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**Development and validation of an RPA-CRISPR-Cas12b assay for rapid visual
detection of *Solanum rostratum***

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Abstract

Solanum rostratum is a noxious invasive weed posing risks to crop production and public health. Its seeds are easily concealed in bulk grain commodities, serving as the primary pathway for long-distance dispersal. However, existing PCR-based detection methods require laboratory equipment and lengthy turnaround, and their suitability for rapid on-site seed quarantine remains limited. In this study, we developed and laboratory-validated an RPA-CRISPR/Cas12b assay for detection of *S. rostratum* using leaf DNA and plasmid templates. A reference sequence dataset was constructed from 148 newly generated DNA barcode sequences and publicly available sequences from NCBI and BOLD, covering five marker regions (rbcL, matk, ITS, ITS2, and trnH-psbA). Using the best-performing sgRNA (SR-sgRNA-1) and RPA primer pair (SR-RPA-F1/R1), selected according to fluorescence increase rates, a two-step fluorescence assay was developed. A visual FAM (6-carboxyfluorescein) readout format was also established. Both detection formats were systematically evaluated using plasmid templates and 30 actual samples (14 *S. rostratum* accessions collected across eight Chinese provinces/autonomous regions and 16 non-target *Solanum* species). Both formats detected plasmid DNA down to 10 copies/test. In the tested panel, the assay identified 14/14 *S. rostratum* accessions and 16/16 non-target *Solanum* accessions, with complete concordance between the two formats. This RPA-CRISPR/Cas12b

assay provides a rapid and highly specific detection workflow, effectively addressing the technical bottleneck in seed quarantine and offering a practical solution for source interception of *S. rostratum*.

Keywords: *Solanum rostratum*, RPA CRISPR-Cas12b, visual detection, seed quarantine, invasive alien species

Introduction

Biological invasion has become a major global environmental issue. Among invasive alien species, malignant weeds represent the most direct threat to agricultural production and natural ecosystems in invaded areas, owing to their prolific reproduction, rapid dispersal, and broad ecological adaptability [1].

S. rostratum is native to North America, which is densely covered with conical spines, and contains highly toxic neurotoxic alkaloids such as solanine [2], endangering livestock, crops, and human health. Since its first discovery in Liaoning Province, China, in 1981 [3], the weed has rapidly spread to nine provincial administrative regions, including Heilongjiang, Jilin, Liaoning, Hebei, Shanxi, Beijing, Inner Mongolia, Xinjiang, and Ningxia. Ecologically, it can outcompete and displace over 30% of native plant species [4] and strongly modifies soil microbial communities, which in turn enhances its own nutrient uptake and creates a self-accelerating invasion cycle [5]. Agriculturally, *S. rostratum* damages maize, cotton, and soybean directly, with potential losses to China's maize sector estimated at 2.937–35.083 billion RMB [6], and also acts as a wild primary host for the Colorado potato beetle (*Leptinotarsa decemlineata*) and as an intermediate host for several viruses and plant-parasitic nematodes, thereby worsening the spread of quarantine pests and pathogens.

S. rostratum seeds measure about 2.5 mm by 2.0 mm, are dark brown to black, and bear honeycomb-like surface pits, which allows them to contaminate bulk commodities such as imported soybean and maize. Once mixed seeds enter China with imported grain, whether through spillage during processing and transportation or as discarded byproducts in field environments, they may establish new invasive populations in new areas. Thus, seeds constitute the key vehicle by which this weed

crosses geographic barriers and disperses over long distances. Their inconspicuousness during port inspection makes seed quarantine the weakest and most urgent link in the interception chain. Shifting from a "passive response" to "source interception" therefore requires breaking the technical bottleneck in seed quarantine.

At the molecular level, considerable progress has been achieved. A specific PCR method based on GBSSI gene sequence differences can accurately distinguish *S. rostratum* from closely related solanaceous species within one working day [7]. DNA barcoding (e.g., ITS sequences) and microsatellite markers have provided reliable tools for identifying samples lacking complete morphological characteristics, such as seeds and seedlings, and for population genetic structure analysis [8-10]. The successful decoding of the *S. rostratum* genome (898.42 Mb) in 2023 further provides a data foundation for developing new molecular detection targets [11].

At the field monitoring level, the deep learning model TrackSolanum attained 94.2% precision and 96.5% recall in UAV imagery [12], while a lightweight model derived from YOLO v5 can run on edge devices for vehicle-mounted real-time detection [13]. Paired with UAV hyperspectral remote sensing (accuracy >96%), these tools have begun to form a three-tier surveillance network: satellite census, UAV detailed survey, and ground verification.

However, existing detection technologies still face many practical bottlenecks. Although molecular detection offers high accuracy, it relies on laboratory conditions and specialised equipment, is time-consuming, and cannot meet the timeliness requirements of rapid port clearance. Although UAV remote sensing provides broad coverage, its ability to identify sparsely distributed plants or those morphologically similar to associated vegetation is limited, and it is constrained by weather and lighting conditions [12]; moreover, it is only applicable to monitoring at the adult plant stage.

To address these bottlenecks, a nucleic acid amplification technique is needed that combines high sensitivity, high specificity, simple operation, and suitability for on-site detection. RPA, first described by Piepenburg et al. [14], is an isothermal method that

amplifies targets within 20–30 minutes at a constant 37–42°C, eliminating the need for thermal cycling [15–17]. RPA is simpler to operate, faster, less dependent on sophisticated equipment, and more tolerant of crude samples; consequently, crude or minimally processed samples can often be used directly as DNA templates after simple lysis or dilution [15,18]. In recent years, combining RPA with CRISPR/Cas systems has markedly enhanced detection through dual recognition [19], achieving single-copy sensitivity [20–21]. RPA has already been used to detect diverse invasive organisms, including *Liposcelis bostrychophila* [22], *Trogoderma granarium* [23], and *Tuta absoluta* [24].

Effective RPA assays depend on selecting short, species-specific genomic regions, typically 100–200 bp in length, flanked by conserved primer-binding sites [14, 25]. In *Solanum* DNA barcoding studies, coding genes (rbcl, matk), non-coding intergenic spacers (trnH-psbA, ndhF, trnS-trnG), nuclear genes (ITS/ITS2), and supplementary nuclear genes (waxy/GBSSI) have all been evaluated as candidate markers, each with its own advantages and limitations in terms of variation density, flanking conservation, and amplification success [7, 8, 10,26, 27–32]. However, existing studies have mostly targeted conventional PCR or sequencing applications, and a systematic comparison and evaluation specifically addressing RPA primer design requirements is lacking.

Based on the above analysis, this study aims to develop an RPA-based rapid detection method for *S. rostratum* seeds, with specific objectives: (1) to select a candidate barcode region suitable for RPA primer design in *S. rostratum*; (2) to design specific primers suitable for RPA amplification and optimize the reaction system; and (3) to evaluate the sensitivity, specificity, and laboratory performance of the method. The present study adds an RPA-CRISPR/Cas12b assay to the growing toolkit for field-compatible detection of invasive weeds.

