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Miniaturized Paper-Based Gene Sensor for Rapid and Sensitive Identification of Contagious Plant Virus

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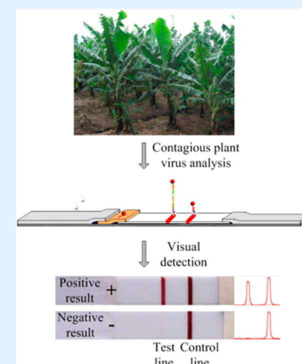
ABSTRACT: Plant viruses cause significant production and economic losses in the agricultural industry worldwide. Rapid and early identification of contagious plant viruses is an essential prerequisite for the effective control of further spreading of infection. In this work, we describe a miniaturized paper-based gene sensor for the rapid and sensitive identification of a contagious plant virus. Our approach makes use of hybridization-mediated target capture based on a miniaturized lateral flow platform and gold nanoparticle colorimetric probes. The captured colorimetric probes on the test line and control line of the gene sensor produce characteristic red bands, enabling visual detection of the amplified products within minutes without the need for sophisticated instruments or the multiple incubation and washing steps performed in most other assays. Quantitative analysis is realized by recording the optical intensity of the test line. The sensor was used successfully for the identification of banana bunchy top virus (BBTV). The detection limit was 0.13 aM of gene segment, which is 10 times higher than that of electrophoresis and provides confirmation of the amplified products. We believe that this simple, rapid, and sensitive bioactive platform has great promise for warning against plant diseases in agricultural production.



KEYWORDS: *paper-based gene sensor, contagious plant virus, gold nanoparticle colorimetric probes, low-cost diagnostics, visual detection*

■ INTRODUCTION

Plant viruses cause many plant diseases that lead to significant production and economic losses in the agriculture and forestry industries worldwide. Plant viruses are a menace to plant vigor and longevity, and they affect negatively the quality and quantity of crop yields.[1,2] It is estimated that at least 10% of global food production losses are due to plant diseases, which result in the loss of about $33 billion in the United Stated every year.[3,4] Most plant viruses multiply in insect vectors and are spread by transovarial transmission, which makes their control more difficult.[5−9] The insect vectors are distributed widely in the world and migrate over long distances, even across oceans.[10−12] Moreover, most of the approaches including transgenic processes and drugs to control plant viruses are plant-species-limited or virus-species-limited and are not very effective.[13] Therefore, efficient and early identification of contagious plant viruses is critical to diminishing losses through proper management strategies such as dislodging of viruses from infected material and control of vectors through pesticide applications.

Bananas and plantains (*Musa spp.*) are grown as table fruits and staple food items, major cash crops, and significant export crops in many tropical and subtropical areas of the world.[14] Banana plantations are subjected to a variety of natural calamities, although diseases, particularly viral diseases, are the major threats to this crop worldwide. Among all viral infections, banana bunchy top virus (BBTV) (genus *Babuvirus*, family *Nanoviridae*) causing banana bunchy top disease (BBTD) is responsible for massive reductions in crop yields.[15] It was first recorded in Fiji as early as 1889 and has since spread throughout the Asia-Pacific basin and a few countries in Africa through infected plant material.[16] BBTV, an icosahedral virion 18−20 nm in diameter, has a multicomponent genome containing at least six circular single-stranded DNA (ssDNA) molecules with a size of approximately 1.1 kb each.[17] The virus is transmitted by the banana aphid (*Pentalonia nigronervosa* Coq.) in a persistent manner.[18] BBTV is also spread through infected banana plant suckers and other tissues used in banana propagation.[19] The symptoms of BBTD include the presence of dark green streaks on the undersurface of the leaf, stunted and malformed leaves, standing upright and growing progressively shorter, giving the "bunchy top" appearance from which its name derives.[20] When plants are infected early, they do not bear fruit, and when they are infected later, the fruits are typically stunted and unmarketable.[21] Currently, the recommended strategies for controlling BBTV include identifying and removing infected plants as early as possible, replanting with virus-free plants, and controlling banana aphids through pesticide applications.[22] As such, a proper management strategy depending on the rapid and sensitive identification of infected plants is needed, so that continuing sources of BBTV can be destroyed promptly to prevent any further spread.

