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Abstract

Low-cost and quick detection of biotic stresses is critically important for protection of staple food crops such as maize in smallholder farms in developing countries, where access to improved seed varieties, fertilizers, and pesticides is limited due to financial and geographical reasons. Here, we report a new lateral flow detection technology directly integrated in a maize leaf, in which microspheres conjugated with analyte-specific capture antibodies are non-invasively injected. The antibody-conjugated microspheres capture and detect an analyte in a concentration-specific manner. In this study, we optimized microsphere size for effective infiltration and immobilization in the leaf, and further demonstrated detection of a fluorescent mock biomarker, fluorescein, in a live maize plant. This *in planta* lateral flow biosensor is the first of its kind and is expected to provide a low-cost and user-friendly detection method for biotic stresses in the field.

Keywords

biosensor, lateral flow assay, microsphere, plant biotechnology, maize

Introduction

Maize is one of the most widely grown staple crops globally, and it is especially critical in developing regions of sub-Saharan Africa and Mesoamerica, where maize alone comprises more than 20% of food calories and up to 73% of total maize production is used as a food source.¹ Despite high demand, maize growth suffers from significant losses at both pre- and post-harvest stages due to biotic stresses such as viruses, fungi, bacteria, and other pests and pathogens. Numerous methods to improve maize growth and yield, including adoption of improved seed varieties, use of fertilizers, and application of pesticides, have been suggested to alleviate recurrent food shortages; however, these have not been widely used by smallholder farmers due to their high cost and lack of accessibility.^{2,3}

Quick and efficient detection of plant pathogens is desirable for timely intervention of plant diseases. Standard methodologies in plant diagnostics range from physical examination of symptoms to the use of molecular diagnostics such as enzyme-linked immunosorbent assay (ELISA) or polymerase chain reaction (PCR).^{4–6} ELISA and PCR offer high sensitivity and specificity; however, both these approaches require expensive laboratory facilities and trained operators. In contrast, physical examination can only offer a diagnosis well past the onset of pathogenic infection.

Recently, lateral flow devices have gained much attention as inexpensive, user-friendly, in-field diagnostic tools.⁷ Lateral flow devices typically incorporate the use of a

nitrocellulose test strip and capture antibodies to detect the presence of an analyte in a two-step sandwich assay. Wicking of an analyte-containing sample solution through a nitrocellulose substrate results in capture of the analyte by the first antibody, conjugated to a colored particle. The analyte–antibody pair then flow downstream to a second analyte-specific antibody that is immobilized on the surface of the test strip (the detection zone). An accumulation of the initial analyte–antibody pairs at the detection zone results in a positive test indicated by an aggregation of colored particles visible to the naked eye.⁷ Although lateral flow devices have recently been developed to detect plant pathogens,⁵ these devices require the user to initiate testing and are likely used for rapid confirmation of pathogenic infection already suspected from physical examination. An ideal detection platform would provide notification at the first onset of pathogenic infection. Such a platform would benefit from a biodetection strategy that is integrated directly within the host plant itself.

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Syringe agroinfiltration is a method of gene delivery into plant hosts using *Agrobacterium*.^{8,9} A needleless syringe is filled with an *Agrobacterium*-containing solution. The tip of the syringe is then placed against the abaxial (lower) side of an intact plant leaf, and the solution is manually injected into the leaf interstitium. A temporary color change from light to dark green indicates successful infiltration of *Agrobacterium* into the leaf.^{10,11} Syringe agroinfiltration has been demonstrated successfully in several plant species.^{11,12} Although there are other methods of transient gene expression in live plant hosts, including biolistics (microprojectile bombardment)¹³ and microneedle injection,¹⁴ the need for minimal equipment and simplicity of procedure has made syringe agroinfiltration a popular choice in recent years.