Methods

Plant materials and sampling

To evaluate the specificity of the RPA-CRISPR assay developed in this study, a panel of

30 *Solanum* accessions was assembled. The target weed *S. rostratum* was collected from 14 natural populations in eight Chinese provinces/autonomous regions (Heilongjiang, Jilin, Liaoning, Beijing, Hebei, Shanxi, Inner Mongolia, and Xinjiang). Non-target taxa for specificity testing included 16 closely related *Solanum* species (Table 1).

All plant materials were formally identified by Dr. Weidong Fu and Dr. Guoliang Zhang. Both are experienced plant taxonomists with expertise in the identification of invasive *Solanum* species. Identification was based on diagnostic morphological characters following Flora of China and standard Solanaceae taxonomic keys.

Voucher specimens for all 30 accessions are maintained at the Invasive Alien Plant Laboratory, Institute of Environment and Sustainable Development in Agriculture, Chinese Academy of Agricultural Sciences, Beijing, China. All accessions have corresponding voucher numbers, which are listed in Table 1.

Young leaves were harvested, immediately snap-frozen in liquid nitrogen, and maintained at -80°C until DNA extraction. All experimental research on plants and field studies complied with institutional, national, and international guidelines. Collection activities were conducted in accordance with local legislation of the People's Republic of China.

Construction of target sequence datasets

DNA barcode amplification and sequencing

Leaf genomic DNA was isolated using a modified CTAB method involving chloroform-isoamyl alcohol extraction [33]. Using the extracted genomic DNA as template, PCR amplification of five barcoding regions was performed on the 30 plant accessions. Each 25 μL reaction contained 1 μL template DNA, 1 μL each of forward and reverse primers, 12.5 μL of 2 $\times$ Taq Master Mix (Tiangen, ET101), and 9.5 μL ddH₂O. The primer pairs and thermal profiles were as follows:

rbcl: rbcLa-F (ATGTCACCACAAACAGAGACTAAAGC) and rbcLa-R (GTAAATCAAGTCCACCRG); 95 $^{\circ}\text{C}$ for 4 min; 35 cycles of 94 $^{\circ}\text{C}$ for 30 s, 55 $^{\circ}\text{C}$ for 1 min, and 72 $^{\circ}\text{C}$ for 1 min; final extension at 72 $^{\circ}\text{C}$ for 10 min [34].

matk: primers matk-1RKIM-f (ACCCAGTCCATCTGGAAATCTTGTTTC) and matk-3FKIM-r

(CGTACAGTACTTTTGTGTTTACGAG); annealing temperature 52 °C, otherwise as above [35].

ITS2: ITS_S2F (ATGCGATACTTGGTGTGAAT) and ITS_S3R (GACGCTTCTCCAGACTACAAT); annealing temperature 55 °C, otherwise as above [36].

Complete ITS region (ITS1–5.8S–ITS2): ITS1 (TCCGTAGGTGAACCTGCGG) and ITS4 (TCCTCCGCTTATTGATATGC); 95 °C for 5 min; 37 cycles of 94 °C for 50 s, 50 °C for 50 s, and 72 °C for 105 s; final extension at 72 °C for 10 min [37].

trnH-psbA intergenic spacer: trnH(GUG) (CGCGCATGGTGGATTACAATCC) and psbA (GTTATGCATGAACGTAATGCTC); 95 °C for 5 min; 35 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 1.5 min; final extension at 72 °C for 10 min [38–39].

All PCR products were examined by electrophoresis on 1.5% agarose gels, and the target bands were sequenced by Beijing Tsingke Biotech Co., Ltd.

Public database sequence retrieval and combined dataset construction

To assess conservation of candidate RPA primer binding sites and verify primer specificity, *Solanum* barcode sequences were downloaded from NCBI GenBank [40] and BOLD Systems [41] for five markers (ITS1, ITS2, matK, trnH-psbA, and rbcL). All sequences were manually inspected and filtered to remove poor-quality, unassigned, or duplicated records, yielding non-redundant sets. Public sequences for the five markers were also retrieved to aid evaluation of candidate primer sites. Along with sequences from our 30 accessions, these data formed a combined dataset used to guide RPA primer design by assessing conservation at candidate annealing sites and evaluating primer specificity.

sgRNA design and screening

sgRNAs were designed bioinformatically based on the target sequence of the target species, taking into account PAM proximity, GC content, intra-molecular secondary structures, local structural accessibility, base-position preferences, and predicted specificity [42–43]. The spacer sequences were subjected to NCBI Primer-BLAST analysis [44], with the specificity criteria set as 100% intra-species coverage and < 15 consecutive nucleotides matching any non-target organism. Three candidate sgRNAs (SR-sgRNA-1, SR-sgRNA-2, SR-sgRNA-3, Table 2) were selected and synthesized.

For sgRNA performance testing, specific PCR primers (SR-PCR-F: CGAAAACAAAAGATTCAAGTTCGA, SR-PCR-R: CATATTCCTAATATTTTACTGACATAG) were designed within 100 bp flanking regions of the sgRNA binding sites. The target fragment was amplified from plasmid template in a 50 µL reaction containing 44 µL of 1.1× Gold Master Mix (Tsingke, DLP202), 2 µL each of forward and reverse primers, and 2 µL of plasmid template. The amplification program was: 98 °C for 2 min; 30 cycles of 98 °C for 10 s, 50 °C for 15 s, and 72 °C for 10 s; with a final extension at 72 °C for 5 min. The resulting amplicons were then used for subsequent sgRNA performance testing.

The CRISPR trans-cleavage reaction (50 µL) contained 2 µL AaCas12b (SynSor, XS-R-002), 1 µL sgRNA, 5 µL 10× AaCas12b Buffer, and 0.5 µL CRISPR ssDNA Reporter (12b-FAM) (SynSor, XS-R-201), with ddH₂O added to bring the final volume to 50 µL. Then 1 µL of PCR product was added to the reaction, which was incubated at 43 °C for 15 min in a qPCR instrument with FAM fluorescence acquisition every minute. The performance or specificity of each sgRNA was preliminarily evaluated based on the changes in the fluorescence curves. A clear difference in the endpoint fluorescence values between the target amplicon and the negative control was required for a positive hit. The sgRNA exhibiting the highest fluorescence increase rate (SR-sgRNA-1) was selected for subsequent experiments.

Fluorescence data were quantitatively evaluated using the Fluorescence Slope, calculated as:

$$\text{Fluorescence Slope} = \frac{R_n - R_1}{R_1}$$

where R_n is the fluorescence signal at the n-th minute, and R_1 is the fluorescence signal of the negative control at the first minute.

A sample was scored as positive if its Fluorescence Slope was significantly higher than that of the negative control, and if the negative control exhibited less than 20% fluorescence change within 15 min.

RPA primers design and screening

RPA primers were designed within the target sequence, with primer lengths of 30-35 bp, GC content of 40%-60%, and amplicon sizes of 100-200 bp [15, 18]. Three forward primers (F1-F3) and three reverse primers (R1-R3) were designed (Table 2). All primer sequences were subjected to NCBI Primer-BLAST to verify amplification coverage and specificity.

