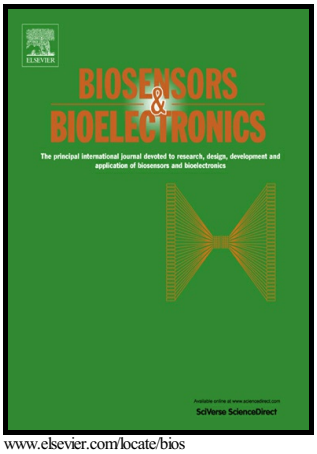

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Article

On-site detection of stacked genetically modified soybean based on event-specific TM-LAMP and a DNAzyme-lateral flow biosensor

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Abstract

Stacked genetically modified organisms (GMO) are becoming popular for their enhanced production efficiency and improved functional properties, and on-site detection of stacked GMO is an urgent challenge to be solved. In this study, we developed a

cascade system combining event-specific tag-labeled multiplex LAMP with a DNAzyme-lateral flow biosensor for reliable detection of stacked events (DP305423 × GTS 40-3-2). Three primer sets, both event-specific and soybean species-specific, were newly designed for the tag-labeled multiplex LAMP system. A trident-like lateral flow biosensor displayed amplified products simultaneously without cross contamination, and DNAzyme enhancement improved the sensitivity effectively. After optimization, the limit of detection was approximately 0.1% (w/w) for stacked GM soybean, which is sensitive enough to detect genetically modified content up to a threshold value established by several countries for regulatory compliance. The entire detection process could be shortened to 120 min without any large-scale instrumentation. This method may be useful for the in-field detection of DP305423 × GTS 40-3-2 soybean on a single kernel basis and on-site screening tests of stacked GM soybean lines and individual parent GM soybean lines in highly processed foods.

Keywords: Stacked genetically modified soybean; event-specific TM-LAMP; DNAzyme-lateral flow biosensor.

1. Introduction

Soybean is a very important oil and food crop, and it is planted worldwide to meet domestic demand, especially in America, Brazil, and China (Leff et al., 2004; USDA, 2016). Since the first genetically modified (GM) "Roundup-Ready®" soybean from

Monsanto was commercialized in the United States in the mid-1990s, modern biotechniques have been widely developed and commercialized in soybean as a primary trend in the past two decades (http://www.isaaa.org/gmapprovaldatabase). However, several controversial issues are being discussed, including food safety, environmental risk, and ethical concerns (Wynne et al., 2001; Bawa et al., 2013; Yu et al., 2016). Consequently, more than 40 countries have established a series of laws and rules for GMO labeling to protect consumers' freedom of choice. For instance, the threshold for labeling of GM material in a non-genetically modified (non-GM) background is defined as 0.9% in the European Union (European Commission Regulation, 2003), 3% in Korea (Ministry of Agriculture and Forestry of Korea, 2000), 5% in Japan (Food and Marketing Bureau, Ministry of Agriculture, Forestry and Fisheries of Japan, 2000), and zero in China (Ministry of Agriculture of the People's Republic of China, 2002). As another tendency, the planting of GM soybean with two or more transgenic traits, commonly called 'stacks', is rapidly increasing throughout the world (Parisi et al., 2016). These products can be expected to reduce cost and meet the multiple needs of researchers, farmers and consumers (Qi et al., 2012). The GM percentage in a soybean sample containing stacked traits as determined by the currently available methodology is prone to be overestimated compared to the actual percentage if we do not know the sample contains stacked traits (Appenzeller et al., 2009; Parisi et al., 2016). However, estimating

the number and nature of stacks is difficult compared with specific GM events because a stacked traits GM kernel contains the same traits as two separate GM soybean kernels from two individual parent GM lines (Akiyama et al., 2005). Once stacked trait GM kernels in a real sample have been homogenized or turned into processed foods to perform the analysis, they cannot be differentiated from separate kernels in the mixture of its parent lines, which is a practical problem for daily monitoring and international trade for experimental fields and customs (Akiyama et al., 2005; Holst-Jensen, 2009). In addition, in some jurisdictions (e.g., the EU, Argentina, Japan, Korea and the Philippines) a commercial stack, even if it results from two authorized single GM events, requires a separate risk assessment (Parisi et al., 2016). In other countries (e.g., Australia, Brazil, Canada, China, New Zealand, India and the United States), the need for a separate risk assessment for commercial stacks is evaluated on a case-by-case basis (Parisi et al., 2016). Therefore, the enforcement of these threshold values and the predicament of actual supervision have both created a demand for the development of stacked GMO analysis methods.

