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Rapid diagnosis of *Diaporthe helianthi* in sunflower using RPA/CRISPR-Cas12 and lateral flow assay

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Abstract

Diaporthe helianthi is one of the main fungal pathogens responsible for causing phomopsis stem canker and significant yield losses of sunflowers. In this study, the calmodulin (*Cal*) gene of *D. helianthi* was selected to develop a rapid detection method involving recombinase polymerase amplification (RPA) combined with CRISPR/Cas12a-based detection at 37 °C. The developed detection system could complete the specific detection of *D. helianthi* in 45 min, including 25 min for RPA and 20 min for CRISPR/Cas12 reaction. The detection system could be coupled with both lateral flow test strips and fluorescence signal reading modes. The detection limit for lateral flow assay was 1 pg/μL genomic *D. helianthi* DNA (14 copies/μL); The detection limit for fluorescence signal was 0.1 pg/μL genomic DNA (1.4 copies/μL), approximately 100 times higher than that of the real-time PCR. Thus, the developed RPA/CRISPR-Cas12a system meets the need for portable detection of *D. helianthi* on-site at ports and in the field.

Keywords *Diaporthe helianthi*, RPA, Cas12a, Lateral flow assay, Rapid

Introduction

Background

Sunflower (*Helianthus annuus* L.) is one of the main oil crops in the world (Hussain et al. 2018), and serves as a source of medicine and cosmetics (Kaya et al. 2012; Adeleke and Babalola 2020). In addition, non-oilseed sunflower is of ornamental value (Cvejic et al. 2016), and the seeds are also popular snacks and foods for birds and small pets (Rauf et al. 2017). However, fungal diseases, such as phomopsis stem canker, have been reported to result in significant yield losses in Europe, the United States, and Australia. With the increased demand for sunflower as a food, oil source, and ornamental variety, early detection of plant pathogens plays a key role in preventing the spread of disease (Gulya et al. 2019), strengthening the quarantine inspection of imported sunflower seeds and field planting monitoring (Qian et al. 2022). *Diaporthe helianthi* (*D. helianthi*) is a destructive fungus on sunflower plants and a plant quarantine pest in China and many other countries (Zhang et al. 2013). It mainly infects the leaves, stems, and inflorescences of the sunflower plants (Degener et al. 1999). In the early stage of the disease, gray-brown spots appear at the junction of the stem and petiole, and then the spots quickly spread and circle the stem, causing the upper part of the stem

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to become withered and necrotic, the leaves wilted, the flower disc dysplastic, and immature seeds (Debaeke and Estragnat 2009). *D. helianthi* was first discovered in Yugoslavia in 1980 and subsequently spread to France (Viguie et al. 1999), the United States (Mathew et al. 2015), Italy, and other countries (Pecchia et al. 2004), resulting in a high disease incidence and significant yield loss. The disease in France in 1984 resulted in a 40% loss of field sunflower crop yields, which greatly harmed sunflower production (Says-Lesage et al. 2002). This pathogen is transported long distances from one area to another (Li and Duan 2020), introducing a high risk of pathogen introduction by seed trade (Chen 2012).

Rapid and accurate detection technologies offer strong technical support to mitigating the introduction and spread of quarantine pests (Martinelli et al. 2015). Several methods have been developed for the detection of *D. helianthi*, including morphological identification (Zhang et al. 2013), polymerase chain reaction (PCR) (Chen et al. 2012), multiplex PCR assays based on dual priming oligonucleotide (DPO) primers (Qian et al. 2022), real-time TaqMan PCR (Elverson et al. 2020), and isothermal detection methods such as recombinase polymerase amplification (RPA) (Qian et al. 2021). Traditional approaches of identifying plant fungal pathogens involve isolating and culturing diseased tissues on a culture medium, selecting suspicious single fungal colonies, re-culturing, observing morphology, and sequencing PCR products (Ward et al. 2004). However, these steps are often time-consuming (Ray et al. 2017) and require expensive equipment and technical expertise, which restricts their diagnostic application outside the laboratory (Boonham et al. 2008). Therefore, it is imperative to establish a rapid, sensitive, and field-friendly detection method for the diagnosis of *D. helianthi* in plant tissues.

Clustered regularly interspaced short palindromic repeats (CRISPR)-CRISPR-associated enzyme systems (Cas) develop novel nucleic acid detection methods (van Dongen et al. 2020) that have been used for pathogen detection (Zhou et al. 2018; Lei et al. 2022). CRISPR-Cas12a, an RNA-guided endonuclease, recognizes a T-rich protospacer-adjacent motif (PAM) (TTTN) sequence under the guidance of CRISPR RNA (crRNA) (Wang et al. 2019) and possesses collateral cleavage activity (Dai et al. 2019). When the Cas12a/crRNA complex binds to the target nucleic acid at the PAM site, the activated collateral cleavage ability cleaves ssDNA modified with indicator molecules to construct various detection methods (Xiong et al. 2020). Based on this property, DNA endonuclease-targeted CRISPR trans-reporter (DETECTR) fluorescence detection (Chen et al. 2018), Cas12a-based Visual Detection (Cas12VDet) visual detection (Duan et al. 2022), one-pot lateral flow assay (Lei et al. 2022), and other detection platforms have been developed to detect the amplification products of bacteria (Liang et al. 2019), virus (Dronina et al. 2022), and fungi (Lei et al. 2022; Kang et al. 2021) using recombinase polymerase amplification (RPA) techniques. RPA, an isothermal nucleic acid amplification technique, requires only a pair of primers to rapidly and exponentially amplify DNA at a constant temperature of 37–42 °C (Li et al. 2019). It is an ideal companion with CRISPR-Cas12a detection for rapid on-site diagnosis of pathogens (Wang et al. 2020).

Here, we developed a rapid, accurate and promising method for detecting the fungal pathogen *D. helianthi* in sunflower tissues by combining RPA and CRISPR-Cas12a. Using the FAM-ssDNA-Quencher and FAM-ssDNA-Biotin as the reporter, the developed system can be detected using fluorescence detection and lateral flow strips, respectively. At the same time, a portable metal incubator with constant temperature was equipped for RPA and CRISPR reactions in order to better accommodate on-site testing. The current study aimed to develop a promising, rapid, and accurate identification strategy based on the RPA/CRISPR-Cas12a system, which is expected to enable port quarantine and early field diagnosis of *D. helianthi* in sunflower products.

Results

The strategy of RPA/CRISPR-Cas12a assay for *D. helianthi*

The genomic DNA of *D. helianthi* or total DNA of *D. helianthi* infected plant tissues was extracted using plant genomic DNA extraction kit with a magnetic bead or fast DNA extraction kit (Fig. 1a). The target DNA was amplified using RPA basic amplification kit at 37 °C for 25 min (Fig. 1b). The RPA products were detected with CRISPR-Cas12a fluorescence detection (Fig. 1c) or lateral flow assay (Fig. 1d). When positive RPA products were present and discriminated by the sequence-specific Cas12a/crRNA in the CRISPR-Cas12a reaction buffer, the CRISPR-Cas12a-based collateral cleavage activity was triggered, thus cleaved the FAM-ssDNA-BHQ1 (FQ reporter) or FAM-ssDNA-Biotin (LF reporter) reporters, generating FAM or biotin molecules. The released FAM molecules from FAM-ssDNA-BHQ1 generated green fluorescent signals when excited with 488 nm light, but the intact FAM-ssDNA-BHQ1 did not generate detectable fluorescence due to the quenching of BHQ1 (Fig. 1c). As for the lateral flow strip detection, the FAM group of FAM-ssDNA-Biotin combined with anti-FAM-antibody-modified gold nanoparticles (GNP) immobilized on the sample pad. For the negative sample, the FAM-ssDNA-Biotin was intact, and the GNP-FAM-ssDNA-Biotin conjugates were captured by streptavidin in the control area to form the control line (Fig. 1d); For the positive sample, the FAM-ssDNA-Biotin was cleaved, and the FAM-GNP

