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Research Article

CRISPR-Cas12a-Assisted Multicolor Biosensor for Semiquantitative Point-of-Use Testing of the Nopaline Synthase Terminator in Genetically Modified Crops by Unaided Eyes

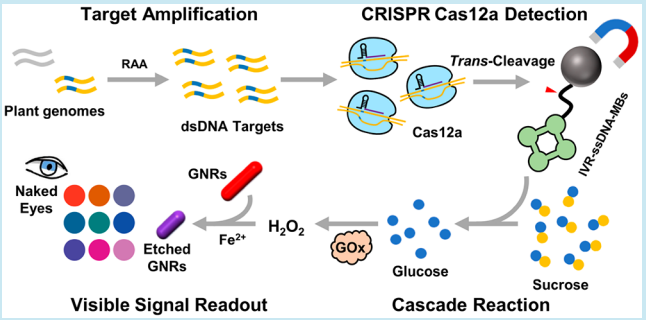
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ABSTRACT: Existing methods of detecting foreign genes and their expression products from genetically modified organisms (GMOs) suffer from the requirement of professional equipment and skillful operators. The same problem stays for the CRISPR-Cas12a system, although it has been emerging as a powerful tool for nucleic acid detection due to its remarkable sensitivity and specificity. In this report, a portable platform for the visible detection of GMOs based on CRISPR-Cas12a was established, which relies on a color change of gold nanorods (GNRs) caused by the invertase-glucose oxidase cascade reaction and the Fenton reaction for signal readout. A nopaline synthase (NOS) terminator was employed as a model target commonly existing in foreign genes of GMOs. With the help of recombinase-aided amplification, this platform achieved comparable sensitivity of DNA targets (1 aM) with that of a fluorescence reporting assay. As low as 0.1 wt % genetically modified (GM) content in Bt-11 maize was visually observed by unaided eyes, and the semiquantitation of GM ingredients can be obtained within the range of 0.1 to 40 wt % through the absorption measurement of GNRs. Furthermore, five real samples were tested by our method, and the results indicated that the GM ingredient percentages of GMO samples were 2.24 and 24.08 wt %, respectively, while the other three samples were GMO-free. With the advantages of a simple procedure, no need for large or professional instruments, high sensitivity, and selectivity, this platform is expected to provide reasonable technical support for the safe supervision of GMOs.



KEYWORDS: *CRISPR-Cas12a, visible point-of-use testing, genetically modified organism, NOS terminator*

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Genetic modified (GM) crops have brought huge economic, environmental, and agricultural benefits to the world since their commercialization at the end of the 19th century.[1] According to the accomplishment report from the International Service for the Acquisition of Agri-biotech Applications (ISAAA), there were 191.7 million hectares of GM crops, covering more than 10% of total arable lands in the world and involving 17 million farmers in 26 countries in 2019.[2] Since transgenic technology and GM crops have been widely and increasingly applied in recent years, their uncertain biosecurity, especially the food safety of GM crops, has received unprecedented high attention.[3] Under this circumstance, many countries and international organizations such as the Food and Agriculture Organization of the United Nations have made various evaluation criteria to ensure the safe usage of GM crops, and thereby, highly sensitive analytical techniques are essential to execute regulatory policies.[4]

Several detection methods have been established to satisfy the requirement of strict monitoring of GM crops. These methods can be generally divided into two categories according to different detection targets.[5,6] In the first category, nucleic acid molecules in GM crops such as foreign genes or specific promoters and terminators were applied as detection targets.[7,8] Through techniques such as polymerase chain reaction (PCR) and loop-mediated isothermal amplification (LAMP), a target nucleic acid was amplified and analyzed by fluorescence readout or sequencing and finally compared with GM crop banks to ensure the presence of specific foreign genes. For instance, on the basis of real-time quantitative PCR (qPCR), Košir et al.[9] have established a duplex droplet digital PCR assay to analyze the endogenous soybean genes and GM-soybean MON 40-3-2 simultaneously, and they achieved the quantitative detection of GM ingredients in complex samples. In the second category, the expression products of foreign genes were tested by immunological methods or mass spectrometry.[10] For example, by combining an immunological

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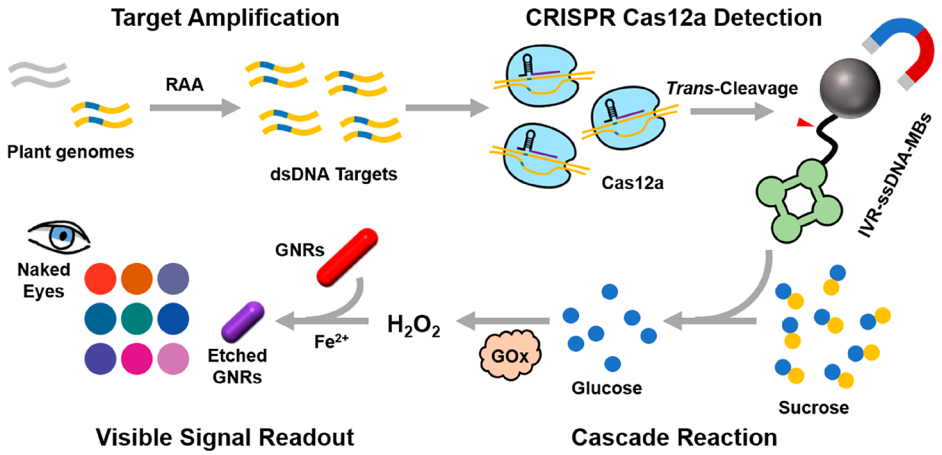


Figure 1. Working principle of CRISPR-Cas12a-assisted multicolor biosensor for POU testing of NOS terminator in GM crops by unaided eyes.

reaction and a screen-printed carbon electrode, Ocaña et al.[11] have developed a bioanalytical device for detecting 5-enolpyruvylshikimate-3-phosphate synthase from *Agrobacterium* species strain CP4 (CP4-EPSPS) with a limit of detection (LOD) of 0.9 wt %. Although these existing analytical methods applied on GM crops have the merits of high sensitivity and reliability, they are still limited in practice for monitoring GM crops in remote areas. Specifically, the requirement of special instruments and multistep operations make these methods only applicable in the lab setting but not suitable for the point-of-use (POU) testing, which should be low-cost, portable, and user-friendly.[12−14] Also, the screening of antibodies specific to various targets in foreign expression products is time-consuming and expensive.

Clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated (Cas) enzymes constitute one of the most prominent technologies in synthetic biology.[15] Besides its outstanding genome editing capability, CRISPR Cas effectors also showed remarkable potential in biosensing of nucleic acid detection.[16,17] For instance, owing to the RNA-guided nonspecific single-stranded DNA (ssDNA) cleavage activity when binding the target DNA,[18,19] various CRISPR-Cas12a biosensors have been introduced for detecting different nucleic acid targets, including cancer mutations,[20] pathogenic bacteria and viruses,[21,22] bacterial resistances,[23] and others.[24,25] Chen et al.[26] have combined the CRISPR-Cas12a system with recombinase polymerase amplification and fluorometric substrates to create a novel detection method termed DETECTR and achieved aM level sensitivity enabling rapid and specific testing of human papillomavirus. However, such a fluorescence-based CRISPR-Cas12a system still required a large instrument for signal readout and was impractical to fulfill the real POU testing. Afterward, Tsou et al.[27] have introduced colloidal gold test strips to read results by unaided eyes for the development of a CRISPR-Cas12a system as POU testing devices. Although the detection process was simplified by test strips, this kind of single-color display strategy could only produce qualitative results and lacked sensitivity and accuracy toward visible detection, which is critical for POU testing, especially in the strict supervision for GM crops. Therefore, a more flexible and accurate signal readout method, such as a multicolor display, is highly preferred.