The two-step RPA-CRISPR assay was performed as follows. The isothermal amplification lyophilized pellet was resuspended thoroughly in 48 μ L buffer A (SynSor, XS-R-101). Twelve microliters of this solution was placed on the cap of an 8-tube strip, and 1.6 μ L nucleic acid template was added. A separate 8-tube strip was prepared with 0.2 μ L each forward and reverse primer and 1.0 μ L magnesium acetate in the tube. The cap containing template was placed onto the reaction tube, briefly centrifuged to collect contents, and vortexed 10 s. The tube was incubated immediately at 42°C for 30 min for RPA. The CRISPR detection system (10 μ L) contained 0.4 μ L AaCas12b, 0.2 μ L sgRNA, 1 μ L 10 $\times$ AaCas12b Buffer, and 0.1 μ L CRISPR ssDNA Reporter (12b-FAM), brought to 10 μ L with water. This mixture was placed on the cap of a fresh 8-tube strip. After RPA, the original cap was replaced with the CRISPR-containing cap (avoiding liquid entry). The tube was incubated at 43°C for 30 min. After mixing by brief centrifugation and vortexing for 10 s, the reaction was further incubated at 43°C for 30 min with FAM reading every minute.

For primer screening, plasmid templates diluted to 1×10^4 and 1×10^3 copies/test were used, and the optimal primer pair was selected based on fluorescence intensity and fluorescence increase rate for subsequent experiments.

Performance evaluation of the RPA-CRISPR detection system

Sensitivity testing and limit of detection determination

Ten-fold serial dilutions of the plasmid template containing the target fragment (1×10^4 , 1×10^3 , 1×10^2 , 1×10^1 , 1×10^0 copies/test) were tested using the two-step RPA-CRISPR system, with three replicates per concentration. Ten replicates at concentrations near the expected LOD (1×10^1 and 5×10^0 copies/test) were performed.

The plasmid copy number was calculated using the following formula:

$$N_{\text{plasmid}} = \frac{N_A \times C \times 10^{-9}}{(2710 + N_i) \times 660}$$

Where N_{plasmid} is the plasmid copy number concentration (copies/ μL), N_A is Avogadro's constant (6.02×10^{23}), C is the plasmid concentration (ng/ μL), N_i is the number of inserted fragment bases in the plasmid, 2710 is the length of the pUC57 vector, and 660 is the average molecular weight of a base pair.

Specificity testing

Specificity was evaluated with 30 actual samples (14 *S. rostratum* positives and 16 non-target samples including close relatives and other non-target taxa, as listed in Table 1) using the optimized two-step RPA-CRISPR system. Each sample was tested in three technical replicates.

Establishment of the visual detection system

Visual detection system setup. Based on the fluorescence detection system, the final concentration of ssDNA Reporter in the CRISPR reaction was increased to 120 $\mu\text{mol/L}$, while the other components remained the same. The visual CRISPR reaction system (10 μL) contained 0.4 μL AaCas12b (2.5 $\mu\text{mol/L}$), 0.25 μL sgRNA (100 ng/ μL), 1 μL 10 $\times$ AaCas12b Buffer, and 1.2 μL CRISPR ssDNA Reporter (12b-FAM), made up to 10 μL with ddH₂O. The CRISPR mixture was placed onto the cap of a fresh 8-tube strip. Positive and negative results were determined by high-concentration FAM-associated colour observed by eye.

Sensitivity testing of the visual detection system. Ten-fold serial dilutions of the plasmid template (1×10^4 , 1×10^3 , 1×10^2 , 1×10^1 , 1×10^0 , 1×10^{-1} copies/test) were tested using the visual detection system, with three replicates per concentration.

Sensitivity confirmation of the visual detection system. To confirm the sensitivity, two concentrations near the expected LOD (1×10^1 and 5×10^0 copies/test) along with the negative control were selected for 10 replicate tests each.

Evaluation of the visual detection system with actual samples. The visual detection system was evaluated using 30 actual samples (comprising 14 target *S. rostratum* samples and 16 non-target samples, Table 1). Each sample was tested in three replicates, and the visual results were compared with those obtained from the

in-tube fluorescence assay.

Results

Reference sequence dataset construction and species-specific marker identification

To obtain reference sequences for species-specific primer and sgRNA design, five DNA barcode regions (rbcl, matK, ITS, ITS2, and trnH-psbA spacer) were amplified and sequenced from all collected specimens. A total of 148 sequences were generated and deposited in GenBank under accession numbers PZ890009–PZ890044 for rbcl, PZ890045–PZ890079 for matk, PZ858580–PZ858601 for the complete ITS region, PZ858602–PZ858622 for ITS2, and PZ890080–PZ890113 for the trnH-psbA spacer.

To supplement these newly generated sequences and enable comprehensive evaluation of candidate primer binding sites, *Solanum* barcode sequences were also retrieved from NCBI GenBank and BOLD Systems. After manual quality filtering and deduplication, the NCBI dataset comprised 1,487 ITS1 sequences (representing 693 species), 1,726 ITS2 sequences (697 species), 552 matk sequences (179 species), 397 trnH-psbA sequences (116 species), and 687 rbcl sequences (167 species). The BOLD dataset comprised 370 ITS1 sequences (216 species), 612 ITS2 sequences (374 species), 470 matk sequences (150 species), 158 trnH-psbA sequences (47 species), and 325 rbcl sequences (73 species). Together with the 148 sequences generated in this study, these data formed a combined reference dataset used to identify diagnostic SNP sites specific to *S. rostratum* and to guide rational design of RPA primers and CRISPR sgRNAs. Selection of trnH-psbA was guided by published evidence and by the practical performance observed in this study.

sgRNA screening and selection

Three candidate sgRNAs (SR-sgRNA-1, SR-sgRNA-2, and SR-sgRNA-3) were designed and evaluated for their CRISPR trans-cleavage activity using the same PCR-amplified target fragment as the substrate. The results demonstrated that SR-sgRNA-1 exhibited superior performance, with a faster fluorescence increase rate, and was therefore selected for subsequent RPA primer screening (Fig. 1A, 1B). No significant

fluorescence rise was observed in the NTCs for any of the sgRNAs, indicating that the observed signals were specifically target-dependent. SR-sgRNA-1 was therefore used in all subsequent RPA-CRISPR experiments.

RPA primer screening and selection

Nine RPA primer combinations from three forward (F1–F3) and three reverse (R1–R3) primers were screened against plasmid templates at two concentrations (1×10^4 and 1×10^3 copies/test) using the two-step RPA-CRISPR system with SR-sgRNA-1 fixed. The F1/R1 combination gave the strongest signals at both concentrations, with the highest endpoint fluorescence and fastest slope increase (Fig. 1C, 1D, 1E). Other combinations, especially those containing F3 or R3, showed much weaker signals, particularly at the lower concentration. F1/R1 was therefore chosen for further characterization.