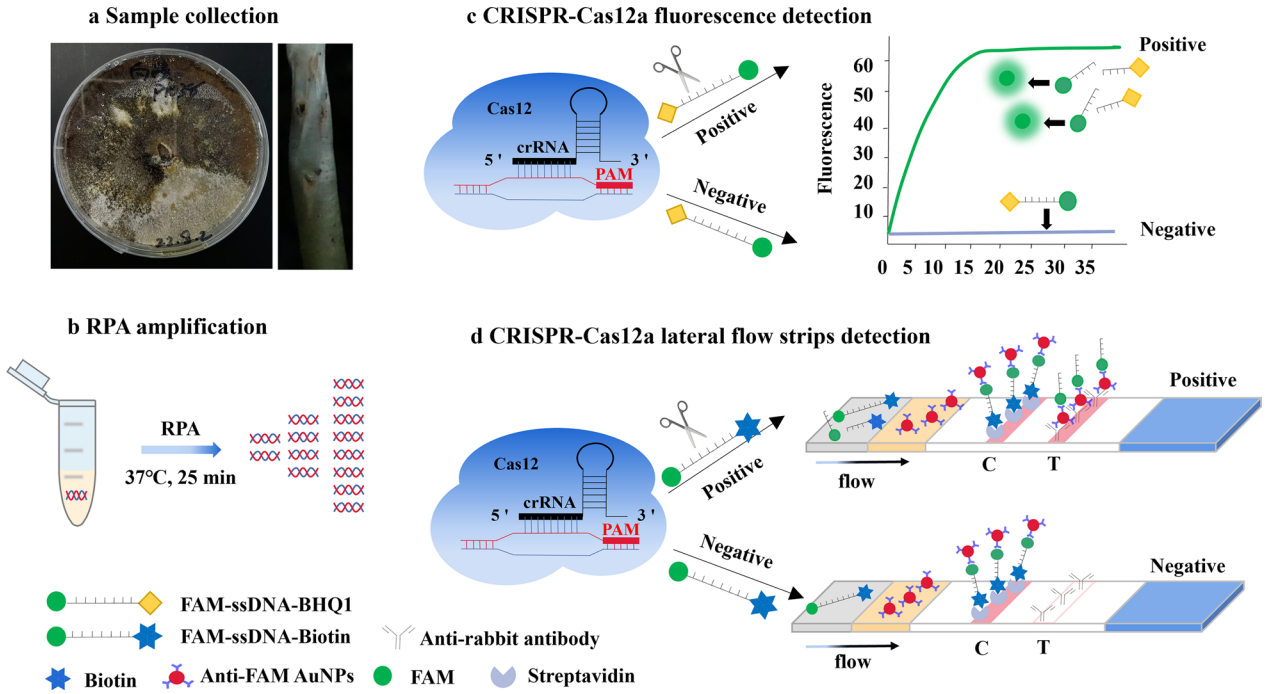

Fig. 1 Schematic illustration of the principle of the RPA/CRISPR-Cas12a system

conjugates passed the control area to arrive the test area, where the anti-FAM antibody were captured by the anti-rabbit antibody to form the test line (T) (Fig. 1d).

RPA primer screening

The products obtained by RPA using four pairs of primers (Additional file 2: Table S1) from *D. helianthi* genomic DNA and other fungi genomic DNA were analyzed by 1.5% agarose gel electrophoresis, and the results showed that primer Dh-Cal-F1/Dh-Cal-R1 could specifically amplify *D. helianthi* genomic DNA (Additional file 1: Figure S1), produce the best sensitivity and detect 200 pg *D. helianthi* genomic DNA (Fig. 2). Therefore, primer Dh-Cal-F1/Dh-Cal-R1 was chosen to amplify D. *helianthi* genomic DNA, and crRNA was designed according to the RPA products. The partial sequence of crRNA was converted from 20 bp

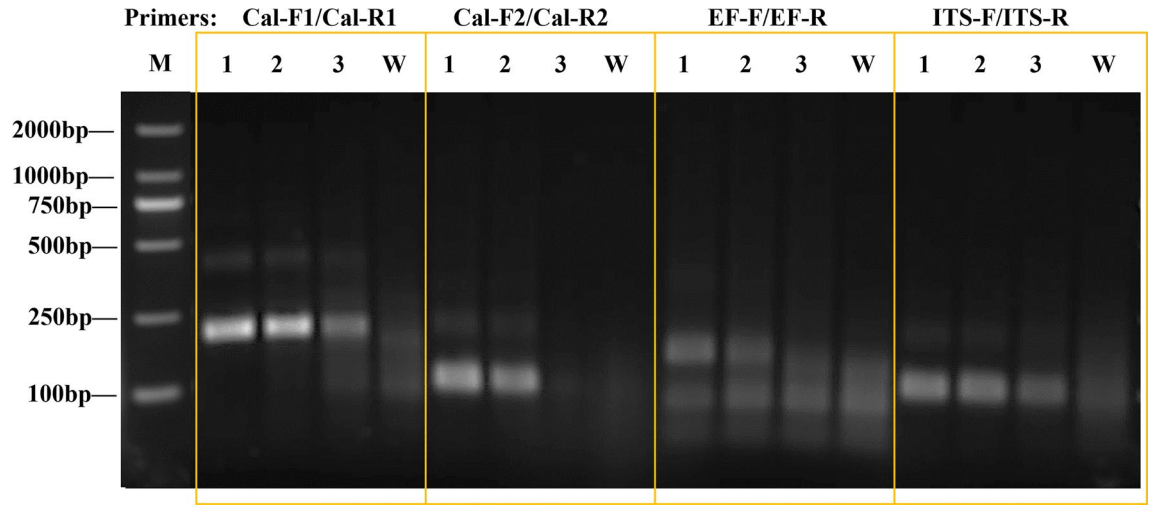

Fig. 2 Sensitivity evaluation of four primer sets designed based on different gene sequences of *D. helianthi*. Lane 1, 20 ng of *D. helianthi* genomic DNA; Lane 2, 2 ng of *D. helianthi* genomic DNA; Lane 3, 200 pg of *D. helianthi* genomic DNA; Lane W, sterile water; M, DL 2000 DNA marker

nucleotides (5′-TGCCTGCAGGATAAGGACGGC-3′) following the PAM site (TTTN) to be 5′-UGCCUGCAGGAUAAGGACGGC-3′ (Additional file 2: Table S1).

Optimization of RPA/CRISPR-Cas12a detection

The RPA products obtained with amplification time of 10, 20, 25, 30, 40 min were detected using CRISPR-Cas12a-based fluorescence. The results indicated that the 40 min RPA amplification produced the highest fluorescence intensity using CRISPR-Cas12a-based fluorescence, and 25 min and 30 min of RPA amplification time produced almost the same fluorescence intensity, while negative control with water as the template did not produce any fluorescence signal. Therefore, 25 min of RPA amplification time was chosen (Fig. 3a). The higher concentration of FQ reporter in CRISPR-Cas12a reaction buffer produced higher fluorescent intensity, but more FQ reporter cost more, so 0.2 μmol/L FQ reporter was used in the following experiments (Fig. 3b).

The CRISPR-Cas12a reaction time has obvious effects on the test line and control line of the lateral flow strips. The results showed that 5 min of CRISPR-Cas12a reaction produced a detectable weak test line (T), and 20 min of reaction time produced an obvious test line, while the corresponding negative control had no test line (Fig. 3c); thus, 20 min of CRISPR-Cas12a reaction time was used in the following experiments. In addition, a higher concentration of LF reporter produced a deeper test line (Fig. 3d). When the concentration of LF reporter was lower than 0.2 μmol/L, the test line was weak. Therefore, the concentration of LF reporter involved in the CRISPR-Cas12a-based lateral flow strip was 0.2 μmol/L.

