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Development of an RPA-based CRISPR/Cas12a assay in combination with a lateral flow strip for rapid detection of toxigenic *Fusarium verticillioides* in maize

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Abstract

Fusarium verticillioides is an important phytopathogenic fungus that poses a threat to maize yield and quality in global maize-growing regions by causing *Fusarium* ear and stalk rot. The fungus is known to produce fumonisins, which are toxic secondary metabolites and have been associated with high incidences of esophageal cancer. The *FUM1* gene is responsible for producing a crucial polyketide synthase required for fumonisin biosynthesis and is present in all pathogenic strains of *F. verticillioides*. This study aims to develop a rapid and accurate detection assay for *F. verticillioides*, by utilizing Chelex-100 resin for DNA extraction, recombinase polymerase amplification coupled with CRISPR/Cas12a cleavage and lateral flow detection (RPA-Cas12a-LFD) assay based on the *FUM1* gene. The developed RPA-Cas12a-LFD assay exhibited remarkable specificity for *F. verticillioides*, and the lowest limit of detection was 2 ag DNA of *F. verticillioides*. The entire diagnostic process was completed in just 73 minutes, including sample DNA extraction, RPA reaction, Cas12a cleavage, and result readout. Furthermore, RPA-Cas12a-LFD assay was found to be equivalent to single-spore isolation and partial translation elongation factor 1α gene (*TEF-1α*) sequencing in identifying diseased samples in the field. In summary, this accurate and portable detection equipment has great potential for detecting and recognizing *F. verticillioides*, especially in areas where advanced lab equipment is not available.

Keywords

Fusarium verticillioides, recombinase polymerase amplification (RPA), Cas12a, lateral flow detection, maize

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1. Introduction

Fusarium verticillioides (teleomorph: *Gibberella moniliformis*) is a major pathogen that affect maize crop worldwide, leading to significant qualitative and quantitative losses (Liu et al., 2022). In China, *F. verticillioides* is commonly responsible for maize ear and stalk rot (Duan et al., 2016; Li et al., 2019). The infection of *F. verticillioides* can disrupt the structure of maize starch and altering its physicochemical qualities, reducing its suitability for both food and non-food industries (Wei et al., 2022). It is important to note that the maize kernels infected by fungi are highly dangerous for human consumption, as they contain fumonisins that are carcinogenic (Braun & Wink, 2018). According to the 2022 World Mycotoxin Survey, the prevalence of fumonisin contamination in China is alarmingly high at 93% (DSM, 2022). Additionally, most of the maize produced worldwide is used as animal feed, which not only poses a threat to animal health but also increases the likelihood of indirect mycotoxin contamination in humans. Therefore, early diagnosis techniques to identify *F. verticillioides* are crucial in preventing of maize ear and stalk disease, thus benefiting the food and feed industry.

In the past, identifying *F. verticillioides* through its morphological characteristics was a lengthy and complex process, that required expensive equipment and well-trained staff (Guarro & Gené, 1992). As an alternative approach, contemporary nucleic acid amplification techniques (NAATs) such as real-time PCR, nested PCR, or a combined culture and NAAT approach provides fast and accurate results as compared to culture-based methods (Campos et al., 2019; Omori et al., 2018; Patiño et al., 2004). For example, loop-mediated isothermal amplification (LAMP) and recombinase polymerase amplification (RPA) are accurate and sensitive isothermal amplification technologies (Wigmann É et al., 2020; Xu et al., 2022). Performing LAMP can be quick and efficient, taking only 30-60 minutes at 65 °C. However, when using 4-6 primers to target 6 or 8 regions within a small region, there is an increased chance of primer-

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primer hybridization, leading to template-free amplification. Additionally, the high efficiency of the LAMP technique increases the risk of carry-over contamination, which can cause false-positive results (Nzelu et al., 2019). Compared to LAMP technology, RPA employs two primers to obtain amplicons within 30 min at a lower temperature leading to less false-positive results, making it a suitable amplification method for this study (Fig.1 "RPA reaction").

Recent studies have shown that, the clustered regularly interspaced short palindromic repeats (CRISPR)-Cas (CRISPR-associated proteins) system-based nucleic acid detection technology has the ability to accurately recognize target sequences better than other previously developed methods (Gootenberg et al., 2018; Li et al., 2018). This detection method is based on the crRNA-guided Cas12a (Cpf1) protein that has shown non-target DNase activity to single-stranded DNA (ssDNA) when binding to target DNA containing a protospacer-adjacent motif (PAM) site (5'-TTTN-3') (Fig. 1 "Cas12a cleavage") (Chen et al., 2018). RPA product with the PAM site aforementioned could activate Cas12a cleavage (Fig. 1). Combining Cas12a cleavage with RPA reaction products has created a versatile rapid and specific platform for pathogens detection, such as HPV16 (25/25 agreement) and HPV18 (23/25 agreement) using DNA Endonuclease-Targeted CRISPR Trans Reporter (DETECTR) with fluorescence readout, *Mycoplasma* contamination detection with 100% accuracy from cell culture using Cas12a-based Visual Detection (Cas12VDet) with fluorescence readout, 10 aM of African swine fever virus DNA detection using Cas12a-based On-site and Rapid Detection System (CORDS) along with lateral-flow strip readout, multiplexed nucleic acid detection platform with Specific High-sensitivity Enzymatic Reporter unlocking version 2 with fluorescence and lateral-flow strip readout (SHERLOCKv2), citrus scab diagnosis using an RPA-CRISPR/Cas12a combined with a lateral flow assay, 0.3072 fg/μl of *Alternaria* DNA detection using RPA-CRISPR/Cas12a combined with rolling circle amplification and so on (Bai et al., 2019; Chen et al., 2018;

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Gootenberg et al., 2018; Liu et al., 2023; Shin et al., 2021; Wang et al., 2019). The lateral-flow strip readout is superior to the fluorescence readout as it doesn't require any laboratory equipment.