Gold nanorods (GNRs) with different aspect ratios could exhibit diverse colors due to the unique transverse surface plasmon resonance (TSPR) and longitudinal surface plasmon resonance (LSPR) patterns in the absorption spectrum. These characteristics make GNRs attractive as an excellent substrate in a chromogenic reaction for multicolor signal readout.[28−30] For instance, Mao et al.[31] have established a portable and visible detection method based on GNRs, which were synthesized with the protection of hexadecyl trimethylammonium bromide (CTAB) in the horizontal direction. With the existence of H_2O_2, GNRs could be etched along the longitudinal direction and resulted in the presence of different colors, which were distinguished by unaided eyes. The distinctive blue shift of the LSPR peak in the absorption spectrum of GNRs during the color change enabled the semiquantitative exhibition of detection results. This signal readout platform based on a multicolor display has the potential to be developed as a biosensor for POU testing when combined with the CRISPR-Cas12a system.

Inspired by this progress, we integrated the target recognition system, which comprised recombinase-aided amplification (RAA) and CRISPR-Cas12a techniques, with the cascade reaction induced multicolor signal readout strategy based on GNRs, to finally establish a sensitive and visible POU testing method for GM crops. First, invertase was anchored on functional magnetic beads (MBs) *via* the mediation of ssDNA. The nopaline synthase (NOS) terminator was chosen as a model DNA target commonly used in GM crops that is nonexistent in the genomes of ordinary plants. With the presence of target DNA, the unique collateral DNase activity of CRISPR-Cas12a was activated with the assistance of CRISPR RNA (crRNA). Then invertase was released from conjugated MBs when the ssDNA linker was cut by activated Cas12a. After magnetic separation, free invertase catalyzed the decomposition of sucrose to produce glucose, which was then oxidized by glucose oxidase (GOx) to generate a large amount of H_2O_2. After the cascade reaction for signal amplification, an Fe^{2+}-mediated Fenton reaction converted H_2O_2 to ·OH, which possesses higher redox potentials than Au(I)/Au(0) (+1.69 V).[32] Thus, GNRs were etched by ·OH to cause the decrease of the aspect ratio and the resulting blue shift of LSPR peak. Therefore, different colors depending on the origin concentrations of the target DNA can be easily observed by unaided eyes. The biosensor developed in this work is promising to be applied for portable POU testing of GM crops in remote areas.

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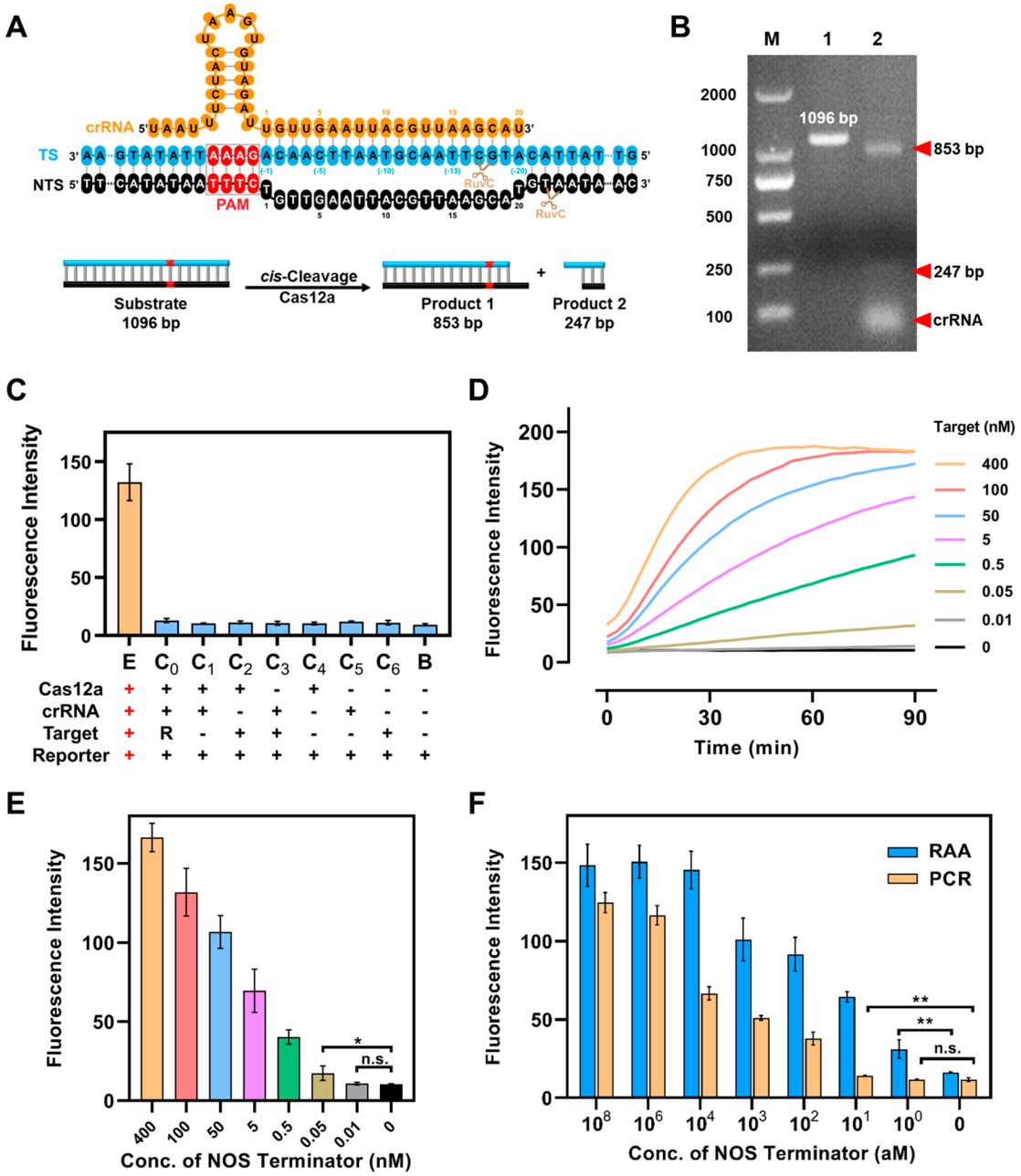


Figure 2. Establishment of NOS terminator detection system based on fluorescence reporting assay of Cas12a. (A) Principle of Cas12a-mediated *cis*-cleavage of linear dsDNA target containing NOS terminator. (B) Gel image of *cis*-cleavage products (lane 1: 1096 bp dsDNA without *cis*-cleavage, lane 2: two segments of *cis*-cleavage products). (C) Feasibility of *trans*-cleavage of Cas12a guided by crRNA in fluorescence reporting assay. Experiment group (E) and control group (C_0) possessed a complete reaction system with NOS terminator dsDNA target and random sequence dsDNA, respectively, the other control groups (C_1−C_6) possessed defective reaction systems, and the blank group (B) possessed a reporter only (with three technical replicates; error bars represent the mean ± SEM). (D) Reaction kinetics of NOS terminator detection based on the Cas12a-assisted fluorescence reporting assay. Serially diluted dsDNA with the NOS terminator was employed as the target, and the fluorescence intensity was measured every 3 min (average values of three technical replicates were shown in the reaction kinetics curves). (E) Sensitivity of the fluorescence reporting assay at 30 min indicates the LOD was as low as 0.05 nM (with three technical replicates; error bars represent the mean ± SEM; $^*P < 0.05$, and "n.s." means no significance in "Student's t test"). (F) Sensitivity of the fluorescence reporting assay combining with RAA or PCR at 30 min indicates the LOD was 1 aM with RAA and 10 aM with PCR (with three technical replicates; error bars represent the mean SEM; $^{**}P < 0.01$, and "n.s." means no significance in "Student's t test").

