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Development of a portable DNA extraction and cross-priming amplification (CPA) tool for rapid in-situ visual diagnosis of plant diseases

Jie Li[1†], Juan Du[1†], Shengzhican Li[1], Jiali Dong[1], Jiahan Ying[1], Yuehao Gu[1], Jie Lu[2], Xinyu Zeng[3], Philip Kear[4], Daolong Dou[1,5] and Xiaodan Wang[1*]

Abstract

Plant pathogens cause severe losses to crop yields and economic returns in agriculture. Despite plant tissue DNA extraction of typically constituting a preliminary step in nucleic acid-based molecular diagnostics, such lab-based methods can be time-consuming and arduous to complete many samples. To mitigate these challenges, we developed an inexpensive portable DNA extraction technique that is lightweight and suitable for deployment in sampling locations, such as fields. It includes a DNA extraction device fabricated with a Steel Microneedle Array (SMA) and a simple high-efficiency DNA extraction buffer. As a result, DNA extraction times can be reduced to within ~ 1 min, and the eluted DNA is demonstrated to be suitable for subsequent molecular biological analyses without requiring additional purification. Cross-priming amplification (CPA) technology was first established to detect *Phytophthora infestans*, which achieves sensitivity attainment of 10^{-7} ng/μL. The detection result can be conveniently estimated with naked-eye visual inspection using fluorescent dsDNA binding dye. CPA was demonstrated to be more feasible than PCR-based approaches and performed well in species-specific and practicability tests. This study elucidates a novel integrated pathogen detection technique coupled with SMA-Device extraction and a modified visual CPA assay to establish and verify various field-based samples infected with multiple pathogens. Altogether, the total sample-to-answer time for pathogen detection was reduced to ~ 1.5 h, making field-based analysis affordable and achievable for farmers or extension workers inside and outside the laboratory.

Keywords DNA extraction device, Steel microneedle array, Cross-priming amplification, *Phytophthora infestans*, Pathogen detection, Sample-to-answer

[†]Jie Li and Juan Du contributed equally to this work.

*Correspondence:
Xiaodan Wang
xdwang@cau.edu.cn
Full list of author information is available at the end of the article

Background

Solanaceae is the third most important family in the plant kingdom, comprises of over 3000 species. The family includes economically important crop species such as potato (*Solanum tuberosum*), tomato (*Solanum lycopersicum*), eggplant (*Solanum melongena*), and pepper (*Capsicum frutescens*), contributing to a sizeable proportion of human dietary and medicinal applications (Knapp et al. 2004; Lu et al. 2021). However, these crops are frequently attacked by a wide range of pathogens. For instance, the late blight pathogen *Phytophthora infestans* is the most devastating, whereas *Botrytis cinerea* causes gray mold and soil-borne *Phytophthora* blight oomycete *P. capsica* incur significantly quantitative and qualitative crop losses every year (Fry et al. 2015; Kamoun et al. 2015; Petrasch et al. 2019; Sabbadin et al. 2021). Therefore, accurate and timely diagnosis of diseases is an essential precursor to effectively controlling diseases for plant quarantine, monitoring, and forecasting (Martinelli et al. 2015; Calicioglu et al. 2019).

Conventional pathogen diagnosis methods, which use biological phenotyping that require the recognition of disease symptoms, isolation, and culture, scarcely meet the current demands for in-field DNA extraction because they are time-consuming and experience-dependent (Fang and Ramasamy 2015). Furthermore, it is difficult to differentiate diseases with highly similar symptoms, especially the initial symptoms. In light of the difficulty of targeted pathogen detection, a range of polymerase chain reaction (PCR)-based molecular detection methods have been developed. The use of PCR in identification is largely due to its ubiquity in standardized laboratory applications. Examples of identification methods include nested PCR (nPCR), multiplex PCR, and real-time quantitative PCR (qPCR) (Vincelli and Tisserat 2008; Garibyan and Avashia 2013; Keller et al. 2015; Navarro et al. 2015). However, these techniques are time-consuming, highly costly, and laboratory- or operator-dependent, resulting in significant bottlenecks in developing in-situ applications (Lau and Botella 2017; Noh et al. 2019). Therefore, developing a portable in-situ diagnosis method (e.g., for in-field analysis) is the focus of current research and is of significant practical application.

The first step toward PCR amplification is the rate-limiting extraction of nucleic acid. For instance, CTAB- or kit-based methods are laborious, involving toxic solvents, time-consuming, expensive, and therefore are unsuitable for outside the laboratory environment (Khiyami et al. 2014; Moeller et al. 2014; Abdel-Latif and Osman 2017; Ristaino et al. 2020). Furthermore, despite commercial methodologies like paramagnetic beads easing large batch processing, the complex wash and elution steps remain too cumbersome for efficient applications (Esser et al. 2006; Moeller et al. 2014). Such difficulties in the purification process can be simplified by applying nucleic acid binding substrate-based DNA extraction (e.g., aluminum oxide membrane). Nevertheless, these techniques are tied to the need for complex equipment and fabrications (Kim et al. 2010). Likewise, additional methods of nucleic acid isolation, such as plastic sample preparation bags, Flinders Technology Associates (FTA) cards- or cellulose-based paper system (e.g., Whatman No.1 paper, cellulose dipstick), and microneedle patches, do not isolate nucleic acids at high concentrations, and have a small finite-sized storage capacity, nor do they have sufficient quality for molecular identification despite being cost-effective sample preparation solutions (Qiu and Mauk 2015; Paul et al. 2019; Mason and Botella 2020; McCoy et al. 2020). Consequently, developing a rapid and economical plant DNA extraction platform or a large-scale portable DNA extraction device for real-time monitoring of crop diseases in the field is imperative.

However, the isothermal amplification of nucleic acids is a straightforward process. No PCR-based thermocycling is required as amplification occurs at a constant temperature. Furthermore, it is widely used due to its simple, quick operation and high accuracy (Craw and Balachandran 2012). Cross-priming amplification (CPA) is currently applied for the isothermal amplification of nucleic acids (Xu et al. 2012). With this method, at least one cross primer is used, making the sequences of the amplified product form a single-stranded semi-loop or branched structure. The targeted DNA sequence is continually amplified at a constant temperature by *Bst* DNA polymerase with the property of chain replacement at a constant temperature. According to the different number of cross primers in the system, this technology can be divided into Single-crossing CPA and Double-crossing CPA, which can increase the utilization rate of the primer region while ensuring the same capacity for amplification efficiency (Xu et al. 2012). Thus, the requirement of template conservatism in primer design is reduced. As a result, CPA was more attractive and dependable for the rapid detection of pathogens and has been successfully applied in animal- or plant-pathogen monitoring (e.g., *Mycobacterium tuberculosis, Acidovorax citrulli*), plant quarantine, food safety inspection (e.g., *Enterobacter sakazakii*), and the identification of genetically modified crops (Fang et al. 2009; Zhao et al. 2010; Zhang et al. 2012, 2014). Nevertheless, despite CPA having much potential in laboratories requiring tools of basic pathogen detection, especially those found in economically deprived areas, its utility in detecting filamentous pathogens is under-reported.

Here, we report the development of a portable in-situ DNA extraction device fabricated with a Steel

Microneedle Array (hereafter abbreviated to SMA-Device) to overcome the bottlenecks associated with conventional DNA extraction methods, shortening extraction times to within 1 min. Its usage does not rely on any specialized training or lab equipment. Firstly, equipped with a highly efficient DNA extraction buffer, the DNA extracted from the SMA-Device was confirmed through multi-trial analysis to be of suitable quality for later molecular analyses. Secondly, the coupled CPA assay was established to be suitable for the requirements of in-situ disease detection. Lastly, we demonstrated that the optimized CPA combined with the SMA-Device extraction was equally suited to laboratory and non-laboratory environment since it successfully detected large numbers of infected field samples in-situ. The established platform could allow farmers or extension workers to perform screening assays of plant diseases directly in the field. The application of the coupled CPA-SMA Device in pathogen detection reduced total sample-to-answer time to about 1.5 h.

