A Microfluidic Assay for Identifying Differential Responses of Plant and Human Fungal Pathogens to Tobacco Phylloplanins

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ABSTRACT

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Phylloplanins are defensive glycoproteins secreted onto leaf surfaces by trichome-bearing plants such as tobacco (*Nicotiana tabacum*). They are of interest because of their antimicrobial properties, but like other natural product bioactives, the assessment and screening of phylloplanins biological activity is impeded by limited availabilities of active compounds. Here we report an inexpensive microfluidic approach that requires ≤ 20 microliters of tobacco phylloplanins to assess spore germination inhibition of plant and human fungal pathogens. Spores of

Colletotrichum coccodes and *Aspergillus fumigatus* suspended in solutions containing tobacco phylloplanins did not germinate at 48 and 30 h post-treatment, respectively. Tobacco phylloplanins transiently inhibited spore germination of *Fusarium sambucinum*, but had no detectable activity against *Alternaria solani* or *Verticillium albo-atrum* at the concentrations tested, demonstrating differential sensitivity of fungi to tobacco phylloplanins.

INTRODUCTION

Trichomes are epidermal protuberances developing outwards on the surface of various plant organs (19) and can be broadly classified as simple/non-glandular and glandular secreting types. Glandular secreting trichomes are present in about 30% of vascular plants and secrete secondary metabolites such as terpenoids and phenylpropanoids that can provide disease and pest resistance (7,18). Shepherd et al. (15) discovered that tobacco plants produce and secrete to their leaf surfaces antifungal plant proteins termed phylloplanins. Native phylloplanins of *Nicotiana tabacum* referred to as leaf water wash in earlier studies (8,15) predominantly consist of T-phylloplanin. Tobacco phylloplanins can be collected by gently agitating undamaged leaves in water, lyophilizing the resulting solution, and resuspending the lyophilized product in sterile water. With SDS-PAGE, T-phylloplanin is visible as four bands with molecular masses of 16 kDa, 19 kDa, 21 kDa, and 25 kDa. The four bands all have the same N-terminal amino acid sequence and are believed to arise from glycosyl additions to a single 13 kDa core polypeptide encoded by the *T-phylloplanin* gene (Accession AY705384).

Analyses of the T-phylloplanin promoter with a reporter gene indicate that it directs expression solely in short glandular secreting trichomes, thus revealing a highly specialized mechanism for localization and delivery of proteins to leaf surfaces (15). Short glandular trichomes are believed to continually secrete T-phylloplanin onto the leaf surface, although

factors such as leaf age might influence secretion. Native tobacco phylloplanins have inhibitory activity against spore germination and hyphal growth of the basidiomycete *Rhizoctonia solani*, and the ascomycete *Pyricularia oryzae* (8). A recombinant 13 kDa T-phylloplanin polypeptide produced in *Escherichia coli* inhibited spore germination of *Peronospora tabacina*, the causal agent of blue mold disease in tobacco (15). More recently, *T-phylloplanin-GFP* fusion gene when expressed in a susceptible tobacco variety conferred resistance to blue mold disease (10,12).

Mechanized methods for harvesting tobacco phylloplanins from tobacco leaves on a large scale are not yet available. This impeded our ability to screen for their antimicrobial properties and led us to explore techniques that are compatible with low reagent quantities. Microfluidic platforms are reputed for temporal and spatial control of microenvironments concurrently allowing assays to be performed in micro-channels using smaller quantities of samples (11). To date, these microsystems have been successfully used for applications in drug discovery, cell culturing, and characterization of fungal secondary metabolites (2,3,5). In this study, we developed a simple microfluidic channel array that enabled the in vitro study of antifungal effects of tobacco phylloplanins on plant and human pathogens.