Currently, numerous protein- and DNA- based methods have been developed to test for stacked GMOs (Ahmed, 2002). Protein-based assays can be used to detect multiple characters, but they do not have the ability to detect transformation events because the

same exogenous protein can be expressed in different crops or different events of the same crop (Zhang et al., 2012). Event-specific DNA-based methods are more precise because they use unique sequences at the junctions of the gene construct and the 5' or 3' flanking genomic DNA sequences, which are created by random insertion of the introduced gene (Shin et al., 2013; Kim et al., 2014; Bhoge et al., 2015). Multiplex event-specific PCR methods have been widely investigated because they are capable of detecting stacked traits simultaneously with lower cost and higher throughput. For example, Xu et al. (2009) showed a universal primer multiplex PCR (UP-M-PCR) for the simultaneous detection of Bt11 × GA21 in maize mixtures. Shi et al. (2013) developed event-specific detection for five genetically modified maize lines using multiplex-PCR. However, traditional PCR requires specific instruments, harsh experimental conditions, and high experimental cost, dramatically hampering its implementation for wider and more versatile applications. Compared to conventional PCR, loop-mediated isothermal amplification (LAMP), an isothermal nucleic acid amplification technique developed by Notomi et al. (2001), is simpler due to its excellent efficiency and on-site applicability. Consequently, multiplex event-specific LAMP methods are suitable for simultaneous detection and discrimination of stacked GM events. Guan et al. (2010) reported a visual event-specific LAMP detection method for two events of GM soybean (GTS 40-3-2 and MON89788) by employing SybrGreen I dye. Di et al. (2014) developed real-time LAMP

detection systems for two GM soybean strains (GTS 40-3-2 and MON89788) using a turbidimeter. Despite the progress made with LAMP amplification protocols, the product detection technologies still retain some inherent limitations (Njiru, 2010). For instance, the tube cap has to be opened to add SYBR green I dye after reaction, which may easily cause aerosol pollution (Cheng et al., 2015). Moreover, low resolution of the turbidimeter makes determination hard when the amount of LAMP products is very low (Dhama et al., 2014). Additionally, these methods cannot show detection results for stacked traits in a single reaction tube, making them not applicable for on-site use. Hence, it is advisable to choose an easy-to-use and sensitive method for event-specific LAMP product detection from stacked GMOs simultaneously by means of one reaction tube.

Accordingly, the lateral flow biosensor (LFB), a kind of alternative rapid detection method, was proposed to overcome these shortcomings due to being user-friendly, rapid, robust, pollution-free and able to display multiple results simultaneously using one reaction tube (Li et al., 2010; Sajid et al., 2015). However, its wider application is still greatly limited due to the two following reasons. One is that the gold nanoparticle (AuNP) based detection sensitivity is not high enough, thus some efforts toward enzyme- and silver- based enhancement strategies are highly desirable (He et al., 2011; Parolo et al., 2013; Hou et al., 2012). DNAzymes with peroxidase-like activity were first reported by Sen and co-workers in the late 1990s (Li and Sen, 1997; Travascio et al., 1998), and they

have several advantages over classical enzymes, such as greater simplicity, better stability and relatively low cost. As an excellent signal element, DNAzymes-based methods have been widely used in the field of sensing technologies (Guo et al., 2012; Leung et al., 2013; Ma et al., 2014; Lin et al., 2015; Du et al., 2016; Wang et al., 2016; Liu et al., 2017; Zhou et al., 2017). Moreover, since a single AuNP can be loaded with hundreds of DNAzymes (Niazov et al., 2004; Wang et al., 2015), DNAzyme functionalized AuNPs could enhance the sensitivity of lateral flow biosensors effectively. The other reason is its incompetence for stacked trait detection, and multiplexing capacity is critical for improving detection efficiency, enhancing the detection dimension and reducing analysis cost. Hence, the combination of DNAzyme-enhanced AuNPs and multiplex event-specific LAMP could provide a novel and excellent way to solve the above problems.

In this article, we attempted to establish an elevated method to identify stacked GM soybean events based on unique and specific integration junction sequences between the host plant genome DNA and the integrated gene, using tag-labeled multiplex LAMP (TM-LAMP) together with a DNAzyme-lateral flow biosensor (DLFB), which allows for the simultaneous detection of combined GM soybean traits. The event of DP305423 × GTS 40-3-2 was selected as the target analyte, which presents the stacked traits of glyphosate herbicide tolerance, sulfonylurea herbicide tolerance, and modified oil/fatty

acid (Qi et al., 2012). An endogenous gene of soybean, *lectin*, was also used in this study for species identification. The proposed cascade system was reliable for accurate detection of stacked GM soybean in a single soybean kernel and was rapid for screening of stacked GM soybean lines and individual parent GM soybean lines in highly processed foods in an on-site manner.

2. Materials and methods

2.1 Materials

The GM plant events (DP305423 × GTS 40-3-2 soybean, DP305423 soybean, GTS 40-3-2 soybean, MON 87701 soybean, MON 89788 soybean, MON 87705 soybean, MON863 maize, GA21 maize, and Bt11 maize) and non-GM plants (soybean and maize) were provided by Sigma-Aldrich Trading Co., Ltd. (Shanghai, China), the DuPont Company (Boston, MA, USA) and the Monsanto Company (St. Louis, MO, USA). Real samples of soybean products (soybean oil, soybean milk powder, tofu, and dried tofu) were purchased from a local market in Beijing, China.