Sensitivity of the RPA-CRISPR fluorescence assay

To determine the LOD, ten-fold dilutions of target plasmid (1×10^4 , 1×10^3 , 1×10^2 , 1×10^1 , and 1×10^0 copies/test) were tested. Fluorescence was clearly detectable down to 1×10^1 copies/test, with signal intensity decreasing proportionally with template concentration (Fig. 2A, 2B). At 1×10^0 copies/test, the signal was only marginally above negative control, suggesting this concentration is near the practical detection limit. To confirm the LOD, ten independent replicates were run at 1×10^1 and 5×10^0 copies/test. All ten replicates at 1×10^1 copies/test gave consistently positive signals with low variability, whereas at 5×10^0 copies/test only a subset of replicates amplified (Fig. 2C, 2D). The robust LOD of the RPA-CRISPR assay is therefore 1×10^1 copies/test.

Specificity of the RPA-CRISPR assay

The specificity of the optimized assay was evaluated using 30 genomic DNA samples, comprising 14 *S. rostratum* specimens and 16 non-target *Solanum* species (Table 1). As shown in Fig. 3, all 14 *S. rostratum* samples produced strong, clearly positive fluorescence signals, while all 16 non-target samples yielded signals indistinguishable from the no-template controls. These results demonstrate that the RPA-CRISPR assay has 100% sensitivity (14/14) and 100% specificity (16/16) for the detection of *S.*

rostratum under the tested conditions.

Development and validation of the visual detection system

To facilitate on-site detection without specialized instrumentation, we adapted the fluorescence-based assay into a visual FAM readout format by increasing the ssDNA reporter concentration to 120 $\mu\text{mol/L}$. The feasibility of this visual system was first tested using 1×10^4 copies/test plasmid and a no-template control. As shown in Fig. 4A, the positive sample (PTC) displayed a clear colour change distinguishable by the naked eye, while the no-template control (NTC) remained colourless, confirming the visual system's feasibility.

Sensitivity testing of the visual system across a ten-fold dilution series (1×10^4 to 1×10^{-1} copies/test) revealed that the visual signal was consistently detectable down to 1×10^1 copies/test, with no visible signal at concentrations below this threshold (Fig. 4B). Ten-replicate confirmation tests at 1×10^1 and 5×10^0 copies/test showed that 9 out of 10 replicates at 1×10^1 copies/test yielded clearly positive visual signals, whereas only 4 out of 10 replicates were positive at 5×10^0 copies/test (Fig. 4C). Based on a criterion of $\geq 90\%$ positive detection rate, the robust LOD of the visual format was determined to be 1×10^1 copies/test.

Finally, the practical applicability of the visual system was assessed using the same panel of 30 genomic DNA samples. All 14 *S. rostratum* samples yielded clearly positive visual signals, whereas all 16 non-target samples remained negative (Fig. 5). These visual results were fully consistent with those obtained from the fluorescence-based assay, demonstrating that the visual detection system retains both sensitivity and specificity while offering the advantage of readout without a fluorescence excitation light source or optical filters.

Discussion

Systematic evaluation of candidate regions for RPA primer design

In this study, we systematically evaluated the suitability of each candidate region as an RPA target from four aspects: nuclear ITS/ITS2, chloroplast coding genes (*rbcl*, *matk*), chloroplast non-coding regions (*trnH-psbA*), and supplementary nuclear genes

(waxy/GBSSI). The rationale for selecting the trnH-psbA intergenic spacer as the final target is elucidated.

Nuclear ITS/ITS2. In *Solanum* DNA barcoding studies, the nuclear ribosomal DNA internal transcribed spacer (ITS/ITS2) is often considered an important supplement to core barcodes due to its relatively fast evolutionary rate and accumulated variation. However, the practical application of this region in *Solanum* is constrained by multiple factors, particularly presenting structural obstacles for RPA primer design. Cadavid et al. [26] evaluated 222 nrITS sequences from *Solanum* subgenus *Leptostemonum* and found only 73% amplification success and 51% sequencing success. They attributed the main limitation of nrITS to poor primer universality, linked to its multi-copy nature and sequence variation among copies. From an RPA primer design perspective, poor universality of ITS flanking sequences makes stable amplification primers difficult to design. Although ITS2 secondary structure information can improve species resolution, Yang et al. [45] found that including it increased parsimony-informative sites by 88.57% and distinguished three morphologically similar species, the complexity of secondary structures also complicates target screening. Therefore, while ITS/ITS2 has some theoretical discriminatory potential in *Solanum*, its poor flanking conservation and multi-copy complications make it a less practical RPA target in this study.

Chloroplast coding genes rbcL and matk. rbcL and matk are the core plant barcodes recommended by the CBOL Plant Working Group [46], with strong primer universality and high amplification success, but both exhibit limited polymorphism in *Solanum* and are not suitable for distinguishing closely related species. Among nine species of *Solanum* subgenus *Solanum*, the mean interspecific K2P distance of rbcL was only 0.0150 [8]. Tegally et al. [27] similarly demonstrated that rbcL could not distinguish *S. violaceum* from *S. anguivi*. matk shows better polymorphism than rbcL but remains limited. Cadavid et al. [26] reported a species identification rate of 75.7% (28/37 species) for matk in subgenus *Leptostemonum*, with nucleotide diversity (π = 0.01248) and mean interspecific genetic distance (1.42%) significantly lower than those of trnH-psbA (π = 0.03224, 2.6%). From the RPA primer design perspective, its

overall low variation level makes it difficult to ensure stable discrimination between *S. rostratum* and all closely related species. Therefore, the insufficient polymorphism of *rbcL* and *matK* limits their practical suitability as RPA targets.

Chloroplast non-coding regions (*trnH-psbA*). Chloroplast non-coding regions generally evolve faster than coding genes and offer unique advantages in identifying closely related species. The *trnH-psbA* intergenic spacer is one of the most variable non-coding regions. In subgenus *Leptostemonum*, Cadavid et al. [26] showed that *trnH-psbA* had significantly higher nucleotide diversity ($\pi = 0.03224$) than *matK* ($\pi = 0.01248$), and a higher mean interspecific genetic distance (2.6% vs. 1.42%). In *Physalis*, Feng et al. [47] evaluated 36 *trnH-psbA* sequences and reported 100% PCR amplification and sequencing success, 100% species identification success, interspecific genetic distances of 0.002~0.922, and intraspecific distances of only 0.000~0.008, showing a clear barcoding gap and demonstrating that *trnH-psbA* combines high variation density with high operational reliability. In summary, among chloroplast non-coding regions, *trnH-psbA* shows favourable characteristics in variation density, fragment length suitability, and amplification success, making it a practical candidate for RPA application in this study.

Supplementary nuclear gene. *waxy* (GBSSI) is a single-copy nuclear gene that avoids ITS multi-copy complications. Levin et al. [48] found that *waxy* was particularly suitable for phylogenetic inference, with high parsimony-informative site proportion and low homoplasy. Zhang et al. [10] reported that *waxy* provided 642 variable sites (42.04%) and a mean interspecific K2P distance of 0.0453. Liu et al. [7] designed species-specific PCR primers based on GBSSI that achieved specific amplification of *S. rostratum* among five closely related species, indicating that *waxy*/GBSSI does contain regions suitable for specific detection. However, unlike core barcoding regions, *waxy*/GBSSI requires constructing a dedicated reference database and validating against numerous closely related species, an effort far beyond the scope of a single study, given *Solanum* comprises approximate 1,500~2,000 species.