Herein, we report the first development of a biosensing technology that uses a live maize leaf as a lateral flow “test strip.” By modifying the established agroinfiltration procedure, we inject and immobilize capture-antibody-conjugated microspheres in the leaf interstitium. Our one-step lateral flow detection platform aims to target fluorescent biomarkers (e.g., aflatoxins) or to incorporate the use of detection-sensitive, colorimetric polydiacetylene (PDA) vesicles^{15,16} to eliminate the need for two-step sandwich assays for nonfluorescent biomarkers. In this study, we successfully detect a mock pathogen, fluorescein, using infiltrated microspheres conjugated with anti-fluorescein antibody (Fig. 1A).

Materials and Methods

Materials

Maize seeds were purchased from Carolina Biological Supply Company (Burlington, NC). One milliliter needleless syringes were purchased from Fisher Scientific (Waltham, MA). Fluorescent microspheres were purchased from Spherotech (Lake Forest, IL). Biotinylated anti-fluorescein antibody was purchased from eBiosciences (San Diego, CA). All other chemicals used were research grade and were purchased from Sigma-Aldrich (St. Louis, MO).

Maize Growth

Maize seeds were germinated in coarse vermiculite in 1020 half flat trays in a greenhouse maintained within a temperature range of 16–27 °C (61–81 °F). The trays were filled with Peters Excel 21-5-20 Multi Purpose fertilizer solution (125 ppm N) from Everris (Dublin, OH) once a day.

Infiltration of Microspheres

Fluorescent microspheres were washed in 1× phosphate-buffered saline (PBS) and resuspended in an infiltration buffer (10 mM MgCl₂, 10 mM MES-potassium, pH 5.6). Microsphere solutions (0.5% v/v solids) were infiltrated

into the abaxial side of intact maize leaves. Immediately after infiltration, the abaxial epidermis was gently wiped with deionized water to remove residual uninfiltrated microspheres. Infiltrated leaves were examined from the adaxial (upper) surface under a Leica DM2000 fluorescence microscope following infiltration and every 24 h for 72 h. Fluorescence intensities were determined as a ratio of the signal intensity of the target region to the leaf background. Retention rate was calculated as a ratio of fluorescence intensity at 72 h after infiltration and immediately following infiltration.

Lateral Flow Detection

Anti-fluorescein antibody-coated red fluorescent microspheres (AF microspheres) were prepared by mixing 0.5 μm streptavidin-coated red fluorescent microspheres with biotinylated anti-fluorescein antibodies, washed, and resuspended in the infiltration buffer. The AF microsphere solutions (0.5% v/v solids) were injected into the second leaf from the bottom of 15- to 20-day-old maize plants, examined for initial microsphere content, and transferred to fluorescein solutions (0, 3.2, 8, 20, or 50 μM fluorescein in PBS adjusted to pH 6.8). The plants were grown in the fluorescein solutions for 72 h to allow for sufficient fluorescein uptake by the plants, and the solutions were changed every 12 h. The plants were examined again at the end of the 72 h experiment. For the duration of the experiment, plant roots were fully immersed in the fluorescein solutions and the solutions were protected from light. Fluorescence intensities were determined as a ratio of the mean signal intensity in a region of interest (ROI) to the leaf background. The ROI is defined per plant as a fixed rectangular area that encompasses the spot where red fluorescent microspheres have been infiltrated. The *leaf background* is defined as a corresponding region lateral to the ROI on the same leaf. Normalized fluorescence intensities for test plants were calculated as a ratio of the fluorescence intensities to the mean intensity of control plants grown in the 0 μM fluorescein solution.

Results and Discussion

Infiltration of Microspheres in Leaf Tissue

A pressure-driven infiltration technique, inspired by agroinfiltration, was developed to noninvasively introduce microspheres into the leaf. A pressure gradient, introduced by the syringe on the leaf surface, propels bio-reagents through pores in the leaf abaxial epidermis and into the interstitial space (Fig. 1B). Infiltration in maize leaves is successfully demonstrated without damaging the epidermis (Fig. 1C). In the proposed technology, microspheres are used to provide stationary surfaces through which antibodies are effectively immobilized within the leaf tissue. Without such surfaces, antibodies cannot be retained at a fixed spot inside the leaf.