The observation of characteristic symptoms caused by a virus is a common practical method for plant virus detection in the

Received: September 29, 2014
Accepted: November 20, 2014
Published: November 20, 2014

 © 2014 American Chemical Society dx.doi.org/10.1021/am506695g | ACS Appl. Mater. Interfaces 2014, 6, 22577−22584

Table 1. Oligonucleotide Sequences

oligonucleotide	sequence (5′−3′)
primer 1 (P1)	GGCAGGAGGAAGTATGGA
primer 2 (P2)	GATGGCTATGTTCAGGTT
block oligonucleotide 1	GTAGTCGTGGCTTGTTGC
block oligonucleotide 2	ATGTAGTCGGCTGGTTGA
block oligonucleotide 3	CACACCAACAGCATAAAC
detection probe	CTTCCATACTTCCTCCTGCCTTTTTTTTTTTT-C3-SH
capture probe T	biotin-TTTTTTTTTTTTATAAATAAACCTGGTGCTTC
capture probe C	GGCAGGAGGAAGTATGGAAGTTTTTTTTTTTT-biotin

field.[23] However, similar symptoms can be induced by different viruses and by nonpathogenic disorders, including nutritional deficiencies, drought, and insect damage. Furthermore, plants might have been infected too recently to exhibit symptoms, in which case they would not be identifiable by visual means. These facts make it unreliable to diagnose plant viruses by symptom observations. Although many methods can be applied to laboratory diagnostics of these viruses,[24−26] enzyme-linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR) are the most common and widely utilized techniques for the routine identification of plant viruses.[27,28] ELISA, based on proteins produced by viruses, is a reliable method for detecting plant viruses that is suitable for high-throughput testing. However, ELISA requires multiple-step processes involving sample purification, incubation, and washing steps, making this technique time-consuming and labor-intensive. Furthermore, ELISA has to rely on the quality of available virus-specific antisera, which are not easy to obtain. PCR, based on specific nucleic acid sequences of viruses, is more sensitive than ELISA and has been used to detect plant viruses. However, PCR requires an additional step of visualization by gel electrophoresis, which requires use of the mutagen ethidium bromide. Moreover, electrophoretic methods do not provide enough specific information about the sequence of the amplified fragments. As a result, nonspecific PCR products of similar size can lead to erroneous interpretation. Real-time PCR, which eliminates the need for laborious gel electrophoresis,[29] although highly accurate, requires extensive equipment and highly skilled operators, which is uncommon for developing countries and resource-limited and remote regions and is generally not suitable for use in the field. Thus, additional efforts are needed to create more broadly applicable approaches that allow accurate, rapid, sensitive, and selective identification of plant viruses at early stages to control the development and spread of plant diseases.

Recently, an emerging lateral flow biosensor, also called the dipstick strip biosensor, that combines chromatography with conventional immunoassay has received increasing attention in biomarker analysis and clinical diagnosis.[30−34] The format is similar to that of ELISA, and the base substrate, made of nitrocellulose membrane, consists of immobilized capture binding protein (usually antibody or antigen).[35,36] Labels such as colloidal gold are allowed to concentrate and form a visible result (test line and control line).[37−40] A milestone in the field is the commercial application of the pregnancy test strip with the identification of human chorionic gonadotropin (HCG).[41,42] Compared with the techniques mentioned above, the lateral flow biosensor has several advantages, including a user-friendly format, a very short assay time (generally less than 10 min), less interference owing to chromatographic separation, long-term stability over a broad range of climates, cost-effectiveness, and the lack of a need for skilled professionals.

In the present work, we report a low-cost paper-based gene sensor based on a miniaturized lateral flow platform and gold nanoparticle colorimetric probes for rapid, sensitive, and visual identification of a plant virus. It allows visual detection of the amplified products within several minutes and, in contrast to previously described methods, does not require special instrumentation. Moreover, the gene sensor is a dry-reagent system, which avoids the multiple incubation, separation, and washing steps commonly performed in most current assays. Qualitative judgment can be realized by observing the color change of the test line, and quantitative data can be obtained by recording the optical intensity of the test line. The feasibility of the sensor was evaluated by identification of the BBTV in banana plants. The promising properties of the sensor are reported in the following sections.

MATERIALS AND METHODS

Chemicals. Gold(III) chloride trihydrate was purchased from Sigma-Aldrich (St. Louis, MO). Streptavidin (SA) from *Streptomyces avidinii*, tris(2-carboxyethyl)phosphine hydrochloride (TCEP), bovine serum albumin (BSA), and Tween 20 were all obtained from Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. (SSBE; Shanghai, China). X−Z two-dimensional dispenser HM3020 and Goldbio cutting module GD300 were purchased from Shanghai KinBio Tech Co., Ltd. (Shanghai, China). Cellulose fiber sample pads, conjugate pads, plastic adhesive backing pads, absorption pads, and nitrocellulose membranes were purchased from Millipore (Billerica, MA). Taq DNA polymerase was purchased from TaKaRa Biotechnology Co., Ltd. (Dalian, China). Other chemicals employed were of analytical reagent grade and were used as received. When required, all chemical solutions and buffers were prepared in ultrapure water (18.2 MΩ/cm). All oligonucleotides used in this work were synthesized and purified by high-performance liquid chromatography (HPLC) at SSBE, Shanghai, China. Their sequences are listed in Table 1.