In this study, we present an approach to rapidly detect *F. verticillioides* on-site using CRISPR/Cas12a system. This involves combining rapid nucleic acid extraction, RPA reaction, Cas12a cleavage after target recognition, and lateral flow dipstick visualization. The whole procedure can be completed within 73 min and results can be visualized by the naked eye (Fig. 1). Due to its portability, this method can improve quality management in the field and in storage facilities to without expensive equipment.

2. Materials and methods

2.1. Fungal strains source

The *Fusarium* and non-*Fusarium* strains were obtained from diseased maize stalks and kernels that were collected from different regions of China including Jilin Province, Inner Mongolia, Shandong Province, Liaoning Province, Shanghai City, Gansu Province, Henan Province, Shanxi Province, and Yunnan Province and were maintained in our laboratory (Table 1). All of the *Fusarium* strains used in this study were identified based on their morphological characteristics and partial sequences of the translation elongation factor 1α gene (*TEF-1α*), Whereas all of the non-*Fusarium* strains were identified on the partial sequences of internal transcribed spacer (*ITS*).

2.2. Preparation of DNA extracts for pathogen detection

The strains were grown at 25 °C until the PDA plates were full. For crude DNA extraction, the hyphae were scraped with toothpicks into the tube containing 100 μl of 5% Chelex-100 solution (Chelex® 100 sodium form, Sigma-Aldrich, Germany) and incubated at 65 °C for 20

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min. The supernatant was collected and used as crude DNA for RPA reaction and detection. To detect the sensitivity, DNA was extracted using the CTAB (Cetyltrimethylammonium bromide) method (Pahlich & Gerlitz, 1980). The quality and quantity of the extracted DNA were assessed using P200/P200+ microvolume spectrophotometers (Pultton Technology, California, USA). The DNA concentration was adjusted to 10 ng/μl with ddH_2O (double distilled water) and stored at -20 ℃ for further use.

2.3. Design of primers and crRNA

The obtain the partial *FUM1* sequence of *F. verticillioides* LNF15-11, the primers FUM1-F and FUM1-R were used to amplify it (Table 2). A polymerase chain reaction (PCR) assay was conducted in the thermal cycler with a reaction mixture was 25 μl 2× TaKaRa Ex Taq®, 1 μl forward primer (FUM1-F), 1 μl reverse primer (FUM1-R), 1 μl *F. verticillioides* LNF15-11 DNA, and final volume was adjusted by adding 22 μl ddH_2O. The PCR products were purified using the HiPure Gel Pure DNA Mini Kit (Magen Biotech, Guangzhou, China) and sequenced by Sangon Biotech (Beijing, China). Homologous sequences of the *FUM1* gene for alignment were obtained from National Center for Biotechnology Information (NCBI) website (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The sequence alignment was performed using Clustal X version 2 and Jalview version 2.11.2.5 (Larkin et al., 2007; Waterhouse et al., 2009). After that,the conservative region of *F. verticillioides* but variant region for other strains were chosen to design three primer pairs RPA-FUM1-1-F/R, RPA-FUM1-2-F/R, and RPA-FUM1-3-F/R according to the manual of TwistAmp® DNA Amplification Kits (Fig. 2). To evaluate the specificity of designed primer pairs, the genomic DNA of *F. verticillioides*, *F. fujikuroi*, *F. proliferatum*, *F. temperatum*, and *F. graminearum* were amplified by using three primer pairs in the RPA reaction. The amplified PCR products were then electrophorized on 2% agarose gel.

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Additionally, we ensured that the ideal amplicon contained at least one protospacer adjacent motif (PAM) site (5'-TTTN-3') to facilitate recognition by Cas12a (Chen et al., 2018).

For Cas12a cleavage, the crRNA for the CRISPR detection system was designed based on the amplified product from *F. verticillioides* and synthesized by Sangon Biotech. As in Figure 2, the noted "PAM" site "CAAA" was not the actual PAM site. The reverse complementary sequence "TTTG" was the true PAM site. The complete sequence of crRNA included a direct repeat sequence for cas12a recognition "5'-UAAUUUCUACUAAGUGUAGAU-3' " (scaffold sequence) and spacer sequence "5'-GGAUUCGGCGUAGAAAAGUUG-3' " (guide sequence).

2.4. RPA-Cas12a-LFD reaction

The RPA reaction was prepared using the TwistAmp™ basic Kit (TwistAmp® basic kits. TwistDx™, Cambridge, United Kingdom). The reaction mixture was prepared by adding RPA enzyme powder followed by 2.4 μl forward primer (10 μM), 2.4 μl reverse primer (10 μM), 29.5 μl primer free rehydration buffer, and 11.2 μl ddH_2O. The resulting mixture was thoroughly mixed and then centrifuged, and the volume was divided into five RPA reactions (9.1 μl per reaction). Then, 0.4 μl crude DNA and 0.5 μl Magnesium Acetate (MgOAc, 280 mM) were added to the RPA reaction. The mixture was then incubated on a dry heating block at 40 °C for 20 min. Next, a single Cas12a-crRNA reaction was prepared by combining 16.875 μl ddH_2O, 0.125 μl crRNA (10 μM), 2 μl 10×NEBuffer™ r2.1, and 1 μl EnGen® Lba Cas12a (Cpf1) (1 μM) (New England Biolabs, Ipswich, MA, USA) were incubated at 37 °C for 10 min. Afterward, 17 μl Cas12a-crRNA reaction, 2 μl RPA product, and 1 μl ssDNA reporter were transferred into a new tube. The mixture was incubated at 37 °C to perform CRISPR/Cas12a cleavage assay. After 30 min' s incubation, 30 μl ddH_2O was added to 50 μl of the product.

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Then, the lateral flow strip (Cat. No. JY0301; Tiosbio Biotechnology Co, Ltd., Beijing, China) was inserted into the tube and incubated at RT (room temperature) for 3 min. If two lines appeared on the strip, it indicated a positive result, while a single line meant a negative result. The strip was then removed for inspection and photographed using Canon EOS 80D camera.