RESULTS AND DISCUSSION

Working Principle of the Biosensing System. The visible biosensing assay targeted on a NOS terminator was mainly composed of four parts (Figure 1): nucleic acid amplification, a Cas12a *trans*-cleavage system, a cascade reaction, and a Fenton-reaction-based visible signal readout. First, the NOS terminator in samples was amplified by highly sensitive RAA to produce target DNA in high copy numbers. Then, the introduced target DNA was hybridized with the crRNA to trigger the *trans*-cleavage activity of the CRISPR-Cas12a system. Thereby the ssDNA linkers between functional MBs and invertase were cut by Cas12a, in which the amount of

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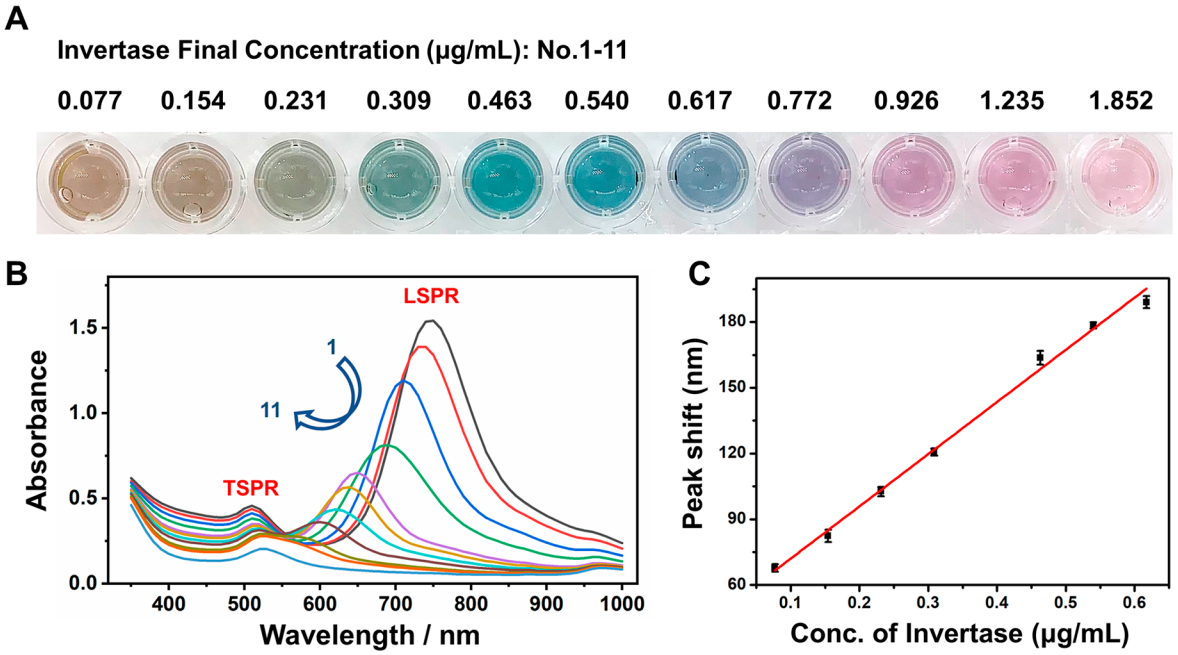


Figure 3. Sensitivity of visible GNR etching by cascade reaction. (A) Images of responses of GNRs to different concentrations of invertase in the color reaction system (1.852, 1.235, 0.926, 0.772, 0.617, 0.540, 0.463, 0.309, 0.231, 0.154, and 0.077 μg/mL) after 30 min of etching. (B) Response of UV−vis spectrum of the GNRs and different concentrations of invertase in the color reaction system. (C) Linear response of the peak shift of LSPR and invertase concentration in the color reaction system with standard deviations obtained from three measurements (R^2 = 0.996).

free invertase was related to the concentration of activated Cas12a by the target DNA. After the magnetical removal of residual MBs, the released invertase in the supernatant was subjected to a cascade reaction to catalyze the hydrolysis of sucrose, and the produced glucose was immediately oxidized by GOx to generate H_2O_2. Under an acidic environment, an Fe^{2+} induced Fenton reaction occurred to convert H_2O_2 into an active intermediate (·OH) rapidly. Finally, GNRs were etched by ·OH mainly along the longitudinal axis due to the protection of CTAB, and the resulting obvious change of length−diameter ratio could directly determine the color of the solution. In consequence, the final color was efficiently correlated with the original content of target DNA and could be facilely distinguished by unaided eyes. More importantly, the peak wavelength of LSPR shifts could be applied for the semiquantification of target DNA by UV−vis spectrophotometry, making this platform convenient and sensitive for visible detection of a NOS terminator through a series of signal transductions.

Feasibility of *cis*- and *trans*-Cleavage of Cas12a Guided by crRNA. As shown in Figure 2A,B, a 1096 bp double-stranded DNA (dsDNA) target was completely cut into two segments (853 and 247 bp) with the presence of Cas12a and corresponding crRNA. The length of the segments was consistent with the predesign of PAM sites and confirmed the *cis*-cleavage activity of Cas12a. An F-ssDNA-Q reporter (Table S1) was applied as the substrate for *trans*-cleavage catalyzed by Cas12a. The intact experimental group consisting of 50 nM Cas12a, 150 nM crRNA, and 100 nM NOS terminator dsDNA exhibited an obvious fluorescence signal increasing after 30 min of incubation, while no change was observed in other control groups either using random dsDNA (C_0) or lacking some of the components (C_1−C_6) (Figure 2C). These results indicated that specific dsDNA complementary with crRNA could trigger the sequence-independent DNase activity of Cas12a to cut the F-ssDNA-Q reporter and lead to a corresponding increase of fluorescence intensity, which showed good potential in NOS terminator detection.

Sensitivity of NOS Terminator Detection System Based on Cas12a. To evaluate the detection sensitivity of a NOS terminator by a Cas12a system, 2 μL samples with different concentrations of the NOS terminator (400, 100, 50, 5, 0.5, 0.05, 0.01, and 0 nM) were respectively analyzed by a preoptimized fluorescence reporting assay (Figure S1). The fluorescence intensity was measured every 3 min, and the obtained reaction kinetics indicated that the concentration of the dsDNA target, the *trans*-cleavage activity of Cas12a, and the fluorescence intensity were positively correlated (Figure 2D). Moreover, the group with the 0.05 nM dsDNA target still exhibited a significant difference in fluorescence intensity (*P < 0.05) compared to the blank group, which suggested that the fluorescence reporting assay had a good sensitivity to detect as low as 0.05 nM NOS terminator without any amplification process.

