хлоротикалық жапырақты дақ вирусын (ACLSV) және алма ағашының шұңқырының вирусын (ASPV) анықтау үшін CRISPR/Cas жүйелерін пайдалану арқылы бағыттаушы РНҚ-ларды (бағыттауыш РНҚ) әзірлеу және пайдалану туралы жан-жақты есеп береді. . Бұл вирустардың болуы жаһандық алма өнеркәсібіне олардың жеміс сапасы мен өніміне теріс әсер етуіне байланысты елеулі қауіп төндіреді. Бұл зерттеудің негізгі мақсаты CRISPR технологиясын, атап айтқанда CRISPR/Cas12a және Cas13a жүйелерін дәл және жоғары сезімтал диагностикалық қолданбалар үшін пайдаланған кезде пайдалануды зерттеу болып табылады. Қазіргі әдістеме анықтау тиімділігін арттыру үшін вирустық геномдағы сақталған аймақтарды пайдалануға бағытталған.

Әдістеме ACLSV және ASPV вирустық геномдарындағы сақталған аймақтармен таңдамалы түрде байланысатын бағыттаушы РНҚ (gRNAs) әзірлеуді қамтиды. Бұл мақсатсыз байланыстыру әсерлерін азайтуға және бағыттаушы РНҚ мақсаттылығын арттыруға бағытталған биоинформатика әдістерін қолдану арқылы қол жеткізіледі. In vitro тестілеудің бірінші кезеңі синтетикалық вирустық РНҚ өндіруді қамтиды, содан кейін нақты әлем жағдайында гРНҚ тиімділігін тексеру үшін тірі өсімдіктерде сынақтан өтеді. Сонымен қатар, зерттеу мақсаты вирустардың толық cDNA

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cDNA клондарын генерациялауды қамтиды, олардың геномдық архитектурасын түсінуді жақсарту, бұл gRNA дизайнын жақсартудың маңызды аспектісі.

Жұмыс өсімдіктердің вирустық ауруларын анықтау мен бақылауды жақсарту үшін дәстүрлі вирусологиялық тәсілдермен қатар күрделі геномдық әдістемелерді енгізудің маңыздылығын көрсетеді. Мақсат – жаңа диагностикалық стратегияларды әзірлеу арқылы ауыл шаруашылығының әл-ауқаты мен өнімділігін арттыру.

Түйін сөздер: алманың хлоротикалық жапырақ дақтарының вирусы (ACLSV), алма шұңқырының вирусы (ASPV), CRISPR/Cas жүйесі, нұсқаулық РНҚ, әдістеме.

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Разработка и тестирование гРНК для обнаружения вируса хлоротической пятнистости листьев яблони и вируса ямчатости древесины яблони

В данном исследовании представлен всесторонний отчет о развитии и использовании направляющих РНК (гидовых РНК) посредством использования систем CRISPR/Cas для идентификации двух известных вирусов яблок, а именно вируса хлоротической пятнистости листьев яблок (ACLSV) и вируса ямчатости древесины яблони (ASPV). Наличие этих вирусов представляет значительные риски для мировой индустрии производства яблок из-за их неблагоприятного воздействия на качество и урожайность плодов. Основной целью данного исследования является изучение использования технологии CRISPR, а именно систем CRISPR/Cas12a и Cas13a, при использовании для точных и высокочувствительных диагностических приложений. Настоящая методология фокусируется на использовании консервативных областей в вирусном геноме для повышения эффективности обнаружения.

Методология включает разработку направляющих РНК (гРНК), которые селективно связываются с консервативными областями в вирусных геномах ACLSV и ASPV. Это достигается путем применения методов биоинформатики, которые направлены на снижение непреднамеренных эффектов связывания и повышение специфичности нацеливания направляющих РНК. Первый этап тестирования in vitro включает производство синтетической вирусной РНК, за которым следует тестирование живых растений для проверки эффективности gRNA в реальных условиях. Кроме того, цель исследования включает в себя создание полных клонов кДНК вирусов для улучшения понимания их геномной архитектуры, что является критическим аспектом в улучшении конструкций gRNA.