Specificity of RPA/CRISPR-Cas12a detection system

Using the optimal experimental conditions, the DNA samples of *D. helianthi* and other pathogens were amplified by

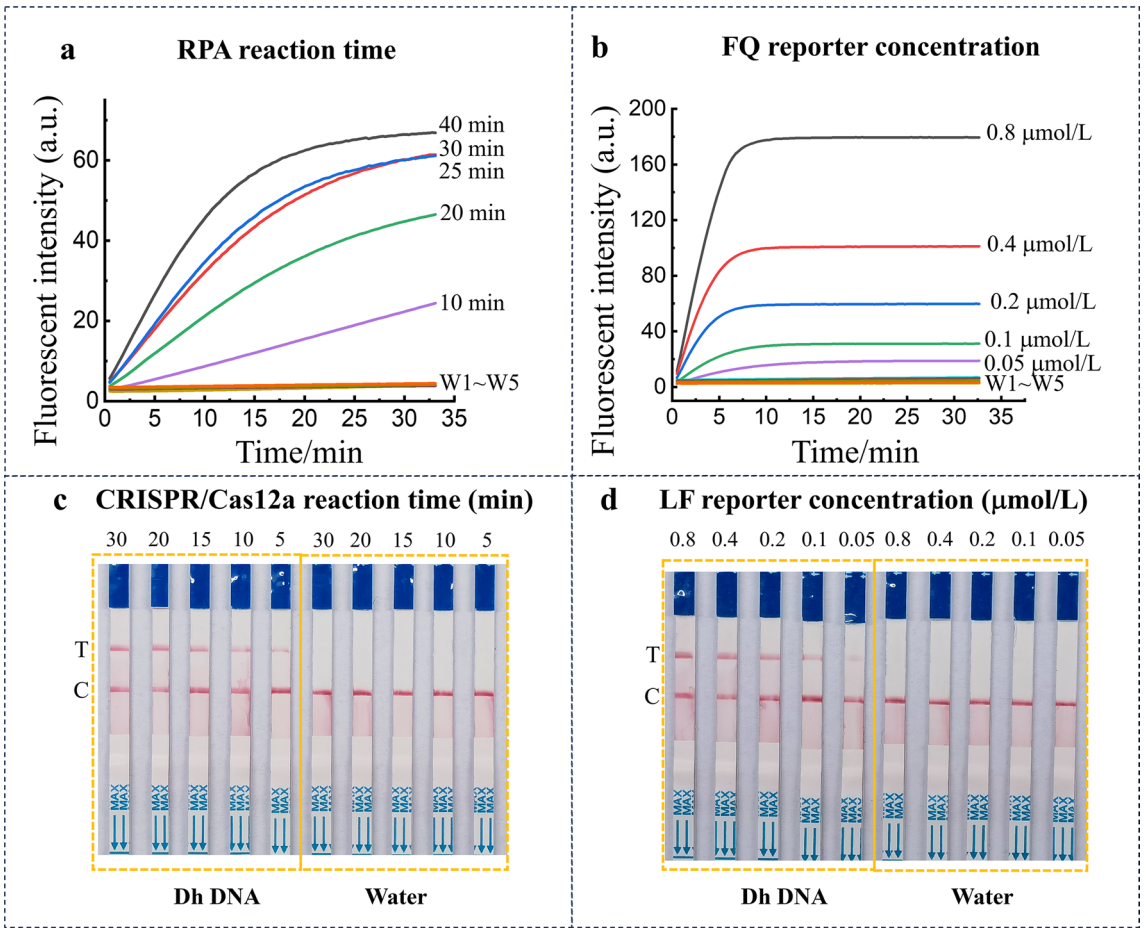

Fig. 3 Optimization of RPA/CRISPR-Cas12a detection method. **a** Fluorescence intensity of RPA products with different RPA reaction time. W1–W5 are the corresponding negative controls. **b** Fluorescence intensity with different FQ reporter concentrations in the CRISPR/Cas12a reaction system. W1-W5 are the corresponding negative controls. **c** The lateral flow strips of CRISPR/Cas12a reaction products with different reaction time. **d** The lateral flow strips of CRISPR/Cas12a reaction products with different LF reporter concentrations. T, test line; C, control line

RPA and detected by the CRISPR-Cas12a method. The fluorescence results showed that only the genomic DNA of *D. helianthi* produced obvious fluorescence intensity, while other fungal genomic DNA had very low fluorescence signals (Fig. 4a). The CRISPR-Cas12a-based lateral flow strips detected only DNA sample of *D. helianthi*, having obvious test line (T), while other fungal DNA samples and negative control had no test lines (Fig. 4b), indicating that the RPA/CRISPR-Cas12a detection method can accurately distinguish *D. helianthi* from other fungal species.

Sensitivity of RPA/CRISPR-Cas12a detection method

The tenfold serially diluted *D. helianthi* genomic DNA (10 ng/μL) was detected using the developed RPA/CRISPR-Cas12a method (Fig. 5a, b). The results showed that the RPA/CRISPR-Cas12a fluorescence method could detect 0.1 pg/μL genomic DNA(1.4 copies/μL) (Fig. 5a), while the RPA/CRISPR-Cas12a lateral flow strip could detect 1 pg/μL genomic DNA (14 copies/μL) (Fig. 5b). The real-time PCR detection method could detect 10 pg/μL (1.4×10^2 copies/μL) genomic DNA (Fig. 5c), indicating that the sensitivity of the newly developed RPA/CRISPR-Cas12a system is approximately 100 times higher than that of the real-time PCR method.

Applicability of RPA/CRISPR-Cas12a for plant samples

The DNA extracted from the spiked plant tissues (leaves, stems, and seed coat of healthy sunflower) with ground *D. helianthi* mycelia powder were detected using RPA/CRISPR-Cas12a, and the results (Fig. 6) showed that *D. helianthi* mycelia mixed with plant tissues could be detected using both RPA/CRISPR-Cas12a fluorescence detection (Fig. 6a, d, g) and lateral flow strips method (Fig. 6b, e, h). The fluorescence intensity of spiked leaves was between 73.5% and 84.0% of that of *D. helianthi* mycelia (Fig. 6c). The fluorescence intensity of

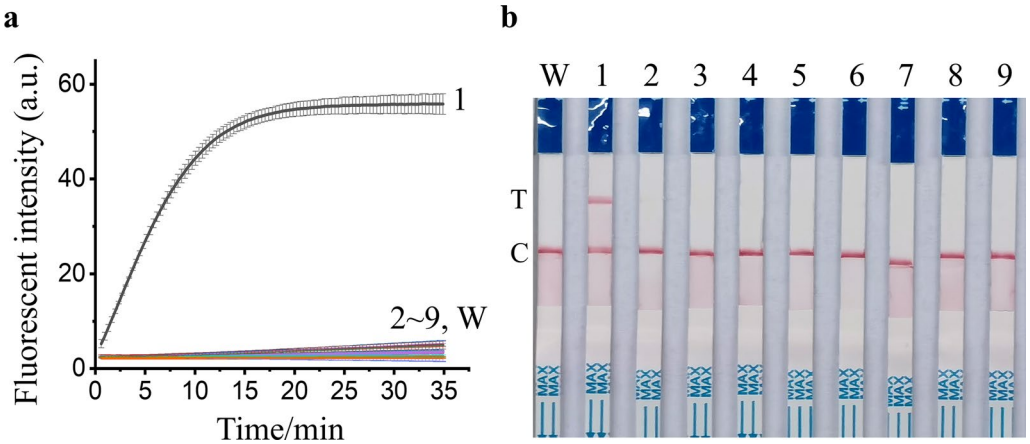

Fig. 4 Specificity evaluation of RPA/CRISPR-Cas12a detection method. **a** CRISPR-Cas12a-based fluorescence detection of genomic DNA. **b** RPA/CRISPR-Cas12a-based lateral flow strips of genomic DNA. 1, *D. helianthi;* 2, *Leptosphaeria lindquistii;* 3, *Leptosphaeria maculans;* 4, *Leptosphaeria biglobosa;* 5, *Diaporthe phaseolorum* var. *Caulivora;* 6, *Diaporthe phaseolorum* var. *Meridionalis;* 7, *Sclerotinia sclerotiorum;* 8, *Verticillum dahlia;* 9, Sunflower leaves; W, sterile water. T, test line; C, control line