2.5. Specificity and sensitivity of the RPA-Cas12a-LFD assay for *F. verticillioides* detection

In order to confirm the accuracy of the RPA-Cas12a-LFD assay developed for detecting *F. verticillioides* strain, crude DNA from various *F. verticillioides* strains, as well as additional *Fusarium* strains, and other fungal species from different regions of China were analyzed. (Table 1). The results were then visualized by using RPA-Cas12a-LFD assay.

To determine the sensitivity of the RPA-Cas12a-LFD assay for *F. verticillioides* detection, different concentrations of genomic DNA was tested, i.e., 10 ng/μl, 1 ng/μl, 100 pg/μl, 10 pg/μl, 1 pg/μl, 100 fg/μl, 10 fg/μl, 1 fg/μl, 100 ag/μl, and 10 ag/μl. The genomic DNA was extracted using CTAB method and then it was diluted with ddH_2O and the sensitivity of the RPA reaction was tested by adding 0.2 μl DNA. The amount of DNA used in RPA reaction was 2 ng, 200 pg, 20 pg, 2 pg, 200 fg, 20 fg, 2 fg, 200 ag, 20 ag, and 2 ag, respectively. The results were visualized using the RPA-Cas12a-LFD assay.

2.6. Detection of *F. verticillioides* in artificially diseased maize stalks and kernels

To ensure the application of RPA-Cas12a-LFD assay for *F. verticillioides* detection in the field, we artificially inoculated *F. verticillioides* into maize stalks and kernels. At the 10^{th} leaf stage, 100 μl of the conidial suspension with a concentration of 1×10^6 conidia/ml was inoculated into the maize stalks. For maize kernels, 100 μl of the aforementioned conidial suspension was inoculated into 5 g of the sterilized maize kernels and cultured at 25 °C and then collected at different inoculation times including 24 h, 48 h, 72 h, 96 h, and 120 h. The sterilized maize

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kernels were prepared in accordance with the method described by Liang et al (2022). Maize stalks and kernels inoculated with ddH_2O served as a negative control. Three replications of each experiment were made. After inoculation, the crude genomic DNA was extracted from stalks at 5 days post inoculation and from kernels at different times using a 5% Chelex-100 solution, respectively. The RPA-Cas12a-LFD assay was used for visual detection of the results. To determine fungal biomass in maize kernels, total genomic DNA was extracted from kernels at different growth stages, and quantified using qPCR as previously described (Gao et al., 2007). The *β-tubulin* gene of *F. verticillioides* was used to quantify fungal colonization, while the *EF1-α* gene of maize served as endogenous plant control.

2.7. The developed RPA-Cas12a-LFD assay for diagnosis of field maize samples

Field samples were collected from Taiyuan City, Shuozhou City, and Yangquan City in Shanxi Province. The samples were subjected to crude DNA extracted using 5% chelex-100 solution. The detection results were obtained visually using the RPA-Cas12a-LFD assay. Furthermore, fungal isolation, purification, and molecular identification were carried out based on the previous study (Xi et al., 2021). Briefly, the samples were cut into small pieces, surface sterilized, and then cultured on potato dextrose agar plates. Fungal isolates with different morphological characteristics were selected and cultured after single spore purification. To identify fungal isolates, their morphological features were observed on PDA and carnation leaf agar (CLA). Furthermore, molecular identification was done using *ITS* and *EF1-α* sequencing"

2.8. Statistics and reproducibility

All results obtained from at least three independent experiments were presented as the mean ± SD. Statistical analyses and graphing were performed using GraphPad Prism 6.0.1.

3. Results

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3.1. Design and optimization of detection primers and crRNA

Following the guidelines of RPA primer designing, three sets of primer pairs, RPA-FUM1-1-F/R, RPA-FUM1-2-F/R, and RPA-FUM1-3-F/R were manually designed from the *FUM1* gene of *F. verticillioides* (Table 2). To evaluate the specificity of the designed three primer pairs, the RPA amplification was carried out using *F. verticillioides*, *F. fujikuroi*, *F. proliferatum*, *F. temperatum*, and *F. graminearum* as templates. Among the three designed primer pairs, RPA-FUM1-1-F/R was considered the optimal pair due to its lack of non-specific amplification and optimal amplicon length as compared to other fragments (Fig. 2A). After selecting the detection primer pairs, one of the PAM sites (5'-TTTG-3') in the amplicon was chosen and the following sequence was selected as the target sequence of crRNA for *F. verticillioides* detection (Fig. 2B).

3.2. Specificity and sensitivity of RPA-Cas12a-LFD assay for detection of *F. verticillioides*

The specificity of RPA-Cas12a-LFD assay for *F. verticillioides* was initially tested by mycelial DNA from various *F. verticillioides* strains, which were isolated from diseased maize samples from Jilin Province, Inner Mongolia, Shandong Province, Liaoning Province, Shanghai City, Gansu Province, and Henan Province in China (Table 1). The results showed that the RPA-Cas12a-FLD assay was able to accurately diagnose all strains of *F. verticillioides* regardless of their origins (Fig. 3A). Meanwhile, the RPA-Cas12a-LFD assay was tested for its capability to differentiate other nine species. The test results showed that there was no cross-reaction among all the tested strains, including seven different *Fusarium* species and two non-*Fusarium* species (Fig. 3A). Based on these findings, it can be concluded that the RPA-Cas12a-LFD assay could successfully distinguish *F. verticillioides* from other *Fusarium* and non-*Fusarium* fungal species.