Results

The SMA-Device-based in-situ DNA extraction protocol

A portable Steel Microneedle Array (SMA) device was developed to establish an in-situ plant DNA extraction method. Without laboratory equipment, it could conveniently complete the process with a simple plant DNA extraction buffer (S-buffer). A flowchart to summarize the process is shown in Fig. 1a. Furthermore, the scanning electron microscopy image of the SMA is displayed in Fig. 1b. More detailed information on the fitting procedure and parameter dependencies are described in "Methods".

The syringe was installed with the down tube after drawing in about 500 µL S-buffer. Then, the inner tube was inserted into the collecting tube. Next, soft plant tissue of interest (e.g., an infected leaf sample) was placed inside the inner tube and subsequently surface-punctured continuously by the SMA while injecting the S-buffer into the tube. Finally, gravity pulls the crude DNA solution downwards into the collection tube (Fig. 1a). Significantly, the whole DNA extraction process would take less than 1 min, and afterward, the inner tube and the extractor could be reused after cleaning. The reuse of materials gave rise to reduced component usage, which we consider a tremendous environmental and financial benefit, in addition to the field-applicable advantages compared with the 3–4 h conventional CTAB method.

Comparison of amounts and quality of DNA extracted by different S-buffers

The amounts and effects of DNA captured from two kinds of S-buffers were compared to optimize the extraction system. The corresponding appearances of UV absorption spectra were plotted, and both buffers showed significant absorption at 260 nm, illustrating a large amount of DNA from plant leaves. Among them, S-buffer 2 (~4.05, 179.28 ng/µL) possessed a remarkably higher absorption peak and concentration of DNA compared with S-buffer 1 (~1.30, 66.30 ng/µL), which is closer to the CTAB method (~10.50, 509.53 ng/µL) (Fig. 2a, b, Additional file 1: Table S1). Although the A260/A280 (DNA/protein) and A260/A230 (DNA/polysaccharide) ratio of S-buffers revealed the presence of interfering substances (Additional file 1: Table S1), gel electrophoresis of target PCR reactions with isolated DNA showed successful amplifications in all treatments without further purification (Fig. 2c). Therefore, S-buffer 2, which presented stable amplifications and comparable PCR products with the CTAB method, was prepared for further SMA-Device extraction, indicating the applicability to capture in planta pathogen DNA from infected plant leaves.

Assessment of the extraction efficiency of the SMA-Device to isolate DNA when equipped with the S-buffer 2

We further validated whether the DNA extracted from the SMA-Device extraction is suitable for PCR amplification (Fig. 3a). Notably, almost no bands were detected in the Flat Patch (schematic shown in Fig. 3a) group when applying a similar extraction procedure with S-buffer 2. Consistent with this result, a ~3.85 times higher concentration and a ~1.31 cycles lower cycle threshold (Ct) value were recorded with the application of the SMA-Device in comparison to the Flat Patch extraction, indicating a significant DNA yield increase and a slightly smaller yield than with the CTAB method (Fig. 3b, c).

Optimization of the CPA Assay

In this study, we took potato late blight as an example to explore the applicability of the CPA assay. To achieve both applicable and specific features of CPA primers, five primers targeting a representative, conserved region of *P. infestans* (ITS region) were designed (Fig. 4a). To further optimize the reaction system of CPA, yields of DNA product under different reaction conditions were monitored by gel electrophoresis. The results showed that the remarkable amplification occurred from 40 min and grew linearly over the subsequent period in which 60 min was the optimal reaction time (Fig. 4b). Besides, as shown in Fig. 4c, the amplification displayed a unimodal curve with an initial rising trend, followed by a decrease. Therefore, 63°C was chosen as the optimal reaction temperature as it sat at the peak value within the trend.

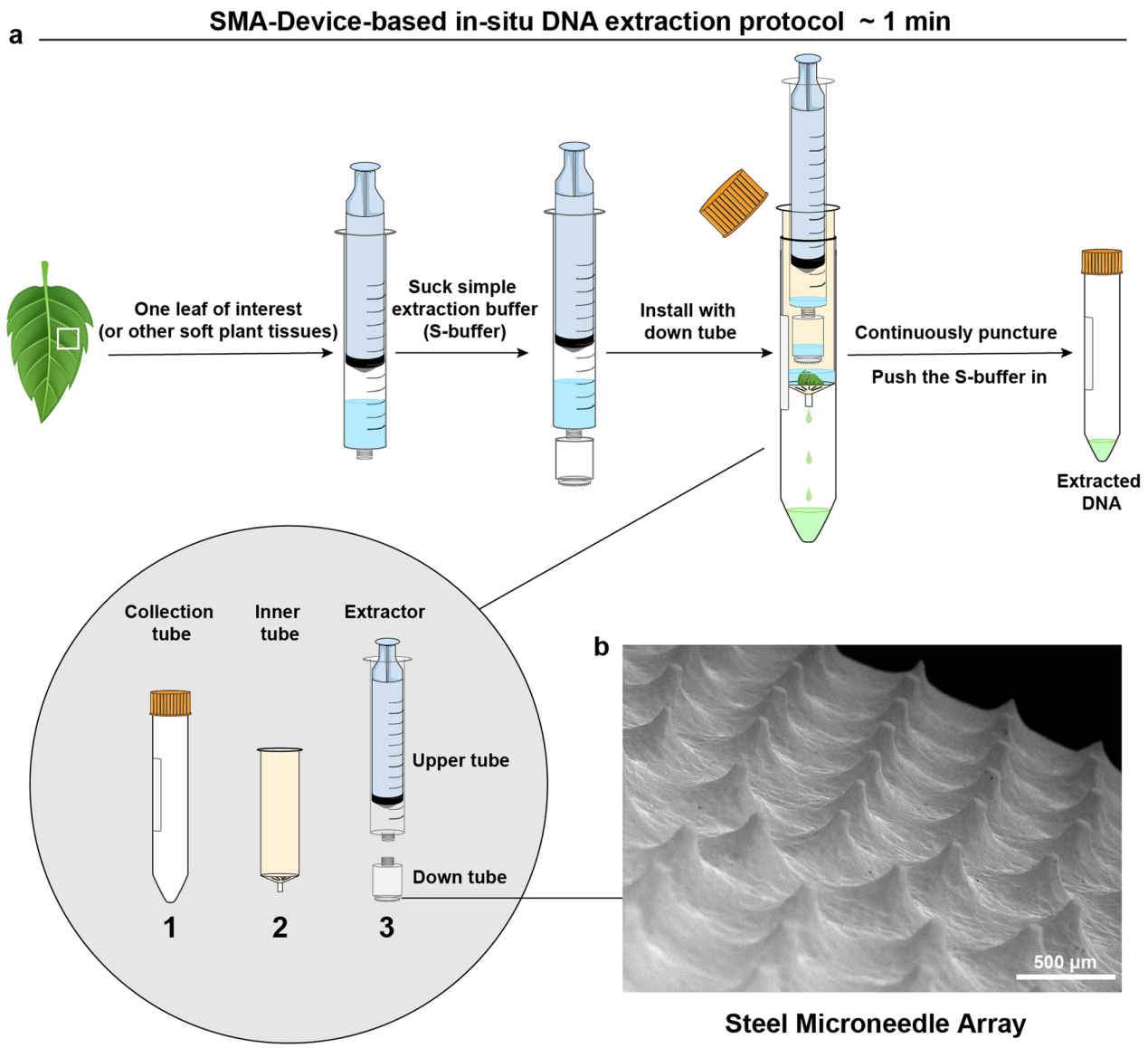

Fig. 1 SMA-Device-based in-situ DNA extraction protocol. **a** Schematic of the experimental setup of the portable SMA-Device and the entire equipment-free DNA extraction process. The blue liquid represents the S-buffer, the pale-yellow tube represents the inner tube, and the green liquid represents the crude extract of DNA. **b** Scanning electron microscopy image of Steel Microneedle Array. Scale bar = 500 μm