MICROFLUIDIC CHAMBER FABRICATION

Microfluidic channel arrays were fabricated using a soft-lithography method (Fig. 1). The mold was fabricated using a 150-mm diameter silicon wafer upon which was spun 2 layers of SU8 epoxy photoresist sequentially, the first with a thickness of 150 μm and the second of 500 μm . Masks for patterning the designs in the photoresist were drawn on Adobe Illustrator (Adobe, San Jose, CA) and printed at Imagesetter Inc. (Madison,

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FIGURE 1

Schematic representation of microfluidic device fabrication process. (A) A multilayered mold is fabricated by spinning sequential layers of SU8 negative photoresist on a silicon wafer. Each layer is patterned using UV photolithography with ink-jet printed masks and a simple 365 nm UV light source. (B) Using soft-lithography, PDMS polymer is cured on the mold to create a replicate of the channel array. (C) The PDMS layer is bonded to glass by activating both the glass and PDMS surfaces using an oxygen-plasma and placing them in contact for 15 min. (D) The result is a platform containing an array of microscale channels on a microscope slide.

WI). The soft-lithography process was performed by mixing polydimethylsiloxane (PDMS) in a ratio of 1:10, degassing the mix in a vacuum desiccator, and pouring onto the mold. Following the application of PDMS, a transparent sheet, a rectangle of silicone rubber, a rectangle of rigid polyester, and 4 kg of weights were placed on the mold. The molds were baked at 80°C for 4 h, after which the PDMS was removed from the mold and bonded to a glass microscope slide following an oxygen plasma surface activation treatment (FEMTO, Diener, Germany) (4).

TOBACCO PHYLLOPLANINS COLLECTION

Tobacco (*Nicotiana tabacum* TI 1068) plants were grown in 15.2-cm diameter plastic pots in a greenhouse. MetroMix (Sun Gro Horticulture, BC, Canada) was used as potting soil and a slow-releasing fertilizer (Osmocote) was applied at the rate of 7.5 g per pot. Tobacco phylloplanins were collected by washing 25 to 30 fully expanded leaves in 200 ml of distilled water with gentle agitation for 15 sec. Leaves with visible damage were not washed to avoid contamination from leaf sap. The wash solution was then passed through a Miracloth (EMD Millipore, Billerica, MA, USA) to filter debris and the filtrate was lyophilized to

dryness. The lyophilized powder was resuspended in 1 ml of sterile Milli-Q water, and centrifuged at 12,000 × g for 5 min at 24°C. The supernatant was filtered using a PVDF membrane syringe filter (13 mm / 0.22 µm; Fisher Scientific, PA, USA) and stored at 4°C until further use. All experiments were replicated thrice and each experiment consisted of a new batch of plant leaf washes.

Proteinase treatment: Proteinase K immobilized on Eupergit C beads (200 mg, Sigma-Aldrich, MO, USA) was placed into mini-spin filters and 500 µl of lyophilized samples were added to these filters. The filters were then placed in a 1.5 ml microcentrifuge tube and incubated at 37°C overnight. Flow-through was collected by centrifuging the tubes at 5000 × g for 10 min, which was again incubated at 95°C for 30 min followed by a second centrifugation at 5000 × g for 5 minutes to pellet any precipitate.

SPORE GERMINATION ASSAYS

Plant and human fungal pathogens used for in vitro testing of tobacco phylloplanins activity are listed in Table 1. *Aspergillus fumigatus* was grown on glucose minimal medium agar at 37°C for 3 days. *A. fumigatus* spore suspensions were made by applying 10 ml of sterile 0.01% Tween-20 solution to the plate

TABLE 1 Fungi tested in this study.				
Fungus	Strain	Fungal family	Disease	Host
<i>Alternaria solani</i>	Asol11	Pleosporaceae	Early blight	Plants
<i>Verticillium albo-atrum</i>	Ac186	Plectosphaerellaceae	Wilt	Plants
<i>Fusarium sambucinum</i>	PD1	Nectriaceae	Dry rot	Plants
<i>Colletotrichum coccodes</i>	DRN-MK	Glomerellaceae	Black dot	Plants
<i>Aspergillus fumigatus</i>	Af293	Trichocomaceae	Invasive aspergillosis	Humans

and subsequently agitating conidiophores with a cell spreader. *Alternaria solani* was grown on clarified V8 juice agar, *Fusarium sambucinum* on quarter strength potato dextrose agar (PDA), and *Colletotrichum coccodes* and *Verticillium albo-atrum* on full-strength PDA. Spore suspensions were prepared by scraping 10 to 15 day old colonies into a small volume of sterile Milli-Q water. Spore suspensions were prepared independently thrice and quantified using a haemocytometer and appropriate dilutions were made.