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To determine the sensitivity of the RPA-Cas12a-LFD assay, the genomic DNA of *F. verticillioides* with different concentrations was used (Fig. 3B). The test results showed that when the DNA concentration was adjusted from 2 ng to 2 fg, two clear bands were observed, which were indicated by the strong color signal at the test line on the lateral flow dipsticks. However, when the DNA concentration was further decreased to 2 ag, the test lines became blurred but still visible on the lateral flow dipsticks (Fig. 3B). Based on these findings, it can be concluded that RPA-Cas12a-LFD assay is capable of successfully detecting DNA concentration as low as 2 ag DNA of *F. verticillioides*.

3.3. Analytical performance of RPA-Cas12a-LFD assay on maize stalks and kernels artificially infected with *F. verticillioides*

The RPA-Cas12a-LFD detection assay was used to verify the feasibility of detecting *F. verticillioides* in maize samples (Fig. 4A). Maize stalks inoculated with *F. verticillioides* were tested, and the assay successfully identified the presence of the target strain in the infected sample, as indicated by the appearance of a second line on the lateral flow dipstick (Fig. 4B). Therefore, the maize genomic DNA did not affect the detection results and RPA-Cas12a-LFD assay could identify *F. verticillioides* in the maize stalks.

F. verticillioides not only infects the maize kernels in the field, but also causes devastation losses during storage. To study this, maize samples were collected at different time points after inoculation (Fig. 4C). The results revealed that there were no disease symptoms on the maize kernels at 24 h and 48 h post-inoculation, similarly to the control (water). Additionally, the fungal relative biomass showed no significant difference between infected samples at 24 h, 48 h, and healthy samples, which resulted in negative results on the lipsticks test line (negative results) (Fig. 4D and 4E). However, the diseased samples showed positive results when the

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relative biomass showed a significant difference between the inoculated maize kernels at 72 h, 96 h, 120 h, and control (Fig. 4C, D, and E). Based on these results, the RPA-Cas12a-LFD assay was found to be efficient for detecting *F. verticillioides*-infected maize kernels for more than 3 days.

3.4. Diagnosis of diseased maize ears in the field

To rapidly detect *F. verticillioides* in the field, the RPA-Cas12a-LFD assay equipment was further optimized as shown in Fig. 5A. This equipment allows for the entire detection process to be completed in just 73 min, which includes 20 min for DNA extraction, 20 min for RPA reaction, 30 min for Cas12a cleavage, and 3 min of lateral flow detection (Fig. 1). The efficacy of the detection kit was confirmed by collecting diseased maize ears from different cities of Shanxi province (as shown in Fig. 5B and C). Furthermore, *F. verticillioides* strains were successfully isolated and identified from those two positive samples. Based on these results, it can be concluded that the kit is capable of detecting *F. verticillioides* in the field.

4. Discussion

F. verticillioides is the most prevalent strain responsible for maize stalk and ear rot in almost all maize-growing region globally (Munkvold, 2003). However, other strains such as *F. proliferatum*, *F. fujikuroi*, *F. graminearum*, *F. oxysporum*, *F. temperatum*, *F. subglutinans*, *F. andiyazi*, *Aspergillus niger*, and *Alternaria sp.* have also been isolated from diseased maize samples (Li et al., 2019; Qin et al., 2014; Sun et al., 2017; Xi et al., 2021; Yong Gang, 2019; Zhang et al., 2014). The prevalence of different pathogens varies by region, which is primarily influenced by geographic location and climate. In China, *F. verticillioides* is considered the most important pathogen of maize ear rot (Duan et al., 2016; Qiu et al., 2015).

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To date, various molecular methods have been used to detect *F. verticillioides* based on different genes. The conventional PCR method, using the VER1/2 primer (based on calmodulin gene sequence), has successfully detected *F. verticillioides* with a sensitivity of 12.5 pg of pure total genomic DNA (Mulè et al., 2004). VERTF-1 and two reverse primers (VERTR and VERTF-2) have been used for the early detection of *F. verticillioides* in 1:100 diluted positive cereal samples by conventional PCR and semi-nested PCR (N et al., 2016; Patiño et al., 2004). For specific detection of *F. verticillioides* isolates, a PCR-ELISA assay was developed, that showed 100-fold higher sensitivity than conventional PCR (Omori et al., 2018). However, these methods are difficult to apply in the field because of the need for various equipment such as PCR machine, microwave, electrophoresis tank, and gel imaging systems. Other amplification methods are commonly used for DNA detection, including LAMP, RPA, HDA (Helicase-Dependent Amplification), and E-SDA (Exponential Strand Displacement Amplification). Among these methods, LAMP and RPA have higher efficiency (Zhao et al., 2015). A *FUM1*-specific assay based on LAMP has been developed to detect multiple fumonisin-producing strains with a detection limit of 5 pg genomic DNA at 65 ℃ in 60 min (Wigmann É et al., 2020). However, the RPA-LFD assay demonstrated the capability to detect as low as 20 fg DNA of *F. graminearum* at 40 ℃ within 25 min, surpassing the detection limits, time duration, and operating temperature of LAMP methods (Liang et al., 2022). In this study, the *FUM1* target region, which is present in all *F. verticillioides* strains and different from other fumonisin-producing strains like *F. proliferatum*, was selected for primer design and this assay gave a relatively specific result for *F. verticillioides* by RPA reaction. Nevertheless, the aforementioned assays, as described by Mulè et al. (2004), Patiño et al. (2004), and Wigmann É et al. (2020), require the utilization of DNA extraction kits to acquire amplified templates. In order to efficiently implement such assays in the field, it becomes imperative convenient

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method for obtaining sufficient DNA from sample for RPA reaction. In this study, we have successfully devised a rapid extraction protocol employing chelate-100 resin, enabling the acquisition of a satisfactory quantity of total DNA for detection within 20 min. This approach helps the developed RPA-Cas12a-LFD to be suitable in field situations.