Specificity and applicability of the developed CPA assay

P. infestans strains, other oomycetes (including species of the genera *Phytophthora* and *Pythium* spp.), and major *Solanaceae* disease-causing pathogens were used to conduct the CPA assays, respectively. The results showed that *P. infestans* strains alone were amplified, indicating that CPA primers successfully displayed high specificity (Fig. 5a, b). Furthermore, the developed CPA method could detect *P. infestans* adequately in both single and mixed samples when simulating a more complicated sample environment that could be associated with high-throughput screening (Fig. 5c). To evaluate the applicability of the CPA assay, an individual tuber inoculation and leaf detached assay of potato were conducted as described (Fig. 5d, f). In addition, a trypan blue assay for inoculated leaves was performed after the emergence of initial symptoms (Fig. 5f). After dividing each sample into four representative physiological regions according to disease symptoms, visual CPA assays were carried out with the corresponding genomic DNA, respectively, and detection results were distinguished based on the color change. We confirmed that amplifications occurred in samples taken from the

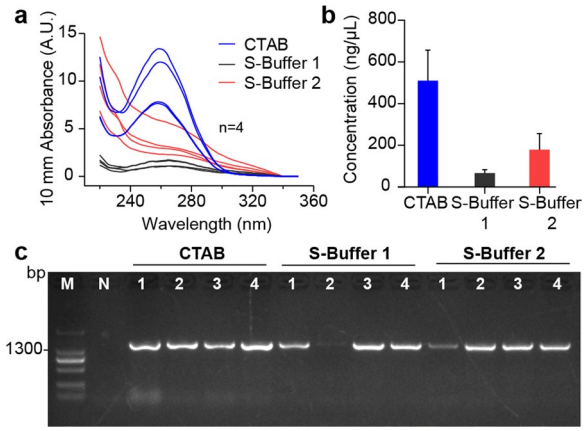

Fig. 2 Total DNA amounts and extraction effects of different S-buffers. **a** UV absorption spectra measurement of DNA solutions using Nanodrop from CTAB-, S-buffer 1-, S-buffer 2-extraction. n = 4. **b** Comparison of DNA concentration based on two kinds of S-buffers. **c** Direct PCR amplification of a fragment of the *P. infestans* ITS region with different S-buffers from infected potato leaf tissues. N, negative control

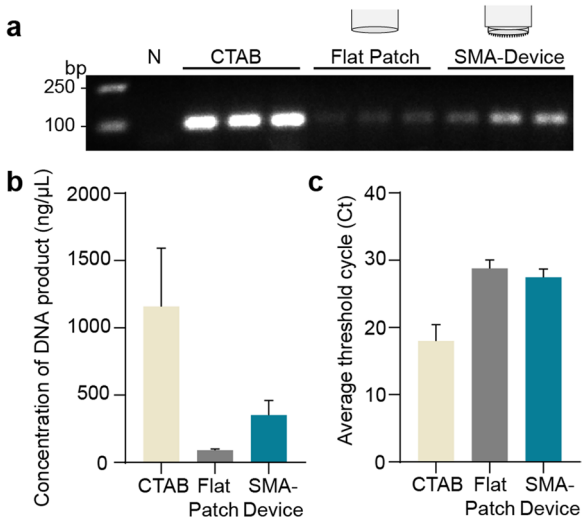

Fig. 3 DNA extraction efficiency assessment of modified SMA-Device extraction method. Comparison of PCR amplification efficiency (**a**), concentration (**b**), and Ct values (**c**) of extracted DNA using CTAB, Flat Patch, and SMA-Device extraction. **a** Gel electrophoresis showing amplification products of the *StEF1a* gene. N, Negative control. **b** A Nanodrop spectrophotometer determined the DNA concentrations. **c** Ct values of extracted DNA were calculated through qPCR analysis

juncture between healthy and diseased areas (Region II, V) and the diseased areas (Region III, VI) (Fig. 5e, g).

Analytical sensitivity between visual CPA and other assays

Ten-fold serially diluted genomic DNA of *P. infestans* (ranging from 10^{2} to 10^{-8} ng/μL) was used to assess the sensitivity of CPA and other conventional methods. As shown in Fig. 6a, at 10^{-7} ng/μL, CPA's initial sensitivity was recorded by an observable transition in color from brown to green in positive samples. In contrast, cPCR was limited to a sensitivity of 10^{-1} ng/μL, as assessed by gel electrophoresis (Fig. 6b). However, the sensitivity of nPCR was found to increase by an additional 100-fold compared with the cPCR (Fig. 6c). Furthermore, the sensitivity of qPCR ranged from 10^{2} to 10^{-2} ng/μL when positive samples were interpreted as ≤ 35 Ct values and the coefficient of determination was ≥ 0.99 (Fig. 6d, e). The melting and amplification curves were presented in Fig. 6d, demonstrating individual signal peaks and specific amplification products. Moreover, a standard curve showing a good linear relationship was generated correlating with high amplification efficiency and coefficient of determination ($y = -0.4220x + 38.76$, $R^2 = 0.9976$) (Fig. 6e). In aggregate, we verified that the newly established CPA assay possessed tremendous detection accuracy compared with traditional PCR based methods.

Detection of field samples using the SMA-Device and visual CPA

To determine the coupled performance of the SMA-Device and the visual CPA method, *P. infestans*-infected potato leaves of different disease severities were randomly collected from the field for sampling (Fig. 7a). As the results showed that disease severity remained constant with the amount of gene amplification, pathogenic DNA collected from the in-situ SMA-Device extraction could be successfully amplified and quantified by PCR (Fig. 7b). In addition, *P. infestans* was positively detected by utilizing the developed visual CPA assay with corresponding DNAs (Fig. 7c). To further test the direct diagnostic efficiency in field applications, the detection rates of three primary *Solanaceae* diseases following the principle of the combined technique were calculated. It revealed that more than 90% of the challenging plant leaves' diseases were defined without ambiguity (Fig. 7d, e; Additional file 2: Figure S1). As a result of these observations, we can confirm that the coupled technique offers immense field applicability (Fig. 8).

Discussion

Precise molecular diagnosis of pathogens is a prerequisite for making suitable disease management strategies (Ali et al. 2021). Although PCR-based molecular diagnoses are widely available, most require additional equipment for genotyping techniques such as electrophoresis, fluorescence-based nucleic acid identification, sequencing and mass spectrometry. Furthermore, conventional methods must be operated by trained personnel, relying on complex procedures, supported by expensive facilities,