The spore germination assay was conducted in microfluidic channels prepared according to the described method (Fig. 1). Each micro-channel was loaded with 20 µl or 15 µl (*A. fumigatus*) total fluid volume, containing 10 µl or 13.5 µl (*A. fumigatus*) of tobacco phytoplanins solution and 10 µl or 1.5 µl (*A. fumigatus*) spore suspension, premixed in microcentrifuge tubes. The final spore concentration in each micro-channel was 4×10^2 for *Alternaria solani*, 1.5×10^3 for *A. fumigatus*, 2×10^3 for *C. coccodes* and *F. sambucinum*, and 2×10^4 for *V. albo-atrum*. Tobacco phytoplanins concentration was estimated using a bicinchoninic acid (BCA) protein assay kit (Thermo Scientific Pierce, Rockford, IL) and the final concentration in each micro-channel was either 0.5 mg/ml (*Alternaria solani*, *V. albo-atrum*, *F. sambucinum*, *C. coccodes*) or 0.9 mg/ml (*A. fumigatus*). Channels were incubated at 24°C in a moist chamber to prevent evaporation from the channels. Germination rates were quantified microscopically at various time points depending on the fungus by counting 200 spores per treatment. A spore was considered germinated if the length of the germ tube exceeded half the length of the spore or multiple germ tubes developed. Each experiment was performed three times and statistically analyzed using Welch's t-test. All statistical analyses were performed with R software version 2.15.3. Statistical significance was set at $P < 0.05$.

EFFECTS OF TOBACCO PHYTOLPLANINS ON SPORE GERMINATION

Tobacco phytoplanins inhibited *C. coccodes* and *A. fumigatus*, transiently inhibited *F. sambucinum*, and were inactive against *Alternaria solani* and *V. albo-atrum* at the concentrations tested, thereby indicating a spectrum of pathogen sensitivity (Table 2). *C. coccodes* spores did not germinate in the presence of tobacco phytoplanins at 48 h post inoculation (Table 2). Very few of the *A. fumigatus* spores germinated when incubated with the tobacco phytoplanins, and this effect persisted for at least 30 h post

inoculation (Table 2, Fig. 2). The inhibition of *A. fumigatus* and *C. coccodes* spore germination was eliminated when proteins were digested with proteinase-K, suggesting that tobacco phytoplanins were necessary for inhibition (*data not shown*). Similar relief in the inhibition of *Peronospora tabacina* spore germination with proteinase digestion of tobacco phytoplanins was noted by Shepherd et al. (15). Unlike *C. coccodes* and *A. fumigatus*, *F. sambucinum* spore germination was only transiently inhibited by tobacco phytoplanins. *F. sambucinum* spores germinated in controls at 12 h post inoculation (hpi), but not in the presence of tobacco phytoplanins. However, at 24 hpi, this inhibitory effect was not evident and extensive hyphal growth was observed (Table 2, Fig. 3).

The inhibitory effect of varying concentrations of tobacco phytoplanins against *C. coccodes* and *A. fumigatus* spores was shown in Fig. 4. There was zero percent germination at 48 hpi when *C. coccodes* spores were suspended in tobacco phytoplanins overnight and washed twice with sterile water. However, a similar effect was not observed with *A. fumigatus* (germination percentage of 82.93 ± 3.57 at 30 hpi; *data not shown*). Therefore, the effect of tobacco phytoplanins was irreversible with *C. coccodes* but not with *A. fumigatus*, suggesting a different mode of action between these two fungi.