Banana plant samples infected with banana bunchy top virus (BBTV), banana streak virus (BSV), and cucumber mosaic virus (CMV) and a virus-free tissue-cultured banana plantlet (used as a healthy control) were all obtained from South China Agricultural University (Guangzhou, China).

Synthesis of Gold Nanoparticles (AuNPs) and Preparation of DNA-Modified AuNPs. Approximately 13-nm citrate-capped gold nanoparticles (AuNPs) were synthesized by the reduction of $HAuCl_4$.[43] All glassware and a mechanical stirrer used in the procedure were thoroughly cleaned in freshly prepared aqua regia, rinsed with ultrapure water, and then oven-dried prior to use. For the synthesis, 100 mL of 1 mM $HAuCl_4$ was heated to boiling while being vigorously stirred in a round-bottom flask equipped with a reflux condenser. Subsequently, 10 mL of 38.8 mM trisodium citrate solution was added quickly, which led to a change in solution color from light yellow to dark red. After boiling for an additional 15 min, the solution was slowly cooled to room temperature with continuous stirring and stored in dark bottles at 4 °C before use. The resulting red solution of 13-nm-

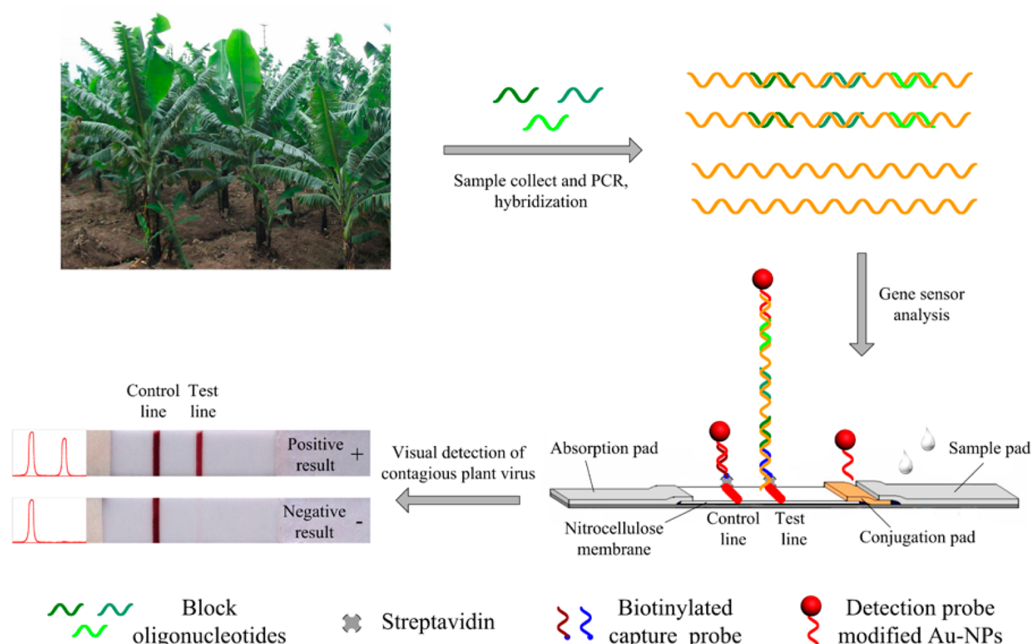


Figure 1. Operating principle of the paper-based gene sensor for identification of contagious plant virus.

diameter AuNPs exhibited a characteristic absorption maximum at 520 nm.

DNA-modified AuNPs were prepared according to published protocols with slight modifications.[44] Briefly, a thiolated detection probe was activated by treatment with 10 equiv of TCEP at pH 5.2 for 1 h at room temperature. Then, the probe was added to a AuNP solution (10 nM) at a final concentration of 500 nM, and the mixture was incubated for at least 16 h under gentle shaking. The solution was aged by slow addition of NaCl to a final concentration of 100 mM in Tris acetate buffer (5 mM, pH 8.2) and allowed to stand for another 24 h. After being washed and purified twice by centrifugation at 12000 rpm for 20 min with 10 mM Tris-HCl buffer (pH 7.4), the detection-probe-modified AuNPs were resuspended in a solution containing 20 mM Na_3PO_4, 5% BSA, 0.25% Tween-20, and 10% sucrose. A desired volume of this suspension was dispensed onto a glass fiber pad (used as the conjugation pad), dried at room temperature, and then stored in a desiccator until future use.