Comprehensive assessment: *trnH-psbA* as the optimal RPA target. Based on the systematic comparison above, the chloroplast *trnH-psbA* intergenic spacer shows the

most balanced performance across primer design feasibility, variation density, flanking conservation, amplification success, and operational convenience, making it the optimal RPA target in this study. This conclusion is strongly supported by our own reference sequence dataset. We constructed a dataset covering five barcoding regions (rbcL, matk, ITS/ITS2, trnH-psbA), comprising 148 newly sequenced accessions and approximate 6,800 public sequences from NCBI and BOLD, and performed systematic sequence alignment and SNP site screening.

The rationale for the selection of trnH-psbA is as follows: (1) it combines high inter-specific variation density with extremely low intra-specific variation, ensuring the barcoding gap; (2) flanking sequences are highly conserved, ensuring primer universality and amplification stability; (3) amplification and sequencing success approach 100%, avoiding ITS/ITS2 multi-copy complications; (4) variation density is several times higher than matk and rbcL.

Intraspecific variation validation: the importance of multi-population sampling for RPA primer design

Beyond interspecific specificity, RPA primer design requires thorough evaluation of intra-specific variation, an evaluation that is critical because RPA specificity depends on the number and distribution of mismatches [15] and that has been addressed by aligning multiple target sequences to capture intra-specific diversity and extract conserved regions as candidate targets [25]. Accordingly, successful RPA primers must not only distinguish interspecific polymorphic sites but also ensure that primer-binding regions are sufficiently conserved across all populations of the target species. Otherwise, underestimated intraspecific polymorphism can cause mismatches in certain geographical populations, leading to false-negative results, a critical failure in quarantine applications.

Based on this consideration, we adopted a multi-population sampling strategy throughout the assay development process. During the sequence analysis phase, trnH-psbA sequences covering the major geographical distribution range of *S. rostratum* were included as comprehensively as possible to evaluate the conservation of candidate primer-binding sites. This follows the principle of using

multi-sequence alignment to identify conserved regions across diverse target sequences [25]. Crucially, during the validation phase, we tested *S. rostratum* samples from 14 geographically distinct populations across eight provinces and autonomous regions. The fact that all populations consistently tested positive experimentally confirms the high intraspecific conservation of the selected target region and the broad applicability of the designed primers across the species' Chinese distribution range.

This result agrees with the extremely low intraspecific genetic distance (0.000~0.008) observed for trnH-psbA in *Physalis* by Feng et al. [47], and aligns with the principle that robust field-deployable diagnostics must be validated against geographically diverse populations to ensure reliable performance across potential invasion sites and port interception scenarios. Thus, by combining in silico multi-sequence screening with experimental validation across geographically representative populations, our multi-population validation approach provides a useful framework for evaluating practical reliability of RPA-based quarantine diagnostics.

Superiority of the RPA-CRISPR/Cas12b Dual-Specificity Recognition Mechanism

The RPA-CRISPR/Cas12b detection system established in this study significantly improves detection reliability through a dual-specificity recognition mechanism, which is particularly important in port quarantine scenarios.

Mechanism of dual recognition. In traditional RPA, non-specific amplification is a major cause of false positives [15, 25, 49]. With the CRISPR/Cas12b system, even if RPA produces non-specific products, specific recognition between sgRNA and the target sequence is required to activate Cas12b's trans-cleavage activity [50]. This is consistent with the mechanism of Cas12a, in which target binding triggers nonspecific ssDNA trans-cleavage [19]. This design is intended to add a "precise verification" step after RPA's "coarse amplification": the first specificity layer comes from RPA primers, the second from base pairing between crRNA and target. Both must be satisfied to generate a positive signal, eliminating false positives from non-target DNA interference.

Experimental validation in this study. The specificity evaluation results of this study

validated this advantage. Among the 30 tested materials, no cross-reactions were observed in other *Solanum* related species. This indicates that the dual screening of RPA primers and crRNA effectively excluded interference from non-target species. For port quarantine, the dual-specificity recognition mechanism is particularly important. Imported grains may contain a mixture of various *Solanum* weed seeds (e.g., *S. carolinense*, *S. torvum*) that may resemble *S. rostratum* in seed morphology. Dual-specificity recognition ensures that only *S. rostratum* produces a positive signal, avoiding misjudgment and clearance delays in complex sample detection.

Comparative Advantages and Application Prospects of the RPA-CRISPR/Cas12b Detection System in Seed Quarantine

The RPA-CRISPR/Cas12b visual detection system established in this study demonstrates significant advantages in multiple aspects, making it particularly suitable for port seed quarantine scenarios.

Detection speed. RPA reactions proceed under isothermal conditions (37~42°C) without thermal cycling equipment. Under the optimized conditions of this study, the complete workflow from DNA extraction to result interpretation can be completed within approximately 1 hour, much faster than traditional PCR methods (typically requiring 2~3 h). This time advantage is particularly critical in port quarantine scenarios, which rapid clearance requires detection to be completed during cargo stay.

Operational simplicity and instrument-free readout. RPA does not require precision thermal cyclers; only portable heating devices (such as small metal baths, portable incubators) are needed to complete the reaction. Detection results are interpreted directly through color change in the reaction tube, positive reactions display a visible color change discernible by the naked eye, without the need for any fluorescence excitation light source, optical filters, or lateral flow dipsticks. This homogeneous visual detection system achieves truly instrument-free readout, greatly simplifying the operational workflow, reducing consumable costs.

Simple sample processing and single-seed detection capability. RPA has low requirements for DNA purity and can directly use crude DNA extracts or even

heat-treated lysates as templates [15, 18], greatly simplifying the pretreatment steps for hard samples such as seeds—without the need for complex DNA purification, only simple lysis and dilution are required to obtain templates suitable for RPA amplification. This not only saves time but also reduces the requirement for professional skills of operators. In terms of sensitivity, the fluorescence-based detection system achieved a robust limit of detection of 10 copies/test, and the visual detection system reached the same level of 10 copies/test. This sensitivity level means that the method can effectively detect a single *S. rostratum* seed, although small (approximately 2.5 mm × 2.0 mm), its genomic DNA far exceeds the detection limit. Single-seed detection capability is critical for port quarantine because weed seeds in imported grain typically occur as sporadic individuals rather than in large aggregates. This method effectively detected *S. rostratum* from 14 different geographical populations, further indicating high intraspecific conservation of the target region and good general applicability.

In summary, the establishment of this method effectively provides a promising tool in existing detection technologies for seed quarantine, provides key technical support for "source interception" of *S. rostratum*, and also offers a referable technical pathway for rapid detection of other quarantine weeds in the genus *Solanum*.