Chloroauric acid tetrahydrate ($HAuCl_4 \cdot 4H_2O$), NaCl, hemin, hydrogen peroxide, trisodium citrate, Triton X-100, Tween-20, 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 3,3',5,5'-tetramethylbenzidine (TMB), 3-amino-9-ethylcarbazole (AEC),

di-azo-aminobenzene (DAB), o-phenylenediamine (OPD), bovine serum albumin (BSA), phosphate buffered saline (PBS, pH 7.4, 0.01 M), sodium chloride–sodium citrate (SSC) buffer (20 concentrate, pH 7.0), Tris-HAc buffer (pH 7.4, 10 mM) and borate buffer (BB, pH 9.0, 0.1 M) were purchased from Sigma Chemical Company (St. Louis, MO, USA). The anti-biotin antibody, anti-FITC antibody, anti-digoxin antibody, anti-hex antibody and goat anti-mouse IgG were purchased from Abcam Inc. (Cambridge, MA, USA). Double distilled water (ddwater) was used throughout the experiments.

Backing cards (HF000MC100), glass fiber sample pads (CFSP001700), conjugation pad (GFCP000800), nitrocellulose membranes (135s) and absorbent pads (CFSP001700) were purchased from Millipore (Bedford, MA, USA).

2.2. Event-specific TM-LAMP procedure

2.2.1 Genomic DNA exaction and quantification

For DNA isolation from raw and mildly processed foods, the genomic DNA rapid extraction was carried out as reported in 40 min (Garcia et al., 2016). The DNA of the highly processed samples was extracted with the cetyltrimethyl ammonium bromide (CTAB) method as reported (Arun et al., 2013). The quantity and quality of purified DNA samples were measured and evaluated using the NanoDrop 1000 UV/Vis Spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, USA). Additionally,

the quality of DNA was analyzed by 1% (w/v) agarose gel electrophoresis (data not shown). A total of 100 ng genomic DNA was used as template in each reaction.

2.2.2 Design of event-specific TM-LAMP primers

A set of four primers containing two inner primers (FIP and BIP) and two outer primers (F3 and B3) was designed for each target sequence. FIP contains the F1c (complementary to F1) and the F2 sequence. BIP contains the B1c sequence (complementary to B1) and the B2 sequence. The detailed locations of the event-specific LAMP primers in their targets are shown in **Fig. S1**. All the TM-LAMP primers used in this work were newly designed using specific software (https://primerexplorer.jp/lampv5/index.html). All primers used in this study are listed in **Table S1** and were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China).

2.2.3 Event-specific TM-LAMP assays

Event-specific TM-LAMP assays were performed in a 25 μL total reaction mixture containing 20 mM Tris-HCl (pH 8.8), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 5 mM $MgSO_4$, 0.1% Triton X-100, 0.5 M betaine (Sigma), 1.2 μM each FIP and BIP, 0.2 μM each F3 and B3, 400 μM each dNTP. We normalized the primer concentration for each set of primers so that the reaction time for each set was the same for a set quantity of template DNA (Nagamine et al., 2014). After adding 2 μL of template DNA, the mixture was

incubated for 5 min at 95 °C and cooled on ice, and then 8 U *Bst* DNA polymerase large fragments (New England Biolabs) was added. The mixture was incubated at 65 °C for 40 min in a conventional metal heating block. Event-specific TM-LAMP assays were carried out in triplicate for each template DNA, and a no template control contained water instead of the template.

2.3 DNAzyme-lateral flow biosensor procedure

2.3.1 Fabrication of DNAzyme-AuNPs-antibody conjugate

Au-NPs were synthesized following a previous method with little modification (Cheng et al., 2016). Briefly, a 50 mL aqueous solution of 2.4×10^{-4} M $HAuCl_4$ was heated to boiling, and then 1.67 mL of 1% trisodium citrate was added. The boiling solution was stirred for another 30 min, and then stored at 4℃ prior to use.

The DNAzyme was synthesized by Sangon Biotech Co., Ltd. (Shanghai, China), and the sequence is listed in **Table S1**. DNAzyme-functionalized AuNPs were prepared as reported (Cheng et al., 2016). Briefly, 20 μL 100 mM thiolated modified DNAzyme solution and 980 μL 10 nM AuNP solution (pH 8.0) were mixed under stirring, slowly brought to 50 mM NaCl, and incubated for another 12 h. Subsequently, the conjugate was collected by centrifugation at 12,000 × g for 20 min.

The DNAzyme-AuNPs-antibody conjugate was prepared as reported with a little modification (Zhu et al., 2011). A 20 μL aliquot of purified goat anti-biotin antibody (1 mg/mL) was added to 1 mL DNAzyme-AuNPs solution (pH 8.0-8.5). After gentle stirring for 15 min, 20 μL of 10% BSA aqueous solution was added to block excess reactivity of DNAzyme-AuNPs, followed by stirring for another 30 min. The mixture was maintained at 4°C for 2 h and centrifuged at 12000 × g for 20 min to remove excess antibody. The precipitate was washed twice with Tris-HAc buffer. Finally, the DNAzyme-AuNPs-antibody conjugate (pH 8.5) were stored in Tris-HAc buffer at 4°C before experiments.