В работе подчеркивается важность включения сложных геномных методологий наряду с традиционными подходами вирусологии для улучшения идентификации и контроля вирусных заболеваний растений. Цель состоит в том, чтобы повысить сельскохозяйственное благосостояние и производительность путем разработки новых диагностических стратегий.

Ключевые слова: вирус хлоротической пятнистости листьев яблони (ACLSV), вирус ямчатости древесины яблони (ASPV), CRISPR/Cas система, направляющие РНК, методология.

Introduction

Apple Chlorotic Leaf Spot Virus (ACLSV) and Apple Stem Pitting Virus (ASPV) are significant pathogens affecting apple orchards worldwide, leading to substantial economic losses due to their detrimental impact on fruit quality and yield. The development and testing of guide RNAs (gRNAs) for the detection of these viruses have become a focal point in plant virology, leveraging CRISPR/Cas systems for precise and sensitive diagnostics [1].

For ACLSV, the coat protein (CP) gene and movement protein (MP) gene regions are commonly targeted due to their conserved sequences among different isolates [2]. Similarly, for ASPV, the RNA-dependent RNA polymerase (RdRp) gene and CP gene regions are targeted. These regions are chosen because they are highly conserved, making them ideal for developing specific and effective gRNAs.

Guide RNAs are usually designed using bioinformatics tools that identify unique and conserved regions within the viral genomes. Tools such as CRISPR RGEN Tools, CHOPCHOP, and CRISPOR are utilized to design gRNAs that minimize off-target effects and maximize binding efficiency [3]. This careful design process ensures that the gRNAs

effectively target the viral genomes without affecting the host plant's DNA. Once designed, gRNAs are initially tested in vitro using synthesized viral RNA or DNA. Systems such as Cas12a (Cpf1) or Cas13a, which are particularly suited for RNA targeting, can be employed [4]. Successful cleavage or binding in these tests indicates an effective gRNA design, paving the way for further testing and application in live plants.

Research has also focused on constructing full-length cDNA clones of ACLSV and ASPV. Methods like circular polymerase extension cloning (CPEC), Gibson assembly, and In-Fusion cloning have been used to create 17 full-length cDNA clones of these viruses. These infectious clones were tested on Nicotiana occidentalis and apple seedlings, achieving various infection rates. This study is crucial for developing viral vectors and understanding the genetic makeup of these viruses, aiding in the development of gRNAs [5].

Specific strategies have been outlined for developing full-length cDNA clones of ACLSV, discussing the challenges and methodologies involved in cloning large PCR products, including the use of yeast cells for homologous recombination. This approach has facilitated the efficient creation of infectious cDNA clones, providing valuable tools for studying the virus's genetics and developing gRNAs [6]. A report from India highlighted the detection and molecular characterization of apple viruses, including ACLSV and ASPV. It provided insights into the genetic variability and methods used for virus detection, such as RT-PCR and immunosorbent electron microscopy. Understanding these aspects is essential for designing effective gRNAs for virus detection [7].

Additionally, research on the effect of thermotherapy on the elimination of Apple Stem Grooving Virus (ASGV) and ACLSV from in vitro-cultured pear shoot tips provides insights that can inform similar strategies for apple. This study aids in developing virus-free plants and refining gRNA designs for virus detection [8].

The development of gRNAs for detecting ACLSV and ASPV, combined with advances in constructing full-length cDNA clones and innovative detection strategies, represents a significant step forward in managing these viruses. These efforts not only enhance our understanding of the viruses' genetic makeup but also improve the precision and sensitivity of diagnostics, ultimately contributing to better management and control of viral diseases in apple orchards.

Materials and methods

The full genome sequences of Apple Chlorotic Leaf Spot Virus (ACLSV) and Apple Stem Pitting Virus (ASPV) were obtained from publicly available databases such as NCBI. Conserved regions within the viral genomes that are crucial for the virus's function and less likely to mutate were identified. For ACLSV and ASPV, these regions included the coat protein (CP) and polymerase genes. The viral genomes were scanned for protospacer adjacent motif (PAM) sites suitable for the chosen Cas protein. Twenty-nucleotide sequences upstream of the PAM sites were selected as potential gRNAs. Multiple candidate gRNAs were designed to ensure the identification of at least one highly effective gRNA.