Detection of NOS Terminator Combining with PCR and RAA Amplification. To investigate whether RAA was suitable for NOS terminator detection based on Cas12a, templates with different concentrations of a NOS terminator (10^8, 10^6, 10^4, 10^3, 10^2, 10^1, 10^0, and 0 aM) were respectively amplified either by traditional PCR or RAA. The amplification products were characterized by agarose gel electrophoresis and grouped by the concentration of templates (Figure S2A,C). Subsequently, 2 μL of PCR and 0.5 μL of RAA products from the same group were respectively analyzed by the fluorescence reporting assay using 50 nM Cas12a and 150 nM crRNA, and the fluorescence intensities from each group were obtained at 30 min for comparison. Notably, RAA products exhibited much more significant fluorescence intensity than the PCR products in the same group, and the LOD of RAA (10^0 aM) was only 1/10 of the LOD of PCR (10^1 aM) (Figure 2F). In

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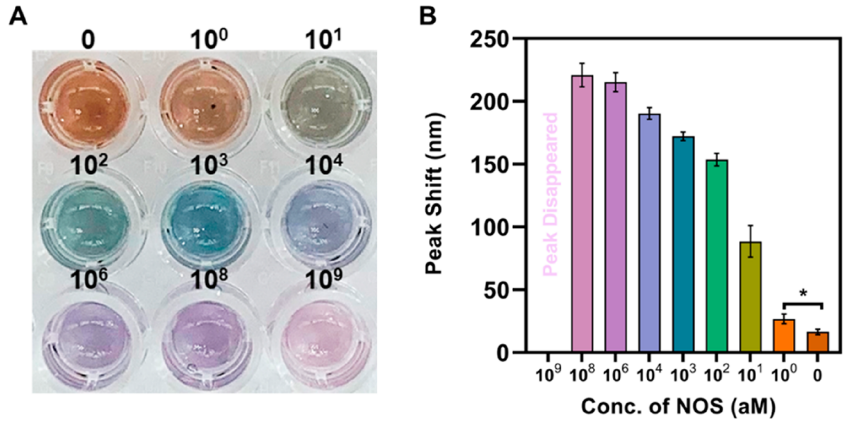


Figure 4. Sensitivity of the visible detection assay of the NOS terminator. (A) Images of responses of GNRs to RAA amplified products from different concentrations of the template (10^9, 10^8, 10^6, 10^4, 10^3, 10^2, 10^1, 10^0, and 0 aM) in visible detection after 30 min of etching. (B) Relationship between the peak shift of LSPR and NOS terminator template concentration in the RAA system after 30 min of etching (with three technical replicates; error bars represent the mean ± SEM; *P < 0.05, and "n.s." means no significance in "Student's t test").

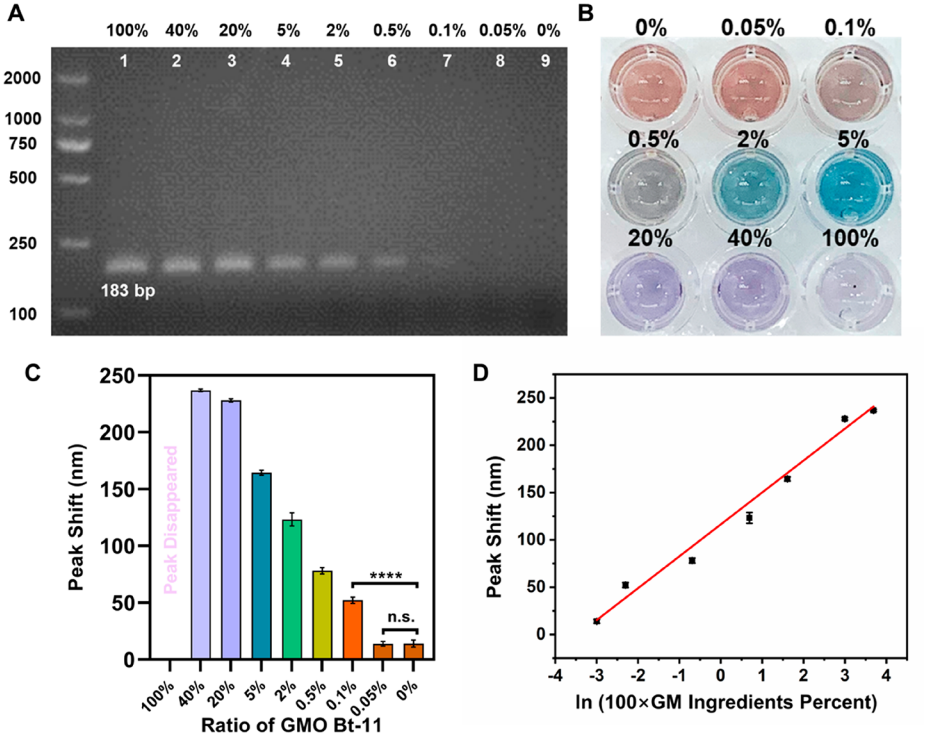


Figure 5. Specificity and sensitivity of the visible assay in NOS terminator detection. (A) Gel image of RAA amplified products. Lanes 1−9 represent Bt-11 maize containing 100, 40, 20, 5, 2, 0.5, 0.1, 0.05, and 0 wt % of transgenic ingredients, respectively. (B) Images of responses of GNRs to different transgenic ingredients in visible detection after 30 min of etching. (C) Relationship between peak shift of LSPR and different transgenic ingredients after 30 min of etching (with three technical replicates; error bars represent the mean ± SEM; ****P < 0.0001, and "n.s." means no significance in "Student's t test"). (D) Linear response of peak shift of LSPR vs ln (GM ingredients percent ×100) in the visible assay (with three technical replicates; error bars represent the mean ± SEM; R^2 = 0.988).

addition, RAA could achieve the exponential amplification of a NOS terminator within a constant low temperature (around 37−42 °C) without the requirement of professional equipment, while PCR required a precise control system for denaturation, annealing, and extension at different temperatures to obtain similar results (Table S2). Since RAA was more preferred for POU testing, it was adopted for NOS terminator detection in the subsequent studies.