2.3.3 Preparation of the DNAzyme-lateral flow biosensor

The DLFB consisted of the following five components: a sample pad, a conjugate pad, a nitrocellulose membrane, an absorbent pad, and backing. The sample pad was made from glass fiber and saturated with buffer (pH 8.0) containing 0.25% Triton X-100, 0.1 M BB, and 0.15 M NaCl. Then, the pad was dried at 37 °C for 2 h and stored in a desiccator. A test line and control line were prepared by dispensing anti-FITC antibody (1.0 mg/mL) solution (or anti-digoxin antibody solution, or anti-hex antibody solution) and goat anti-mouse IgG (1.0 mg/mL) solution at different locations on the nitrocellulose membrane using a BioDot BioJet BJQ 3000 dispenser (Irvine, CA). The distance between

each line was approximately 5 mm. The nitrocellulose membranes were then dried overnight at 37°C and stored at 4°C. The conjugate pad and absorbent pad were assembled on backing with an overlap between them of approximately 1-2 mm to ensure that the solution could migrate through the biosensor. LFBs were cut at a width of 3.5 mm using a Bio-Dot Paper Cutter module CM4000 (Irvine, CA). Finally, three LFBs with different test lines were assembled together, like a trident, using the sample pad as linker and either used immediately or sealed and stored under dry conditions at room temperature until use.

2.4 Detection of stacked genetically modified soybean

2.4.1 Preparation of Running Buffer

First, 5 μL of DNAzyme-AuNPs-antibody conjugate was dispersed in 140 μL of basic buffer. Then, 10 μL of hemin (1 μM) was added to the solution and mixed, then incubated for 20 min at 37 °C to form DNAzyme-hemin complexes. Finally, 10 μL of a mixture of enzymatic substrate (2 μM) and hydrogen peroxide (2 μM) was added and incubated for 10 min.

2.4.2 Cascade System

Following event-specific TM-LAMP, 175 μL of freshly prepared running buffer was mixed with 25 μL of the amplified product solution; then, the mixture was applied to the

sample pad of the trident-like DLFB and migrated up by capillary force. The lines were evaluated visually within 5 min. The naked-eye detection was performed simply by observing the color change of the three test lines, which differed between genetically modified soybean and non-genetically modified soybean samples. The entire detection process could be shortened to 120 min without any large-scale instrumentation.

3. Results and discussion

3.1 Principles

3.1.1 Event-Specific TM-LAMP

As illustrated in **Scheme 1A,** the aim of event-specific TM-LAMP was to generate numerous biotin- and FITC-attached duplex DNAs, biotin- and digoxin-attached duplex DNAs, and biotin- and hex-attached duplex DNAs, which indicated the existence of GM soybean event DP305423, GM soybean event GTS 40-3-2, and an endogenous soybean reference gene, respectively. A total of three sets of event-specific TM-LAMP primers were successfully designed and verified by 2% (w/v) agarose gel electrophoresis (**Fig. S2 and)**. The sensitivity and stability of the designed assay were also verified and presented in **Fig. S3.** The accuracy of multiplex reaction was based on the approximately equal amplification efficiency of the primers, which was examined by means of real-time LAMP**.** According to previous reports (Nagamine et al., 2014), when three primer sets

were added to a reaction mixture, LAMP could efficiently amplify each target with levels reaching the detection limit in approximately time, they hold equal amplification efficiency. As shown in **Fig. S4**, very efficient and nearly identical amplification was obtained in this design, which indicates that this assay is very reliable and well suited for simultaneous measurements.

3.1.2 DNAzyme-AuNPs-antibody Conjugation

A DNAzyme-AuNPs-antibody conjugate was prepared as a signal element in the proposed biosensor, and the modified process is illustrated in **Scheme 1B.** DNAzymes can effectively catalyze the hydrogen peroxide-mediated oxidation of ABTS, TMB, AEC, OPD or DAB to yield a colored peroxidase product. Followed by applying these enzymatic substrates and hydrogen peroxide in running buffer, the enzymatic reactions between the DNAzyme and substrate produce colored products, which are deposited on the AuNP surface to enhance the visual effect and the intensity of lines.

3.1.3 DNAzyme-Lateral Flow Biosensor

The detection of the amplified duplex DNA complexes using a DLFB is illustrated in **Scheme 1C**. The principle of the basic LFB measurement is based on the sandwich type of "(anti-biotin-AuNPs)-(target DNA complexes)-(relevant antibody)" hybridization reactions, which is the same as in our previous work (Xu et al., 2016; Chen et al., 2016). The enhancement of LFB using DNAzymes brought an updated version to this design. A

red band on the 1st test line indicated a “DP305423 positive” result, while no red band indicated a “DP305423 negative” result. Similarly, red bands on the 2nd test line indicated a “GTS 40-3-2 positive” result, while no red bands indicated a “GTS 40-3-2 negative” result. The 3rd test line for *lectin* detection was used for verification of the species of soybean, the absence of amplification inhibitors, and the good running of the biosensor. All of the control lines indicated that the biosensor worked properly.

3.2.4 Trident-like DNAzyme-Lateral Flow Biosensor

Multiplexing capacity is critical for improving detection efficiency, enhancing the identification precision and reducing analysis cost. However, the design of such multi-detection systems requires careful consideration of cross-reactivity and the limitation of Washburn's theory (Fenton et al., 2008; Li et al., 2016). Thus, the sensitivity of multiplexed lateral flow detection is usually lower than that of the individual single-plex detections. An alternative solution is to band these single-plexes together in a parallel way instead of adding more lines in a single array along the device, which proved to be a good prototype for conventional lateral flow biosensors (Li et al., 2016). In our study, the principle of trident-like DLFB is illustrated in **Scheme 1D**. A screening analysis was simply performed by observing the color change of the three test lines, and the measurements were reliable for the specific detection of separate kernels and screening of stacked GM soybean lines and individual parent GM soybean lines by

matching with **Table 1**. Therefore, the different features of the three test lines reflect the various ingredients in soybean samples, indicating that the trident-like DLFB can detect stacked GM soybean in a single soybean kernel and highly processed foods.