Assembly of Paper-Based Gene Sensor. The bioactive paper-based gene sensor prepared in this work consisted of four sections: sample pad, conjugation pad, nitrocellulose membrane, and absorption pad (Figure 1). The sample pad was made of glass fibers; it was saturated with a desired volume of saline−sodium citrate (SSC) buffer (4×) and then dried and stored in a desiccator at room temperature, to ensure optimal conditions for the analyte throughout the flux. The conjugation pad prepared previously was used as storage for the labels, where the binding reaction between the analyte and the labels started. The nitrocellulose membrane was used to dispense streptavidin-biotinylated capture probe T and streptavidin-biotinylated capture probe C (complementary to the AuNP-labeled detection probe) with the HM3020 dispenser at different locations to form the test line and control line, where the signal was developed. The streptavidin-biotinylated probe was prepared by mixing 50 μL of 100 μM biotinylated probe with 150 μL of 1 mg/mL streptavidin and incubating for 1 h at room temperature. The distance between the test line and control line was around 4 mm. The membrane was dried at room temperature and stored in a dry state. The absorption pad was a strip of thick absorbent paper used to wick the fluid through the membrane. All of these sections were laminated on a plastic adhesive backing in an orderly manner with an overlap of 2 mm to facilitate capillary flow. Finally, the whole assembled plate with a 4-mm width was cut using the GD300 Goldbio cutting module and stored in a desiccator for the subsequent assays.

Virus Source and Genomic DNA Extraction. Samples were collected from banana plants exhibiting typical symptoms associated with infection by BBTV and other viruses (BSV, CMV) at South China Agricultural University (Guangzhou, China). Banana tissue was cut into fine pieces (approximately 0.2 g) using a sterile scalpel and used immediately or stored at −80 °C for long-term storage. Genomic DNA was extracted from approximately 0.2 g of infected or uninfected plant sample using the cetyltrimethylammonium bromide (CTAB) method described by Doyle and Doyle.[45]

Polymerase Chain Reaction. The PCR was carried out in an Eppendorf thermal cycler with Taq DNA polymerase in a total volume of 25 μL. Two oligonucleotide primers (Table 1) designed within conserved sequences of the BBTV coat protein gene (GenBank: U97526). The reaction conditions were as follows: denaturation at 94 °C for 4 min; 35 temperature cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s; and a final extension time of 6 min at 72 °C.

Hybridization with Block Oligonucleotides and Analytical Procedure. The hybridization procedure was performed by adding block oligonucleotides with a final concentration of 100 nM each to the amplification products. The mixture was then incubated at 95 °C for 5 min, 55 °C for 30 s, and finally 4 °C for 30 s in the PCR system. The resulting mixture was then applied to the sample pad with the desired volume of 4 × SSC buffer, and allowed to migrate toward the absorption pad. After 5 min, another 50 μL of 4 × SSC buffer was applied to wash the sensor. The test line and control line were evaluated visually within 10 min. Quantitative measurements were performed by photographing the sensor with a cell phone camera and then determining the peak area of test line using ImageJ.

RESULTS AND DISCUSSION

Principle. The operating principle of the bioactive paper-based gene sensor for the identification of contagious plant viruses is illustrated in Figure 1. DNA is the preferred analyte for the screening of complex samples, because it is much more stable than proteins.[46−49] Genomic DNA is extracted from the healthy or virus-infected plant samples, and then a specific gene is amplified; only the virus-infected samples can be amplified. Because the amplification products are double-stranded DNA (dsDNA), three short oligonucleotides are designed for use as block oligonucleotides to mix with the target dsDNA. The block oligonucleotides have sequences complementary to one

strand of the target dsDNA that hybridize near the capture and detector oligonucleotides and much higher concentrations and diffusion rates than the target dsDNA strands in the mixture. The mixture is thermally denatured. Then, the block oligonucleotides anneal to the target DNA strands and prevent them from rehybridizing. The resulting mixture is then applied to the sample pad. The mixture migrates by capillary action and rehydrates the detection-probe-modified AuNPs. The target DNA hybridizes with the detection probe to form a complex and then continues to migrate along the sensor. The complexes are captured by the capture probe T immobilized on the test line through a second hybridization with the target DNA. The accumulation of AuNPs on the test line is visualized as a characteristic red band. By migrating further and passing the control line, the excess detection-probe-modified AuNPs are captured on the control line by hybridization with capture probe C, thus forming a second red band. In the absence of the right amplification products, no red band is observed on the test line. In this case, a red band on the control line shows that the sensor performed properly. Qualitative results are obtained simply by observing the color change of the test line, and quantitative analysis is realized by recording the optical intensity of the test line with ImageJ.

Optimization of the Block Oligonucleotide Concentration. The block oligonucleotides were designed to consist of short sequences complementary to one strand of the target dsDNA that hybridize near the capture and detector oligonucleotides and had much higher concentrations and diffusion rates than the target dsDNA strands. The higher concentration of the block oligonucleotides was used to enhance the accessibility of the target sequence and improve the hybridization efficiency, thereby greatly increasing the intensity of the test line. The concentration of the block oligonucleotides was optimized in the range 0−1000 nM (Figure 2). It was found that the intensity of the test line increased with the concentration of block oligonucleotides and reached a relatively stable plateau at a concentration of 100 nM. This indicates that an increased block oligonucleotide concentration is beneficial for the hybridization efficiency, and that an oligonucleotide concentration of 100 nM block provides a satisfactory result for the analysis. Therefore, a block oligonucleotide concentration of 100 nM was selected to provide excellent performance in the following experiments.