Feasibility of Invertase-ssDNA-Functionalized MBs in Visible Assay of Cas12a. In order to establish the visible detection assay, the feasibility of GNR etching induced by the Fenton reaction (Figures S3 and S4) as well as the good reproducibility in the GNR synthesis process and the color change process (Figure S5) were first confirmed. To test whether this signal readout platform was suitable for the sensitive detection, a certain concentration of invertase was incubated with 30 μL of 1 M sucrose, 4.8 μL of 100 U/mL of GOx, 6 μL of Buffer C (100 mM sodium citrate buffer, pH 5.6), and 4.2 μL of ddH_2O at 37 °C for 30 min, followed by reacting with the color-substrate solution (75 μL of GNR storage solution, 7.5 μL of 20 mM $FeSO_4{\cdot}7H_2O$, and 11.25 μL of 4 M HCl) at 37 °C for another 30 min. The color change

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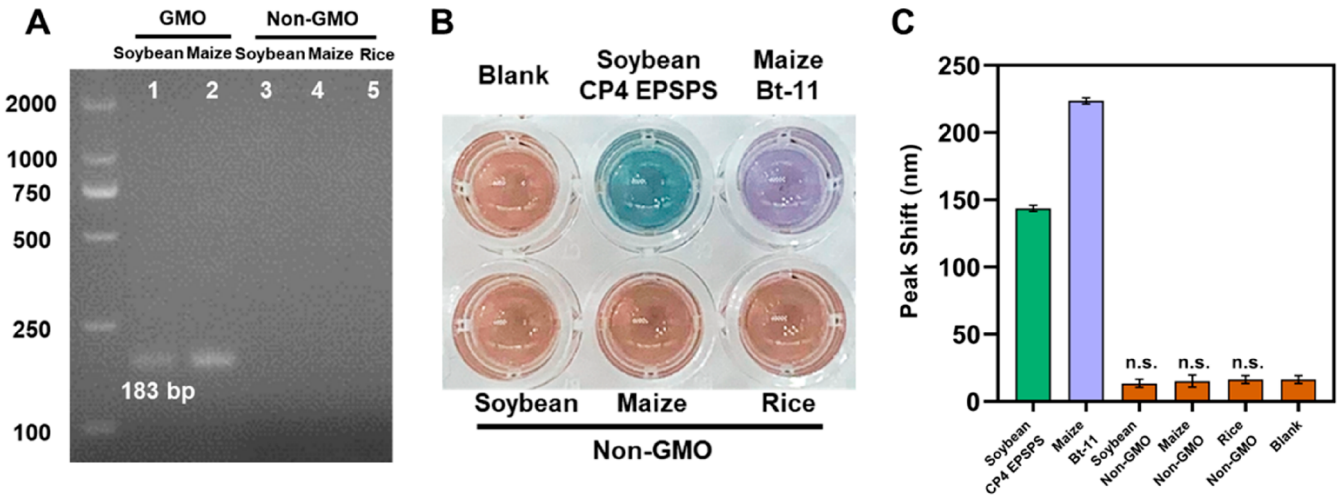


Figure 6. Detection of GM ingredients in real samples using the visible assay. (A) Gel image of RAA amplified products. Lanes 1−5 represent CP4-EPSPS soybean, Bt-11 maize, and GMO-free soybean, maize, and rice, respectively. (B) Images of responses of GNRs to different real samples in visible detection after 30 min of etching. (C) Relationship between peak shift of LSPR and different real samples after 30 min of etching (with three technical replicates; error bars represent the mean ± SEM; "n.s." means no significance in "Student's *t* test").

caused by 0.077 μg/mL invertase can be obviously distinguished by unaided eyes (Figure 3A). With the help of a UV−vis spectrum (Figure 3B,C), the LOD was calculated as 0.0217 μg/mL by the definition of 3σ/slope, where σ is the standard deviation of blank samples, with a dynamic range from 0.077 to 0.617 μg/mL and an R^2 value of 0.996.

Subsequently, the conjugation of invertase-ssDNA was prepared and characterized by SDS-PAGE, which exhibited a broad band with a larger molecular weight since a single invertase molecule could conjugate with several ssDNA strands (Figure S6). After the preparation of invertase-ssDNA-functionalized MBs, different concentrations of the NOS terminator were tested to confirm the practicability of the visible assay of Cas12a, and DNase I possessed similar nonspecific ssDNA cleavage activity, as Cas12a was also involved to verify the feasibility of the visible detection system. The color change was observed when the concentration of the NOS terminator was increased (Figure S7). With the presence of 100 U/mL of DNase I or 50 nM Cas12a activated by 400 nM target, ssDNA linkers on the surface of MBs were cleaved to release enough invertase, which led to the quick etch of GNRs and the resulting disappearance of the LSPR peak in the absorption spectrum. The etching time for the visible detection of NOS terminator was further optimized to reduce the time consumption (Figure S8). Therefore, the visible assay based on the CRISPR-Cas12a system and subsequent color reaction was feasible to detect the NOS terminator in a portable way with unaided eyes.

Sensitivity of Visible Detection of NOS Terminator. To test the detection sensitivity of the visible assay, the same RAA products amplified from different concentrations of the NOS terminator were evaluated by the visible assay. Similarly, different concentrations of target DNA can be clearly distinguished by unaided eyes (Figure 4A). In addition, a significant difference in peak shift between the 10^0 aM group and the blank group was observed by the absorption spectrum (Figure 4B), which was comparable to the LOD of RAA in the fluorescence reporting assay. Thus, we concluded that this novel visible assay could achieve sensitive detection of the NOS terminator that is as good as the fluorescence reporting assay.

Specificity and Sensitivity of Visible Assay in GMO Detection. To test the sensitivity of the visible assay in GMO detection, a series of reference substance powders containing 100, 40, 20, 5, 2, 0.5, 0.1, 0.05, and 0 wt % of Bt-11 transgenic content were respectively employed as detection samples. The genomes of samples were extracted to amplify the NOS terminator by RAA following the protocol mentioned in the Supporting Information. The extracted amplification products using the phenol−chloroform method were characterized by 3% agarose gel electrophoresis, and the LOD of the GM ingredients through identification in the gel image was 0.1 wt % (Figure 5A). Subsequently, 0.5 μL of amplification products without extraction was analyzed by our established visible assay based on the CRISPR-Cas12a system and subsequent color reaction. The gradient color change responding to 0−100 wt % of GM ingredients was clearly visible (Figure 5B), and the color change caused by 0.1 wt % GM ingredients can be easily observed by unaided eyes. These results were consistent with those determined by agarose gel electrophoresis mentioned above, which indicated the high selectivity and sensitivity of this visible detection platform in the complex background. More importantly, the peak shift of LSPR was increased linearly with the increasing value of ln (GM ingredients percent ×100), with a correlation coefficient (R^2) of 0.988. Hence, the semiquantitative analysis of GM ingredients was achieved in the visible assay by the formula: $Y = 33.73 * \ln(100 * X) + 116.381$ (Y = peak shift of LSPR (nm); X = GM ingredients percent) within the dynamic range of 0.1 to 40 wt % (Figure 5C,D). Based on the laws and regulations issued by different countries to regulate the GM content in products (Table S3), some of them made a strict regulation, and only the products with lower than 0.5 wt % GM content can be labeled "GMO-free". Therefore, the developed Cas12a-assisted visible detection assay could satisfy the need of GMO detection in most countries. In addition, compared with current GMO detection techniques,[6,9,11,33,34] such as MALDI-TOF mass for protein detection and microchip-PCR for DNA detection, the Cas12a-assisted visible assay achieved quantitative detection capability with similar sensitivity and presented better portability than other methods, which suggests good potential in POU testing.