As *F. verticillioides* could be frequently isolated from symptomless kernels, the need for a more precise and sensitive method to facilitate early diagnosis of *F. verticillioides* infection is needed (Kant et al., 2017). This led to the discovery of advanced techniques like CRISPR/Cas12a and Cas13, SHERLOCK, and HOLMES (one-HOur Low-cost Multipurpose highly Efficient System) for clinical diagnostics (Chen et al., 2018; Gootenberg et al., 2018). Notably, HOLMES exhibited the capability to detect DNA concentration as low as 1-10 aM (1.0E-18 moles), which is lower than the normal RPA reaction (Li et al., 2018). By integrating the CRISPR-Cas12a system, RPA technique achieved the remarkable capability of detecting as low as 1 fg/μl total DNA of *F. graminearum* (Mu et al., 2022). This study further advanced the RPA-Cas12a-LFD assay, enabling the detection of *F. verticillioides* DNA at levels as low as 2 ag. Consequently, it became feasible to identify *F. verticillioides* infections in maize kernels as early as 72 hpi (hours after inoculation). In addition, it gave the same positive results for the 13 different *F. verticillioides* strains isolated from the seven provinces, while yielding negative results for the other nine species because of the high specificity of Cas12a recognition. These findings indicate that the RPA-Cas12a-LFD assay is an extraordinarily sensitive and specific tool for the efficient detection of *F. verticillioides* in the field. Furthermore, two positive samples collected from Shanxi Province have been identified by using RPA-Cas12a-LFD developed in this assay, along with the traditional isolation procedure. However, it is important to note that all the field samples tested were maize ears collected from the same province.

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Therefore, it is necessary to conduct further testing on various samples from different locations in the future to obtain accurate and comprehensive results.

5. Conclusion

In this study, the developed RPA-Cas12a-LFD assay was designed, incorporating four essential components: Chelex-100 solution for rapid DNA extraction, RPA reaction for efficient amplification of target DNA , CRISPR/Cas12a system for cleaving of the target sequence and producing a detectable signals, and lateral flow detection for visualizing the results. The developed RPA-Cas12a-LFD assay exhibits tremendous potential as a valuable tool for swift and on-site diagnosis of *F. verticillioides*, eliminating the requirement of specialized equipment. Furthermore, it can also be deduced that the developed RPA-Cas12a-LFD assay has the potential to evaluate the quality of raw materials in food and feed products, thereby mitigating the risk associated with mycotoxin contamination.

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CRediT authorship contribution statement

Xiaoyan Liang: Methodology, Investigation, Writing – original draft. **Xiu Zhang**: Methodology, Investigation, Collecting Samples. **Kaifei Xi**: Methodology. **M. Haissam Jijakli**: Writing – review & editing. **Yang Liu**: Funding acquisition. **Wei Guo:** Conceptualization, Formal analysis, Methodology, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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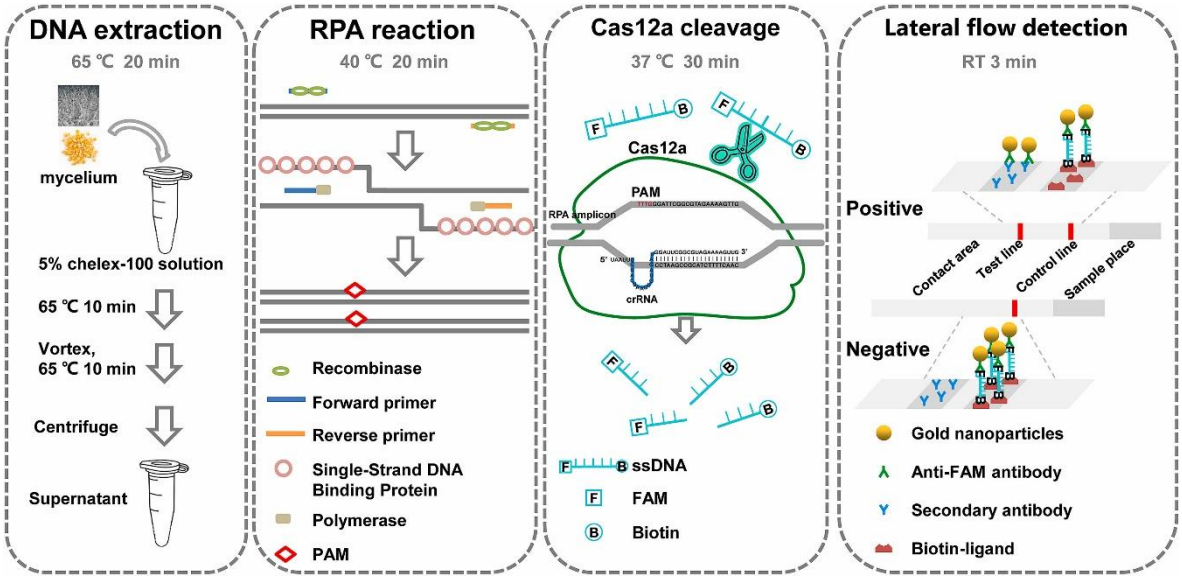

sFig. 1 Schematic diagram of RPA-Cas12a-LFD assay and minimal time required for the detection of *F. verticillioides*. For DNA extraction, the mycelium or ground maize kernels were added to 5% Chelex-100 solution at 65 ℃ in 20 min. After centrifugation, the supernatant was used as the crude DNA template for the RPA reaction. The crude DNA was amplified with RPA enzymes and primers at 40 ℃ for 20 min. For Cas12a cleavage, the pre-heated Cas12a/crRNA complex would recognize resulted RPA amplicon, then trans-cleaved ssDNA reporter with FAM and biotin tags. After the reaction, for a negative result, all the uncut ssDNA reporters were captured by biotin-ligand on the control line. If was a positive result, the cleaved FAM binding with anti-FAM antibody conjugated gold nanoparticles showed a clear band on the test line. The lateral flow detection would finish in 3 min at RT.