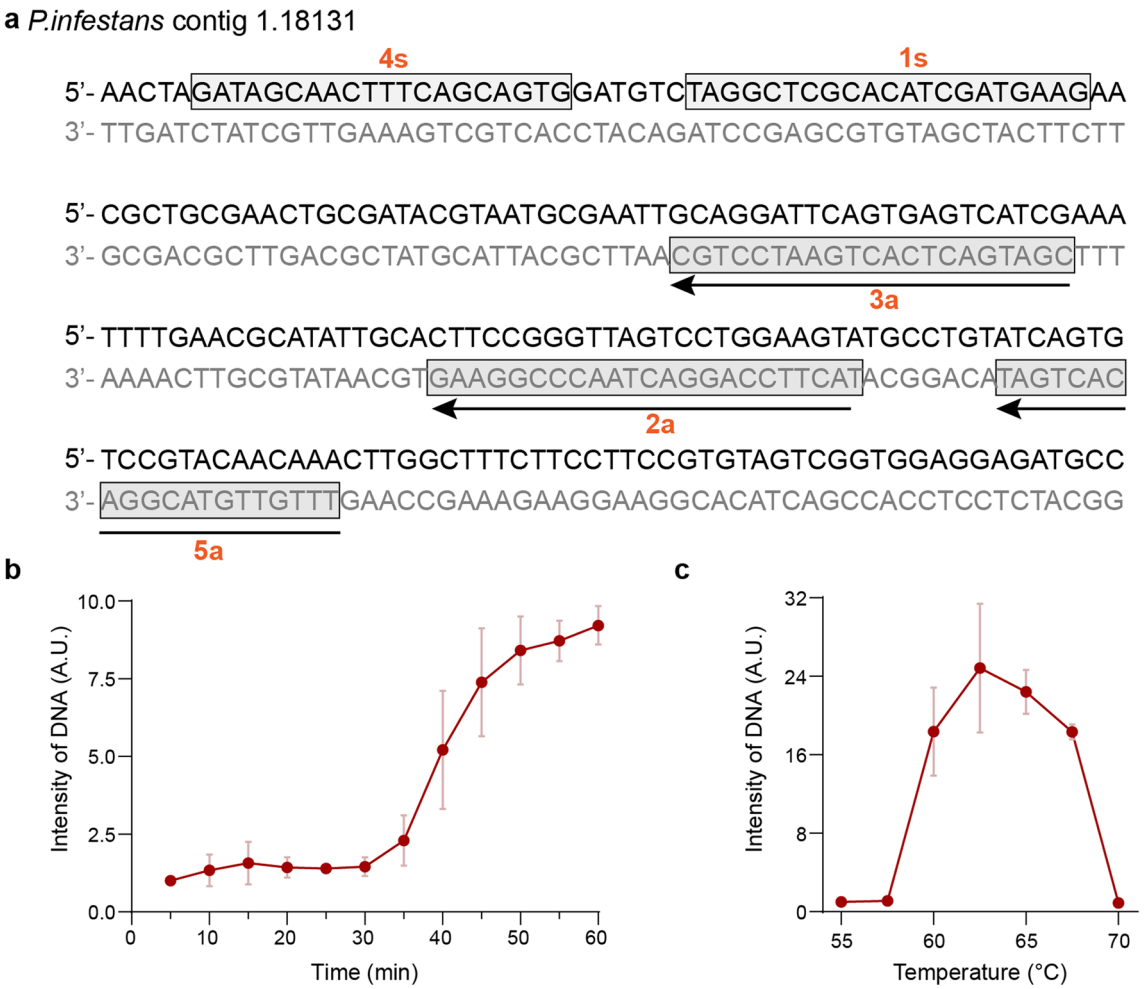

Fig. 4 Optimal conditions of the CPA reaction system. **a** Schematic representation of primer positions in *P. infestans* contig 1.18131. A set of five candidate primers were represented respectively. 2a1s: cross primer. 4s, 5a: displacement primers. **b** and **c** Determination of CPA products derived from primer sets through 2% agarose gel electrophoresis with 10 μg pure DNA of *P. infestans* (1 μL of each) for indicated reaction time or at annotated temperature, respectively. Quantification and analysis of DNA product intensity were performed with ImageJ software. A.U., Arbitrary Unit

infrastructures, and laboratories (Lau and Botella 2017; Ristaino et al. 2020). Therefore, in less developed regions, cost constraints and limited laboratory availability inhibit the suitability of conventional methods (Fang and Ramasamy 2015). In this study, we developed an integrated detection strategy to visualize disease progression and overcome the burden of labor-intensive procedures (Fig. 8). This method allows unskilled workers to achieve early detection of diseases in resource-limited settings.

Obtaining high-quality and -purity DNA is an initial step for all follow-up nucleic acid-based diagnostic methods (Moeller et al. 2014). Although leaf tissues are one of the most common entry points for plant disease screening, natural plant barriers such as the waxy cuticle and rigid cell walls are significant impediments to the extraction of genomic DNA (Anderson et al. 2018). Here, we screened an efficient extraction buffer to capture pathogen DNA from infected potato leaves, overcoming the tedious process of traditional DNA extraction methods. The corresponding DNA was demonstrably adequate for PCR amplification without further purification despite the presence of some interfering substances. The resultant amplification products were comparable to that of the CTAB method.

The use of a microneedle is an emerging technology promoting permeability in cosmetic and therapeutic applications (Larraneta et al. 2016; Than et al. 2017; Mobayed et al. 2020). It may also be applied to the extraction of nucleic acids from plant tissues (Paul et al. 2020). A microneedle (MN) patch, made of a water-swellable polymer (polyvinyl alcohol; PVA), swells with absorbed intracellular water molecules while extraction

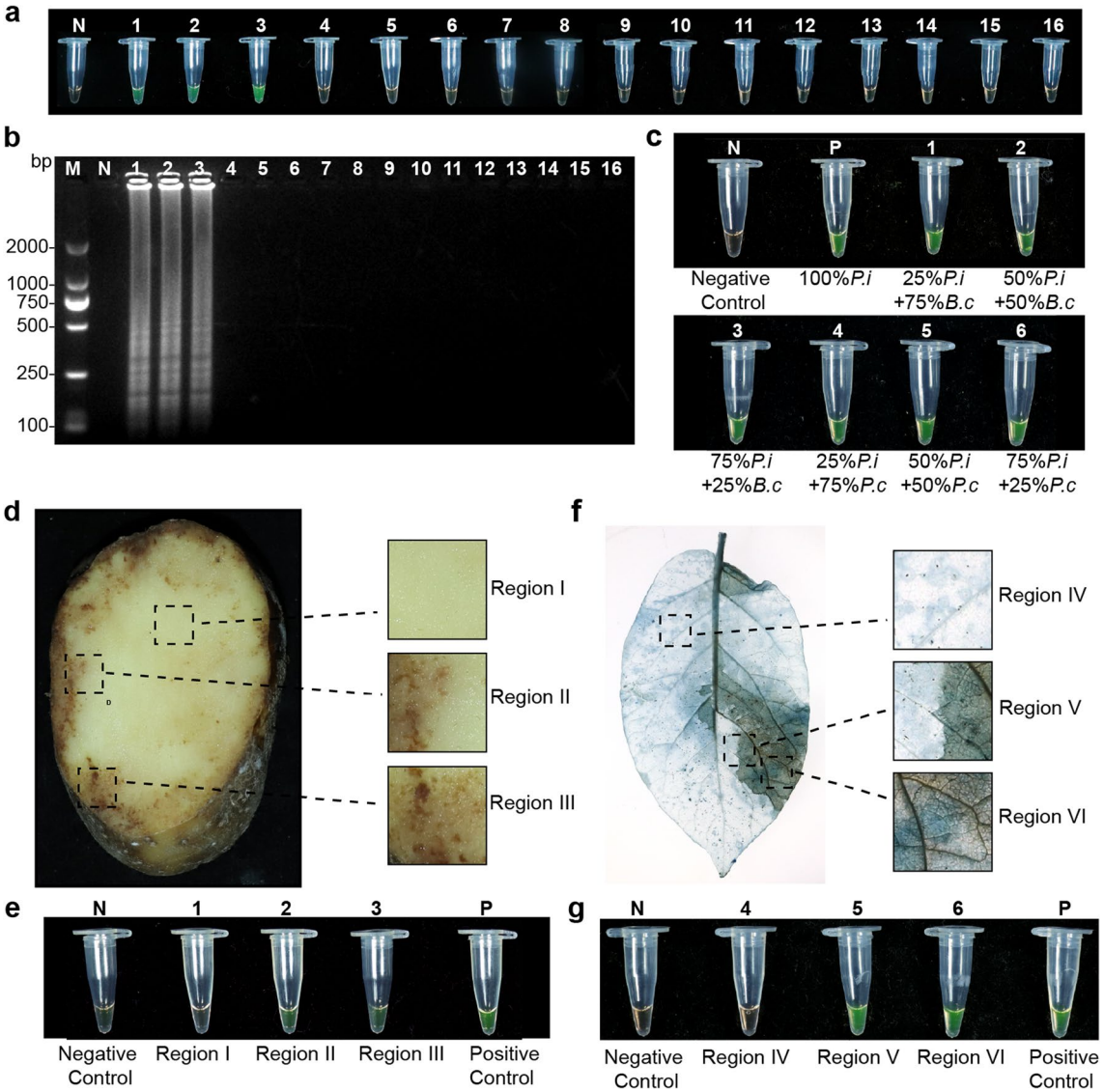

Fig. 5 Specificity test and applicability analysis of developed CPA for *P. infestans* detection. **a** and **b** Colorimetric assessment and gel electrophoresis analysis of the CPA amplificons. Number 1 to 16 indicated *P. infestans* T30-4, *P. infestans* 88069, *P. infestans* MZ, *P. capsica*, *P. nicotianae*, *P. melonis*, *P. drechsleri*, *P. cryptogea*, *P. parasitica*, *P. cactorum*, *Py. ultimum*, *B. cinerea*, *F. culmorum*, *F. solani*, *R. solani*, and *E. carotovora*, respectively. N, negative control. **c** Applicability evaluation of CPA through simulating the complex sample environment in the field. *P.i*, *P. infestans*. *B.c*, *B. cinerea*. *P.c*, *P. capsica*. N, negative control, 0% *P. infestans*. **d**, **f** Representative potato tubers and leaves (after trypan blue staining) were infected with *P. infestans*. Region I to VI represented artificially devised areas varying by stages of infection. **e, g** Detection of *P. infestans* using CPA with DNA extracted from Region I to VI. N, negative control. P, positive control, pure DNA of *P. infestans*

