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Cas12a-based on-site, rapid detection of genetically modified crops[FA]

Genetically modified (GM) crops that impart herbicide resistance, improved tolerance to biotic and abiotic stresses, and enhanced yield or nutritional quality have been developed and grown worldwide (ISAAA Brief 55-2019: International Service for the Acquisition of Agri-biotech Applications (ISAAA), 2019). In response to public concern on genetically modified organisms (GMOs), DNA-based and protein-based methods have been developed to detect GMOs in food/feed samples (Fraiture et al., 2015). These methods, however, require expensive equipment and technical expertise and are unsuitable for field diagnosis (DNA-based methods) or cannot detect non-coding sequences such as promoters or terminators (protein-based methods). Newer clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated protein (Cas)-based nucleic acid detection platforms can detect viral infections and disease-associated single-base mutations with attomolar sensitivities and specificity (Gootenberg et al., 2017; Chen et al., 2018; Li et al., 2018). Because of incompatibility between isothermal amplification and the Cas buffer system, however, most of these methods require an extra step to transfer the amplified product to the Cas detection system, which risks sample contamination. An all-in-one system integrating nucleic acid amplification and CRISPR/Cas-based detection in a single tube was developed to detect SARS-CoV-2 for point-of-care use, but required 70 min and had lower sensitivity than the separate system (Joung et al., 2020). A similarly simple, rapid, specific, and low-cost detection method to detect GMOs, ideally under field conditions, is also needed. Here, we describe a CRISPR/LbCas12a-based nucleic acid detection method that uses crude leaf extracts as samples and is rapid (≤40 min for a full run) and highly sensitive.

Starting with a crude leaf sample and using a modified alkaline polyethylene glycol (PEG) lysis buffer (Hwang et al., 2013) and a handheld tissue homogenizer, we evaluated the lysis time and sample input volume needed to detect the *pCaMV35S* promoter in transgenic papaya (*Carica papaya*) leaves. The shortest effective tissue-grinding time in the lysis buffer was 30 s, and that of the maximum crude sample volume was one-third that needed for the loop-mediated isothermal amplification (LAMP) reaction (Figures 1A, S1). We then applied our CRISPR/LbCas12a detection method to samples containing four different transgenes (*tNOS*, *NPTII*, and *HPT* as well as *pCaMV35S*) to assess its sensitivity. The limit of detection (LOD) was 25 copies per reaction for *pCaMV35S* and *HPT* and 100 copies per reaction for *NPTII* and *tNOS*, and no amplification products were observed from the negative controls (Figures 1B, S2).

Next, to improve the *trans*-cleavage activity of LbCas12a and shorten the nucleic acid detection time, we optimized the reaction buffer and fluorescent probe. Adding Mg^{2+}, bovine serum albumin (BSA), or dithiothreitol (DTT) to the reaction significantly increased the *trans*-cleavage activity. However, increasing the Na^{+} or K^{+} concentration reduced the *trans*-cleavage activity, indicating that neither was required for LbCas12a *trans*-cleavage activity (Figure S3A). We therefore developed a Sun Feng buffer (SF buffer, Tris-OAC, MgOAC, BSA, and DTT), which supported maximal *trans*-cleavage activity within 5 min (more rapidly than with the commercially available NEB CutSmart buffer) (Figure 1C).

When we analyzed the cleavage products of Cas12a *trans*-activity, double-stranded (ds) DNA was degraded by the *trans*-activity of LbCas12a and ErCas12a (MAD7) (Figure 1D), consistent with the reported ability of LbCas12a to degrade ssDNA, dsDNA, and RNA (Fuchs et al., 2019). We therefore designed a hairpin-structure reporter (Figure 1E). Compared with the fluorescence values obtained with the traditional single-stranded (ss) DNA reporter (FAM-TTATT-BHQ1), the fluorescence values were increased significantly with the hairpin-structure reporter, and this stem–loop reporter was only recognized and cleaved by LbCas12a and MAD7 (Figures 1E, S3B). Increasing the length of the unpaired loop further increased the fluorescence signal (Figure S4A).