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Detection of GM Ingredients in Real Samples Using Visible Assay. GM crops from various countries and territories present great diversities, especially those much closer to people's daily lives like soybean, maize, and rice. To verify the practicability of the Cas12a-assisted visible detection platform for the safe supervision of GMOs, two GM samples (CP4 EPSPS soybean and Bt-11 maize) and three samples labeled "GMO-free" (soybean, maize, and rice) were analyzed. Each sample was ground and extracted and then amplified by RAA and characterized by electrophoresis (Figure 6A). After that, 0.2 μL of RAA products from each sample were analyzed by the visible detection assay. In contrast to blank and GMO-free samples, two GM samples both showed obvious color changes, in which the GM content in the maize sample was much higher than that in the soybean sample (Figure 6B). The calculated GM ingredient percentages of CP4 EPSPS soybean and Bt-11 maize samples were 2.24 and 24.08 wt %, respectively, which were consistent with the detection results read by unaided eyes. These results were further confirmed by qPCR (Figure S9), and the quantitative GM ingredient percentages were 2.35 and 25.39 wt %, respectively. Compared to blank, three GMO-free samples showed no obvious color changes and no significant difference regarding the LSPR peak shift (Figure 6C). In addition, the extracted genomes from each sample were also validated by PCR, using lectin gene (*LEC*), invertase gene (*IVR*), and the sucrose phosphate synthase gene (*SPS*) as the reference genes for soybean, maize, and rice, respectively. All expected bands of reference genes were observed (Figure S10), which indicated the correctness of genomes. These results verified that the Cas12a-assisted visible detection platform could accurately and sensitively supervise GMO.

Admittedly, a portable spectrophotometer is needed to achieve more precise quantitative detection in the current Cas12a-assisted visible assay. However, a gradient color chart or an analysis app on the smartphone may be developed in further work to make colorimetric results more accurate and more comfortable to obtain. An integrated detecting platform may also be established based on the current assay with the help of other techniques to improve the usability in practice.[35,36] In this way, the assay is expected to satisfy different POU testing requirements on most using occasions.

CONCLUSION

In summary, we have established a visible and portable detection platform targeting an NOS terminator, which integrated RAA for isothermal amplification of a target, a CRISPR-Cas12a system for target recognition and signal release, a cascade reaction, a Fenton reaction for signal amplification and transduction, and unaided eyes for the final signal readout. Due to the merit of RAA for sensitive amplification of a target at constant temperature, large and professional equipment was avoided in the process. Meanwhile, through the specific signal transition by the Cas12 system, the cascade reaction and Fenton reaction were able to amplify and transduce the signal from the content of target DNA to the final color change that was visible to unaided eyes. In this way, we have addressed the POU limitations of an existing Cas12a-fluorescence reporting assay and made the Cas12a detection system more suitable in POU testing. Our established visible platform exhibited a high detection sensitivity of 1 aM, which was comparative to that of a traditional Cas12a-fluorescence reporting assay. In real applications, as low as 0.1 wt % of GM content in the samples can be distinguished by unaided eyes, and the semi-quantification of GM ingredients could be further achieved by measuring the peak shift of LSPR. Further comparison with the well-established qPCR assay as well as the validation of GM or GMO-free samples has demonstrated the accuracy and practicality of this visible platform. Therefore, with the advantages of high sensitivity and selectivity, user-friendliness, and no need for equipment, this Cas12a-assisted visible detection platform could satisfy the real demand of POU testing and the safe supervision of GMOs. Since only a portable spectrophotometer was required in the established assay for more precise quantitative results, it was more suitable for low-resource laboratories or remote quarantine under current circumstances. However, the assay is promising to be applied in other Cas12a related POU testing systems, such as disease diagnosis and pathogen detection, and used in a broader range of areas, if the usability and integration design get further improvement in future research.

MATERIAL AND METHODS

Material and Reagent. Oligonucleotides used in this article (Table S1) were synthesized from TsingKe Biological Technology (Beijing, China). M-280 Streptavidin Magnetic Beads and the TranscriptAid T7 High Yield Transcription Kit were obtained from Thermo Fisher Scientific (Wuhan, China). Tris (2-carboxyethyl) phosphine (TCEP), 4-(*N*-maleimidomethyl) cyclohexane-1-carboxylic acid, 3-sulfo-*N*-hydroxysuccinimide ester sodium salt (sulfo-SMCC), and invertase from *Saccharomyces cerevisiae* were obtained from Sigma-Aldrich, Inc. (St. Louis, MO, USA). Lba Cas12a and NEBuffer 2.1 were purchased from New England Biolabs Inc. (Ipswich, MA, USA). Magnetic separation devices, glucose oxidase (GOx) from *Aspergillus niger*, and DNase I were bought from Sangon Biotech (Shanghai, China). Chemical reagents were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). The RAA kit was purchased from ZhongCe Bio-Technology Co., Ltd. (Hangzhou, China). DNA polymerase and other PCR reagents were bought from Vazyme Co., Ltd. (Nanjing, China). The RNA clean and concentrator kit was obtained from Zymo Research (LA, CA, USA). The Bt-11 GM reference substance with different fractions of transgenic ingredients and real samples of Bt-11 maize and CP4 EPSPS soybean were obtained from the Beijing Municipal Bureau of Agriculture and Rural Affairs. GMO-free maize, soybean, and rice were bought from the supermarket in Hangzhou, China.

Preparation of Nucleic Acid. The dsDNA target of the NOS terminator used in this article was prepared through annealing of two synthesized ssDNA (shown in Table S1: NOS terminator NTS and TS) and stored at −20 °C for further use. The crRNA was transcribed *in vitro* by the kit. Briefly, the transcription system consisted of 4 μL of reaction buffer, 8 μL of NTP mixtures (10 mM each), 2 μL of the TranscriptAid Enzyme Mix in a kit, and 6 μL of 5 μM dsDNA template, which was annealing of NOS-crRNA-TF and NOS-crRNA-TD containing a T7 promoter, repeat, and spacer. After thorough mixing, the system was incubated at 37 °C for 8 h. Subsequently, crRNA was purified by an RNA clean and concentrator kit, quantified by a microspectrophotometer, and stored at −80 °C for further use.

PCR and RAA Assay for NOS Terminator. PCR and RAA primers were designed according to Primer-BLAST in NCBI. For each PCR amplification system, 25 μL of PCR

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buffer, 1 μL of dNTP mixtures (10 mM each), 4 μL of primer mixtures (5 μM NOS-PCR-F and 5 μM NOS-PCR-D), and 1 μL of DNA polymerase from the kit were thoroughly mixed with 17 μL of H_2O and 2 μL of samples. Then, the PCR assay was carried out with the following process: 95 °C for 5 min, 35 cycles at 95 °C for 15 s, 53 °C for 15 s and 72 °C for 1 min, followed by 72 °C for 5 min for complete elongation. Amplification products were further analyzed by 1% agarose gel electrophoresis. As to the RAA amplification system, the enzyme powder was dissolved by 41.5 μL of buffer A from the kit and blended with 4 μL of primer mixtures (5 μM NOS-RAA-F and 5 μM NOS-RAA-D) and 2 μL samples. After adding 2.5 μL of buffer B, the reaction was started up and incubated at 37 °C for 30 min. Amplification products should be purified by the phenol−chloroform method before analyzed by 3% agarose gel electrophoresis.

***cis*-Cleavage of dsDNA Target with NOS Terminator.** Cas12a processes dsDNA target cleaving activity in a crRNA-guided manner termed *cis*-cleavage. The 20 μL *cis*-cleavage system consisted of 1 μM LbCas12a, 2 μM crRNA, 750 nM linearized dsDNA (1096 bp), which contained the NOS terminator target sequence, 10 U of RNase inhibitor, and 1× NEBuffer 2.1. The system was thoroughly mixed, followed by incubation at 37 °C for 45 min, and the products were analyzed by 1% agarose gel electrophoresis.