occurs. This process delivers DNA to the needles' tips. Subsequently, an elution buffer was used to wash off deposited DNA (Paul et al. 2019). This study used steel-made SMA to promote grinding efficiency and facilitate complete permeation of the S-buffers. Microneedles consisting of bump arrays can quickly open the surface channel pores of leaves (Kim et al. 2018; Lee et al. 2018), so an all-in-one integrated device, coupled with the SMA, was designed to achieve in-situ DNA extraction. In addition, the collection tube provided for subsequent within-tube detection, reduced the possibility of false positive results caused by cross-contamination during fieldwork.

Isothermal nucleic acid amplification technologies (INAMT) have developed rapidly as an alternative to PCR and are considered highly sensitive (Li et al. 2015). Nonetheless, loop-mediated isothermal amplification (LAMP) has been widely used in pathogen detection

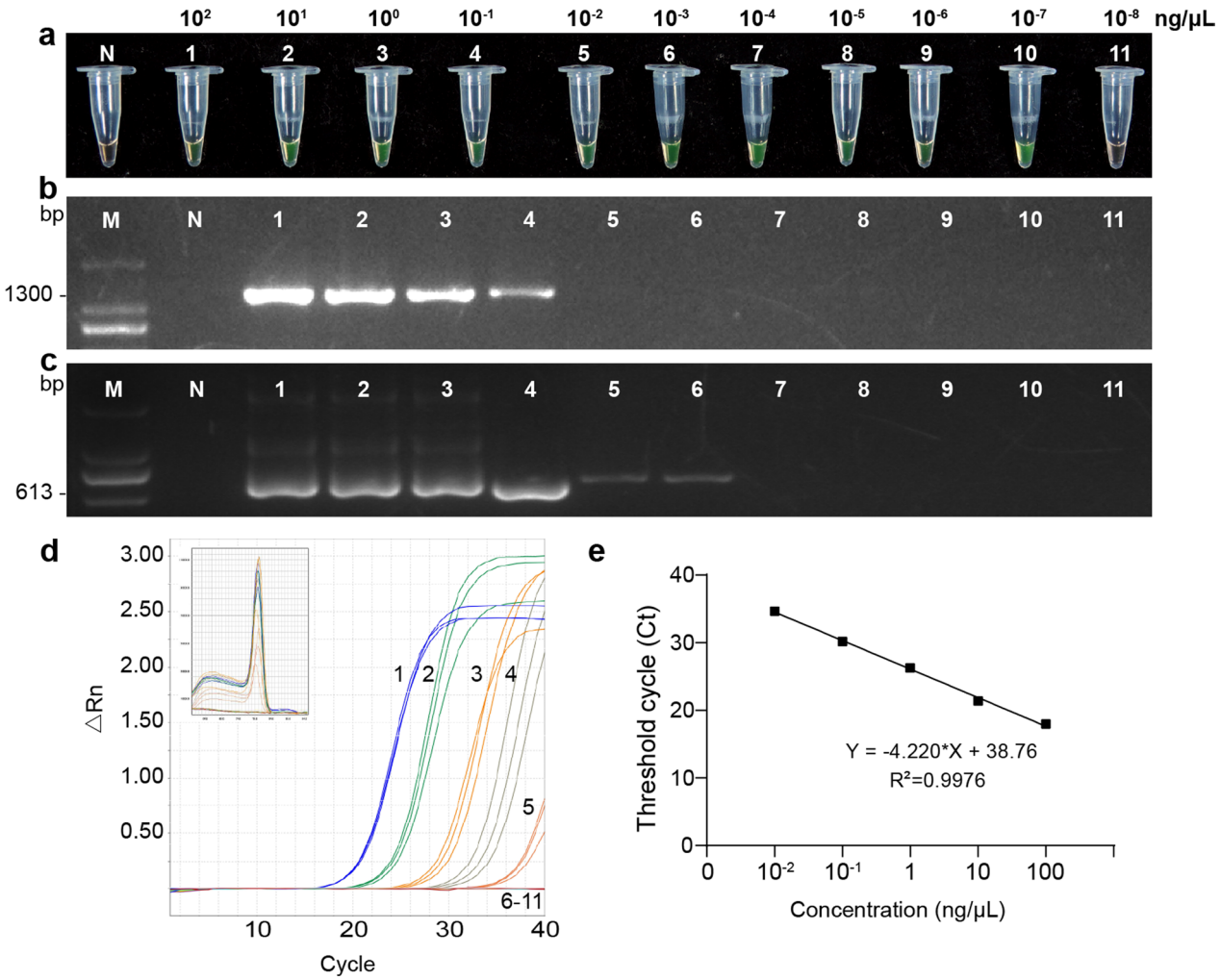

Fig. 6 Comparison of detection sensitivity among CPA and other assays. A serial dilution of *P. infestans* DNA ranging from 10^{2} to 10^{-8} ng/μL was assayed by CPA (**a**), cPCR(**b**), nPCR (**c**), and qPCR (**d**). **d** Melting curve and amplification curve of each sample in qPCR. **e** Standard curve generated by Ct values derived from qPCR analysis

(Wong et al. 2018), despite previous studies concluding that highly conserved nucleotide levels of target genes, ideally single-copy genes, are prerequisites for this technology (Alessi et al. 2020). Furthermore, the reliability of LAMP may be compromised due to some false positives caused by non-specific pairing and contamination, limiting high-throughput applicability (Nagdev et al. 2011; Yamagishi et al. 2017; Deng et al. 2019). However, as an advanced INAMT, CPA can improve accuracy with an improved primer design, which overcomes the reliance on conserved genes (Xu et al. 2012). Therefore, with a limited number of practical applications studied for CPA filamentous pathogen detection, it was used as an application in this study.