Using the optimized reaction conditions, we investigated the efficiency and specificity of CRISPR RNA (crRNA) using the LAMP products of transgenes. In the ssDNA fluorophore-quencher (FQ) assay, the crRNAs for *pCaMV35S*, *tNOS*, and *HPT* (containing the canonical protospacer-adjacent motif [PAM]) had the fastest ssDNA cleavage rate, and the assay plateaued in 5 min, whereas the crRNA for *NPTII* (containing a non-canonical PAM) required 10 min to reach a plateau (Figures 1F, S4B). But all reactions were positive on the later flow steps in 5 min at room temperature (Figure S4C). Furthermore, the crRNAs showed good specificity, with no cross-reactivity among the four targets (Figure S4D). LAMP reactions detected transgenic DNA at a proportion as low as 0.01% of the total DNA input, within 30 min for the *pCaMV35S*, *NPTII*, and *HPT* reactions and 40 min for *tNOS*; we observed no amplification with the non-transgenic mock sample (Figures 1G, S5A).

We then determined the highest ratio of non-GM to GM DNA that could be detected in a mixed sample. Our assay detected one *pCaMV35S* transgenic leaf when mixed with 29 wild-type

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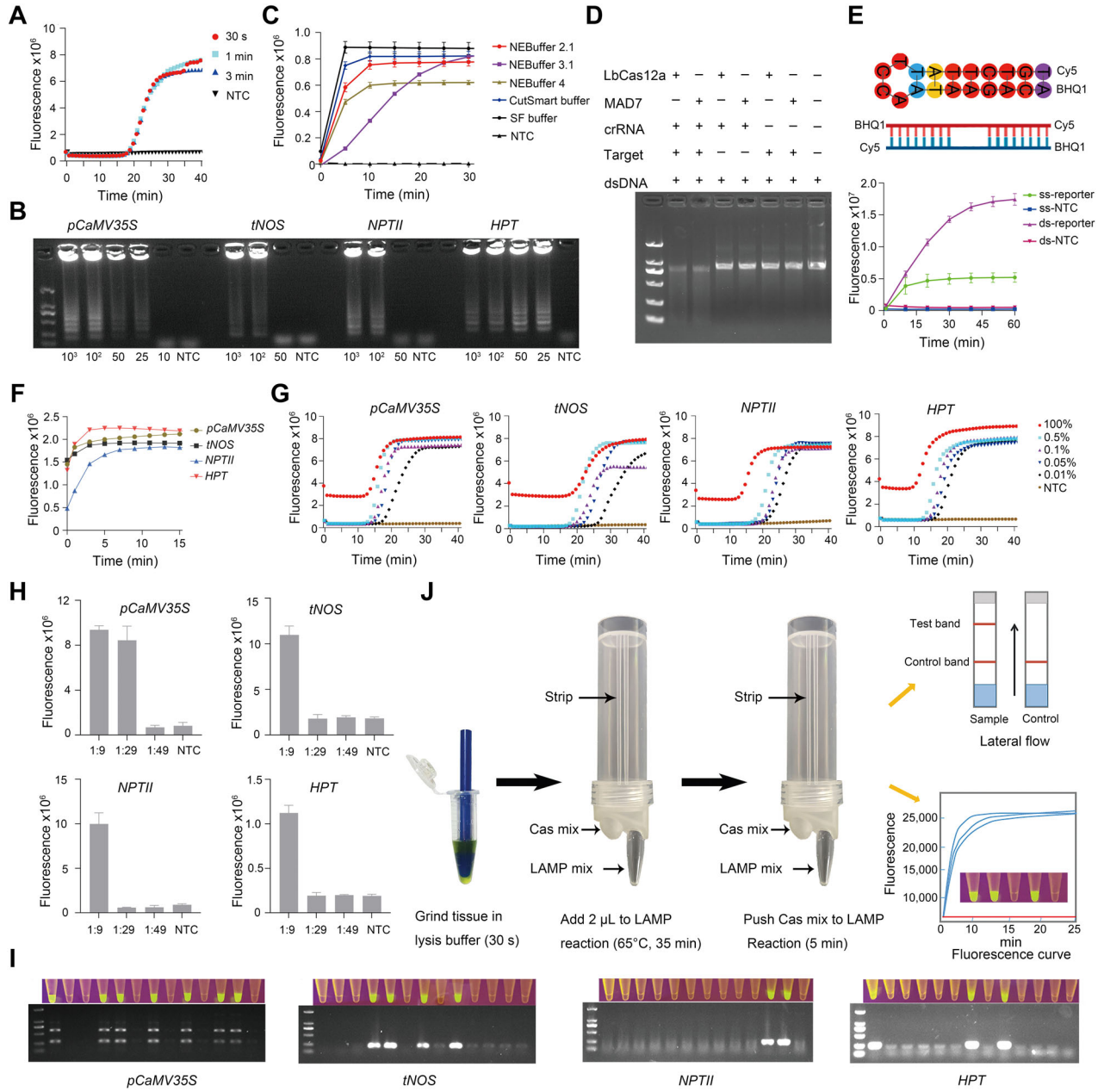