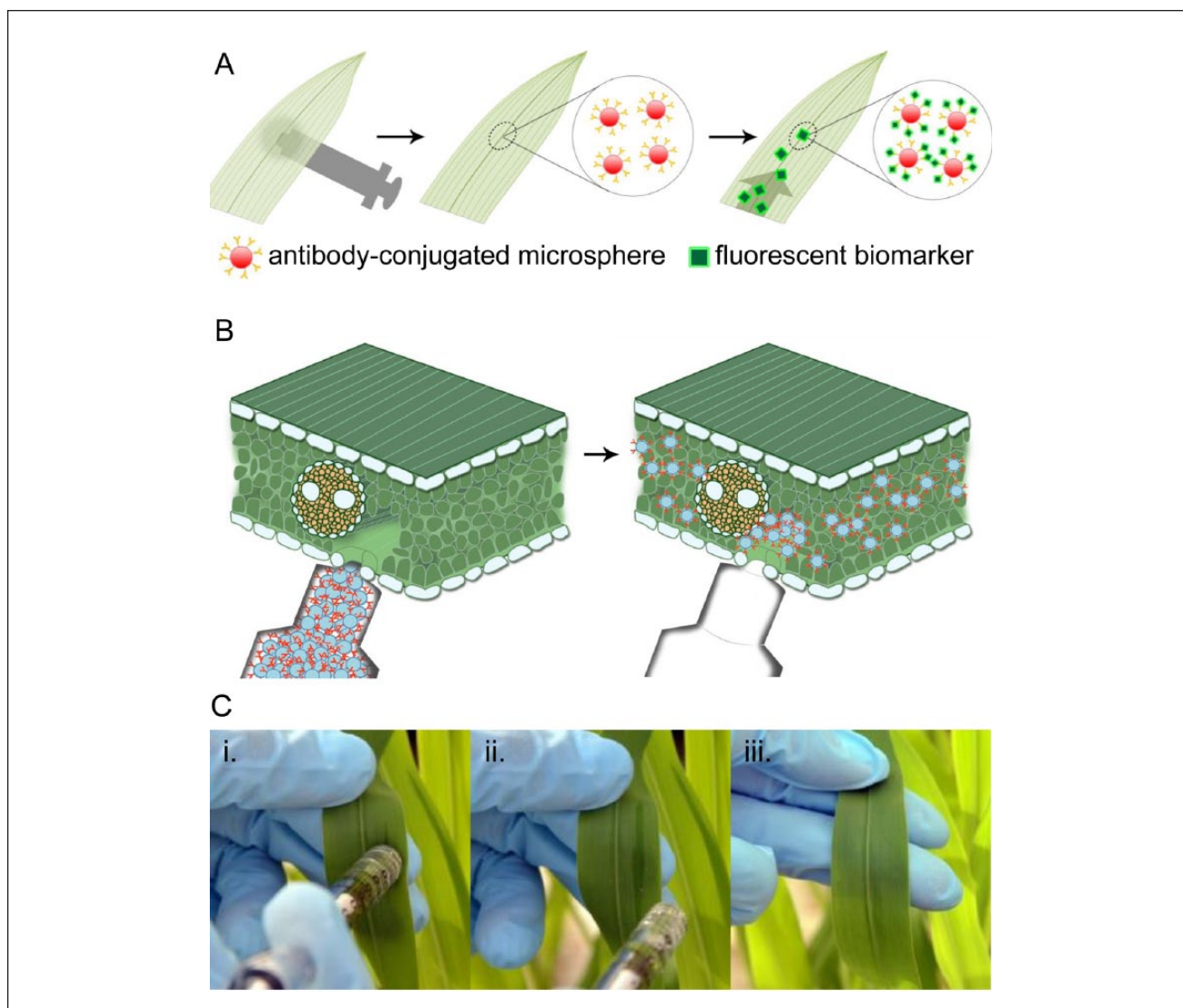


Figure 1. Method of one-step lateral flow detection of pathogen markers in live maize leaves. The present study focuses on detection of a fluorescent biomarker using antibody-conjugated microspheres (**A**), which serves as the basis for future development of the detection of nonfluorescent biomarkers by incorporating stimuli-sensitive colorimetric vesicles (see **Fig. 4**). (**B**) Schematic of infiltration of microspheres into leaf tissue (left) before infiltration and (right) after infiltration. (**C**) Infiltration in a maize leaf: (i) infiltration with a needleless syringe; (ii) immediately after infiltration, when injected buffer solution is visible; and (iii) 10 min after infiltration, when the injected buffer has evaporated without leaving visible marks.