3.2 Characterization

3.2.1 Construction

The AuNPs and DNAzyme-AuNPs-antibody conjugation were characterized first by UV-vis analysis to calculate their concentration, which is approximately 2.5 nM (**Table S2**). Then, they were also visualized by transmission electron microscopy (TEM) to measure their accurate size, resulting in a diameter of approximately 15 nm (**Table S2**). Finally, zeta potential and conductivity measurements further confirmed DNAzyme and antibody functionalization (**Table S3**).

3.2.2 Function

The catalytic function of the DNAzyme-AuNPs-antibody conjugate is a key factor for the performance of the biosensor. To investigate whether the DNAzyme possesses peroxidase-like activity after modification, it was employed to catalyze the oxidation of typical peroxidase chromogenic substrates and produce insoluble pigmented products. As shown in **Fig. 1A**, the results demonstrate that the DNAzyme-AuNPs-antibody conjugate retains similar catalytic activity in solution to unmodified DNAzyme. As shown in **Fig.**

1B, an obvious increase of the visual effect on the test line is presented on the DLFB after treatment with AEC/H_2O_2 and OPD/H_2O_2. The stability of the OPD solution is lower than that of the AEC solution. The AEC/H_2O_2 enzymatic substrate was selected for use in the following experiments, and we confirmed that the DNAzyme-AuNPs-antibody conjugate possesses the function of allowing its visualization by catalyzing a color reaction with peroxidase substrates.

3.3. Optimization

To improve sensitivity for the detection of targets, the conditions of event-specific TM-LAMP and DLFB were optimized systematically.

3.3.1 *Event-Specific TM-LAMP*

The amplification efficiency of TM-LAMP is crucially affected by the reaction time and the amount of Bst polymerase, the concentration of Mg(II), and primer ratios in solution. The reaction initiated by DP305423×GTS 40-3-2 was confirmed by DLFB (**Fig. S5**). As a result, the best performance of event-specific TM-LAMP was obtained with solution containing 8 U Bst polymerase, 5 mM Mg(II), and a 6:1 ratio of inner to outer primers for 40 min reaction.

3.3.2 *Multiplex DNAzyme-Lateral Flow Biosensor*

We further studied the effect of the amount of DNAzyme-AuNPs-antibody conjugate, the amount of running buffer and the type of running buffer by comparing the analytical performance of the DLFB (**Fig. S6**). After optimization, 5 μL of DNAzyme-AuNPs-antibody conjugate and 140 μL of running buffer (4×SSC, 10 mM Tris-HAc, 5 mM Kac, 0.002% Triton X-100, 2% BSA, and 0.05% Tween-20, pH 7.4) were selected to be used in the following experiments.

3.4. Sensitivity

Under optimal experimental conditions, the sensitivity of the proposed system was tested by determining non-GM soybeans spiked with DP305423 × GTS 40-3-2 soybeans at proportions of 0-100% (weight of GM / weight of non-GM, w/w). **Fig. 2A** shows typical images of the trident-like DLFB in response to various proportions of stacked GM soybeans. The color intensity of the test line increased with increasing proportions in the standard samples. False positive results and false negative results were not obtained in any of the tests. The limit of detection (LOD) of the cascade system (defined as the lowest concentration with a visible test line) was 0.1%, roughly considered as 40 copies on the basis of genome size of soybean, which is significantly better than that without DNAzyme enhancement **(Fig. S7)**. The developed assay is user-friendly and sensitive enough to detect GM content up to a threshold value established by several countries for regulatory compliance.

3.5. Selectivity

To study the selectivity of the proposed assay, a series of GM plant DNA samples including DP305423 × GTS 40-3-2 soybean, DP305423 soybean, GTS 40-3-2 soybean, MON 87701 soybean, MON 89788 soybean, MON87705 soybean, MON863 maize, GA21 maize, and Bt11 maize, each with a concentration of 1%, were tested. As demonstrated in **Fig. 3**, only DP305423 × GTS 40-3-2 soybean showed the same color intensity of the three test lines, while this kind of visual signal was hardly detected from the other samples. These results demonstrate excellent selectivity of the proposed assay, attributed to the design of the trident-like DLFB and the highly specific coordination of the junction region between the transgenic insert and the host DNA. This test indicates that the developed cascade assay has good selectivity for DP305423 × GTS 40-3-2 soybean.

3.6. Application

To further test its feasibility in practical use, the introduced event-specific TM-LAMP and DLFB cascade was applied to assess DP305423 × GTS 40-3-2 soybean in 20 real samples, including 8 samples of raw soybean kernels, 8 samples of highly processed foods and 4 artificially mixed samples made by spiking DP305423 × GTS 40-3-2 soybean powder and heating up this mixture. As shown in **Table 2,** 1 out of 8 raw

soybean kernel samples belonged to stacked genetically modified soybean, and none of 8 highly processed food samples potentially containing stacked events of DP305423 × GTS 40-3-2 did so. Moreover, all artificially mixed samples were positive, confirming the reliability of the developed biosensor for detecting stacked GM soybean.