Figure 1. Loop-mediated isothermal amplification (LAMP)-Cas12a field assay for visual detection of genetically modified organisms (GMOs)

(A) Effects of tissue-grinding time on the LAMP reaction. **(B)** Detection sensitivity using different transgene-targeting LAMP primers. NTC, negative template control. Numbers indicate target copy. **(C)** Buffer optimization for LbCas12a *trans*-cleavage activity. **(D)** LbCas12a- and MAD7-specific cleavage of dsDNA. Lanes (numbered left to right): 1, DL2000 marker; 2–7, cleavage assays with ssDNA substrate and different reagents. **(E)** LbCas12a- and MAD7-specific cleavage of the dsDNA reporter. Illustration of dsDNA FQ reporter (top panel), means ± standard deviation (*SD*), three technical replicates. **(F)** Cleavage efficiency of different transgene-targeting crRNAs (*pCaMV35S*, *tNOS*, *NPTII*, *HPT*). LbCas12a ribonucleoproteins (RNPs) made individually with each crRNA were tested against 1 L of LAMP product. **(G)** Amplification curves of LAMP reactions with different proportions of transgenic versus wild-type DNA. **(H)** Fluorescence signals after 20 min of LbCas12a reactions with samples containing one transgenic plus 9, 29, or 49 wild-type leaves using a fast DNA extraction method. (means ± *SD*, three biological replicates). **(I)** LAMP products were detected using LbCas12a RNPs with the appropriate crRNAs (top); PCR assays were simultaneously performed using DNA purified from the same samples (bottom). The last sample was transgene-free. **(J)** Illustration of the new method for visual detection of GMOs using the LAMP-Cas12a assay in the field.

leaves, and one *tNOS*, *NPTII*, or *HPT* transgenic leaf mixed with nine wild-type leaves (Figures 1H, S5B), indicating its sensitivity for detecting transgenic tissue in mixed samples. To detect multiple transgenes in a single tube, we also tested multiplexed LAMP assays. We detected the expected positive bands only with *pCaMV35S* + *HPT*, indicating that *pCaMV35S* and *HPT*, but not *pCaMV35S* + *NPTII* or *pCaMV35S* + *tNOS*, were amplified by the double LAMP assay (Figure S5C).

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To prevent amplicon contamination and improve the device portability, we designed a reaction vessel via 3D printing. The device has three main parts: a reaction tube with a soft rubber chamber, a larger tube, and a plastic clip that holds a flow strip (Figure S6A). The rubber chamber contains the LbCas12a detection mix, and squeezing the chamber manually causes the mix to flow into the LAMP solution. The top of the reaction vessel, which contains a plastic clip to hold a flow strip, has screw threads to accommodate a lid or attachment to the larger tube. The flow strip gives a visible readout, when the LbCas12a detection mixture is combined with the LAMP reaction solution at room temperature for 5 min. Results can also be determined by examining the reaction vessel with a blue LED light (470 nm) (Figure S6B). To validate the assay, we collected 12 samples including maize (*Zea mays*), potato (*Solanum tuberosum*), rice (*Oryza sativa*), and tomato (*Solanum lycopersicum*) samples, from the greenhouse and tested them to detect the four transgenic sequences using this device. Our results obtained using this LbCas12a-based method were 100% consistent with results obtained by PCR (Figure 1I).

In summary, we provide a rapid, simple, low-cost, sensitive, and specific method for on-site detection of GM crops. The single-tube detection vessel prevents contamination and reduces false positives. Moreover, this method detected as little as 0.01% GM tissue in a mixed sample of GM and non-GM tissues within 40 min (Figure 1J). This CRISPR-Cas12a-based method therefore represents an effective alternative tool for field detection of GM plants.

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CONFLICTS OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

AUTHOR CONTRIBUTIONS

Z.L., J.-K.Z., F.L., and Z.D. conceived and designed the experiments; Z.D., X.Y., X.J., and Y.C. performed the detection and optimized the detection system. X.N. designed the reaction vessel. X.N., H.Z., and A.G. provided laboratory support. All authors read the manuscript and approved of the final manuscript.

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SUPPORTING INFORMATION

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