Electronic Supplementary Material (ESI) for RSC Advances.

Supporting Information

Visual detection of *Fusarium proliferatum* based on dual-cycle signal amplification and T5 exonuclease

Materials and Apparatus

2, 2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) and hemin were purchased from Sigma-Aldrich Co., Ltd. (Germany). GeneRuler 100 bp Plus, T4 DNA ligase, phi29 DNA polymerase, and dNTP were purchased from Thermo Fisher Scientific Ltd. (Germany). TwistDx/TwistAmp® Liquid Basic was purchased from TwistDx™ Limited (Cambridge, UK). T5 exonuclease was purchased from New England Biolabs Ltd. (Germany). Roti-Safe and water of HPLC grade with an electrical resistance larger than 18 MΩ was obtained from Carl Roth GmbH + Co. KG (Germany). The DNeasy Plant Mini Kit and QIAquick PCR Purification Kit were purchased in QIAGEN Company (Germany). All other reagents were of analytical grade and used without further purification.

Strains of *F. proliferatum DSM62267* (F120)*, F. equiseti RD13* (F216)*, F. culmorum 3.37 dus Bomm* (F109)*, F. avenaceum borman* (F112), and maize flour contaminated by F120 were kindly provided by Professor Petr Karlovsky of the University of Goettingen. *Ralstonia solancearum* and *Puccinia sorghi* were from the lab of the Tobacco Research Institute of Chinese Academy of Agricultural Sciences. Non-contaminated maize flour was purchased in a local supermarket. Four positive samples and 26 field-collected samples were preserved in Plant Protection Research Center of Tobacco Research Institute of Chinese Academy of Agricultural Sciences.

Primer-F and primer-R were developed from the conserved intergenic sequence of the ribosomal RNA gene cluster in species of *F. proliferatum*, which originally were used in PCR amplification[1, 2]. The trans-complementary sequence of the G-quadruplex was added to the 5′-end of primer-F, and the sequence was then at the end of dsDNA of the RPA product. The RPA product was 278 bp in length. The 5′-terminal of probe 1

was the trans-complementary sequence of primer-F (GGGCGGGTTGGG), and the 3′-terminal of probe 1 was the 5′-terminals of primer-R (CAACACGAATCGC). Probe1 could be hybridised with the ends of one strand of RPA product. Probe 2 was designed according to the conserved sequence of *F. proliferatum*, and it could be hybridised with the RPA product. The oligonucleotides used in this assay are included in Supplemental table 1. They were synthesized by Sigma-Aldrich LLC (Germany).

NanoDrop 2000 (Thermo, USA) was used as absorbance measurements in the assay. The absorption spectra of the resulting solution were monitored within wavelength ranges between 390 nm and 490 nm. The RPA reaction was performed in a heating block (HLC Biotech MHR11, Germany). Electrophoresis apparatus and horizontal electrophoresis (1645052 and Mini-Sub Cell GT Systems, Bio-Rad, USA) were used for target sequence detection. Gel pictures were captured using the EASY® Doc plus imaging system of Herolab GmbH (Germany). Final solutions were captured by camera wit a mobile phone (Huawei, China).

Supplemental table 1 Oligonucleotide sequences used for the assay

Name	Sequences (5′ → 3′)
primer-F	CCCAACCCGCCCTACCCCGGCCACCAGAGGATGTG
primer-R	CAACACGAATCGCTTCCTGAC
probe 1	GGGCGGGTTGGGCAACACGAATCGC
probe 2	TCCCAGACCCATCAGCCAGAGA

DNA extraction

Maize flour of contaminated by *F. proliferatum DSM62267* (F120) (0.1 g) and

uncontaminated maize flour (0.1 g) were suspended by 5 times volumes of buffer containing 10 mM Tris-Cl, 1 mM EDTA (pH 8.0). The suspensions were heated in boiled water for 10 min, and cooled down in ice-water, and vortexed for 1 min. The genomic DNA was released from the cell wall of above mentioned fungi. The supernatants (1 μL) were used as DNA template for RPA. The DNeasy Plant Mini Kit was used to extract and purify genomic DNA of F120*,* which was used for detection of sensitivity and selectivity of the assay. Genomic DNA of F216, F109, F112, *Ralstonia solancearum* and *Puccinia sorghi* was also extracted using the DNeasy Plant Mini Kit.