As for the study target, the devastating disease late blight was used to explore the potential of advanced pathogen detection methods. To the best of our knowledge, we have established the first optimized visual CPA system for this disease with high accuracy, excellent specificity, and little need for additional instrumentation. Furthermore, it can be conducted within a wide range of turnaround times (40–60 min) and temperatures (60°C–67.5°C). All the same, yield results indicated that 60 min at 63°C were optimal reaction conditions. Additionally, CPA primers targeting multi-copy region ITS showed no non-specific amplification for tests on the negative (control) pathogens. Although some *Phytophthora* species have high similarity in ITS sequence, potentially leading to difficulty in primer design and lower specificity (Kroon et al. 2012; Fall et al. 2015), except for *Phytophthora infestans*, other *Phytophthora* spp. rarely infect potatoes (Zhu et al. 2019). However, it has been reported that combining interspecific primers targeting multiple sequences can be used for pathogen

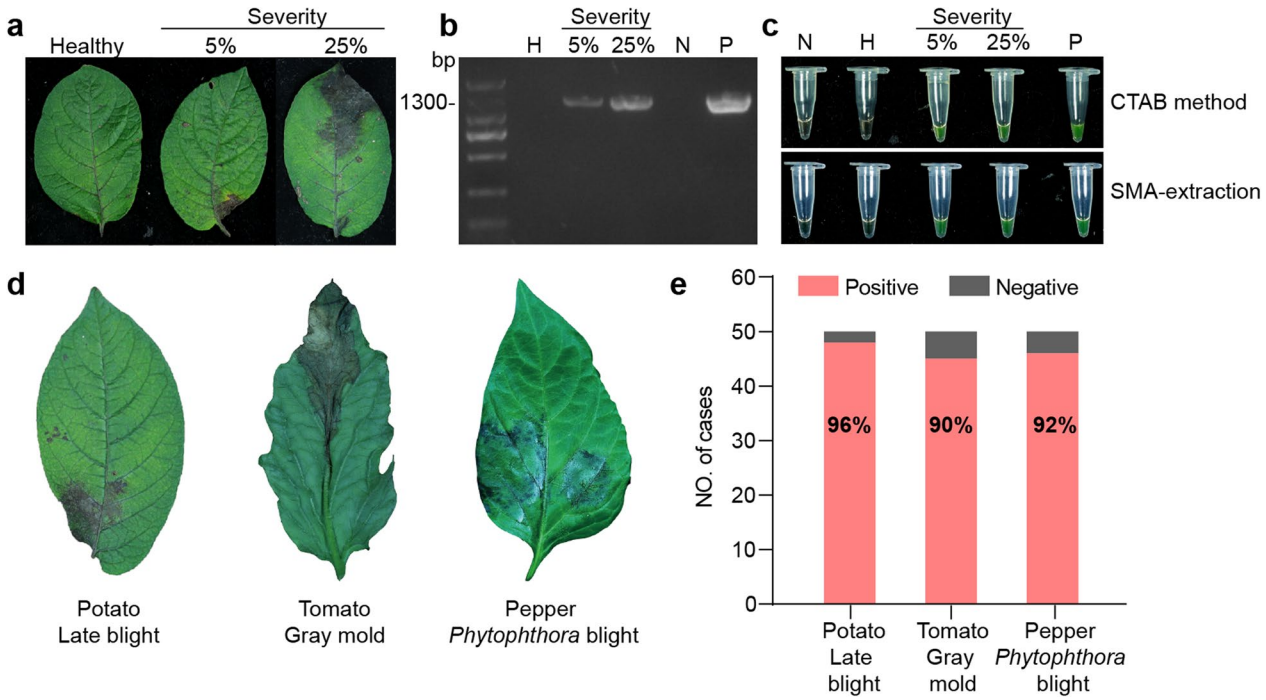

Fig. 7 Detection of diseased field samples using SMA-Device extraction and visual CPA assay. **a** Typical *P. infestans*-infected potato leaves with varying degrees of severity. **b** A fragment of the *P. infestans* genome was amplified by PCR using DNA extracted from **a** following the SMA-Device extraction. **c** Diagnosis of *P. infestans* with visual CPA assay using the corresponding DNAs. **d** Photographs of samples with representative symptoms of potato late blight, tomato gray mold, and pepper *Phytophthora* blight, respectively. **e** Challenging plant leaves collected from the field were subjected to SMA-Device extraction and visual CPA assay, and the detection rate was displayed

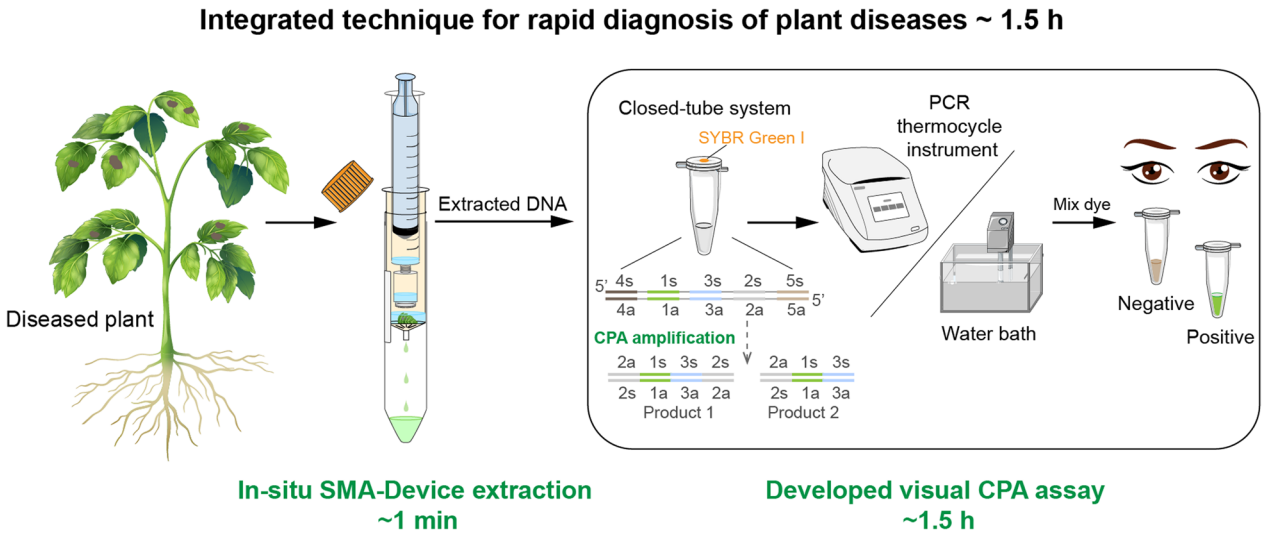

Fig. 8 An integrated workflow to visualize disease detection by utilizing SMA-Device extraction and developed CPA assay

detection, such as the IGS region (Diguta et al. 2010) and the COX gene (Spring et al. 2011). Furthermore, CPA outperformed the other traditional assays by detecting the lowest DNA concentration of 10^{-7} ng/μL, reaching up to 10^6-, 10^4- and 10^5-times; more sensitive than cPCR, nPCR, and qPCR, respectively. This result further illustrates that the application of CPA has enormous potential to improve detection accuracy. In testing, CPA also worked well in the presence of interfering pathogens and

under different disease severities, indicating excellent detection suitability in complex field environments.

Greater awareness of the need to correctly diagnose pathogens and protect crops has been occurring in remote or resource-limited settings, further emphasizing the need for field-portable and cost-effective disease detection systems. The current report unveils a novel integrated pathogen detection technique using an SMA-Device and modified visual CPA to facilitate the field diagnosis of plant diseases. Moreover, the technique was demonstrated to be successfully applied to field diseased samples with multiple pathogens, offering a promising substitute to the conundrum of sampling in large batches.

Conclusions

A rapid, economical, and visual pathogen detection strategy was established, consisting of an equipment-free in-situ DNA extraction method based on an SMA-Device and an improved CPA assay. DNA extraction time was dramatically reduced to ~1 min, and the entire detection process was ~1.5 h. In addition, the extracted DNA exhibited sufficient quality for subsequent molecular detection without further purification, and comparable amplification products were obtained more simply. Also, we developed a fast, accurate, and credible CPA assay system to detect and visualize *P. infestans* for the first time, with increased sensitivity and a higher degree of specificity. Moreover, the SMA-Device extraction and optimized CPA assay were applicable for immediate field diagnosis across large numbers of samples. The established techniques enable in-situ plant disease diagnosis and make future sample-to-answer plant disease detection possible.

Methods

Plant and pathogen cultivation

Potato cv. Favorita was cultivated in the greenhouse at 24°C under a 14-h light/10-h dark photoperiod, with 60% relative humidity.