Fluorescence Reporting Assay Based on *trans*-Cleavage of Cas12a. A 20 μL optimized (shown in Figure S1) fluorescence measuring system was composed of 50 nM LbCas12a, 150 nM crRNA, 1× NEBuffer 2.1, 2 μL PCR products or 0.5 μL RAA products, and 750 nM F-ssDNA-Q reporter as *trans*-cleavage substrate of Cas12a. The reaction system was premixed and transferred to 384-well plates for incubating at 37 °C. The fluorescence signal of the solution was read by Thermo Scientific Varioskan Flash every 3 min (Ex: 535 nm, Em: 556 nm).

Synthesis of GNRs. GNRs used in this article were synthesized by a Ag^+-assisted seed growth strategy.[31] Briefly, to prepare seed solution, 5 mL of 0.2 M CTAB, 5 mL of 1 mM $HAuCl_4{\cdot}3H_2O$, and 1 mL of 6 mM freshly prepared $NaBH_4$ were mixed in a 25 mL flask. After stirring for 2 min, the seed solution was incubated at room temperature for 30 min when the color was turned dark brown. To prepare the growth solution, 10 mL of 0.2 M CTAB and 10 mL of 40 mM sodium oleate were mixed in a 50 mL glass tube and incubated at 30 °C for 10 min, followed by the addition of 840 μL of 4 mM $AgNO_3$ and incubating for another 10 min. After 20 mL of 1 mM $HAuCl_4{\cdot}3H_2O$ and 168 μL of HCl was added, the solution was kept at 30 °C under stirring for 15 min. Then, 100 μL of 64 mM ascorbic acid and 64 μL of seed solution were immediately injected into the growth solution. After vigorous stirring, the mixture was kept undisturbed at 30 °C for 12 h. The synthesized GNRs were centrifuged at 6500 rpm for 30 min to remove excess solution and resuspended in 10 mL of 60 mM CTAB for further characterization and storage. According to the molar absorption coefficient of GNRs (λ = 820 nm, ε = 5.13 × 10^9 M^{-1} cm^{-1}), the concentration of the storage solution was about 0.57 nM.[37]

Preparation of Invertase-ssDNA-Functionalized MBs. According to a procedure in the literature,[38] ssDNA and invertase were conjugated by chemical coupling. Specifically, for thiol activation, 15 μL of 1 mM thiol-ssDNA-biotin, 1 μL of 30 mM TCEP, and 1 μL of buffer A (1 M sodium phosphate buffer, pH 5.5) were mixed and rotated at room temperature for 1 h. The mixture was further purified by Amicon-3K ultrafiltration centrifugation using buffer B (0.1 M NaCl, 0.1 M sodium phosphate buffer, pH 7.3). Simultaneously, 1 mg of sulfo-SMCC was mixed with 400 μL of 20 mg/mL invertase followed by incubation at room temperature for 1 h. The insoluble excess sulfo-SMCC was removed by centrifugation (13 000 rpm, 15 min), and the supernatant solution was then purified by Amicon-100 K ultrafiltration centrifugation using buffer B. Subsequently, purified thiol-ssDNA-biotin was mixed with the the above solution of sulfo-SMCC-invertase and rotated at room temperature for 48 h. Finally, the resulting solution was purified by Amicon-100 K ultrafiltration centrifugation using buffer B. Every obtained 100 pmol of invertase-ssDNA-biotin was mixed with 1 μL of 10 mg/mL streptavidin magnetic beads (SA-MBs) at room temperature for 15 min. Afterward, the invertase-ssDNA-functionalized MBs were washed by PBS buffer and resuspended in 5 μL of buffer B for further analysis.

Visible Detection Assay *via* Multicolor Display. Visible detection of the NOS terminator was achieved by combining the *trans*-cleavage of Cas12a with the color reaction based on functionalized MBs. After discarding the storage buffer, 10 μL of invertase-ssDNA-functionalized MBs was mixed with 20 μL of the Cas12a *trans*-cleavage system containing 50 nM LbCas12a, 150 nM crRNA, 1× NEBuffer 2.1, and 0.5 μL of RAA products and rotated at 37 °C for 30 min. With the help of magnets, the MBs-solution separation was achieved. Afterward, 15 μL of supernate was transferred to a cascade reaction system, which consists of 30 μL of 1 M sucrose, 4.8 μL of 100 U/mL GOx, 6 μL of Buffer C (100 mM sodium citrate buffer, pH 5.6), and 4.2 μL of ddH_2O and incubated at 37 °C for 30 min. Subsequently, 11.25 μL of solution from the cascade reaction system was injected into a 93.75 μL of a color-substrate solution (75 μL GNRs storage solution, 7.5 μL 20 mM $FeSO_4{\cdot}7H_2O$, and 11.25 μL 4 M HCl). Finally, after 30 min of etching at 37 °C, the color change was visible to the unaided eyes. 100 μL solution was further transferred to 96-well plates, and the absorption was read by Thermo Scientific Varioskan Flash.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssynbio.0c00365.

Oligonucleotide sequences and abbreviations used in this study; optimization of the crRNA/Cas12a ratio in the fluorescence reporting assay; comparison of the performance of PCR and RAA in the fluorescence reporting assay; feasibility of visible GNR etching by Fenton reaction; practicability of functionalized MBs in the visible assay; optimization of the etching time in the visible assay; different labeling policies of GMOs; accuracy of our assay compared with qPCR; extraction of plant genomic DNA and PCR verification; comparison with other detection methods for GM crops (PDF)

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Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) Twardowski, T., and Malyska, A. (2015) Uninformed and disinformed society and the GMO market. *Trends Biotechnol. 33*, 1−3.

(2) International Service for the Acquisition of Agri-biotech Applications (ISAAA). (2019) *ISAAA in 2019: accomplishment report.*

(3) Dinneny, J. R. (2018) Getting it right on GMOs. *Science 360*, 1407−1407.

(4) Vigani, M., and Olper, A. (2013) endogenous policy and the market for information. *Food Policy 43*, 32−43.

(5) Gao, H., Wen, L., Tian, J., Wu, Y., Liu, F., Lin, Y., Hua, W., and Wu, G. (2019) A portable electrochemical immunosensor for highly sensitive point-of-care testing of genetically modified crops. *Biosens. Bioelectron. 142*, 111504.

(6) Huang, L., Zheng, L., Chen, Y., Xue, F., Cheng, L., Adeloju, S. B., and Chen, W. (2015) A novel GMO biosensor for rapid ultrasensitive and simultaneous detection of multiple DNA components in GMO products. *Biosens. Bioelectron. 66*, 431−437.

(7) Milavec, M., Dobnik, D., Yang, L., Zhang, D., Gruden, K., and Zel, J. (2014) GMO quantification: valuable experience and insights for the future. *Anal. Bioanal. Chem. 406*, 6485−6497.

(8) Qian, C., Wang, R., Wu, H., Ping, J., and Wu, J. (2018) Recent advances in emerging DNA-based methods for genetically modified organisms (GMOs) rapid detection. *TrAC, Trends Anal. Chem. 109*, 19−31.

(9) Bogozalec Košir, A., Demsar, T., Stebih, D., Zel, J., and Milavec, M. (2019) Digital PCR as an effective tool for GMO quantification in complex matrices. *Food Chem. 294*, 73−78.

(10) Fu, W., Wang, H., Wang, C., Mei, L., Lin, X., Han, X., and Zhu, S. (2016) A high-throughput liquid bead array-based screening technology for Bt presence in GMO manipulation. *Biosens. Bioelectron. 77*, 702−708.