Declarations

Abbreviations

BOLD: Barcode of Life Data System; bp: base pair; Cas12b: CRISPR-associated protein 12b; CBOL: Consortium for the Barcode of Life; CRISPR: Clustered regularly interspaced short palindromic repeats; crRNA: CRISPR RNA; CTAB: cetyltrimethylammonium bromide; ddH₂O: double-distilled water; FAM: 6-carboxyfluorescein; GBSSI: granule-bound starch synthase I; ITS1: Internal transcribed spacer 1; ITS2: Internal transcribed spacer 2; K2P: Kimura 2-parameter; LOD: Limit of detection; LSC: Large single-copy region; matK: maturase K; Mb: megabase; NCBI: National Center for Biotechnology Information; ndhF: NADH dehydrogenase subunit F; nrITS: nuclear ribosomal internal transcribed spacer; NTC:

No-template control; PAM: Protospacer adjacent motif; PCR: Polymerase chain reaction; PTC: Positive template control; qPCR: Quantitative real-time PCR; rbcL: Ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit; RPA: Recombinase polymerase amplification; sgRNA: single guide RNA; SNP: Single nucleotide polymorphism; ssDNA: single-stranded DNA; trnH-psbA: tRNA-His–photosystem II protein D1 intergenic spacer; trnS-trnG: tRNA-Ser–tRNA-Gly intergenic spacer; UAV: Unmanned aerial vehicle; YOLO: You only look once; π (pi): nucleotide diversity.

Plant collection permission

All 30 *Solanum* accessions used in this study, including the target *S. rostratum* and 16 non-target *Solanum* species, are not listed in the List of National Key Protected Wild Plants of China and are not nationally protected wild plants. The cultivated species were obtained from public germplasm resources or commercial cultivars; the wild-collected target and non-target species are non-protected wild plants or invasive weeds. Under the Regulations of the People's Republic of China on Wild Plants Protection, no specific collection permit was required for any plant material in this study.

Human Ethics and Consent to Participate declarations

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The DNA barcode sequences generated in this study have been deposited in GenBank under accession numbers PZ890009–PZ890044 for rbcL, PZ890045–PZ890079 for matk, PZ858580–PZ858601 for the complete ITS region, PZ858602–PZ858622 for ITS2, and PZ890080–PZ890113 for the trnH-psbA intergenic spacer.

Competing Interests

The authors declare that they have no competing interests.

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Authors' contributions

Conceptualization, W.F., G.Z.; Investigation, W.F., Z.Y., Z.W.; Formal analysis, F.J., Y.Z., Z.Y., C.S.; Supervision, F.J., W.F., Z.S., Z.W., and G.Z.; Resources, Y.Z., W.F., C.S.; Funding acquisition, Y.Z.; Methodology, F.J., Y.Z., Z.Y., Z.S.; Visualization, Y.Z., W.F., C.S., Z.S.; Writing - original draft, Y.Z.; Writing - review & editing, Z.Y., F.J., W.F., G.Z.

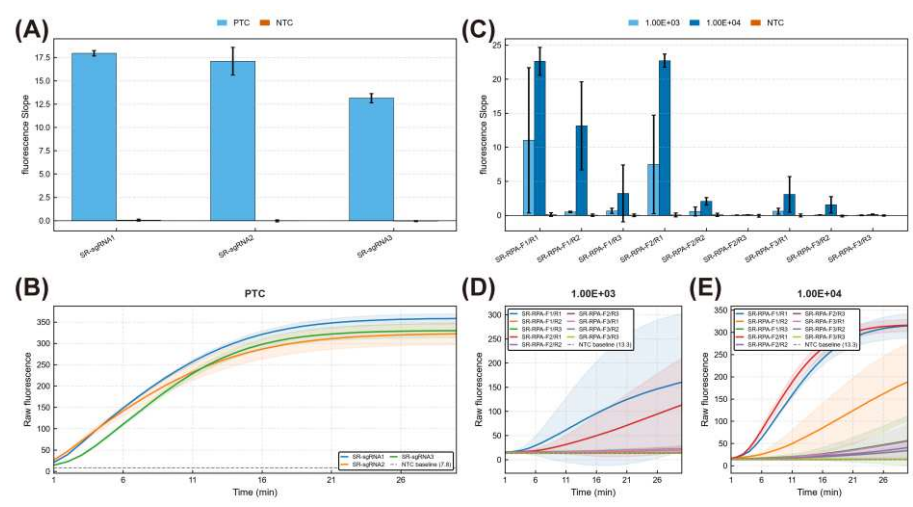


Fig. 1 Screening of sgRNAs and RPA primers for *Solanum rostratum*. (A) Fluorescence increase rate at 6 min during sgRNA screening. (B) Fluorescence curves for different sgRNAs. (C) Fluorescence increase rate for RPA primer screening (30 min). (D) Fluorescence curves for different RPA primer combinations at high concentration (1×10^4 copies/test). (E) Fluorescence curves at low concentration (1×10^3 copies/test). NTC: no-template control (water instead of template).

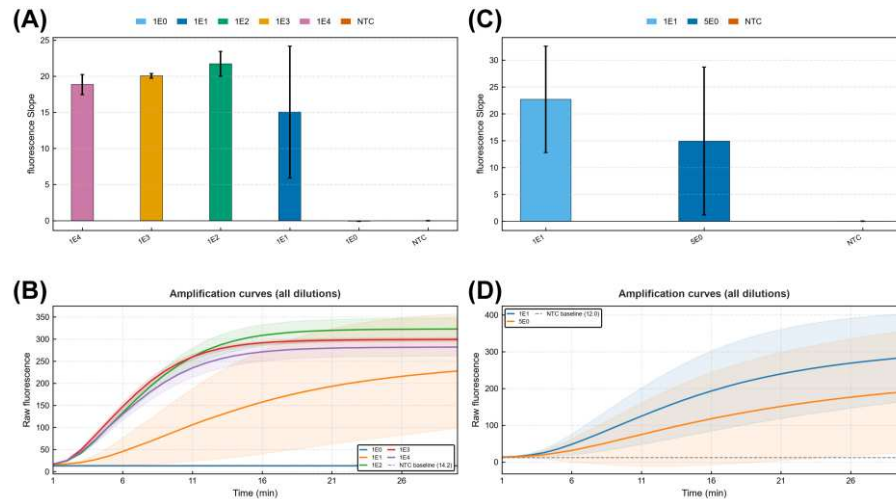


Fig. 2 Sensitivity verification and confirmation of the RPA-CRISPR fluorescence assay for *Solanum rostratum*. (A) Fluorescence increase rate at different concentrations. (B) Fluorescence curves at different concentrations. (C) Fluorescence increase rate at 1×10^1 and 5×10^0 copies/test during confirmation. (D) Fluorescence curves at 1×10^1 and 5×10^0 copies/test during confirmation. NTC: no-template control.