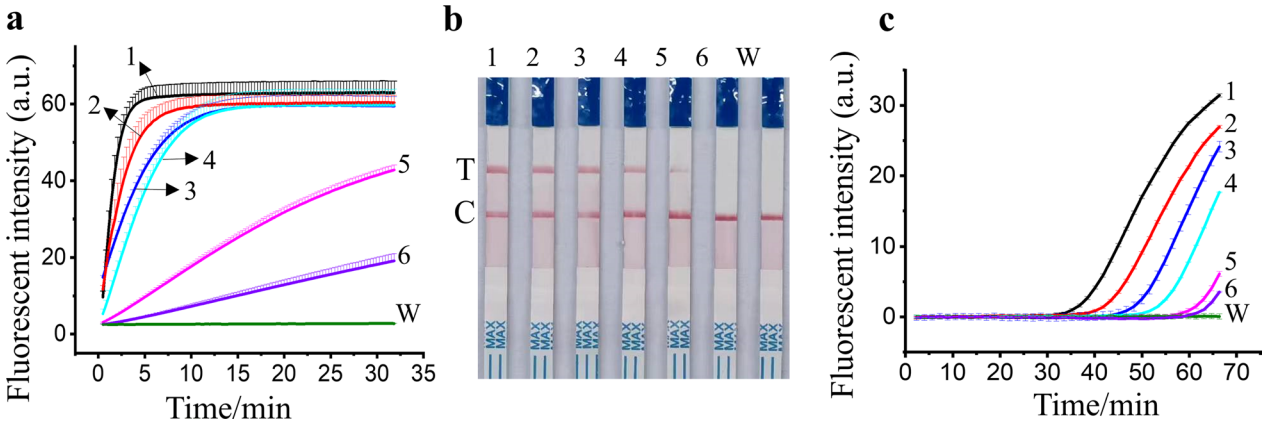

Fig. 5 Sensitivity evaluation of RPA/CRISPR-Cas12a detection method. **a** RPA/CRISPR-Cas12a fluorescence detection method. **b** RPA/CRISPR-Cas12a lateral flow strip method. **c** Real-time PCR. Number 1–6 represented genomic DNA concentrations of 10 ng/μL, 1 ng/μL, 0.1 ng/μL, 10 pg/μL, 1 pg/μL, 0.1 pg/μL, respectively. W, sterile water; T, test line; C, control line

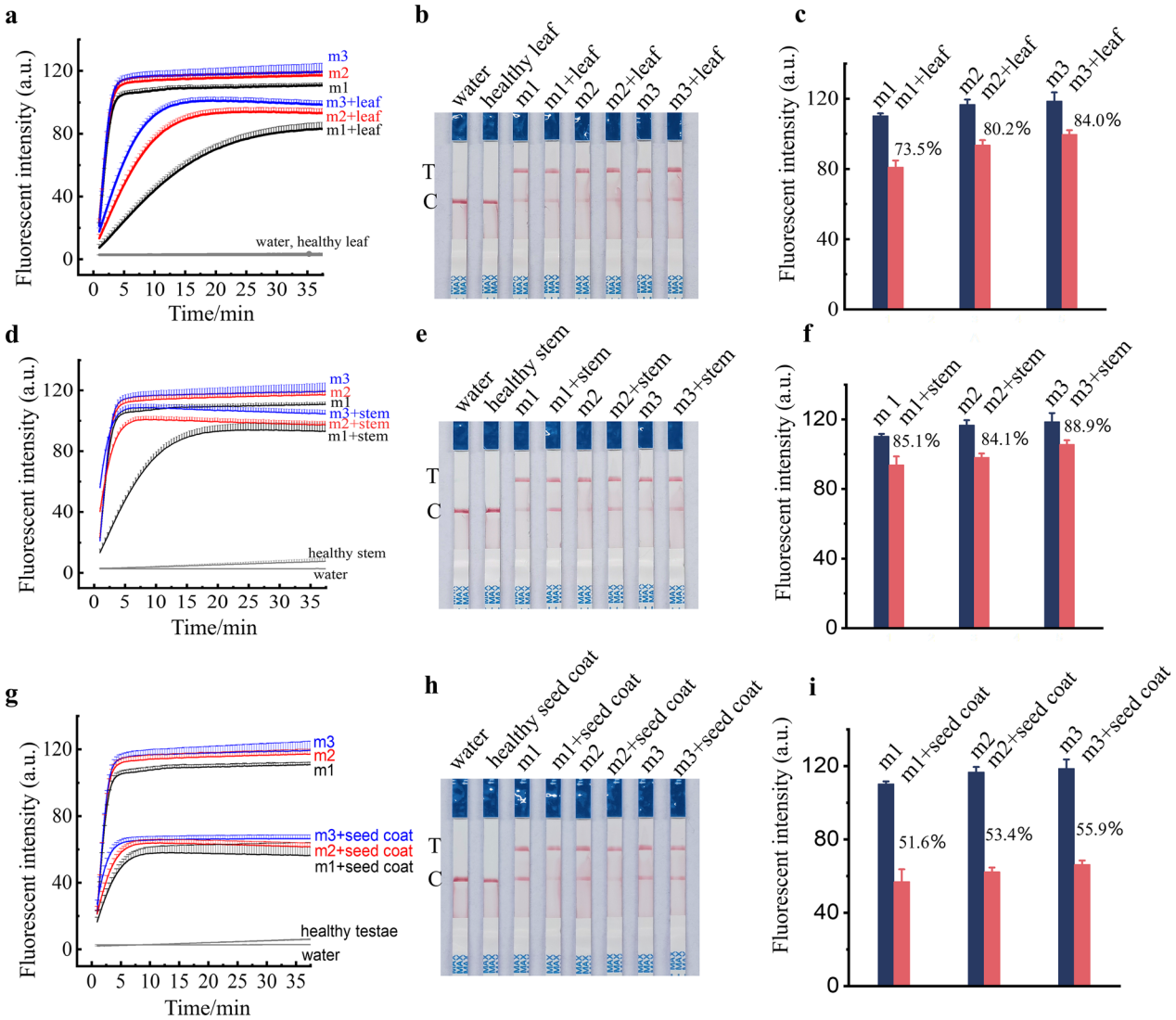

Fig. 6 **a, b** Detection of DNA extracted from *D. helianthi* mycelia spiked healthy sunflower leaves using RPA/CRISPR-Cas12a-based fluorescence detection and lateral flow assay. **c** The fluorescence signals at 30 min detection time. **d, e** Detection of DNA extracted from *D. helianthi* mycelia spiked healthy sunflower stems using RPA/CRISPR-Cas12a-based fluorescence detection and lateral flow assay. **f** The fluorescence signals at 30 min detection time. **g, h** Detection of DNA extracted from *D. helianthi* mycelia spiked healthy seed coat using RPA/CRISPR-Cas12a-based fluorescence detection and lateral flow assay. **i** The fluorescence signals at 30 min detection time. m1 – m3 indicated 40 µL, 20 µL, and 10 µL of *D. helianthi* mycelia in sterile water, respectively; m1 + leaf, mixture of leaf powder and 40 µL *D. helianthi* mycelia in sterile water; m2 + leaf, a mixture of leaf powder and 20 µL *D. helianthi* mycelia in sterile water; m3 + leaf, a mixture of leaf powder and 10 µL *D. helianthi* mycelia in sterile water; m1 + stem, a mixture of stem powder and 40 µL *D. helianthi* mycelia in sterile water; m2 + stem, a mixture of stem powder and 20 µL *D. helianthi* mycelia in sterile water; m3 + stem, a mixture of stem powder and 10 µL *D. helianthi* mycelia in sterile water. m1 + seed coat, a mixture of seed coat powder and 40 µL *D. helianthi* mycelia in sterile water; m2 + seed coat, a mixture of seed coat powder and 20 µL *D. helianthi* mycelia in sterile water; m3 + seed coat, a mixture of seed coat and 10 µL *D. helianthi* mycelia in sterile water. T, test line; C, control line

spiked stems was between 85.1% and 88.9% of that of *D. helianthi* mycelia (Fig. 6f). However, the sunflower seed coat had more interference with the detection results, and the fluorescence intensity of the spiked seed coat was between 51.6% and 55.9% of that of D. helianthi mycelia (Fig. 6i).The stem has the least interference with *D. helianthi*.