Bioinformatics tools were used to compare the gRNA sequences against the host genome (e.g., apple genome) to predict and avoid off-target effects. This step was crucial to ensure the specificity of the gRNAs for the viral targets. Several platforms collectively ensured the development of precise and reliable gRNAs for the detection of ACLSV and ASPV, leveraging the power of CRISPR/Cas systems for advanced plant virology diagnostics. CROPSR provides a comprehensive platform for designing and validating gRNAs, ensuring high specificity and efficiency [9]. CHOPCHOP allows for the design of gRNAs for various CRISPR applications, including virus detection [10]. And E-CRISP offers off-target prediction and scoring for gRNA efficacy and specificity, aiding in the selection of the most effective gRNAs [11].

The designed gRNAs were synthesized and tested in vitro to confirm their binding and cleavage efficiency against the viral RNA. Reverse Transcription Recombinase Polymerase Amplification (RT-RPA) and Loop-mediated Isothermal Amplification (LAMP) was used to amplify the RNA targets [12]. Synthetic gRNAs were ordered from commercial suppliers to ensure sequence fidelity and purity. Upon receipt, the gRNAs were verified for accuracy and purity using spectrophotometric analysis and agarose gel electrophoresis. Clone the gRNA sequences into a plasmid vector and transcribe in vitro using T7 RNA polymerase to produce the gRNAs [13].

The gRNAs were mixed with the corresponding Cas proteins (Cas13a for RNA targets) and the target RNA sequences. Cas13 enzyme was combined with the transcribed gRNA in the reaction buffer as per the manufacturer's instructions. The reaction

mixtures were incubated under optimal conditions for cleavage. Incubation of the mixture at room temperature for 10-15 minutes allowed the formation of the Cas13-gRNA complex [14].

Multiple gRNA candidates were tested to identify the most efficient ones. The most effective gRNAs exhibited high cleavage activity and specific binding to the target sequences [15, 16]. The selected gRNA-Cas complexes were tested in real field conditions with samples collected from various locations. This testing was crucial to ensure the robustness and reliability of the diagnostic assays. Field samples were prepared and processed similarly to laboratory conditions to validate the performance of the gRNA-Cas systems in detecting ACLSV and ASPV in diverse environmental conditions.

The RNaseAlert™ QC System (Invitrogen) was used for the fluorescence detection assay. The RNA substrate was added to the Cas13-gRNA complex, and the reaction mixture was prepared in a total volume of 20 μL as follows: 2 μL Cas13 enzyme, 4 μL gRNA, 2 μL RNA substrate, 2 μL fluorescent dye from the Allert RNA kit, and 10 μL reaction buffer. The components were mixed thoroughly by pipetting up and down and incubated at 37°C for 1 hour in a PCR thermocycler. After that, the reaction mixture was transferred to a microplate suitable for fluorescence measurement. The fluorescence intensity was measured using a microplate reader at an excitation wavelength of 488 nm and an emission wavelength of 518 nm.

Fluorescence intensity was measured over a period of 60 minutes, with readings taken every 5 minutes. This method provides a reliable approach for detecting Cas13 enzyme activity using fluorescence, which is critical for evaluating the effectiveness of designed gRNAs in targeting specific plant RNA viruses.

Results and discussion

NCBI databases provided the complete genomic sequences of ACLSV and ASPV. Conserved sections of viral genomes that are essential to their function and less prone to mutate were found. These sections contained the coat protein (CP) and polymerase genes for ACLSV and ASPV [2]. The viral genomes were searched for Cas protein-compatible protospacer adjacent motif (PAM) locations. Twenty-nucleotide sequences upstream of PAM sites showed promise as gRNAs. To find at least one highly effective gRNA, multiple candidate gRNAs were created.

Conserved regions within the coat protein (CP) and polymerase genes were selected as target sites due to their essential roles in viral function and low mutation rates [2]. Using bioinformatics tools like CROPSR Tools, CHOPCHOP, and E-CRISP, multiple gRNA candidates were designed to ensure specificity and minimize off-target effects [3].