(11) Ocaña, M. F., Fraser, P. D., Patel, R. K., Halket, J. M., and Bramley, P. M. (2007) Mass spectrometric detection of CP4 EPSPS in genetically modified soya and maize. *Rapid Commun. Mass Spectrom. 21*, 319−328.

(12) Gubala, V., Harris, L. F., Ricco, A. J., Tan, M. X., and Williams, D. E. (2012) Point of care diagnostics: status and future. *Anal. Chem. 84*, 487−515.

(13) Boyd, M., and Woolley, T. (2016) Point of care testing. *Surgery 34*, 91−93.

(14) Song, Y., Huang, Y. Y., Liu, X., Zhang, X., Ferrari, M., and Qin, L. (2014) Point-of-care technologies for molecular diagnostics using a drop of blood. *Trends Biotechnol. 32*, 132−139.

(15) Aman, R., Mahas, A., and Mahfouz, M. (2020) Nucleic acid detection using CRISPR/Cas biosensing technologies. *ACS Synth. Biol. 9*, 1226−1233.

(16) Gootenberg, J. S., Abudayyeh, O. O., Kellner, M. J., Joung, J., Collins, J. J., and Zhang, F. (2018) Multiplexed and portable nucleic acid detection platform with Cas13, Cas12a, and Csm6. *Science 360*, 439−444.

(17) Li, Y., Li, S., Wang, J., and Liu, G. (2019) CRISPR/Cas systems towards next-generation biosensing. *Trends Biotechnol. 37*, 730−743.

(18) Swarts, D. C., and Jinek, M. (2019) Mechanistic insights into the cis- and trans-acting DNase activities of Cas12a. *Mol. Cell 73*, 589−600.

(19) Yamano, T., Nishimasu, H., Zetsche, B., Hirano, H., Slaymaker, I. M., Li, Y., Fedorova, I., Nakane, T., Makarova, K. S., Koonin, E. V., Ishitani, R., Zhang, F., and Nureki, O. (2016) Crystal structure of Cpf1 in complex with guide RNA and target DNA. *Cell 165*, 949−962.

(20) Shao, N., Han, X., Song, Y., Zhang, P., and Qin, L. (2019) CRISPR-Cas12a Coupled with platinum nanoreporter for visual quantification of SNVs on a volumetric bar-chart chip. *Anal. Chem. 91*, 12384−12391.

(21) Zhang, M., Liu, C., Shi, Y., Wu, J., Wu, J., and Chen, H. (2020) Selective endpoint visualized detection of Vibrio parahaemolyticus with CRISPR/Cas12a assisted PCR using thermal cycler for on-site application. *Talanta 214*, 120818.

(22) Li, Y., Mansour, H., Wang, T., Poojari, S., and Li, F. (2019) Naked-eye detection of grapevine red-blotch viral infection using a plasmonic CRISPR Cas12a assay. *Anal. Chem. 91*, 11510−11513.

(23) English, M. A., Soenksen, L. R., Gayet, R. V., de Puig, H., Angenent-Mari, N. M., Mao, A. S., Nguyen, P. Q., and Collins, J. J. (2019) Programmable CRISPR-responsive smart materials. *Science 365*, 780−785.

(24) Wang, B., Wang, R., Wang, D., Wu, J., Li, J., Wang, J., Liu, H., and Wang, Y. (2019) Cas12aVDet: a CRISPR/Cas12a-based platform for rapid and visual nucleic acid detection. *Anal. Chem. 91*, 12156−12161.

(25) Qian, C., Wang, R., Wu, H., Zhang, F., Wu, J., and Wang, L. (2019) Uracil-mediated new photospacer-adjacent motif of Cas12a to realize visualized DNA detection at the single-copy level free from contamination. *Anal. Chem. 91*, 11362−11366.

(26) Chen, J. S., Ma, E., Harrington, L. B., Da Costa, M. D., Tian, X., Palefsky, J. M., and Doudna, J. A. (2018) CRISPR-Cas12a target

binding unleashes indiscriminate single-stranded DNase activity. *Science 360*, 436−439.
(27) Tsou, J. H., Leng, Q., and Jiang, F. (2019) A CRISPR test for detection of circulating nuclei acids. *Transl. Oncol. 12*, 1566−1573.
(28) Ma, X., Lin, Y., Guo, L., Qiu, B., Chen, G., Yang, H. H., and Lin, Z. (2017) A universal multicolor immunosensor for semi-quantitative visual detection of biomarkers with the naked eyes. *Biosens. Bioelectron. 87*, 122−128.
(29) Chen, J., Jackson, A. A., Rotello, V. M., and Nugen, S. R. (2016) Colorimetric detection of *Escherichia coli* based on the enzyme-induced metallization of gold nanorods. *Small 12*, 2469−2475.
(30) Zhang, X., Zhou, W., Yuan, Z., and Lu, C. (2015) Colorimetric detection of biological hydrogen sulfide using fluorosurfactant functionalized gold nanorods. *Analyst 140*, 7443−7450.
(31) Mao, Y., Li, J., Yan, J., Ma, Y., Song, Y., Tian, T., Liu, X., Zhu, Z., Zhou, L., and Yang, C. (2017) A portable visual detection method based on a target-responsive DNA hydrogel and color change of gold nanorods. *Chem. Commun. 53*, 6375−6378.
(32) Rachmilovich-Calis, S., Masarwa, A., Meyerstein, N., Meyerstein, D., and van Eldik, R. (2009) New mechanistic aspects of the Fenton reaction. *Chem. - Eur. J. 15*, 8303−8309.
(33) Shao, N., Jiang, S. M., Zhang, M., Wang, J., Guo, S. J., Li, Y., Jiang, H. W., Liu, C. X., Zhang, D. B., Yang, L. T., and Tao, S. C. (2014) MACRO: a combined microchip-PCR and microarray system for high-throughput monitoring of genetically modified organisms. *Anal. Chem. 86*, 1269−1276.
(34) Huang, X., Chen, L., Xu, J., Ji, H. F., Zhu, S., and Chen, H. (2014) Rapid visual detection of phytase gene in genetically modified maize using loop-mediated isothermal amplification method. *Food Chem. 156*, 184−189.
(35) Wang, Y., Ke, Y., Liu, W., Sun, Y., and Ding, X. (2020) A one-pot toolbox based on Cas12a/crRNA enables rapid foodborne pathogen detection at attomolar level. *ACS Sensors 5*, 1427−1435.
(36) Zhang, M., Liu, C., Shi, Y., Wu, J., Wu, J., and Chen, H. (2020) Selective endpoint visualized detection of Vibrio parahaemolyticus with CRISPR/Cas12a assisted PCR using thermal cycler for on-site application. *Talanta 214*, 120818.
(37) Orendorff, C. J., and Murphy, C. J. (2006) Quantitation of metal content in the silver-assisted growth of gold nanorods. *J. Phys. Chem. B 110*, 3990−3994.
(38) Xiang, Y., and Lu, Y. (2011) Using personal glucose meters and functional DNA sensors to quantify a variety of analytical targets. *Nat. Chem. 3*, 697−703.

https://dx.doi.org/10.1021/acssynbio.0c00365
ACS Synth. Biol. XXXX, XXX, XXX−XXX