The inoculated sunflower seedlings were observed and collected at 5, 10, and 15 dpi, respectively (Fig. 7). The canker on the stem aggravated over time, and the nucleic acids extracted from the symptomatic stems were detected using RPA/CRISPR-Cas12a and real-time PCR detection method. The RPA/CRISPR-Cas12a-based fluorescence method could detect positive results for

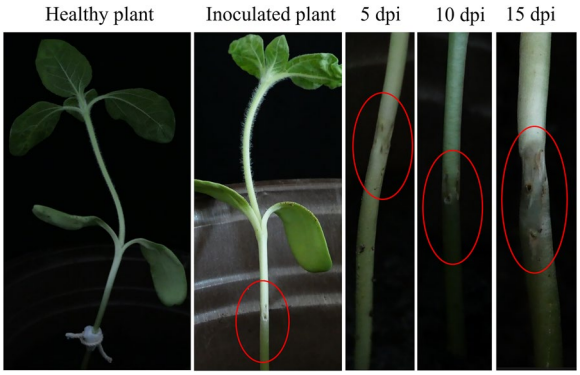

Fig. 7 Symptom development of sunflower seedlings after inoculation with *D. helianthi* spores

D. helianthi in all the diseased sunflower stems (3 repetitions) at 5 dpi and later time (Fig. 8a), while RPA/CRIPSR-Cas12a-based lateral flow assay and real-time PCR could detect positive results for *D. helianthi* in the diseased sunflower stems (3 repetitions) at 10 dpi and later time (Fig. 8b, c).

Discussion

Traditional identification methods for plant pathogenic fungi (Chen et al. 2011) require expensive devices and technical expertise (Borah et al. 2018), and are not suitable for field testing in fields and ports (Raja et al. 2017). Rapid and efficient diagnosis of *D. helianthi* in plant tissues is crucial for controlling the spread of pathogens and early disease management.

The current nucleic acid-based detection methods utilize the internal transcribed spacer (ITS) (Chen et al. 2012), calmodulin (*Cal*) (Qian et al. 2021), and translation elongation factor-1 α (*EF1-α*) (Elverson et al. 2020) as the target gene to detect *D. helianthi*. The ITS region is the most widely sequenced DNA region in fungi, occurs in one to several copies (Iwen et al. 2002), and has often been used as the target gene (Bokulich and Mills 2013). However, the ITS genes of closely related species within the same genus exhibit limited differences due to the high conservative. In this study, the primer targeting calmodulin exhibited the highest sensitivity among the three target genes,

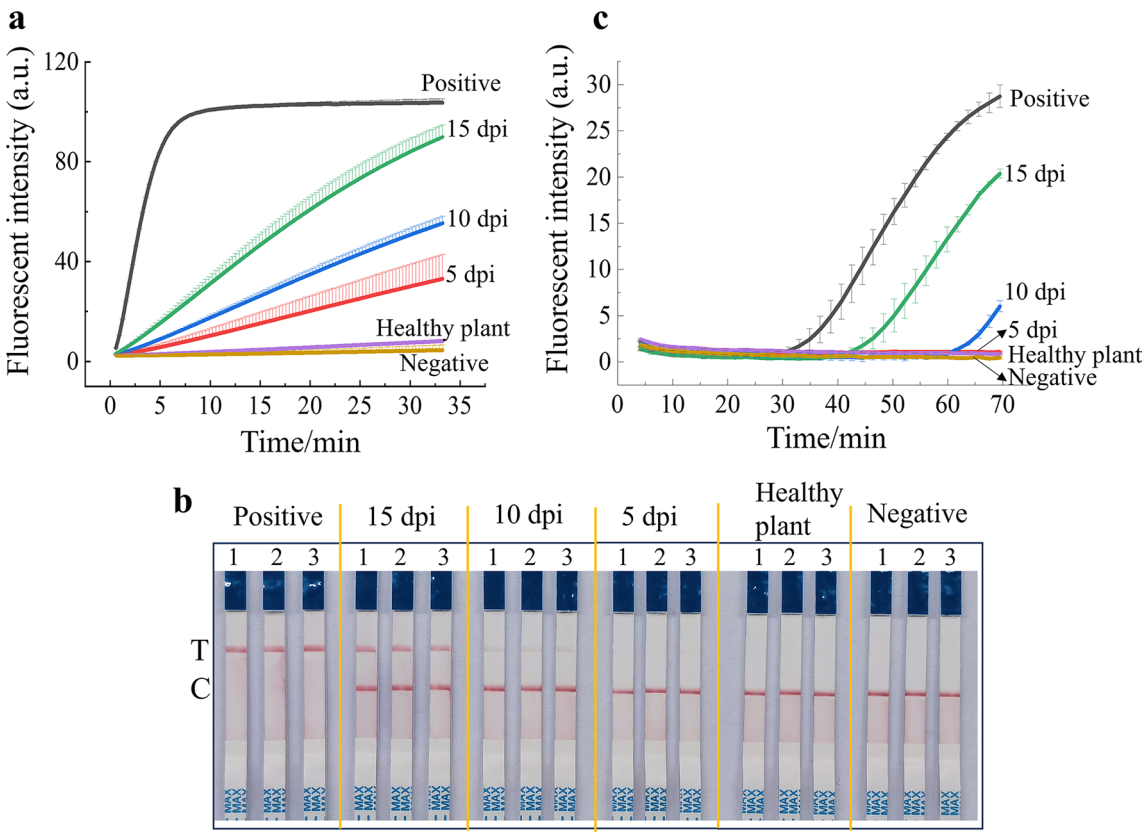

Fig. 8 Detection of DNA extracted from *D. helianthi* inoculated sunflower stems. **a** RPA/CRISPR-Cas12a fluorescence detection method. **b** RPA/CRISPR-Cas12a lateral flow assay method. **c** Real-time fluorescence PCR method. Positive, *D. helianthi* DNA; Negative, sterile water. Numbers 1, 2, 3 indicated the three repetitions. T, test line; C, control line

indicating that the *Cal* gene may have more copies and conserved sites in the *D. helianthi* genomic DNA.

As summarized in Additional file 2: Table S2, although TaqMan real-time PCR has high sensitivity, it requires a temperature higher than 94 °C and thermocycling conditions, which compromise its feasibility for field assay. To overcome the limitations of thermocycling requirements, both recombinase-based isothermal amplification technique and CRISPR-Cas12a reaction system performing at a constant temperature between 37 °C and 42 °C were combined to develop portable detection methods. The required temperature can be achieved in various ways, e. g., warm water, an incubator, and even the human body.

Because of the collateral cleavage activity of the Cas12 enzyme, ssDNA modified with different molecules is used to develop different detection ways. RPA/CRISPR-Cas12a-based fluorescence detection, utilizing FAM-ssDNA-BHQ1 as a reporter, exhibits high sensitivity and requires excitation light and emission light recorder. In addition to the real-time PCR instrument in the laboratory, a portable real-time fluorescence detector (Lei et al. 2019) can also be used for field and port detection. Emitted fluorescence signals can be end-point detected using a portable blue light to observe the results and establish a visual detection (Additional file 1: Figure S2). Lateral flow strip detection does not require cumbersome laboratory equipment and has low environmental requirements. Furthermore, the test results can be directly observed with the naked eye, making it more suitable for field detection.