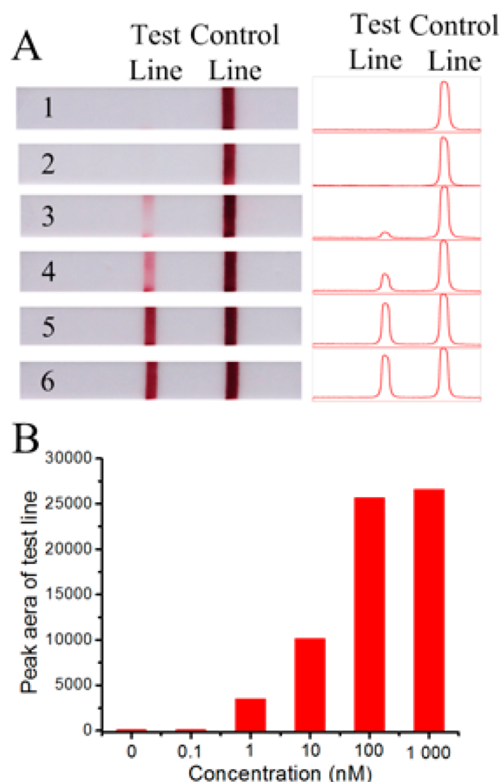


Figure 2. Effect of the concentration of block oligonucleotides on the performance of the paper-based gene sensor. (A) Photographic images (left) and corresponding optical responses (right) of the bioactive paper-based gene sensor with different concentrations of block oligonucleotides. Strips 1−6 represent block oligonucleotide concentrations 0, 0.1, 1, 10, 100, and 1000 nM, respectively. (B) Histogram of the peak area of the test line responding to different concentrations of block oligonucleotides.

Analytical Performance of the Paper-Based Gene Sensor. Under the optimal experimental conditions, we assessed the detection capability of the bioactive paper-based gene sensor with different concentrations of BBTV coat protein gene segment (Figure 3). Figure 3A (left) presents typical photographic images of the sensor in the presence of various concentrations BBTV coat protein gene segment. A red band at the test line was observed with BBTV coat protein gene segment concentrations as low as 80 copy/μL (0.13 aM). For quantitative analysis, the intensities of the test lines were estimated and plotted (Figure 3A, right). Well-defined peaks were obtained, and the peak area of the test line increased with increasing BBTV coat protein gene segment concentration (Figure 3B). The resulting calibration plot of peak area versus BBTV coat protein gene segment concentration was found to be linear over the range from 80 to 8000 copy/μL and is therefore suitable for quantitative work (Figure 3C). A plateau was observed at 8000 copy/μL, and it was not possible to discern differences in the red band at the test line for concentrations over 8000 copy/μL. This result was due to the fact that the amplification products exceed the amount of capture probe T on the test line, so that similar amounts of AuNPs were captured on the test line of the sensor, resulting in red bands that were indistinguishable with the naked eye.

Reproducibility is of great importance in practical applications for the identification of contagious plant viruses. The reproducibility of the approach developed in this work was assessed by testing each sample three times (Figure 3A). Each tested sample gave similar optical responses, which indicates the good reproducibility of the method.

We compared the detection capability of the paper-based gene sensor with that of agarose gel (1%) electrophoresis (Figure 3D). It can be seen that a BBTV coat protein gene segment concentration of 800 copy/μL was detected by electrophoresis. Thus, the sensor developed in this work offers a 10 times higher detection capability than electrophoretic analysis. In contrast to electrophoresis, which provides only the size of the amplified fragment, this analytical platform allows for confirmation of the target sequence by hybridization with specific probes. The detection capability of the sensor was further evaluated by contrasting with real-time PCR (Figure 4). We found that a BBTV coat protein gene segment concentration of 80 copy/μL was detected by real-time PCR. The linear range is from 8×10 to 8×10^6 copy/μL and is thus suitable for quantitative work. One can see that our approach exhibits the same detection limit as real-time PCR, which indicates that this approach provides a satisfactory performance.