Microsphere sizes were characterized for both high infiltration efficiency and post infiltration retention rate. Spheres too large cannot pass through the leaf epidermis, whereas spheres too small are easily removed from the infiltrated spot. Fluorescent microspheres, ranging from 0.1 μm to 1 μm in diameter, were infiltrated into intact maize leaves. Microscopy analysis of the infiltration site during the 72 h period indicates that the 0.5 μm diameter microspheres produce initial signal intensities 2.5, 3, and 4 times higher than those of 0.3, 0.1, and 1.0 μm infiltrated microspheres, respectively (**Fig. 2A and 2B**). In addition, 0.5 μm infiltrated microspheres yield a nearly 100% retention rate

throughout a 72 h period (**Fig. 2C**). These results suggest that a 0.5 μm diameter particle size is desirable for high infiltration efficiency and retention rate, and is appropriate for the immobilization of foreign biomolecules (e.g., antibodies) in the maize leaf tissue.

In Planta Lateral Flow Detection of Fluorescein by AF Microspheres

Detection of fluorescein by AF microspheres was performed as a proof-of-concept demonstration of the proposed *in planta* biosensing technology. AF microspheres

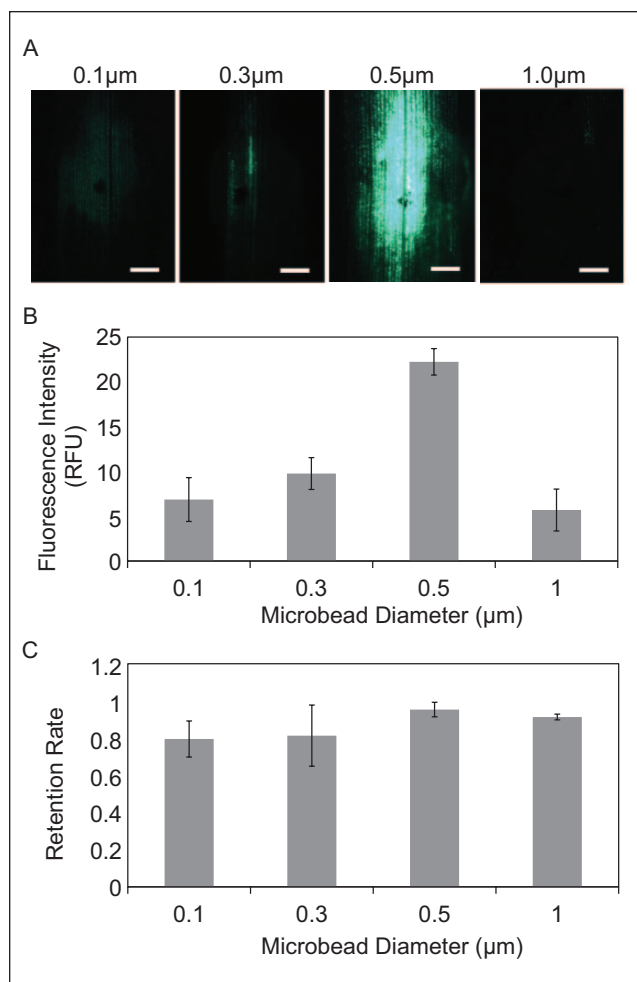


Figure 2. Infiltration of microspheres in leaf. **(A)** Fluorescence images of infiltrated microspheres. **(B)** Fluorescence intensity of infiltrated microspheres by diameter. **(C)** Microsphere retention rate after 72 h. Data represent mean $\pm$ standard deviation ($N = 3$). Scale bars: 1.2 mm.