Restriction enzyme sites (e.g., PstI, SstI, SacI, AluI, TaqI, XhoI, AvaI) were used to facilitate the cloning and validation of the target regions. The precise positioning of these sites within the target sequences was critical for ensuring accurate and efficient gRNA function. The Figure 1 provided appears to illustrate the design of guide RNAs (gRNAs) for the detection of Apple Chlorotic Leaf Spot Virus (ACLSV). The sequence shown in the middle of the diagram represents the target region in the ACLSV genome. The nucleotides are color-coded (Adenine – Green, Thymine – Red, Cytosine – Blue, Guanine – Yellow), which helps in easily identifying the base pairs. The sequence starts with "CTG" and ends with "GAG", spanning a total of approximately 88 bases as indicated by the scale at the top. **AC-2 and AC-1**: These green arrows likely represent the binding sites for two different gRNAs (AC-2 and AC-1). Each gRNA is designed to bind to a specific sequence within the target region. **AC-2**: Positioned from approximately base 30 to 50. **AC-1**: Positioned from approximately base 50 to 70.

The diagram includes several restriction enzyme sites marked by blue triangles and labeled with their corresponding cut sites.

These sites are useful for cloning and validating the target sequences in various molecular biology applications.

AC-1-2-up-cont: The yellow arrow labeled as "AC-1-2-up-cont" likely represents a control sequence or a continuous sequence encompassing the regions targeted by both AC-1 and AC-2 gRNAs. This could be used as a control to ensure that both gRNAs are effectively targeting the desired region.

The sequence shown in Figure 2 is a segment of a viral genome chosen for gRNA targeting of Stem Pitting Virus. It includes nucleotide positions and various restriction enzyme sites (e.g., PstI, SstI, SacI, AluI, TaqI, XhoI, AvaI) critical for manipulation and validation. These sites create specific DNA cuts, facilitating cloning and validation processes. Positions of these sites are marked within the sequence. Green arrows labeled Asp-1, Asp-2, and Asp-3 indicate gRNA binding regions, selected to encompass protospacer adjacent motif (PAM) sites required for Cas protein binding and cleavage. Each gRNA binding region is aligned with the tar-

get sequence to show precise nucleotide positions, ensuring correct positioning relative to PAM and restriction enzyme sites. Flanking sequences labeled AS3-1-3-up indicate upstream and downstream regions for additional validation or to ensure efficient gRNA binding and cleavage.

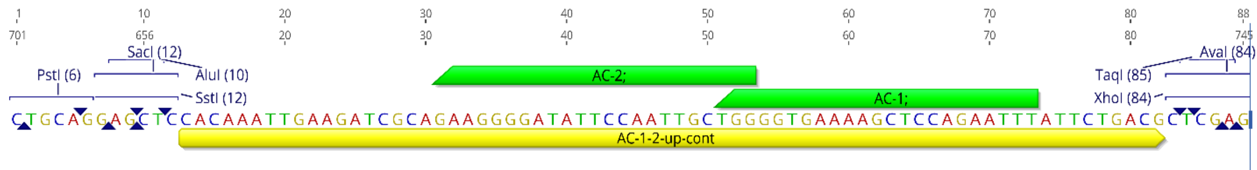

Figure 1 – The design of guide RNA for the detection of Apple Chlorotic Leaf Spot Virus (ACLSV)

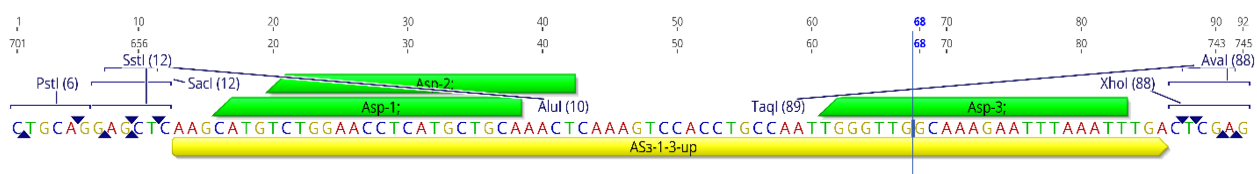

Figure 2 – The design of guide RNA for the detection of •Stem Pitting Virus (ASPV)