4 Conclusions

In summary, we have developed an on-site assay for detection of DP305423 × GTS 40-3-2 stacked events based on event-specific TM-LAMP and DLFB. This method is applicable for accurate detection of stacked genetically modified soybean in raw and screening testing in highly processed food samples due to its high sensitivity, specificity and predictability under isothermal conditions. Furthermore, the portable biosensor can be extended to detect more events by making slight modifications, which shows great promise for in-field supervision in experimental fields and on-site screening for customs.

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Scheme 1 Schematic of the design of the event-specific TM-LAMP and DNAzyme-lateral flow biosensor: (A) Event-specific TM-LAMP; (B) DNAzyme-AuNPs-antibody conjugation; (C) DNAzyme-lateral flow biosensor; (D) Trident-like DNAzyme-lateral flow biosensor.

Fig. 1 Characterisation of DNAzyme/AuNPs/antibody conjugation. (A) Color generation by ABTS, TMB, AEC, OPD and DAB oxidation mediated by DNAzyme and DNAzyme/AuNPs/antibody conjugation. Experiments were performed three times with similar results. (B) DNAzyme enhanced lateral flow biosensor test for the event of DP305423 detection. The proportion of targets: (1) 100%, (2) 10%, (3) 0 %.

Fig. 2 Typical images of trident-like DLFB in response to various proportion of DP305423 × GTS 40-3-2 soybean. The proportion of targets: (A) 100%, (B) 50%, (C) 25%, (D) 10%, (E) 1%, (F) 0.1%, and (G) 0%.

Fig. 3 Typical images of trident-like DLFB in response to a series of GM plant DNA samples (GM proportion: 1%), including (A) DP305423 × GTS 40-3-2 soybean, (B) DP305423 soybean, (C) GTS 40-3-2 soybean, (D) MON 87701 soybean, (E) MON

89788 soybean, (F) MON87705 soybean, (G) MON863 maize, (H) GA21 maize, and (I) Bt11 maize.

Table 1 Comparison of color change of the three test lines for the detection of DP305423 × GTS 40-3-2 in single kernel samples and highly processed food samples using event-specific TM-LAMP and trident-like DLFB.

No.	Samples (copies)	Features of Three Test Lines	Copies of DP305423	Copies of GTS40-3-2	Copies of *Lectin*
A Single Kernel					
1	A (m)	No red band on the 2ed test line	m	0	m
2	B (m)	No red band on the 1st test line	0	m	m
3	C (m)	No red band on the 1st and 2ed test line	0	0	m
4	**A × B (m)**	Same red bands on the three test lines	m	m	m
Highly Processed Food					
1	A (m)	No red band on the 2ed test line and the same red bands on the 1st and 3rd test lines	m	0	m
2	B (m)	No red band on the 1st test line and the same red bands on the 2ed and 3rd test	0	m	m
3	C (m)	No red band on the 1st and 2ed test line	0	0	m
4	**A × B (m)**	Same red bands on the three test lines	m	m	m
1	A (m) + C (n)	No red band on the 2ed test line and different red bands on the 1st and 3rd test	m	0	m + n
2	B (m) + C (n)	No red band on the 1st test line and different red bands on the 2ed and 3rd test	0	m	m + n
3*	A (m) + B (n)	Three different test lines and the thickest of the 3rd one	m	n	m + n
4*	A (m) + B (n) + C (p)	Three different test lines and the thickest of the 3rd one	m	n	m + n + p
5	**A × B (m)** + A (n)	The same red bands on the 1st and 3rd test lines and lighter red band on the 2ed test	m + n	m	m + n
6	**A × B (m)** + B (n)	The same red bands on the 2ed and 3rd test lines and lighter red band on the 1st test	m	m + n	m + n
7	**A × B (m)** + C (n)	The same red bands on the 1st and 2ed test lines and thicker red band on the 3rd test	m	m	m + n
8*	A × B (m) + A (n) + B (p) + C (q)	Three different test lines and the thickest of the 3rd one	m + n	m + p	m + n + p + q

A × B: Stacked Traits Genetically Modified Soybean (DP305423 × GTS40-3-2)

A: Single Traits Genetically Modified Soybean (DP305423)

B: Single Traits Genetically Modified Soybean (GTS40-3-2)

C: Nongenetically Modified Soybean

*: Need Further Confirmation

Table 2 Determination of DP305423 × GTS 40-3-2 by event-specific TM-LAMP and trident-like DLFB cascade in 20 food matrices containing soybean, including 8 samples of raw soybean kernels, 8 samples of highly processed foods and 4 artificially mixed samples.