RPA reaction and ligation

RPA reactions were performed in 50 μL volumes containing 25 μL 2 × reaction buffer, 2 μL dNTP (2.5 mM each), 5 μL 10 × basic E-mix, 2.4 μL primer-F (10 μM), 2.4 μL primer-R (10 μM), 2.5 μl 20 × core reaction mix, and 7.2 μL H_2O and mixed gently before the next step. Then 1μL DNA template and 2.5 μL magnesium acetate (280 mM) were added and spun down to the mix by centrifugal force. The reaction started at the same time. It was performed in a heating block at 42 °C for 20 min. The reaction temperature was then increased to 85 °C for 3 min to inactivate the proteins in the system.

For rapid hybridization, 50 ng RPA product and probe 1 (1 μM) (see Supplemental table 1) were mixed and incubated at 95 °C for 3 min, and cooled down to room temperature. The mix was added to a 10 μL 1×T4 DNA ligase buffer (40 mM Tris-HCl, 10 mM $MgCl_2$, 0.5 mM ATP, 10 mM DTT, pH 7.8 at 37 °C), and 5 U T4 DNA ligase was added. The ligation reaction was performed at room temperature for 30 min.

Exonuclease and RCA reactions

After the ligation reaction, linear DNAs in ligated solution were digested into mononucleotides by T5 exonuclease in 5′→3′ direction. The exonuclease reaction was prepared in a 50 μL 1×NEB buffer 4 (50 mM Potassium Acetate, 20 mM Tris-acetate, 10 mM Magnesium Acetate, 1 mM DTT, pH 7.9). Ligated products (10 μL) and T5 exonuclease (10 U) were added, and the reaction was carried out at room temperature for 5 min. The products were purified with the DNeasy Plant Mini Kit.

RCA was performed in a 20 μL 1×phi29 DNA polymerase buffer (33 mM Tris-acetate, 10 mM Mg-acetate, 66 mM K-acetate, 0.1% (v/v) Tween 20, 1 mM DTT, pH 7.9 at 37 °C). 10 U phi29 DNA polymerase, 1 μL probe 2 (see Supplemental table 1), 3 μL dNTP and 1μL of purified circular ssDNA were introduced into the buffer. The reaction was performed at 30 °C for 30 min.

Oxidation reaction of $ABTS^{2-}$ and H_2O^2 catalyzed by DNAzyme of G-quadruplex

Hemin (0.6 μM, final concentration) and the final solution were mixed in buffer (50 mM Tris-HCl, 150 mM NH_4Cl, pH 7.9, 200 μL), and incubated for 60 minutes at room temperature. $ABTS^{2-}$ and H_2O_2 were then added (2 mM, final concentration), and incubated for 30 min. The specific green color in the final solution was observed and captured by mobile phone. NanoDrop 2000 was used to scan UV-Vis absorptions of these solutions.

Optimization of Detection Conditions

To acquire satisfactory analytical performance, some conditions in the assay needed to be optimized, such as concentration of probe 1, reaction time for ligation and RCA reaction. The amount of genomic DNA of F120 was 1 pg.

Probe 1 was used to hybridize with the 5′-terminal and 3′-terminal of one strand of RPA products. For a certain amount of RPA product, higher amount of probe 1 means a larger amount of hybridization of probe 1/RPA products. The absorbance of the final solution increased with the amount of probe 1 (1 μM) from 0.1 to 1 μL, and decreased for more than 1 μL (Fig. S1A). Redundant probe 1 likely hybridized with each terminal of the RPA product, and the ligation rate was decreased, resulting in low absorbance. Therefore, 1 μL of probe 1 was optimal, and used in the following experiments.