Earlier studies indicated the antimicrobial effects of tobacco phytoplanins (8,9,15) against an oomycete and two fungal pathogens. Here, we show that the antimicrobial activity of tobacco phytoplanins is specific to some fungal species, suggesting that host-pathogen coevolution may select for tobacco phytoplanins resistance in some fungi. The mechanism of action of tobacco phytoplanins still remains unknown. Differential responses to antimicrobial proteins by pathogens, which are likely driven by host-pathogen coevolution, is not uncommon (13,14,16,17). For instance, a 30 kDa protein isolated from leaves of *Engelmannia pinnatifida* strongly inhibited the growth of plant pathogens such as *Fusarium oxysporum*, *Alternaria solani*, and *Gaeumannomyces graminis*, but not the human pathogen *Candida albicans* (6). Knowledge of fungal resistance mechanisms and the ease of acquisition of resistance to phytoplanins would aid in decisions on whether to develop these antimicrobial proteins as a natural product for use in plant / human protection.

TABLE 2 Comparison of the effects of tobacco phytoplanins on fungal spore germination.		
Fungus ^x	Percentage of spore germination ^y	
	In the absence of tobacco phytoplanins	In the presence of tobacco phytoplanins
<i>Alternaria solani</i>	95.4 ± 3.47 a	92.3 ± 0.93 a
<i>Verticillium albo-atrum</i>	98.5 ± 0.76 a	96.7 ± 1.2 a
<i>Fusarium sambucinum</i>	96.8 ± 3.18 a	7.7 ± 10.39 b
<i>Colletotrichum coccodes</i>	95.7 ± 3.28	0 ^z
<i>Aspergillus fumigatus</i>	100 ^z	0.5 ± 0.57

^x Spore germination assessments were made at 6 (*Alternaria solani*), 12 (*Verticillium albo-atrum*, *Fusarium sambucinum*), 30 (*Aspergillus fumigatus*), and 48 (*Colletotrichum coccodes*) hours post inoculation.
^y Means (±SE) followed by the same lowercase letters are not significantly different within a row ($\alpha = 0.05$).
^z No variation in spore germination was observed across three independent experiments.

FIGURE 2
Inhibition of *Aspergillus fumigatus* spore germination by tobacco phytoplanins. (A) *A. fumigatus* spores germinated in minimal media controls at 18 h post-inoculation (hpi). (B) Non-germination of tobacco phytoplanins treated *A. fumigatus* spores at 30 hpi. Bar = 10 µm.

FIGURE 3

Transient inhibition of *Fusarium sambucinum* spore germination by tobacco phytoplanins. (A) *F. sambucinum* spores germinated in water controls at 12 h post-inoculation (hpi). (B) Non-germination of tobacco phytoplanins treated *F. sambucinum* spores at 12 hpi. (C) Extensive hyphal growth observed after treating *F. sambucinum* spores with tobacco phytoplanins at 24 hpi. Bar = 20 μ m.

FIGURE 4

Inhibition curve of *Colletotrichum coccodes* and *Aspergillus fumigatus* spore germination by tobacco phytoplanins. *C. coccodes* and *A. fumigatus* spores were suspended in solutions containing various concentrations of tobacco phytoplanins and incubated for a period of 12 h and 24 h, respectively. Each data point represents an average value from three independent experiments.

CONCLUSIONS

Due to the large volumes of water used for collecting tobacco phytoplanins from leaf aerial surfaces, the samples first needed to be lyophilized to yield a concentrated product highly enriched in protein but very limited in quantities. This aspect of sample recoverability was a significant bottleneck in our ability to perform a large number of assays. Here, we developed a microfluidic channel array enabling the efficient study of tobacco phytoplanins requiring ≤ 20 μ l of reagents. The accessible microscale assay leveraged simple pipette-based pumping methods (1) allowing simple fabrication and use of the channels. The channels positioned on a microscope slide allowed straightforward use and imaging at known locations and devoid of typical meniscus effects that occur in microwell plates. Using this microscale array, we performed tens of assays simultaneously with low quantities of tobacco phytoplanins, and demonstrated that this assay will be broadly useful in further large-scale screening of natural product bioactives against microorganisms.

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