Our RPA/CRISPR-Cas12a detection method has higher sensitivity or equivalent sensitivity compared to the previously reported real-time PCR method (Elverson et al. 2020). The RPA/CRISPR-Cas12a fluorescence detection can detect *D. helianthi* in the stems of inoculated *D. helianthi* at 5 dpi. Meanwhile, this study successfully detected *D. helianthi* in simulated actual fungus-carrying samples by mixing *D. helianthi* mycelia with sunflower tissues, such as leaf, stem, and seed coat. Amongst, the stem has the least interference with the detection of *D. helianthi* in plant tissues. Because the stem is the main infection site of *D. helianthi*, and the canker in a stem is easily observed, we suggested the stem as the best sampling object. Compared with other nucleic acid detection technologies, RPA/CRISPR-Cas12a has advantages in sensitivity, short reaction time, high efficiency, and simplicity. Coupled with the fast extraction of nucleic acid, it can achieve the rapid detection of pathogens in field samples, providing a new technology for port quarantine and early on-site diagnosis.

Conclusions

In conclusion, a rapid, specific, sensitive and visual method based on RPA/CRISPR-Cas12a for detecting *D. helianthin* has been developed. The flexible signal reading modes, including lateral flow strip signal readout and fluorescent signal readout, can well meet the portable requirements of field testing with the employment of portable constant-temperature metal incubator. The developed RPA/CRISPR-Cas12a system for *D. helianthin* has high efficiency and its detection limit is significantly lower than that of the real-time PCR method.

Methods

Materials and reagents

TwistAmp®Basic kit (Cat. No. TABAS03-KIT) and magnesium acetate (280 mmol/L in water) were purchased from TwistDX Co. (Cambridge, UK). PerfectStartII Probe qPCR SuperMix UDG kits (Cat. No. AQ712-01) were purchased from TransGen Biotech Co. Ltd. (Beijing, China). A Qubit® 2.0 Fluorometer and the Qubit® dsDNA HS Assay kit (Cat. No. Q33231) were purchased from Invitrogen (Life Technologies, Carlsbad, CA). A Milli-Q water purification system was obtained from Millipore Corp. (Merck KGaA, Darmstadt, Germany). The ddH_2O obtained from a Milli-Q water purification system was autoclaved for 20 min at 120 °C. Plant DNA extraction kit with magnetic beads (Cat. No. DP342), Rapid DNA Extraction Kit (Cat. No. KG203), DNase/RNase-free water (Cat. No. RT121), and RNase inhibitor (Cat. No. NG209) were purchased from Tiangen Biotech Co. Ltd. (Beijing, China). EnGen®LbaCas12a (Cpf1) (Cat. No. M0653T) and NEBuffer 3.1 (100 mmol/L NaCl, 50 mmol/L Tris–HCl, 10 mmol/L $MgCl_2$, 100 μg/mL) (Cat. No. B6003V) were purchased from New England Biolabs Inc. (Ipswich, MA, USA). Dithiothreitol (DTT) (Cat. No. 43816) was purchased from Sigma Aldrich (St. Louis, MO, USA). The real-time fluorescent signals were recorded with a Roche LightCycler 480 (Roche, USA). The portable incubator was purchased from Chushengdu Co. Ltd. (Chongqing, China). FQ-DNA (FQ reporter: FAM-CCACCC-BHQ1) and LF-DNA (LF reporter: FAM- TTTTTTTTTT-Biotin) were synthesized by Sangon Biotech (Shanghai, China). The detailed sequences are listed in Additional file 2: Table S1.

RPA primer design and screening

The ITS gene, *Cal* gene, and *EF1-α* gene of *D. helianthi* and its closely related were downloaded from the National Center for Biotechnology Information (NCBI) database and aligned to design specific primers for *D. helianthi*. Four pairs of primers were designed to target the corresponding genes (Additional file 2: Table S1), and

the amplicons of recombinase polymerase amplification were separated by 1.5% agarose gel electrophoresis to test the appropriate primers. Primers were synthesized by Sangon Biotech (Shanghai) Co., Ltd.

DNA extraction from *D. helianthi* mycelia or plant samples

The mycelia grown on PDA were collected and ground in liquid nitrogen, and the genomic DNA was extracted using a plant DNA extraction kit with magnetic beads. For plant samples, the stems with suspicious canker or withered leaves or discolored, wrinkled, incomplete, moldy seeds (about 500 mg) were ground into a powder in liquid nitrogen with a mortar and pestle, transferred into a 2 mL tube, and the DNA was extracted using the plant DNA extraction kit with magnetic beads according to the manufacturer's instructions. The extracted pure DNA was quantified using Qubit®2.0 Fluorometer with a Qubit®dsDNA HS Assay kit and stored at −20 °C until further use.

Fast DNA extraction was performed with the Rapid DNA Extraction Kit. First, inoculated plant tissues were collected in a 1.5 mL microcentrifuge tube, then they were immersed in lysis buffer (buffer 1) and ground with a plastic pestle for about 30 s to fully release the DNA. Next, protein precipitation solution (buffer 2) was added and mixed thoroughly. Finally, the solution was centrifuged or left to stand at room temperature for several minutes, then 1 µL of the supernatant was detected with specific *D. helianthi* primers, using fluorescence or lateral flow strip detection.

Recombinase polymerase amplification

The RPA reaction buffer containing 29.5 µL of supplied rehydration buffer, 11.2 µL of nuclease-free water, 2.4 µL of 10 µmol/L forward primer, 2.4 µL of 10 µmol/L reverse primer, 2 µL of DNA, and 2.5 µL of 280 mmol/L magnesium acetate was added to the enzyme pellet and was kept at 37 °C in a portable heating instrument for 25 min.

CRISPR-Cas12a-based fluorescence detection

The Cas12a-mediated fluorescent assay buffer contained 13.4 µL of nuclease-free water, 2 µL of NEBuffer 3.1, 0.4 µL of LbCas12a (5 µmol/L), 0.5 µL of RNase inhibitor (40 U/µL), 0.5 µL of DTT (0.1 mol/L), 0.4 µL of Dh-crRNA (10 µmol/L), 0.8 µL of FL reporter FQ-DNA (5 µmol/L), and 2 µL of RPA reaction product. All components were thoroughly mixed by pipetting up and down, then put in a real-time fluorescent PCR instrument at 37 °C to record the fluorescence signals.

CRISPR-Cas12a-based lateral flow strip detection

The Cas12a-mediated lateral flow strip assay buffer contained 13.4 µL of nuclease-free water, 2 µL of NEBuffer 3.1 (10×), 0.4 µL of LbCas12a (5 µmol/L), 0.5 µL of RNase inhibitor (40 U/µL), 0.5 µL of DTT (0.1 mol/L), 0.4 µL of Dh-crRNA (10 µmol/L), 0.8 µL of LF reporter LF-DNA (5 µmol/L). The mixture was added with 2 µL of RPA amplification product and put in a portable heating instrument at 37 °C for 20 min. The reaction buffer was diluted with 80 µL of ddH_2O, and detected with a lateral flow strip to observe the test line and control line.

Specificity and sensitivity of RPA/CRISPR-Cas12a-based detection method

The specificity of the RPA /CRISPR-Cas12a-based detection method was evaluated using the DNA extracted from *D. helianthi*, *Leptosphaeria lindquistii*, *Leptosphaeria maculans*, *Leptosphaeria biglobosa*, *Diaporthe phaseolorum* var. *caulivora*, *Diaporthe phaseolorum* var. *meridionalis*, *Sclerotinia sclerotiorum*, *Verticillium dahlia,* and healthy sunflower leaves. Each sample was detected three times, and sterile water was used as a negative control.