The reaction time of ligation was related to the amount of circular ssDNA, and then affected absorbance of the resulting solution. As shown in Fig. S1B, the absorbance increased with reaction time, and reached a plateau after 30 min. So, 30 min of ligation time was the optimal time, and used for the ligation reaction below.

The amount of G-quadruplex DNAzyme depended on the reaction time for RCA. The longer time for RCA, the more G-quadruplex sequences were produced. As shown in Fig. S1 C, the absorbance increased from 5 min to 15 min, and then it was nearly flat after 15 min. Therefore, 15 min of RCA reaction was the optimal time and chosen in the following assay.

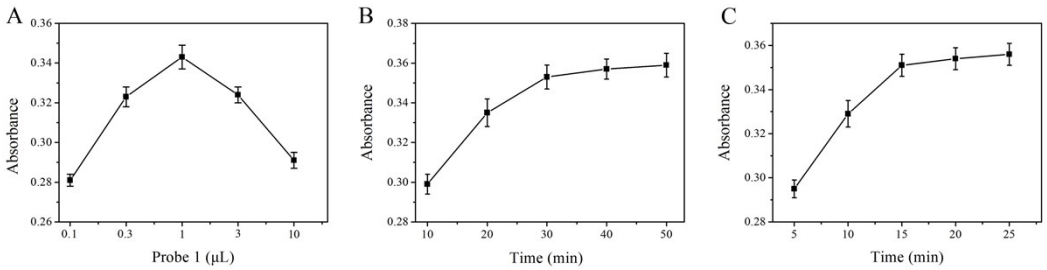

Fig. S1 Optimization of concentration of probe 1 (A), reaction time for T4 DNA ligase (B), and time for RCA reaction (C). The amount of genomic DNA of F120 was 1 pg. Error bars represent the standard deviations of three repeated experiments and the same below.

Reproducibility and stability

The reproducibility of the RPA-RCA-assisted visual strategy was determined. The intra-assay reproducibility was tested every three hours within a day using 1 pg genomic DNA of F120 as template. The relative standard deviation (RSD) value of three times was 3.2 %. The inter-assay reproducibility was tested for three days using 1 pg DNA of *F. proliferatum* each time, and the RSD value was 2.9 %, which showed good reproducibility of the proposed assay. The stability of the assay was monitored by parallel determination of 5 groups with the same amount of F120 DNA. The RSD value of the five groups was 2.8 %, indicating its good stability.

Supplemental table 2**.** Comparison of RPA or RCA-based methods of colorimetric analysis.

Analytical Method	LOD	Ref.
T4 DNA ligase and polynucleotide kinase/phosphatase detection based on RCA and G-quadruplex DNAzyme	3.4×10^{-4} U/mL 3.8×10^{-4} U/mL	[3]
Genotyping method based on allele-specific RPA and colorimetric microarray detection	5-10% genomic SNP	[4]
Fluorometric detection of influenza virus RNA by PCR-coupled rolling circle amplification generating G-quadruplex	4.9 aM	[5]
Rapid detection of *Plasmodium falciparum* with isothermal RPA and lateral flow analysis	100 fg	[6]
New Fpg probe chemistry for direct detection of RPA on lateral flow strips	10-100 copies	[7]
Non-instrumented incubation for the rapid and sensitive detection of proviral HIV-1 DNA	10 copies	[8]
Visual detection of *Fusarium proliferatum* based on asymmetric recombinase polymerase amplification and hemin/G-quadruplex DNAzyme	0.01ng	[9]
Visual detection of *F. proliferatum* based on dual-cycle signal amplification assisted by T5 exonuclease	0.05 pg	This study

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