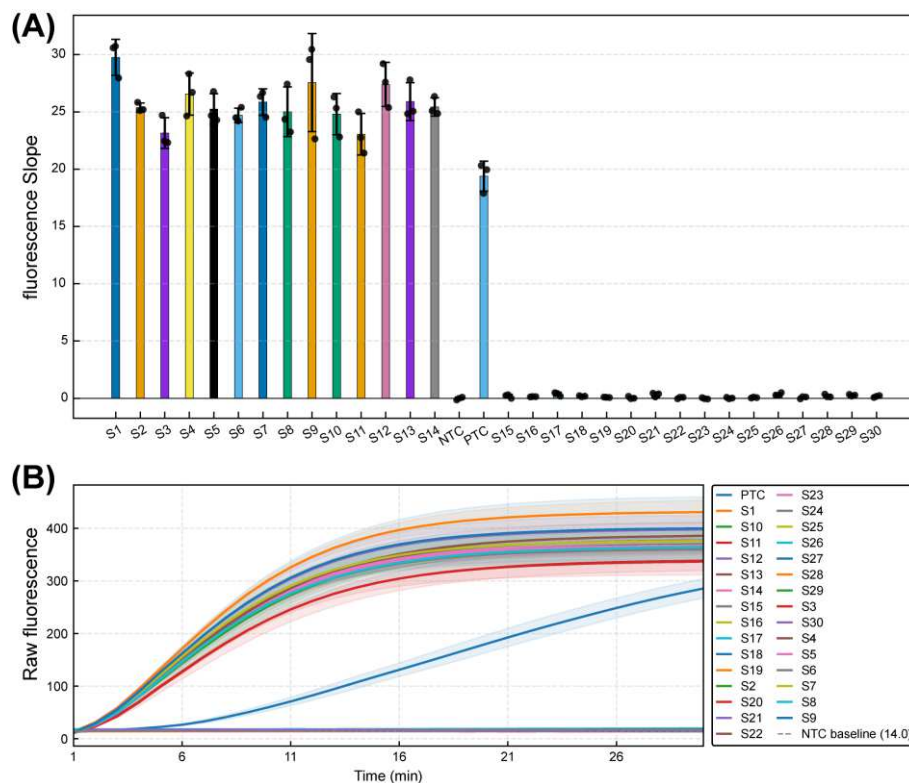


Fig. 3 Validation of the RPA-CRISPR fluorescence assay with actual samples for *Solanum rostratum*. S1-S14: 14 *S. rostratum* samples collected from Heilongjiang (S1, QQHE), Jilin (S2-S3, BCTY/ SYQG), Liaoning (S4, LNCY), Beijing (S5, BJYQ), Hebei (S6,

ZJKSY), Shanxi (S7, DTYG), Inner Mongolia (S8–S13, XAKYZ/ TLKL/ CFBLZ/ HHHT/ BTGY/ BYNE), and Xinjiang (S14, XJCJ). S15–S30: 16 non-target *Solanum* species, including *S. carolinense* (S15), *S. torvum* (S16), *S. viarum* (S17), *S. capsicoides* (S18), *S. mammosum* (S19), *S. violaceum* (S20), *S. melongena* (S21), *S. pseudocapsicum* (S22), *S. americanum* (S23), *S. scabrum* (S24), *S. villosum* (S25), *S. nigrum* (S26), *S. cheesmaniae* (S27), *S. lycopersicum* (S28), *S. tuberosum* (S29), and *S. uleanum* (S30). NTC: no-template control.

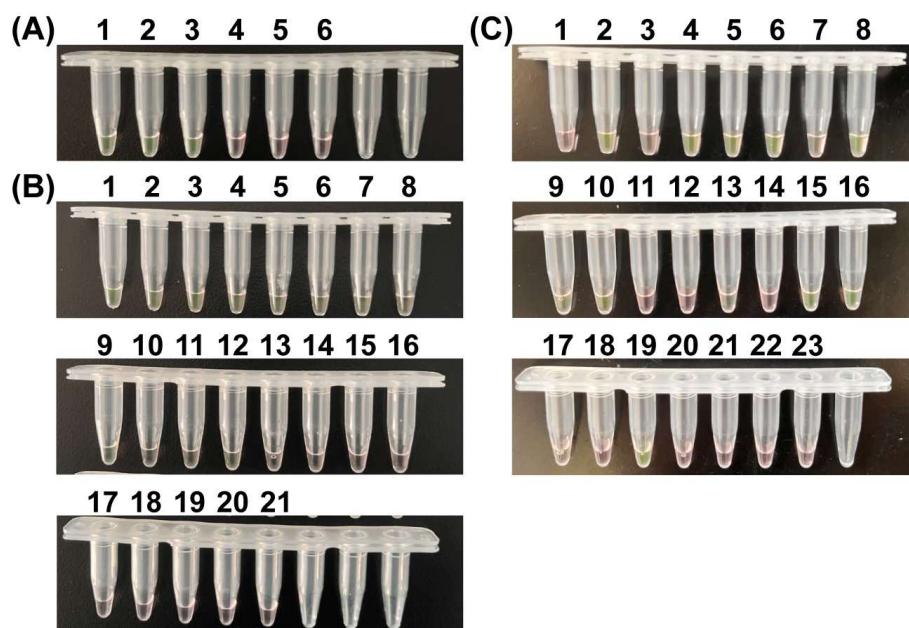


Fig. 4 Establishment and validation of the RPA-CRISPR visual FAM readout system for *Solanum rostratum*. (A) Feasibility test with PTC (positive template control, 1×10^4 copies/test, tubes 1–3) and NTC (no-template control, tubes 4–6). (B) Sensitivity verification at 1×10^4 (tubes 1–3), 1×10^3 (tubes 4–6), 1×10^2 (tubes 7–9), 1×10^1 (tubes 10–12), 1×10^0 (tubes 13–15), 1×10^{-1} (tubes 16–18) copies/test, and NTC (tubes 19–21). (C) Sensitivity confirmation at 1×10^1 (tubes 1–10) and 5×10^0 (tubes 11–20) copies/test with 10 replicates each, and NTC (tubes 21–23).