P. infestans strains T30-4, 88069, and MZ were cultured in the dark on Rye A agar medium (Rye: 60 g/L, Sucrose: 20 g/L, Agar: 15 g/L) at 18°C. Other *Fusarium*, *Rhizoctonia,* and *Botrytis* species used in this study were routinely maintained in the dark on Potato Dextrose Agar (PDA) medium (Potato: 200 g/L, Glucose: 20 g/L, Agar: 20 g/L) at 25°C. *Erwinia carotovora* was grown overnight on solid Luria–Bertani media (LB) medium (Tryptone: 10 g/L, Yeast extract: 5 g/L, NaCl: 10 g/L, Agar: 15 g/L) at 28°C. *P. capsica*, *P. nicotianae*, *P. melonis*, *P. drechsleri*, *P. cryptogea*, *P. parasitica*, *P. cactorum*, and *Pythium ultimum* were cultivated in the dark on 10% vegetable (V8) juice agar medium (V8 juice: 10% v/v, $CaCO_3$: 1 g/L, Agar: 15 g/L) at 25°C. In all cases, the pathogens cultivated in petri dishes were maintained in different constant-temperature incubators (PHCbi, China) (Additional file 2: Figures S2, S3).

Inoculation assay

Culture plates of the *P. infestans* strain MZ were scraped by an L-shaped sterile spreader with 5 mL pre-cooled ddH_2O to make a sporangia suspension. Then, according to the concentration determined by a hemacytometer, the suspension was diluted to the desired density with sterile water.

For the detached leaf assay, four-week-old healthy potato leaves were evenly sprayed for inoculation at a concentration of 6×10^3 sporangia/mL. For the potato tuber inoculation assay, healthy and uniform potatoes were harvested and thoroughly surface-sterilized with 75% alcohol. Individual potato tubers were soaked in spore suspension at 5×10^4 sporangia/mL density for 5 min. Both inoculated leaves and tubes were kept inside sealed plastic boxes to maintain high humidity and placed at 18°C in a climate chamber (routinely a 16 h/8 h light/dark cycle after 24 h continuous darkness) for about 7 and 14 days, respectively.

Assembly of the SMA-device

The device consists of three parts: a collection tube, an inner tube and an extractor (Fig. 1a). The collection tube is a standard centrifuge tube (10–50 mL), and the inner tube can be inserted into it. The bottom of the inner tube is used to support samples to withstand piercing, and there are extract outflow holes at the bottom to allow samples containing DNA to flow into the collection tube. The extractor consists of an upper tube (a syringe) and a down tube with SMA, both mounted and fixed through a screw interface. SMA was imaged by the Tabletop Scanning Electron Microscope (TM-4000Plus, Hitachi, Japan). The SMA was evenly distributed on a 1 cm × 1 cm circular patch. Each needle was 500 μm in base radius, 80 μm in tip radius, and 350 μm in height, featuring excellent scratch and chemical resistance, and robust mechanical properties (Fig. 1b).

Comparison of different simple DNA extraction buffers

Two kinds of simple DNA extraction buffers (S-buffer 1: 20 mM Tris, pH 8. 0, 25 mM NaCl, 2.5 mM EDTA, 0.05% SDS; S-buffer 2: 10 mM Tris, pH 9.5, 0.5 mM NaCl, 0.5 mM EDTA, 0.1 mM KCl, 0.1% Triton X-100) with modifications, according to the research of Zou et al. (2017) and Li et al. (2020), were chosen to test the procedure acceptance rate. Potato leaves infected with *P. infestans* were fully ground in each of the S-buffers,

respectively, with four replicates and 12 samples. Screening outcomes (UV absorption spectra, concentration, and purity analysis) were measured and documented by the Nanodrop ND-1000 spectrophotometer (Thermo Scientific, USA). Furthermore, PCR amplifications for the *P. infestans* ITS region were performed to verify the integrity of isolated DNA. Primer sequences and running programs of PCR were shown in Additional file 1: Table S2 and the corresponding results were presented by gel electrophoresis.

SMA-Device-based in-situ DNA extraction equipped with S-buffer

A flowchart outlining the protocol of the in-situ DNA extraction using an SMA-Device is presented in Fig. 1a. Step-by-step instructions are as follows: First, using the syringe, draw up about 500 µL of efficient S-buffer 2. Then, the Up- and Down- tube were installed following the standard requirements. Next, soft plant tissue (e.g., leaf) was placed inside the device and punctured with S-buffer 2. Simultaneously, the released crude DNA solution could be collected.

For each extraction, the SMA-Device could be reused after a thorough cleaning. The concentration and quality of DNA were recorded by Nanodrop ND-1000 spectrophotometer (Thermo Scientific, USA).

CTAB-based DNA extraction

Hyphal or plant tissues were collected and homogenized with 700 µL pre-warmed 2×CTAB extraction buffer (2% w/v CTAB, 100 mM Tris–HCl, pH 8.0, 20 mM EDTA, pH 8.0, 1.4 M NaCl, 40 mM β-mercaptoethanol). The mixture was vortexed for 30 s and incubated at 65°C for 30 min in a water bath. Next, one volume of phenol/chloroform/isoamyl alcohol (25:24:1) was added, and then the mixture was centrifuged at 13,400 *g* for 10 min and followed by a repeat of the previous step after transferring the supernatant. Next, one volume of pre-cooled isopropyl alcohol and one-tenth volume of 3 M NaAc was mixed with the aqueous phases and kept for 30 min on ice for DNA precipitation. Then the samples were centrifuged for 10 min, 13,400 *g* at 4°C. Next, the supernatants were discarded, and the precipitated DNA pellets were washed twice with 1 mL of cold 70% ethanol. Finally, the dry pellets were resuspended in 30–50 µL ultrapure water, and the respective concentrations were determined using a Nanodrop ND-1000 spectrophotometer (Thermo Scientific, USA).

Trypan blue assay

Lactophenol trypan blue staining solution (4 mg/mL trypan blue, 10% phenol, 10% glycerol, 10% lactic acid, 60% absolute ethyl alcohol, 10% ddH_2O) was prepared to reflect the morbidity. First, infected potato leaves were immersed into the staining solution and heated twice in boiling water for 2 min with an interval of solution cooling. After that, inoculated leaves were soaked overnight in the staining solution. Then the trypan blue solution was replaced with 2.5 g/mL chloral hydrate solution and followed by another overnight incubation at room temperature for destaining.

Development of CPA assay to detect *P. infestans*

A CPA reaction system for detecting *P. infestans* was initially established in this study. Genome assembly sequence of contig 1.18131 (NCBI accession No. AATU01018131.1) in *P. infestans* T30-4 genome, which contains a sufficient variation of ITS region in a lot of *Phytophthora infestans* strains after BLAST analysis (Zhu et al. 2019), was used. Five specific primers, including 2a1s, 2a, 3a, 4 s, and 5a (2a1s: cross primer, 4s, 5a: displacement primers) for a single crossing CPA assay, were developed from the target region using DNAMAN software and shown in Fig. 4a. Each reaction in a total volume of 25 µL consisted of DNA template (10 copies or more), primer 2a1s (0.5 µM), primers 2a and 3a (0.3 µM), primers 4s and 5a (0.1 µM), MgSO4 (6 mM), dNTP mix (0.8 mM, Solarbio, China), Betaine (1 M, Sigma, UK), 1×ThermoPol Buffer, 8 U *Bst* DNA Polymerase Large Fragment (Vazyme, China), and nuclease-free water, was gently mixed by pipetting before use.

To determine the optimal conditions of the CPA assay, we used 20 ng pure DNA of *P. infestans* as the template for each reaction to carry out the amplification. Firstly, the mixtures were conducted at different times in 5 min intervals for up to 60 min at 63°C individually. The amplification products were immediately analyzed on a 2% agarose gel, and a ladder-like pattern of bands was observed in positive groups. The Image J software quantified the band intensities of yield DNA, and each treatment was independently amplified in triplicate. Secondly, we performed the CPA assay at temperatures ranging from 60 to 70°C with a gradient of 2.5°C. Finally, the optimal temperature for incubation was determined using similar methods as previously described.