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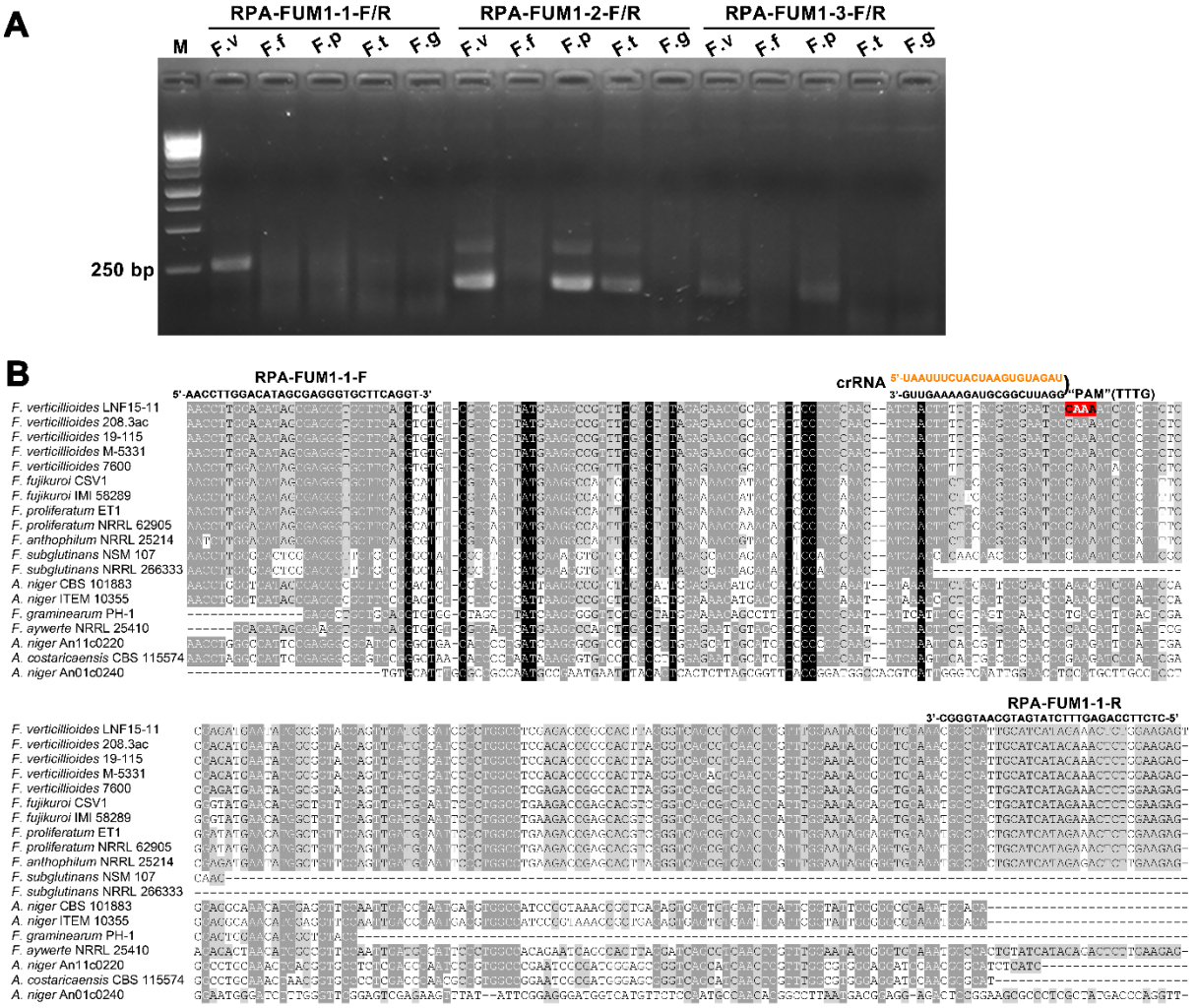

Fig. 2 The primer and crRNA design for *F. verticillioides*. (A) The optimal amplification primer pairs screening using recombinase polymerase amplification (RPA). The agarose gel electrophoresis showed the amplification results of the three designed primer pairs using RPA. Each designed primer pair name is indicated on the top. *F. v*, *F. f*, *F. p*, *F. t*, and *F. g* indicate *Fusarium verticillioides*, *F. fujikuroi*, *F. proliferatum*, *F. temperatum*, and *F. graminearum*, respectively. M represents a marker of 1 Kb molecular weight. (B) Location of the primer pair and crRNA in the *FUM1* gene of *F. verticillioides*. The noted "PAM" site "CAAA" was not the real protospacer-adjacent motif (PAM) site, rather than the reverse complementary sequence "TTTG".

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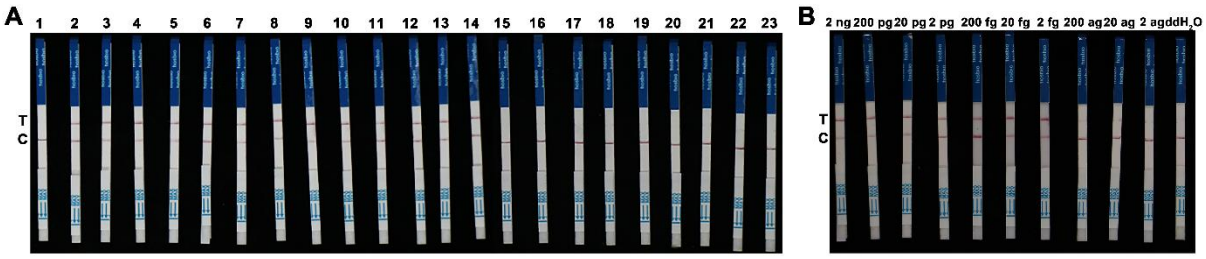