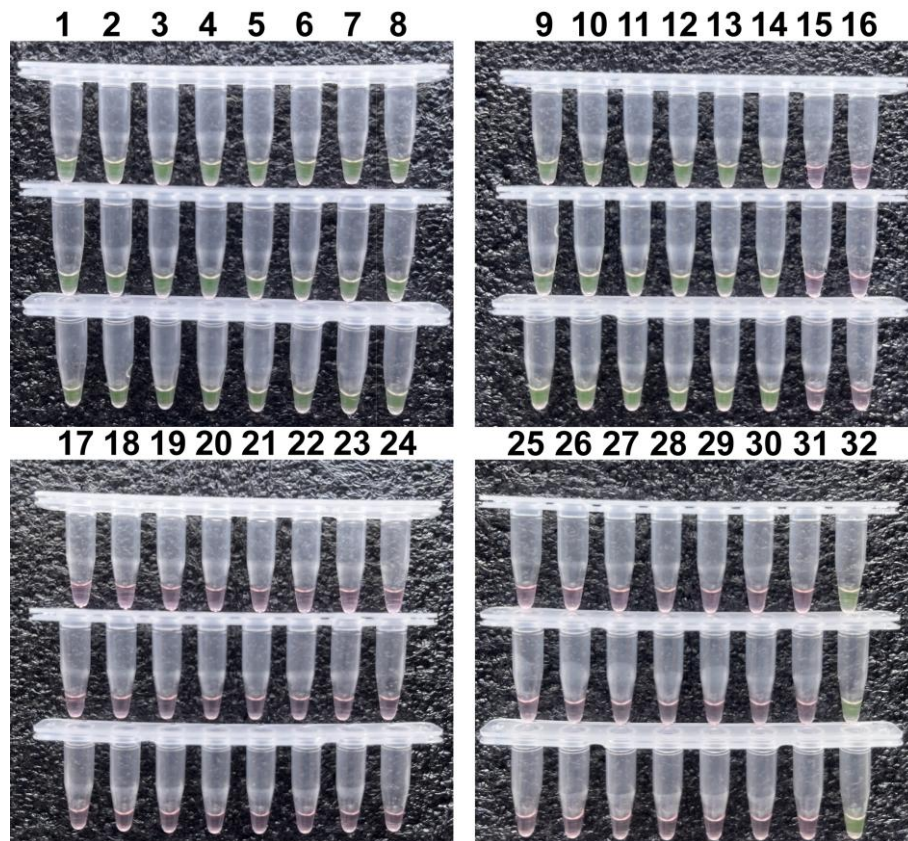


Fig. 5 Validation of the RPA-CRISPR visual FAM readout system with actual 30 samples for *Solanum rostratum*, with three replicates per sample. Tubes 1–14: 14 *S. rostratum* actual samples collected from Heilongjiang (tube 1, QQHE), Jilin (tubes 2–3, BCTY/ SYQG), Liaoning (tube 4, LNCY), Beijing (tube 5, BJYQ), Hebei (tube 6, ZJKSY), Shanxi (tube 7, DTYG), Inner Mongolia (tubes 8–13, XAKYZ/ TLKL/ CFBLZ/ HHHT/ BTGY/ BYNE), and Xinjiang (tube 14, XJCJ). Tubes 15–30: 16 non-target *Solanum* species, including *S. carolinense* (tube 15), *S. torvum* (tube 16), *S. viarum* (tube 17), *S. capsicoides* (tube 18), *S. mammosum* (tube 19), *S. violaceum* (tube 20), *S. melongena* (tube 21), *S. pseudocapsicum* (tube 22), *S. americanum* (tube 23), *S. scabrum* (tube 24), *S. villosum* (tube 25), *S. nigrum* (tube 26), *S. cheesmaniae* (tube 27), *S. lycopersicum* (tube 28), *S. tuberosum* (tube 29), and *S. uleanum* (tube 30). Tube 31: NTC (no-template control). Tube 32: PTC (positive template control).

Table 1 *Solanum* plant materials used for RPA primer design and specificity validation

Species	Collection site	Voucher specimen
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		number
<i>Solanum rostratum</i>	Qiqihar, Heilongjiang (QQHE)	IEDA_Sol_ros_QQHE01
	Baicheng, Jilin (BCTY)	IEDA_Sol_ros_BCTY01
	Songyuan, Jilin (SYQG)	IEDA_Sol_ros_SYQG01
	Chaoyang, Liaoning (LNCY)	IEDA_Sol_ros_LNCY01
	Yanqing, Beijing (BJYQ)	IEDA_Sol_ros_BJYQ01
	Zhangjiakou, Hebei (ZJKSY)	IEDA_Sol_ros_ZJKSY01
	Datong, Shanxi (DTYG)	IEDA_Sol_ros_DTYG01
	Xing'an, Inner Mongolia (XAKYZ)	IEDA_Sol_ros_XAKYZ01
	Tongliao, Inner Mongolia (TLKL)	IEDA_Sol_ros_TLKL01
	Chifeng, Inner Mongolia (CFBLZ)	IEDA_Sol_ros_CFBLZ01
	Hohhot, Inner Mongolia (HHHT)	IEDA_Sol_ros_HHHT01
	Baotou, Inner Mongolia (BTGY)	IEDA_Sol_ros_BTGY01
	Bayannur, Inner Mongolia (BYNE)	IEDA_Sol_ros_BYNE01
	Changji Hui Autonomous Prefecture, Xinjiang (XJCJ)	IEDA_Sol_ros_XJCJ01
<i>Solanum carolinense</i>	Nanjing, Jiangsu	IEDA_Sol_car_JSNJ01
<i>Solanum torvum</i>	Chuxiong, Yunnan	IEDA_Sol_tor_YNCX01
<i>Solanum viarum</i>	Qiannan Buyi and Miao Autonomous Prefecture, Guizhou	IEDA_Sol_via_GZQN01
<i>Solanum capsicoides</i>	Nanjing, Jiangsu	IEDA_Sol_cap_JSNJ01
<i>Solanum mammosum</i>	Shenzhen, Guangdong	IEDA_Sol_mam_GDSZ01
<i>Solanum violaceum</i>	Chuxiong, Yunnan	IEDA_Sol_vio_YNCX01
<i>Solanum melongena</i>	Cultivar	IEDA_Sol_mel_CULT01
<i>Solanum pseudocapsicum</i>	Cultivar	IEDA_Sol_pse_CULT01
<i>Solanum americanum</i>	Nanning, Guangxi	IEDA_Sol_ame_GXNN01
<i>Solanum scabrum</i>	Wenchang, Hainan	IEDA_Sol_sca_HNWC01
<i>Solanum villosum</i>	Changchun, Jilin	IEDA_Sol_vil_JLCC01
<i>Solanum nigrum</i>	Shijiazhuang, Hebei	IEDA_Sol_nig_HBSJZ01

<i>Solanum cheesmaniae</i>	Cultivar	IEDA_Sol_che_CULT01
<i>Solanum lycopersicum</i>	Cultivar	IEDA_Sol_lyc_CULT01
<i>Solanum tuberosum</i>	Cultivar	IEDA_Sol_tub_CULT01
<i>Solanum uleanum</i>	Cultivar	IEDA_Sol_ule_CULT01

623

624 Table 2. sgRNA and RPA primer sequences used in this study

Component	ID	Sequence (5'→3')
sgRNA	SR-sgRNA-1	GUCUAAAGGACAGAUUUUCAACGGGUGUGCCAAUGG
		CCACUUUCCAGGUGGCAAAGCCCGUUGAACUUCAAGC
		GAAGUGGCAC AAUUAUUUUCAAAGAUAU
	SR-sgRNA-2	GUCUAAAGGACAGAUUUUCAACGGGUGUGCCAAUGG
		CCACUUUCCAGGUGGCAAAGCCCGUUGAACUUCAAGC
		GAAGUGGCAC AAUGAUAGUAUUUUCAAU
RPA	SR-sgRNA-3	GUCUAAAGGACAGAUUUUCAACGGGUGUGCCAAUGG
		CCACUUUCCAGGUGGCAAAGCCCGUUGAACUUCAAGC
		GAAGUGGCAC UCUAAUGUAAACAAAAAGU
	SR-RPA-F1	TCAAGTTCGAATATTTCTCTTCTTTCTTAT
	SR-RPA-F2	CGAATATTTCTCTTCTTTCTTATTTCACCT
	SR-RPA-F3	TTCTTATTTCACCTATTTCATGATAGTAT
	SR-RPA-R1	CATTTACACAAAAAATCTAATTTTGACTT
	SR-RPA-R2	TTGATTTCAATTCACACAAAAAATCTAATT
	SR-RPA-R3	TTACTATATCTTTTGATTTCAATTCACAC

625 Note: Bold sequences indicate the spacer regions of the sgRNAs.

626

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