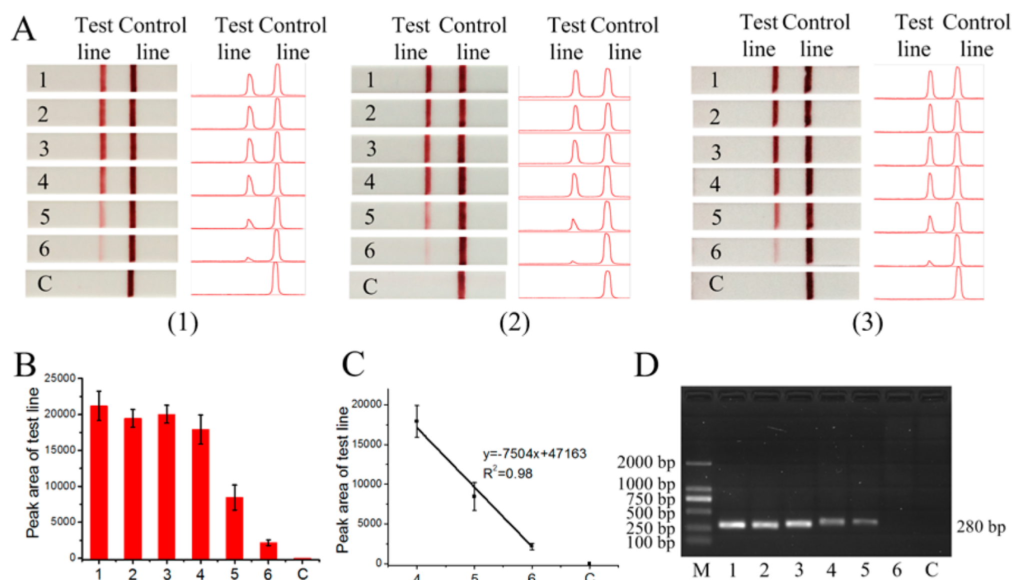


Figure 3. Sensitivity assay of the paper-based gene sensor for identification of BBTV. (A) Photographic images (left) and corresponding optical responses (right) of the paper-based gene sensor with different concentrations of BBTV coat protein gene. Strips 1−6 correspond to concentrations of 8×10^6, 8×10^5, 8×10^4, 8×10^3, 8×10^2, and 8×10 copy/μL; strip C is a negative control. Panels 1−3 represent the results for the three times the test was repeated for each sample solution. (B) Histogram presenting the corresponding peak areas of the test line. (C) Calibration plot of the peak area of the test line versus the BBTV coat protein gene concentration. (D) 1% agarose gel electrophoresis of the amplification products.

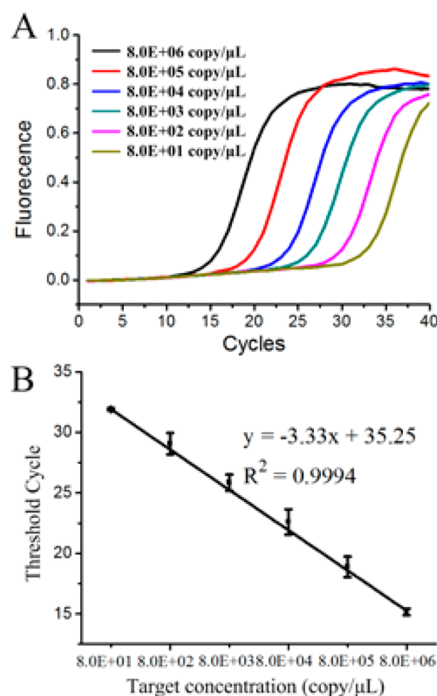


Figure 4. Real-time PCR for the identification of BBTV. (A) Amplification curve of BBTV coat protein gene in real-time PCR. (B) Calibration plot from the real-time PCR.

Specificity is another important parameter in evaluating performance for the identification of contagious plant viruses. In the current study, we examined specificity by testing healthy banana plant samples as well as samples that were infected with BBTV and other viruses (BSV, CMV) (Figure 5). Ultrapure water was used as negative control. As shown in Figure 5A, two clear red bands were observed at the test line and control line with the BBTV-infected banana plant sample, whereas only one red band at the control line was observed with the banana plant samples that were infected with other viruses and healthy, the same as with the negative control. The results were also validated by electrophoresis and showed high consistency (Figure 5C). These results demonstrate that the approach developed in this work has excellent specificity.

Levels of BBTV in Different Banana Plant Tissues. It has been reported that viruses tend to distribute unevenly in plant tissues and that some viruses exist at very low concentrations in their hosts.[50] Therefore, to maximize sensitivity and identify plant viruses in early stages, tissues containing high viral titers should be selected as test samples. We have used this gene sensor to compare BBTV concentrations in different banana plant tissues. Banana suckers infected with BBTV (displaying the characteristic symptoms of BBTV) were used as virus-infected source plants, which were later cultured into banana plants that served as sources of different banana plant tissue. Genomic DNA was extracted from 0.2 g samples of BBTV-infected new leaf, old leaf, pseudostem, and root; dissolved in 100 μL of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0); and then diluted 1000-fold with sterile distilled water for PCR amplification and quantitative analysis. The test results are presented in Figure 6. It can be seen that the optical densities of the new leaf and pseudostem had higher intensities, which indicates higher levels of BBTV. These results suggest that it is better to use new leaf or pseudostem as the sample for identifying BBTV at an early stage.