The *D. helianthi* genomic DNA (Genome size: 63.67 Mb) was tenfold serially diluted from 10 ng/µL (1.4×10^5 copies/µL) to 0.1 pg/µL (1.4 copies/µL), and was detected using the optimized RPA/CRISPR-Cas12a detection method.

The real-time PCR was used as the gold standard to evaluate RPA/CRISPR-Cas12a detection method. The primers Dh4_F/Dh4_R and Dh4_P (Additional file 2: Table S1) (Elverson et al. 2020) were synthesized by General Biosystems (Anhui) Corporation. The 20 µL real-time PCR reaction contained 10 µL 2×TaqMan PCR Master Mix, 0.4 µL Dh4_F (10 µmol/L), 0.4 µL Dh4_R (10 µmol/L), 0.2 µL Dh4_P (10 µmol/L), 1 µL DNA, and 8 µL nuclease-free water. The condition of the real-time PCR reaction is as follows: preincubation using 50 °C for 2 min and 95 °C for 10 min, 40 cycles for 95 °C for 15 s and 60 °C for 1 min.

Application of RPA/CRISPR-Cas12a assay for plant samples

To verify the feasibility of the detection method for plant samples, 200 mg of *D. helianthi* mycelia was ground in liquid nitrogen and dispersed in 400 µL of water. Then, 20, 40, and 60 µL *D. helianthi* mycelia in water were added to 0.2 g of healthy sunflower leaves, stems, and seed coat, respectively. RPA/CRIPSR-Cas12a-based fluorescence method and lateral flow strip method were used to detect *D. helianthi* in healthy plant tissues.

Furthermore, the RPA/CRISPR-Cas12a assay for plant samples was evaluated using inoculated and infected sunflower plants. Healthy sunflower seeds were grown in the greenhouse at 25°C with 12 h light/12 h dark, and the plants with 3 leaves were inoculated with *D. helianthi* spore suspension. The *D. helianthi* cultured on PDA surface was gently scraped off, dispersed in sterilized deionized water, and filtered with sterilized filter paper to obtain spores. The filtrated spores were dispersed in sterile water, and the concentration of suspended spores was measured with a hemocytometer, then adjusted to 1×10^7 conidia/mL with sterile water. The stems of healthy sunflower plants were inoculated using the acupuncture method. Briefly speaking, the stems of sunflower was wounded with a sterilized syringe needle and then perfused with the spore suspension. Sterile water was used as a negative control. The inoculation area was kept moist with a wet cotton wool covering the wound. Sunflower plant tissues were collected and stored at −80 °C at 5, 10, and 15 days post-inoculation (dpi), respectively. Three plants were collected for each group. All the samples were detected using RPA/CRISPR-Cas12a and real-time PCR.

Abbreviations

CRISPR-Cas	CRISPR-associated enzyme systems
Cal	Calmodulin
Cas12VDet	Cas12a-based visual detection
Dh	*Diaporthe helianthi*
DETECTR	DNA endonuclease-targeted CRISPR trans-reporter
dpi	Days post inoculation
EF1-α	Translation elongation factor-1α
ITS	Internal transcribed spacer
PAM	Protospacer-adjacent motif
PCR	Polymerase chain reaction
RPA	Recombinase polymerase amplification

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s42483-024-00310-4.

Additonal file 1: Figure S1. Comparison of the specificity of different *D. helianthi* primer sets based on different gene sequences. **Figure S2. a** Sensitivity evaluation of visual detection of RPA/CRISPR-Cas12a. **b** Specificity of visual detection of RPA/CRISPR-Cas12a.

Additional file 2: Table S1. Primers and sequences involved in this study. **Table S2.** Comparison of different detection methods for *D. helianthin*.

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Author contributions
RK and RL contributed equally to this work. RL and RK designed, performed the majority of the experiments, analyzed data, and wrote original draft. ML, XS, YY, JS, and LY performed the minority of experiments and validated the method. WD provided resources and validated the assay. ML and LL investigated and validated the assay. WS and ZF reviewed the manuscript. WY critically reviewed, and edited the final manuscript. All authors read and approved the manuscript.

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Availability of data and materials
The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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References

Adeleke BS, Babalola OO. Oilseed crop sunflower (*Helianthus annuus*) as a source of food: nutritional and health benefits. Food Sci Nutr. 2020;8(9):4666–84. https://doi.org/10.1002/fsn3.1783.

Bokulich NA, Mills DA. Improved selection of internal transcribed spacer-specific primers enables quantitative, ultra-high-throughput profiling of fungal communities. Appl Environ Microbiol. 2013;79(8):2519–26. https://doi.org/10.1128/aem.03870-12.

Boonham N, Glover R, Tomlinson J, Mumford R. Exploiting generic platform technologies for the detection and identification of plant pathogens. Eur J Plant Pathol. 2008;121(3):355–63. https://doi.org/10.1007/s10658-008-9284-3.

Borah N, Albarouki E, Schirawski J. Comparative methods for molecular determination of host-specificity factors in plant-pathogenic fungi. Int J Mol Sci. 2018;19(3):863. https://doi.org/10.3390/ijms19030863.

Chen Y, Zhang XL, Wang C, Liu B, Wang WZ. Influence of different nutrients and culture condition on growth of *Phomopsis helianthi*. Xinjiang Agric Sci. 2011;48(11):2054–60.

Chen Y, Zhang XL, Wang WZ, Liu B. Research on Detection of *Phomopsis helianthi* by PCR. Xinjiang Agric Sci. 2012;49(3):470–6. https://doi.org/10.6048/j.issn.1001-4330.2012.03.011.

Chen JS, Ma EB, Harrington LB, Da Costa M, Tian XR, Palefsky JM, et al. CRISPR-Cas12a target binding unleashes indiscriminate single-stranded DNase activity. Science. 2018;360(6387):436–9. https://doi.org/10.1126/science.aar6245.

Chen Y. Pest risk analysis for the importation sunfower and research on the rapid detection search on *phomopsis helianthi*. Master thesis, Xinjiang Agricultural University. 2012. Wulumuqi, Xinjiang. https://doi.org/10.7666/d.D236755

Cvejic S, Locic S, Mladenovic E. Inheritance of floral colour and type in four new inbred lines of ornamental sunflower (*Helianthus annuus* L.). J Horticult Sci Biotechnol. 2016;91(1):30–5. https://doi.org/10.1080/14620316.2015.1110989.

Dai YF, Somoza RA, Wang L, Welter JF, Li Y, Caplan AI, et al. Exploring the trans-cleavage activity of CRISPR-Cas12a (cpf1) for the development of a universal electrochemical biosensor. Angew Chem Int Edit. 2019;58(48):17399–405. https://doi.org/10.1002/anie.201910772.

Debaeke P, Estragnat A. Crop canopy indicators for the early prediction of Phomopsis stem canker (*Diaporthe helianthi*) in sunflower. Crop Prot. 2009;28(9):792–801. https://doi.org/10.1016/j.cropro.2009.04.011.