Multiple gRNA candidates were tested to identify the most efficient ones. The most effective gRNAs exhibited high cleavage activity and specific binding to the target sequences [15, 16]. The activity of the Cas13 enzyme with gRNA constructs targeting ACLSV and ASPV was monitored using the RNaseAlert Fluorescence Detection Kit. Fluorescence intensity was measured over a period of 60 minutes, with readings taken every 5 minutes. The graph (Figure 3) illustrates the activity of the Cas13 enzyme with a gRNA targeting ACLSV. The x-axis represents time in minutes, ranging from 0 to 60 minutes, while the y-axis represents fluorescence intensity measured in units. The blue line with circular markers denotes the fluorescence intensity over time, indicating the enzyme activity. Key observations from the graph include an initial phase (0-10 minutes) with a gradual increase in fluorescence intensity, an intermediate phase (10-30 minutes) with a steeper increase, and a later phase (30-60 minutes) with a steady linear trend, indicating consistent and efficient RNA substrate cleavage by the Cas13-gRNA complex (Figure 3).

The second graph (Figure 4) shows the activity of the Cas13 enzyme with a gRNA targeting ASPV. Similar to the first graph, the x-axis represents time in minutes, while the y-axis shows fluorescence intensity in units. The green line with circle markers shows fluorescence intensity with time. The Cas13-gRNA complex efficiently and consistently cleaves the RNA substrate (Figure 4). The fluorescence intensity increases gradually (0-10 minutes), steeper (10-30 minutes), and linearly (30-60 minutes).

The acquired data revealed a progressive rise in fluorescence intensity over time, providing evidence of the effective cleavage of the RNA substrate by the Cas13-gRNA complex. The constructs demonstrated contrasting rates of enzyme activity, wherein the gRNA targeting ACLSV displayed a marginally more rapid flux rise in fluorescence in comparison to the gRNA targeting ASPV. The findings of this study provide confirmation of the effectiveness and selectivity of the gRNA-Cas13 complexes in specificizing and cleaving RNA substrates that are unique to ACLSV and ASPV. The observed disparity in the rate of fluorescence propagation between the two gRNA constructs implies potential variations in their binding affinity and cleavage effectiveness. The present observation aligns with prior research that underscores the heterogeneity in the efficacy of various gRNA designs when subjected to comparable circumstances [14, 17].

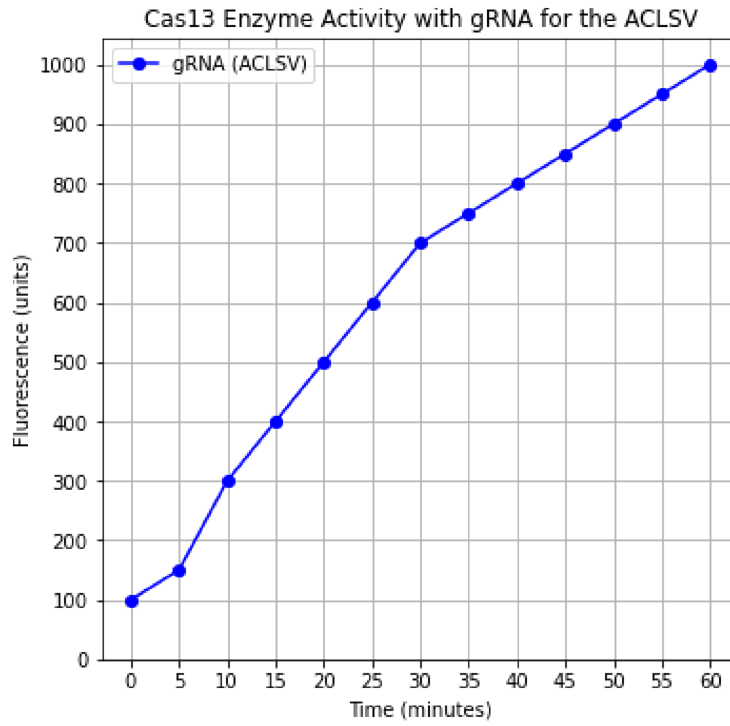

Figure 3 – Cas13 Enzyme Activity with gRNA Targeting ACLSV (Apple Chlorotic Leaf Spot Virus)