Reliability Assesssment. The reliability of the paper-based gene sensor was determined through the identification multiple samples of plants showing BBTV symptoms (Figure 7). Virus-free tissue-cultured banana plantlet was included in the study as a healthy control. One can see that all BBTV-infected banana plant samples presented positive results, whereas the healthy banana plant sample, like the negative control, presented a negative result. These results indicate that this approach is reliable for practical application.

dx.doi.org/10.1021/am506695g | ACS Appl. Mater. Interfaces 2014, 6, 22577−22584

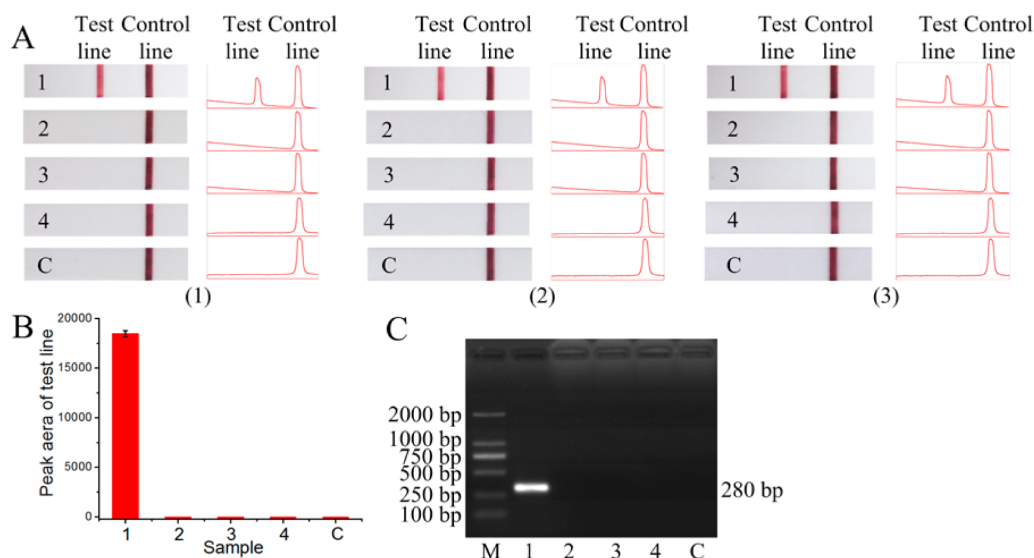


Figure 5. Specificity evaluation of the paper-based gene sensor for the identification of BBTV: 1, BBTV-infected banana leaf; 2, BSV-infected banana leaf; 3, CMV-infected banana leaf; 4, healthy banana leaf; C, negative control. (A) Photographic images (left) and corresponding optical responses (right) of the paper-based gene sensor for the identification of BBTV. Panels 1−3 represent the results for the three times the test was repeated. (B) Histogram presenting the peak areas of the test line responding to different banana plants. (C) 1% agarose gel electrophoresis of the amplification products.