Fig. 3 Specificity and sensitivity of RPA-Cas12a-LFD assay for *F. verticillioides* detection. (A) The lateral flow detection results were performed with different fungal strains. Lane 1, ddH_2O served as negative control; lanes 2 and 3, two *F. verticillioides* strains isolated from Shandong province; lanes 4 and 5, two *F. verticillioides* strains isolated from Jilin province; lanes 6 and 7, two *F. verticillioides* strains isolated from Inner Mongolia; lanes 8 and 9, two *F. verticillioides* strains isolated from Liaoning province; lanes 10 and 11, two *F. verticillioides* strains isolated from Henan province; lanes 12 and 13, two *F. verticillioides* strains isolated from Gansu province; lane 14, *F. verticillioides* 7600; lanes 15, *F. proliferatum*; lane 16, *F. fujikuroi*; lane 17, *F. graminearum*; lane 18, *F. oxysporum*; lane 19, *F. temperatum*; lane 20, *F. subglutinans*; lane 21, *F. andiyazi*; lane 22, *Aspergillus niger*; lane 23, *Alternaria* sp. (B) The sensitivity of the developed RPA-LFD assays was determined using serially diluted genomic DNA of *F. verticillioides* LNF15-11 from 2 ng to 2 ag and ddH_2O served as the negative control.

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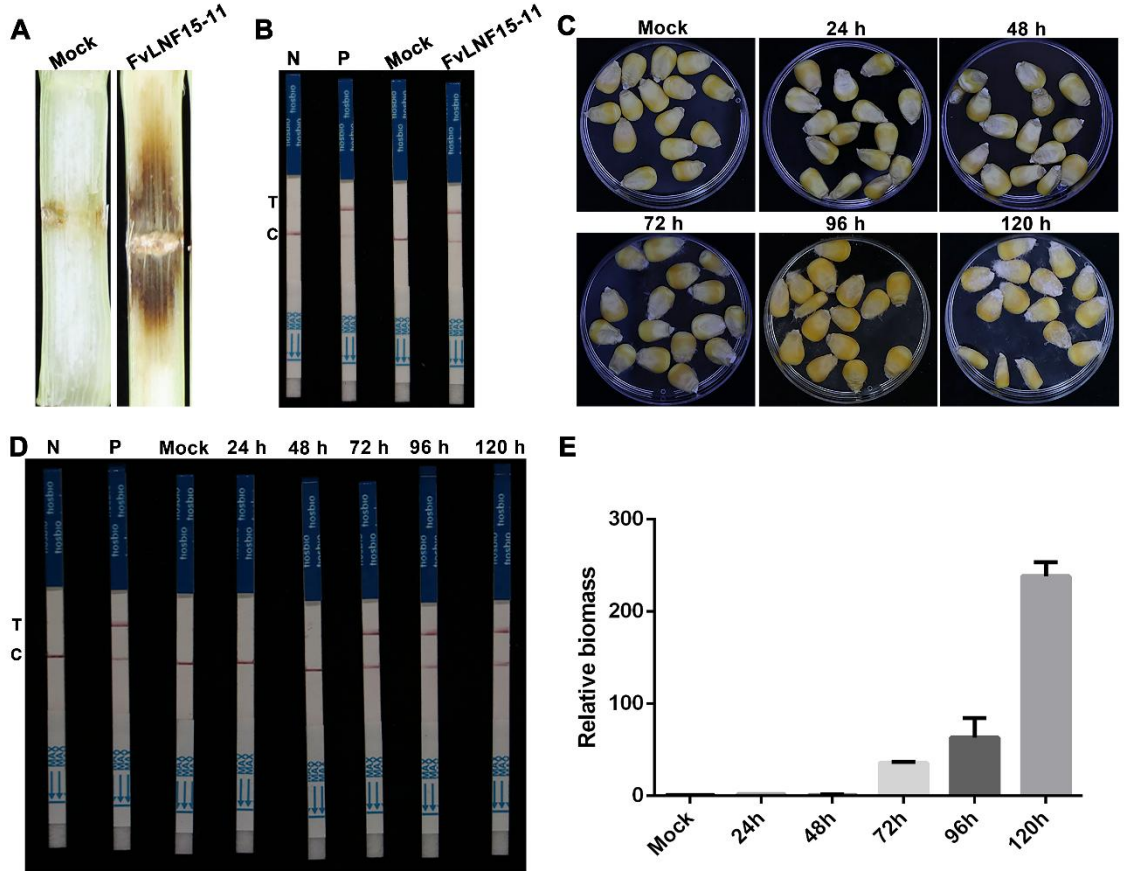

Fig. 4 Early detection of *F. verticillioides* in artificially diseased maize stalks and kernels using the developed RPA-Cas12a-LFD assay. (A) Maize stalks cut longitudinally, showing necrotic lesions around the insertion point at 7 days post-inoculation. No symptoms were observed in control. (B) Detection of *F. verticillioides* in diseased maize stalks according to the developed RPA-Cas12a-LFD assay. Lane 1, ddH_2O served as the negative control (N); lane 2, 10 ng/μl genomic DNA of *F. verticillioides* served as the positive control (P); lane 3, crude DNA of maize stalks inoculated by ddH_2O (Mock); lane 4, crude DNA of maize stalks inoculated by *F. verticillioides*. (C) Symptoms of maize kernels inoculated by *F. verticillioides* at different times (24 h, 48 h, 72 h, 96 h, and 120 h). (D) Detection of *F. verticillioides* in diseased maize kernels according to RPA-Cas12a-LFD assay. Lane 1, ddH_2O served as the negative control (N); lane 2, 10 ng/μl genomic DNA of *F. verticillioides* served as the positive control (P); lane 3, crude DNA extracted from uninfected maize kernels (Mock); lane 4-8, crude DNA extracted from maize kernels of different post-inoculation time (24 h, 48 h, 72 h, 96 h, and 120 h). (C) Relative

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fungal biomasses of the maize kernels of different post-inoculation times were determined by qPCR. Error bars represent standard error.