No.	Samples	DP305423	GTS40-3-2	*Lectin*	Stacked Events Determination
A Single Kernel					
1	Soybean kernel	+	ND	+	Negative
2	Soybean kernel	ND	ND	+	Negative
3	Soybean kernel	ND	ND	+	Negative
4	Soybean kernel	ND	ND	+	Negative
5	Soybean kernel	ND	ND	+	Negative
6	Soybean kernel	ND	+	+	Negative
7	Soybean kernel	ND	ND	+	Negative
8	Soybean kernel	+	+	+	**Positive**
Highly Processed Food					
1	Soybean Oil	ND	ND	+	Negative
2	Soybean Oil	ND	ND	+	Negative
3	Artificially mixed Soybean Oil	+	+	+	**Positive**
4	Soybean Milk Powder	ND	ND	+	Negative
5	Soybean Milk Powder	ND	ND	+	Negative
6	Artificially mixed Soybean Milk Powder	+	+	+	**Positive**
7	Tofu	ND	ND	+	Negative
8	Tofu	ND	ND	+	Negative
9	Artificially mixed Tofu	+	+	+	**Positive**
10	Dried Tofu	ND	ND	+	Negative
11	Dried Tofu	ND	ND	+	Negative
12	Artificially mixed Dried Tofu	+	+	+	**Positive**

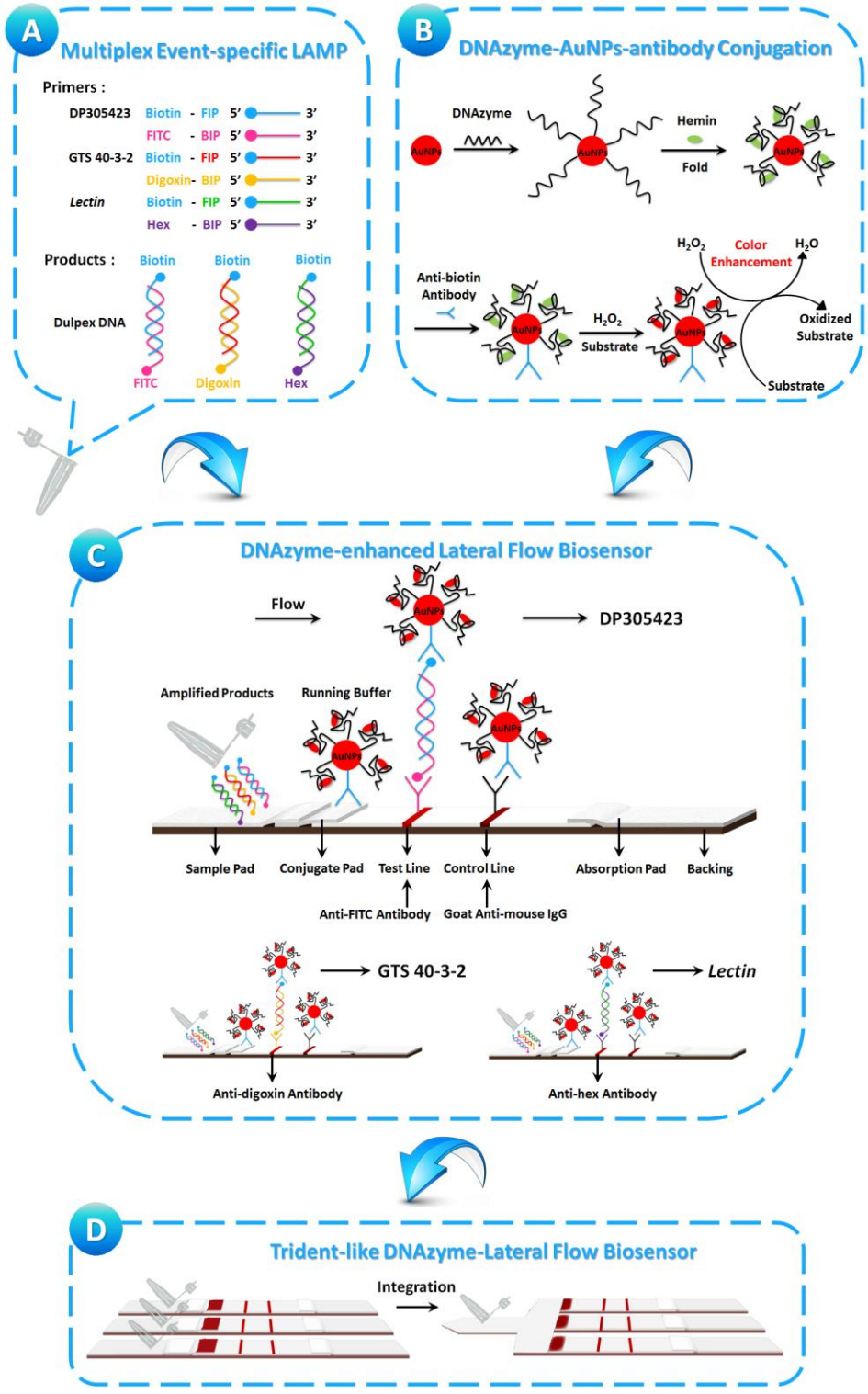
+: Visible red band on the corresponding test line

ND: Not detectable

Highlights

- An assay for in-field supervision and on-site screening of stacked GM soybean.
- 120 min of the entire detection processwithout any large-scale instruments.
- New sets of event-specific TM-LAMP primers for DP305423×GTS 40-3-2 detection.
- The first report of introducing a DNAzyme into a trident-like lateral flow biosensor.
- Different real GMO samples were distinguished with the proposed biosensor.