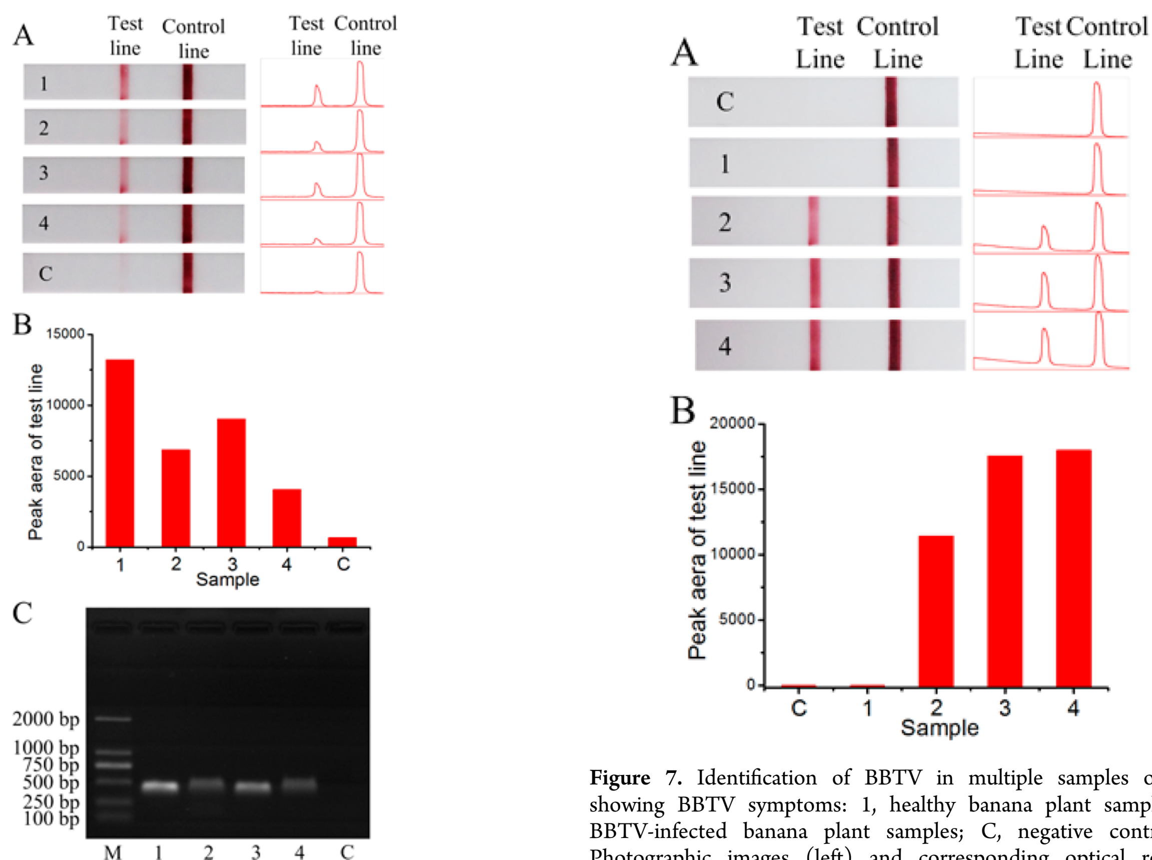


Figure 6. Levels of BBTV in different banana plant tissues: 1, new leaf; 2, old leaf; 3, pseudostem; 4, root; C, negative control. (A) Photographic images (left) and corresponding optical responses (right) of the paper-based gene sensor for the identification of BBTV in different banana plant tissue. (B) Histogram presenting the corresponding peak areas of the test line. (C) 1% agarose gel electrophoresis of the amplification products.

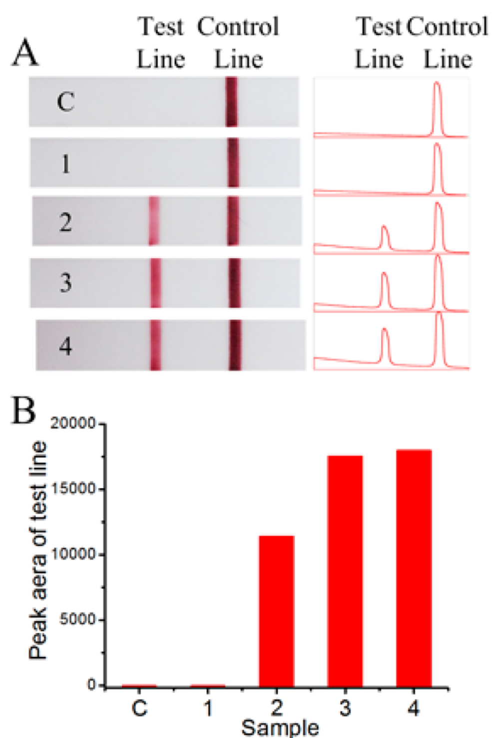


Figure 7. Identification of BBTV in multiple samples of plants showing BBTV symptoms: 1, healthy banana plant sample; 2−4, BBTV-infected banana plant samples; C, negative control. (A) Photographic images (left) and corresponding optical responses (right) of the paper-based gene sensor for the identification of BBTV in multiple banana plant samples. (B) Histogram presenting the corresponding peak areas of the test line.

■ CONCLUSIONS

In summary, we have successfully constructed a low-cost paper-based gene sensor that makes use of hybridization-mediated target capture based on a miniaturized lateral flow platform and

gold nanoparticle colorimetric probes for contagious plant virus identification. It provides qualitative (visual) and quantitative (optical responses of the test line) detection of amplified DNA within minutes with a low background and high specificity without the need for sophisticated instruments or the multiple incubation and washing steps performed in most other assays. The sensor was employed for the identification of BBTV in banana plants with a detection limit of 80 copy/μL (0.13 aM). This detection capability is 10 times higher than that of agarose gel electrophoresis. In contrast to electrophoresis, which provides only the size of the amplified fragment, the sensor developed in this work allows for confirmation of the target sequence by hybridization with specific probes. The gene sensor thus provides a rapid, sensitive, cost-effective, easy-to-use, and quantitative tool for the identification of contagious plant viruses. It shows great promise in practical applications for warning against plant diseases in agricultural production.

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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We are grateful to Prof. Huaping Li and Prof. Guohui Zhou (South China Agricultural University, Guangzhou, China) for kindly providing BBTV-, BSV-, and CMV-infected banana plant samples and virus-free tissue-cultured banana plantlet. This work was supported by the Key Program of NSFC-Guangdong Joint Funds of China (U0931005); the National Natural Science Foundation of China (21475048, 81101121); and the Program of the Pearl River Young Talents of Science and Technology in Guangzhou, China (2013J2200021).

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