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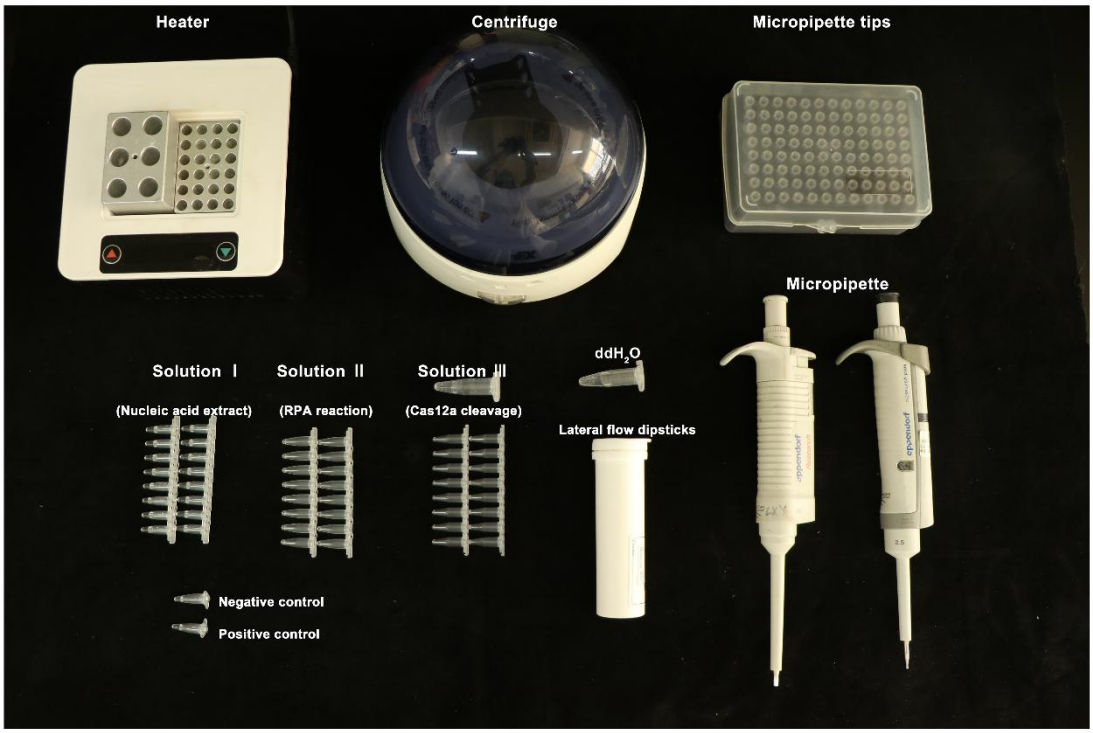
A



B

Diseased maize ears	Location	RPA-Cas12a-LFD results	*F. verticillioides* isolates
Sample 1	Taiyuan, Shanxi province	-	-
Sample 2	Shuozhou, Shanxi province	-	-
Sample 3	Yangquan, Shanxi province	+	+
Sample 4	Taiyuan, Shanxi province	-	-
Sample 5	Taiyuan, Shanxi province	+	+
Sample 6	Shuozhou, Shanxi province	-	-

C

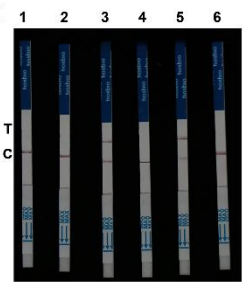


Fig. 5 (A) Minimal instrument requirements of the developed RPA-Cas12a-LFD assay for detecting *F. verticillioides* in the field. (B) The diseased maize ears were collected from three cities in Shanxi province. (C) The sample results were obtained using the developed RPA-Cas12a-LFD assay with the minimal instrument.

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Table 1 Fungal strains used in this study and their results in the RPA-Cas12a-LFD assay.

Species	Number	Host	Location in China	RPA-Cas12a-LFD
***Fusarium* strains**				
F. verticillioides	12	Maize	Jilin, Inner Mongolia, Shandong, Liaoning, Shanghai, Gansu, Henan	+
F. proliferatum	1	Maize	Liaoning, Henan	-
F. fujikuroi	1	Maize	Henan	-
F. graminearum	1	Maize	Jilin	-
F. oxysporum	1	Maize	Liaoning	-
F. temperatum	1	Maize	Yunnan	-
F. subglutinans	1	Maize	Inner Mongolia	-
F. andiyazi	1	Maize	Inner Mongolia	-
***Non-Fusarium* strains**				
Aspergillus niger	1	Maize	Shanxi	-
Alternaria sp.	1	Maize	Shanxi	-

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Table 2 Primers and guide RNA used in this study.

Name	Sequence (5'-3')	Length (bp)
FUM1-F	GCCGGCACGAACCTTGTA	18
FUM1-R	AGGCAACTCCCACACCCTCAA	21
RPA-FUM1-1-F	TAACCTTGGACATAGCGAGGGTGCTTCAGGC	31
RPA-FUM1-1-R	CTCTTCCAGAGTTTCTATGATGCAATGGGC	30
RPA-FUM1-2-F	GGCAAATCGAAGAGAGCATTTGTCGCATCG	30
RPA-FUM1-2-R	CCCATTGGGCACCTTGTCCAGTGAAGATCA	30
RPA-FUM1-3-F	TGGGCAAAGAGCTCATGGACGAATATGAGA	30
RPA-FUM1-3-R	CAAGTGGCTGGGAGAATTCGGCGCGGCCAA	30
RPA-FUM1-crRNA	UAAUUUCUACUAAGUGUAGAUGGAUUCGGCGUAGAAAAGUUG	42
ssDNA reporter	5'-FAM-TTATT-biotin-3'	5
Fv-TUB-F	CCCCGAGGACTTACGATGTC	20
Fv-TUB-R	CGCTTGAAGAGCTCCTGGAT	20
Maize-EF-1α-F	TGGGCCTACTGGTCTTACTACTGA	24
Maize-EF-1α-R	ACATACCCACGCTTCAGATCCT	22