Degener J, Melchinger AE, Hahn V. Resistance in the leaf and stem of sunflower after infection with two isolates of Phomopsis. Plant Breed. 1999;118(5):405–10. https://doi.org/10.1046/j.1439-0523.1999.00410.x.
Dronina J, Samukaite-Bubniene U, Ramanavicius A. Towards application of CRISPR-Cas12a in the design of modern viral DNA detection tools (Review). J Nanobiotechnol. 2022;20(1):41. https://doi.org/10.1186/s12951-022-01246-7.
Duan XY, Ma WD, Jiao ZY, Tian YY, Ismail RG, Zhou T, et al. Reverse transcription-recombinase-aided amplification and CRISPR/Cas12a-based visual detection of maize chlorotic mottle virus. Phytopathol Res. 2022;4:23. https://doi.org/10.1186/s42483-022-00128-y.
Elverson TR, Kontz BJ, Markell SG, Harveson RM, Mathew FM. Quantitative PCR assays developed for *Diaporthe helianthi* and *Diaporthe gulyae* for phomopsis stem canker diagnosis and germplasm screening in sunflower (*Helianthus annuus*). Plant Dis. 2020;104(3):793–800. https://doi.org/10.1094/pdis-09-19-1827-re.
Gulya T, Harveson R, Mathew F, Block C, Thompson S, Kandel H, et al. Comprehensive disease survey of US sunflower: disease trends, research priorities and unanticipated impacts. Plant Dis. 2019;103(4):601–18. https://doi.org/10.1094/pdis-06-18-0980-fe.
Hussain M, Farooq S, Hasan W, Ul-Allah S, Tanveer M, Farooq M, et al. Drought stress in sunflower: physiological effects and its management through breeding and agronomic alternatives. Agric Water Manag. 2018;201:152–66. https://doi.org/10.1016/j.agwat.2018.01.028.
Iwen PC, Hinrichs SH, Rupp ME. Utilization of the internal transcribed spacer regions as molecular targets to detect and identify human fungal pathogens. Med Mycol. 2002;40(1):87–109. https://doi.org/10.1080/714031073.
Kang HX, Peng Y, Hua KY, Deng YF, Bellizzi M, Gupta DR, et al. Rapid detection of wheat blast pathogen magnaporthe oryzae triticum pathotype using genome-specific primers and Cas12a-mediated technology. Engineering. 2021;7(9):1326–35. https://doi.org/10.1016/j.eng.2020.07.016.
Kaya Y, Jocic S, Miladinovic D. Sunflower. In: Gupta S, editor. Technological innovations in major world oil crops, vol. 1. New York: Springer; 2012.
Lei R, Kong J, Qiu YH, Chen NZ, Zhu SF, Wang XY, et al. Rapid detection of the pathogenic fungi causing blackleg of *Brassica napus* using a portable real-time fluorescence detector. Food Chem. 2019;288:57–67. https://doi.org/10.1016/j.foodchem.2019.02.089.
Lei R, Li Y, Li LM, Wang JY, Cui ZH, Ju R, et al. A CRISPR/Cas12a-based portable platform for rapid detection of *Leptosphaeria maculans* in Brassica crops. Front Plant Sci. 2022;13:976510. https://doi.org/10.3389/fpls.2022.976510.
Li XL, Duan WJ. Identification of quarantine fungus *Diaporthe helianthi* from soybean seeds imported from the United States. Chin J Oil Crop Sci. 2020;42(2):292. https://doi.org/10.19802/j.issn.1007-9084.2019238.
Li J, Macdonald J, von Stetten F. Review: a comprehensive summary of a decade development of the recombinase polymerase amplification. Analyst. 2019;144(1):31–67. https://doi.org/10.1039/c8an01621f.
Liang MD, Li ZL, Wang WS, Liu JK, Liu LS, Zhu GL, et al. A CRISPR-Cas12a-derived biosensing platform for the highly sensitive detection of diverse small molecules. Nat Commun. 2019;10(1):3672. https://doi.org/10.1038/s41467-019-11648-1.
Martinelli F, Scalenghe R, Davino S, Panno S, Scuderi G, Ruisi P, et al. Advanced methods of plant disease detection. A review. Agron Sustain Dev. 2015;35(1):1–25. https://doi.org/10.1007/s13593-014-0246-1.
Mathew FM, Alananbeh KM, Jordahl JG, Meyer SM, Castlebury LA, Gulya TJ, et al. Phomopsis stem canker: a reemerging threat to sunflower (*Helianthus annuus*) in the United States. Phytopathology. 2015;105(7):990–7. https://doi.org/10.1094/phyto-11-14-0336-fi.
Pecchia S, Mercatelli E, Vannacci G. Intraspecific diversity within *Diaporthe helianthi*: evidence from rDNA intergenic spacer (IGS) sequence analysis. Mycopathologia. 2004;157(3):317–26. https://doi.org/10.1023/B:MYCO.0000024185.66158.7e.
Qian YK, Wei S, Huang FY, Chen YQ, Chen WM. Recombinase polymerase amplification (RPA) for the detection of *Diaporthe helianthi*. Plant Quar. 2021;35(2):39–43. https://doi.org/10.19662/j.cnki.issn1005-2755.2021.02.004.
Qian YK, Wei S, Zhang N, Yang QQ, Chen WM, Zhang XQ, et al. Rapid detection of 5 fungal diseases in sunflower (*Helianthus annuus*) using dual priming oligonucleotide system-based multiplex PCR and capillary electrophoresis. SLAS Technol. 2022;27(4):253–60. https://doi.org/10.1016/j.slast.2022.03.004.
Raja HA, Miller AN, Pearce CJ, Oberlies NH. Fungal identification using molecular tools: a primer for the natural products research community. J Nat Prod. 2017;80(3):756–70. https://doi.org/10.1021/acs.jnatprod.6b01085.
Rauf S, Jamil N, Tariq SA, Khan M, Kausar M, Kaya Y. Progress in modification of sunflower oil to expand its industrial value. J Sci Food Agric. 2017;97(7):1997–2006. https://doi.org/10.1002/jsfa.8214.
Ray M, Ray A, Dash S, Mishra A, Achary KG, Nayak S, et al. Fungal disease detection in plants: traditional assays, novel diagnostic techniques and biosensors. Biosens Bioelectron. 2017;87:708–23. https://doi.org/10.1016/j.bios.2016.09.032.
Says-Lesage V, Roeckel-Drevet P, Viguie A, Tourvieille J, Nicolas P, de Labrouhe DT. Molecular variability within *Diaporthe/Phomopsis helianthi* from France. Phytopathology. 2002;92(3):308–13. https://doi.org/10.1094/phyto.2002.92.3.308.
van Dongen JE, Berendsen JTW, Steenbergen RDM, Wolthuis RMF, Eijkel JCT, Segerink LI. Point-of-care CRISPR/Cas nucleic acid detection: recent advances, challenges and opportunities. Biosens Bioelectron. 2020;166:112445. https://doi.org/10.1016/j.bios.2020.112445.
Viguie A, Masirevic S, Vear F, Grezes-Besset B, de Labrouhe DT. Comparison of aggressive isolates of *Phomopsis/Diaporthe helianthi* (which causes phomopsis in sunflowers) of French and Yugoslav origin. OCL-Ol Corps Gras Lipid. 1999;6(3):267–72.
Wang B, Wang R, Wang DQ, Wu J, Li JX, Wang J, et al. Cas12aVDet: a CRISPR/Cas12a-based platform for rapid and visual nucleic acid detection. Anal Chem. 2019;91(19):12156–61. https://doi.org/10.1021/acs.analchem.9b01526.
Wang YQ, Ke YQ, Liu WJ, Sun YQ, Ding XT. A one-pot toolbox based on Cas12a/crRNA enables rapid foodborne pathogen detection at attomolar level. ACS Sens. 2020;5(5):1427–35. https://doi.org/10.1021/acssensors.0c00320.
Ward E, Foster SJ, Fraaije BA, McCartney HA. Plant pathogen diagnostics: immunological and nucleic acid-based approaches. Ann Appl Biol. 2004;145(1):1–16. https://doi.org/10.1111/j.1744-7348.2004.tb00354.x.
Xiong Y, Zhang JJ, Yang ZL, Mou QB, Ma Y, Xiong YH, et al. Functional DNA regulated CRISPR-Cas12a sensors for point-of-care diagnostics of non-nucleic-acid targets. J Am Chem Soc. 2020;142(1):207–13. https://doi.org/10.1021/jacs.9b09211.
Zhang WH, Fang L, Luo JF, Li GR. Triplex-PCR molecular detection of two quarantine fungal diseases of sunflower, *Leptosphaeria lindguistii* and *Diaporthe helianthi*. J Southwest Univ. 2013;35(1):10–5.
Zhou L, Peng RX, Zhang R, Li JM. The applications of CRISPR/Cas system in molecular detection. J Cell Mol Med. 2018;22(12):5807–15. https://doi.org/10.1111/jcmm.13925.