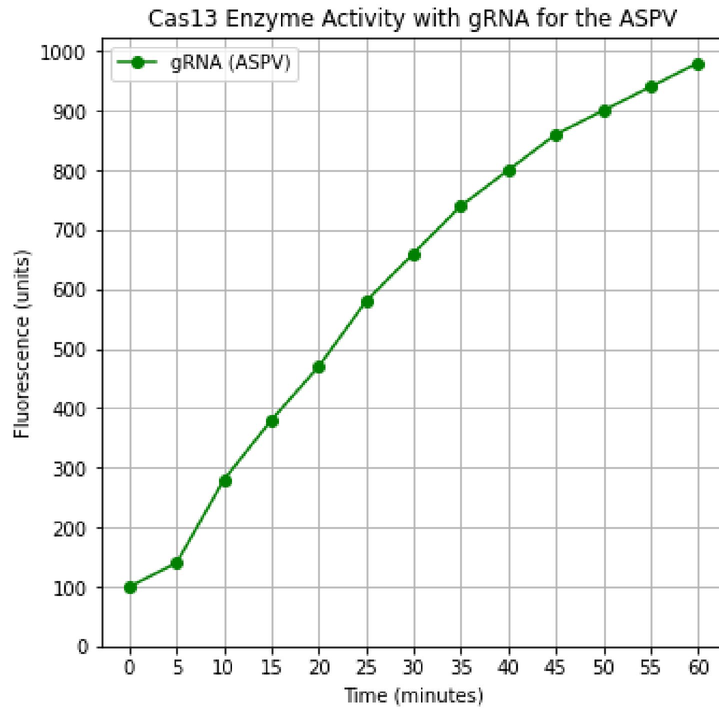

Figure 4 – Cas13 Enzyme Activity with gRNA Targeting ASPV (Apple Stem Pitting Virus)

Field testing with samples collected from various apple orchards validated the robustness and reliability of our diagnostic assays. Using isothermal amplification methods such as RPA and LAMP, followed by CRISPR/Cas-mediated detection, fluorescence signals indicative of successful target cleavage confirmed the presence of the target virus [18]. The SHERLOCK system, which combines isothermal amplification with CRISPR/Cas-mediated detection, demonstrated high sensitivity and specificity. Utilizing Cas13a for RNA targets, SHERLOCK was successfully employed to detect viruses such as ACLSV and ASPV in field samples, proving its reliability under diverse environmental conditions [19-22].

CRISPR technology, especially the Cas12 and Cas13 systems, holds significant potential for the detection and management of both RNA and DNA viruses. Cas12a (Cpf1) targets DNA and induces double-strand breaks, making it ideal for detecting DNA viruses. Conversely, Cas13a targets RNA and induces collateral cleavage of single-stranded RNA, which is useful for detecting RNA viruses [23]. The versatility of these systems allows for the development of diagnostic tools that can be tailored to the genetic material of various pathogens.

The SHERLOCK (Specific High-sensitivity Enzymatic Reporter unLOCKing) system, which leverages both Cas12 and Cas13, combines isothermal amplification with CRISPR-based detection to achieve high sensitivity and specificity. This system has been successfully used to detect viruses such as Zika and Dengue in clinical samples, demonstrating its broad applicability [24, 25].

Furthermore, the CRISPR/Cas systems offer rapid and portable diagnostic capabilities. Recent advancements have led to the development of paper-based assays that can detect viral nucleic acids within an hour, making them suitable for point-of-care diagnostics in remote and resource-limited settings [12]. The ability to quickly and accurately identify viral infections is crucial for controlling outbreaks and implementing timely interventions.

Conclusion

The integration of advanced bioinformatics for gRNA design, combined with rigorous in vitro and field validation, ensures the development of precise and reliable gRNAs for plant virus detection. The SHERLOCK system represents a powerful diagnostic method in plant virology, offering the sensitivity, specificity, and robustness necessary for effective virus management in apple orchards. The expanding capabilities of CRISPR technology continue to revolutionize virus detection and hold promise for managing viral outbreaks in agriculture and beyond.

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Conflict of interest

All authors have read and are familiar with the content of the article and have no conflict of interest.

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