were prepared (**Fig. 3A**) and injected into leaves of live maize plants as described in the “Materials and Methods” section. Infiltrated plants were grown in the fluorescein solutions for 72 h before optical analysis. AF microspheres themselves do not emit green fluorescence (**Fig. 3B**, 0 μ M fluorescein concentration) but can effectively capture the trace amount of fluorescein molecules taken up by the maize plant as evidenced by localized accumulation of green fluorescence signals (**Fig. 3B**, 3.2–50 μ M). Quantitative analysis shows a 22%, 34%, 51%, and 71% increase in normalized fluorescence intensity in plants infiltrated with AF microspheres and grown in the 3.2 μ M, 8 μ M, 20 μ M, and 50 μ M fluorescein solution, respectively, as compared to control plants grown in the 0 μ M fluorescein solution (**Fig. 3C**). In addition, plants infiltrated with control (nonconjugated) microspheres do not show increased

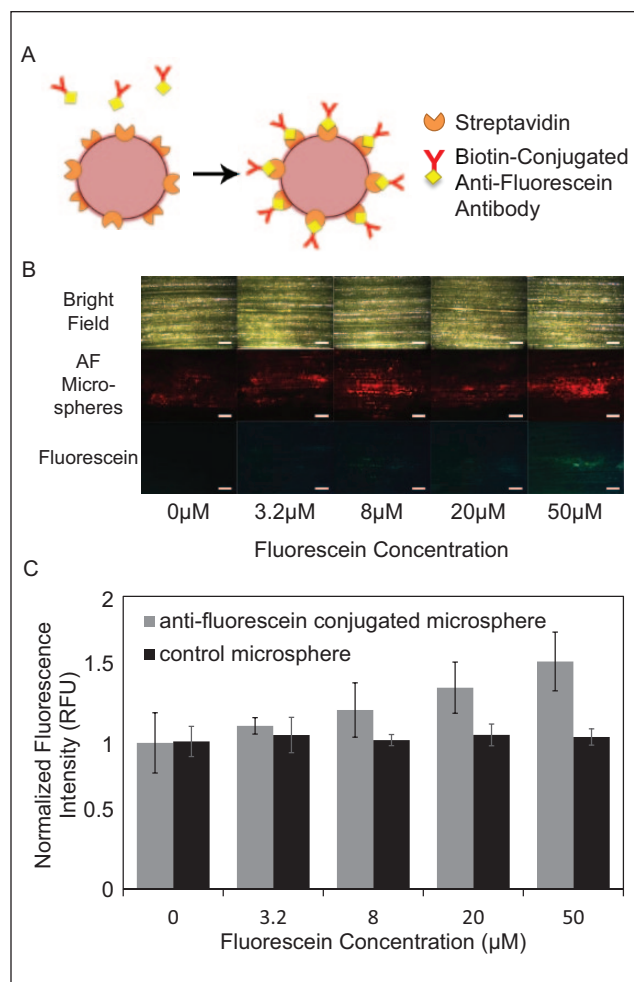


Figure 3. Lateral flow detection of fluorescein in live maize plants after 72 h of growth in fluorescein solutions. **(A)** Immobilization of anti-fluorescein antibody onto red fluorescent microspheres. **(B)** Infiltration of anti-fluorescein antibody-coated red fluorescent microspheres in leaf, followed by capture of fluorescein in plants grown in fluorescein solutions of varying concentrations from 0 to 50 μ M. **(C)** Normalized fluorescence intensity of fluorescein captured by anti-fluorescein conjugated microspheres and control (noncoated) microspheres. Data represent mean $\pm$ standard deviation ($N = 3$). Scale bars: 500 μ m.

fluorescence intensity with increasing fluorescein concentration, further confirming specific detection in the test plants that are infiltrated with AF microspheres. The *limit of detection* (LoD),¹⁷ defined as two standard deviations higher than the arithmetic mean of normalized fluorescence intensity of control plants grown in the 0 μ M fluorescein solution, is 19 μ M. Collectively, these data indicate the feasibility of *in planta* detection of biomolecules in maize based on the proposed technology.