531 **Scheme1**



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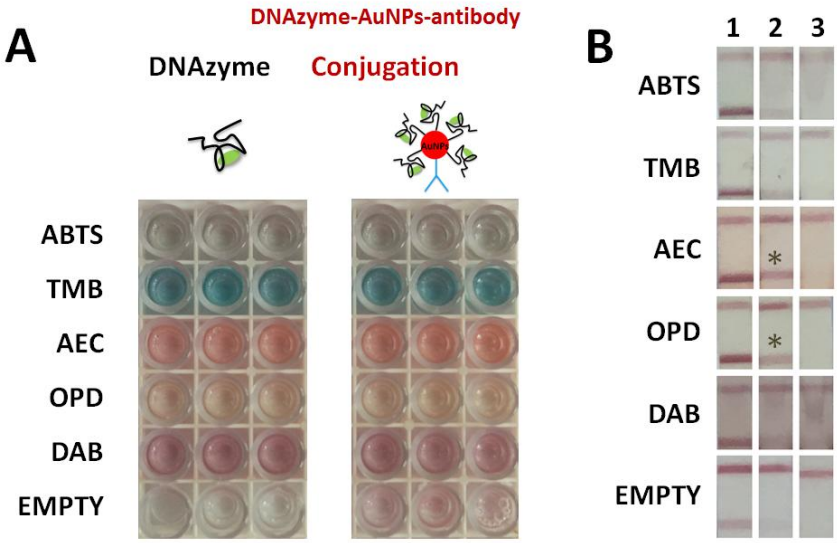
534 **Fig. 1**



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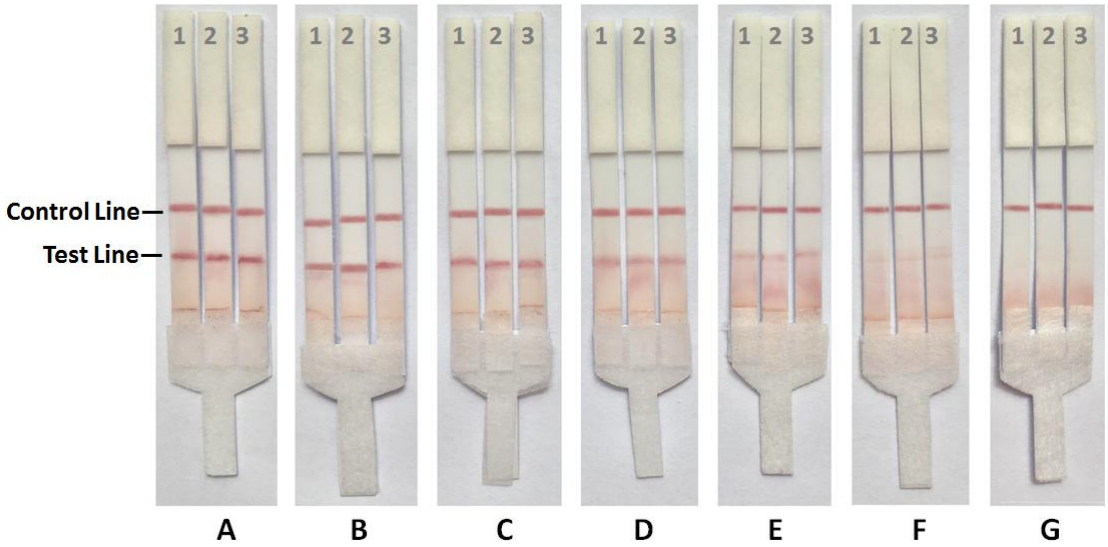
537 **Fig. 2**



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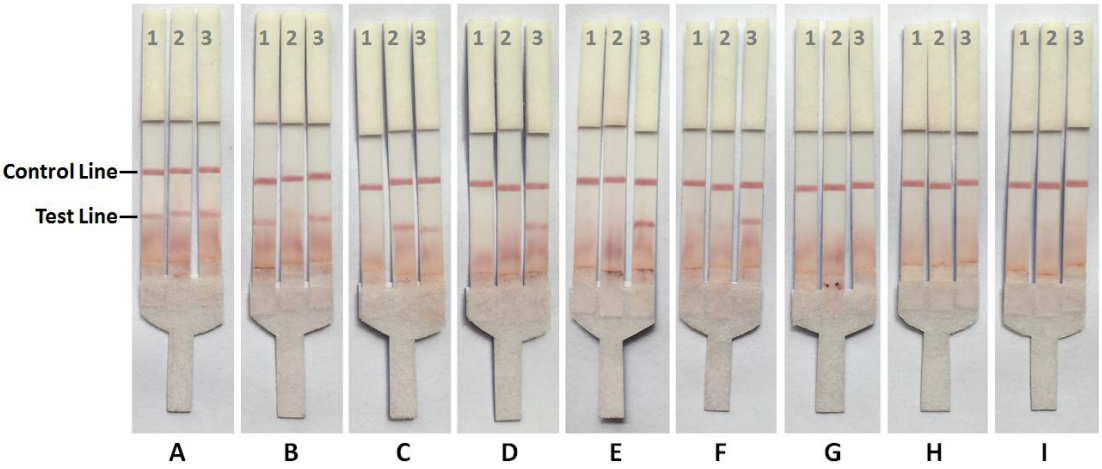
540 **Fig. 3**



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