Visualization of the CPA reaction

A visual CPA assay was developed to inspect the products using the naked eye after staining with an indicator dye. Adding 1 µL of SYBR Green I fluorescent dye (Edley, China) to the cover before starting prevented cross-contamination via aerosol because the tube would be

closed. At the end of the assay, the dye was centrifuged or inverted within the tube to cause mixing with the reaction solutions and then incubated for several minutes. The reaction solution was observed for color change under visible light. The green fluorescence will noticeably increase when bound to double-stranded DNA. Thus, green coloration was interpreted as a positive result, while brown indicated a negative one.

Analytical specificity and suitability of the CPA assay

To determine the analytical specificity of the developed CPA assays, representative pathogens, including *P. infestans* strains, common species from other oomycetes, fungi, and bacteria were evaluated (Fig. 5a). 10 ng genomic DNA was added to each CPA reaction and incubated at 63°C for 60 min. As described above, the amplified CPA products were subjected to visual colorimetric assessment and gel electrophoresis analysis.

For the specificity test, the DNA of *P. infestans* was mixed with interferential pathogens (*B. cinerea* and *P. capsica*) into different proportions (0, 25, 50, 75, 100%), and the mixtures were used as templates. The total amount of DNA in each tube was 10 ng. Visual CPA reactions were performed as described above to represent the amplification efficiency.

In addition, tubers and leaves of potatoes artificially inoculated with *P. infestans* were conducted to test the applicability of the developed CPA assay. Samples were divided into representative health (Region I, IV), the juncture between healthy and diseased (Region II, V), diseased (Region III, VI) areas after typical symptoms emerged (Fig. 5d, f), followed by trypan blue staining of leaves to better exhibit lesions. The genomic DNA of each region was extracted by the CTAB method. The visual CPA assays were performed using 1 µL of extraction as amplification templates from relevant regions. Pure DNA extracted from *P. infestans* was used as a positive control.

Formulation of other amplification assays

Conventional PCR (cPCR)

The 25 µL reaction mix was: 12.5 µL 2×Master Mix (Vazyme, China), 0.25 µM of each primer, 1 µL of gDNA with specific concentration as a template, and sterilized distilled water up to 25 µL. The temperature program was: 2 min at 94°C, 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for varied extension time depending on the expected PCR product length (about 1 min/kb), then a final extension at 72°C for 5 min.

Nested PCR (nPCR)

The 25 µL reaction mix was: 12.5 µL 2×Master Mix (Vazyme, China), 0.25 µM of each primer, 2 µL of cPCR amplification products as a template, and sterilized distilled water up to a final volume of 25 µL. Nested PCR was performed with the same cycling procedures defined for cPCR.

Real-time quantitative PCR (qPCR)

The 20 µL reaction mix was: 10 µL 2×RealStar Fast SYBR qPCR Master Mix (GenStar, China), 0.25 µM of each primer, 1 µL of gDNA with specific concentration, and sterilized distilled water up to 20 µL. The reaction was conducted in the QS6 qPCR instrument (Thermo, US). The temperature program was: 2 min at 95°C, followed by 40 cycles of denaturation at 95°C for 15 s, and annealing at 60°C for 30 s.

For a list of all primer sequences employed in this study, refer to Additional file 1: Table S2. All experiments were repeated at least three times.

The detection sensitivity of CPA and other amplification assays

To compare the detection limits of different detection assays, a series of ten-fold gradient dilutions of *P. infestans* genomic DNA (from 10^{2} to 10^{-8} ng/µL) was used as templates for CPA, cPCR, nPCR, and qPCR separately. After amplification, CPA reaction products were subjected to color reaction as described above, cPCR and nPCR were analyzed using gel electrophoresis. Ct values of qPCR were used to evaluate the sensitivity, and a standard curve was constructed. Further, the corresponding melting-curve analysis and standard curve plotting were used to validate the primer specificity and amplification efficiency.

Detection of diseased field samples using the SMA-Device extraction and visual CPA

In order to verify the applicability of the integrated pathogen detection technique developed in this study, *P. infestans*-infected potato leaves at different degrees of severity were selected from the field, and the total genomic DNA was prepared using the SMA-Device extraction method. Then, PCR amplification targeting the ITS region and the visual CPA assay were performed as described. For each reaction, a mixture of three parallel samples was used as a template to reduce the sample variation. All experiments were repeated three times. Finally, gel electrophoresis was used to analyze related amplification products and show the disease severity.

Moreover, to test whether the integrated technique is suitable for direct diagnostics, a random set of 50 potato late blight, tomato gray mold, and pepper *Phytophthora*

blight infected field samples were collected, respectively. In this way, a total number of 150 samples were stored in a refrigerator at 4°C. Then, following the principle described above, the SMA-Device-based extracted genomic DNA was conducted with the developed CPA assay, and the relevant detection rates were calculated.

Integrated pathogen detection technique coupled with SMA-Device extraction and modified visual CPA assay

A general analysis workflow of an integrated pathogen detection technique that combines both developed assays together was illustrated in Fig. 8. According to the instructions, one leaf of interest (or other soft plant tissues) in the field was first chosen. Then, DNA was extracted in-situ within 1 min by completely puncturing and infiltrating its surface with the SMA-Device and S-buffer. Next, a developed CPA assay with the corresponding DNA was performed with a closed-tube system in a 63°C water bath or a PCR machine. After a 60 min incubation, SYBR Green I dye was mixed with the reaction and left to stand for several minutes. The detection results could be judged by the change of color using the naked eye. The entire process took approximately 1.5 h.

Abbreviations

ITS	Internal transcribed spacer
spp.	Species
COX	Cytochrome-C-oxidase

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s42483-023-00179-9.

Additional file 1: Table S1. Comparison of Nanodrop recordings of DNA solutions extracted by different S-buffers. **Table S2.** List of primer sequences employed in this study.

Additional file 2: Figure S1. Direct diagnostic of field infected samples using visual CPA assay. Direct diagnostic efficiency of visual CPA assay in field application for potato late bight, tomato gray mold, pepper *Phytophthora* blight, respectively. N, negative control. P, positive control. Serial number in white, positive reactions. Serial number in red, negative reactions. **Figure S2.** Colony morphology of different types of pathogens tested in this study. Colony morphology pictures of *P. infestans* T30-4, *P. infestans* 88069, *P. infestans* MZ, *P. capsica*, *P. nicotianae*, *P. melonis*, *P. drechsleri*, *P. cryptogea*, *P. parasitica*, *P. cactorum*, *Py. ultimum*, *B. cinerea*, *F. culmorum*, *F. solani*, *R. solani*, and *E. carotovora* were taken by a camera. Scale bars = 1 cm. **Figure S3.** Morphological characteristics of different types of pathogens tested in this study. Mycelium/thallus morphology pictures of *P. infestans* T30-4, *P. infestans* 88069, *P. infestans* MZ, *P. capsica*, *P. nicotianae*, *P. melonis*, *P. drechsleri*, *P. cryptogea*, *P. parasitica*, *P. cactorum*, *Py. ultimum*, *B. cinerea*, *F. culmorum*, *F. solani*, *R. solani*, and *E. carotovora* were taken by a microscope. Scale bars = 20 μm.

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Authors' contributions

XW and DD conceived and designed the study. JL (Jie Li), JD (Juan Du), SL, JD (Jiali Dong), JY, YG, JL (Jie Lu), and XZ performed the experiments. JL (Jie Li), JD (Juan Du), and XW analyzed the results and wrote the manuscript. PK revised the manuscript. JY modified the manuscript's format. All authors read and approved the final manuscript.

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Availability of data and materials

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Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

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Competing interests

The authors declare that they have no competing interests.

Author details

[1]Department of Plant Pathology and MOA Key Lab of Pest Monitoring and Green Management, College of Plant Protection, China Agricultural University, Beijing 100193, China. [2]Biotechnology Research Institute, Heilongjiang Academy of Agricultural Sciences, Harbin 150028, China. [3]Chongqing Three Gorges Academy of Agricultural Sciences, Chongqing 404100, China. [4]CIP China Center for Asia Pacific (CCCAP), Beijing 100081, China. [5]College of Plant Protection, Academy for Advanced Interdisciplinary Studies, Nanjing Agricultural University, Nanjing 210095, China.

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