The anticipated use of this technology is for the early detection of a disease onset during a plant's lifetime. The conjugated microspheres would therefore be injected into plant

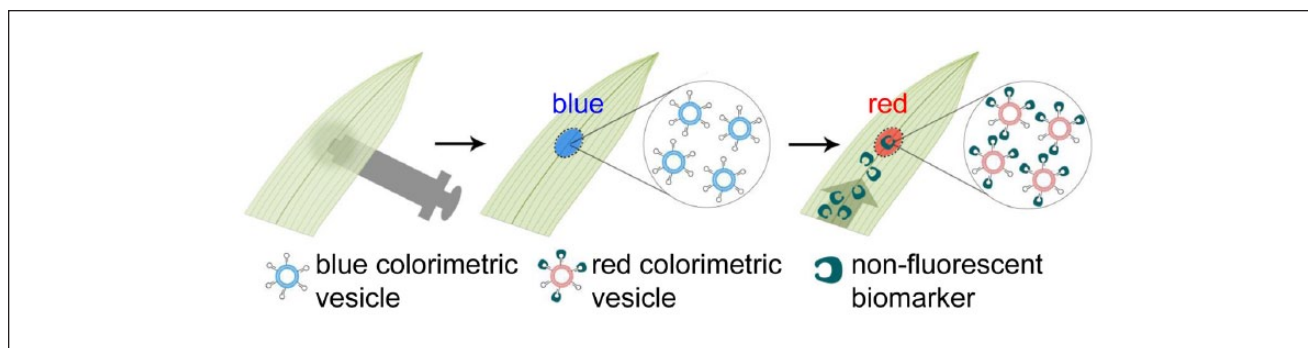


Figure 4. Proposed method of colorimetric, one-step lateral flow detection of pathogens in maize leaves for future development. Infiltrated, stimuli-sensitive, colorimetric vesicles respond to target biomarkers binding to aptamers or antibodies conjugated on the vesicle surface via a color shift (e.g., from blue to red) visible to the naked eye.

leaves at a young and healthy age. Because capture antibodies are likely to degrade and become inactive over time in plant leaves, especially in the field (e.g., in high temperatures and sunlight), more stable capture probes such as DNA aptamers are a desirable alternative for long-term applications. In addition, DNA aptamers are more economical to produce than monoclonal antibodies, thereby allowing for further cost reduction of the proposed low-cost diagnostic method.

Conclusions

We have developed a minimally invasive *in planta* biomolecular detection platform for maize. The design of our detection system incorporates the immobilization of antibody-conjugated microspheres in the leaf to capture analytes and produce a detection signal. Unlike conventional lateral flow assays, which require sample processing (e.g., grinding of plants and extraction of analyte-containing solutions) and are single-use, the proposed, directly integrated technology does not require processing of plants post infiltration and is intended for continuous monitoring of biomarker levels over an extended period of time, potentially enabling early notification of an onset of a disease. This eliminates not only the need for multiple tests per plant but also an external substrate for the lateral flow assay, thereby reducing the costs and resources needed for disease detection. This simple method is minimally invasive, inexpensive, and, to our best knowledge, the first report of a lateral flow biodetection technology that is integrated directly within living plant hosts. Because syringe infiltration is easily adapted to a variety of plants,^{10,11} this proposed technology is expected to be widely applicable. Although this initial report focuses on the demonstration of *in planta* detection of a fluorescent mock pathogen (i.e., fluorescein) only, with further development, particularly with the incorporation of colorimetric, stimuli-responsive PDA vesicles (Fig. 4),^{15,16} this technology is likely to yield an equipment-free and colorimetric *in planta* diagnosis. We anticipate that

such an innovative tool will make a significant impact on improving the yield of healthy food crops by smallholder farmers in developing countries worldwide.

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Declaration of Conflicting Interests

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