



Covering Soybean Leaves With Cellulose Nanofiber Changes Leaf Surface Hydrophobicity and Confers Resistance Against *Phakopsora pachyrhizi*

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Asian soybean rust (ASR) caused by *Phakopsora pachyrhizi*, an obligate biotrophic fungal pathogen, is the most devastating soybean production disease worldwide. Currently, timely fungicide application is the only means to control ASR in the field. We investigated cellulose nanofiber (CNF) application on ASR disease management. CNF-treated leaves showed reduced lesion number after *P. pachyrhizi* inoculation compared to control leaves, indicating that covering soybean leaves with CNF confers *P. pachyrhizi* resistance. We also demonstrated that formation of *P. pachyrhizi* appressoria, and also gene expression related to these formations, such as *chitin synthases* (*CHSs*), were significantly suppressed in CNF-treated soybean leaves compared to control leaves. Moreover, contact angle measurement revealed that CNF converts soybean leaf surface properties from hydrophobic to hydrophilic. These results suggest that CNF can change soybean leaf surface hydrophobicity, conferring resistance against *P. pachyrhizi*, based on the reduced expression of *CHSs*, as well as reduced formation of pre-infection structures. This is the first study to investigate CNF application to control field disease.

Keywords: chitin synthase, Asian soybean rust, cellulose nanofiber, hydrophobicity, *Phakopsora pachyrhizi*, pre-infection structure

INTRODUCTION

Diseases in important crop plants have a significant negative impact on agricultural productivity. For example, Asian soybean rust (ASR) caused by *Phakopsora pachyrhizi*, an obligate biotrophic fungal pathogen, is the most devastating soybean production disease worldwide, with an estimated crop yield loss of up to 90%. ASR has impacted the South American economy in recent years. Godoy et al. (2016) reported that the losses caused by ASR were over 2 billion US dollars in Brazil annually between 2003 and 2014. Although most rust fungi have a high host specificity, the *P. pachyrhizi* host range is broad and can infect diverse leguminous plant leaves in the field (Slaminko et al., 2008). The infection process starts when urediniospores germinate to produce a single germ-tube with an appressorium. Unlike cereal rust fungi that penetrates through stomata (Bolton et al., 2008), *P. pachyrhizi* directly penetrates into host plant epidermal cells by an appressorial peg. After

penetration, *P. pachyrhizi* extends the infection hyphae and forms haustoria (feeding structures) in the mesophyll cells 24–48 h after infection (Goellner et al., 2010). Five to eight days after infection, *P. pachyrhizi* then produces urediniospores by asexual reproduction (Goellner et al., 2010). Urediniospores can be dispersed by wind and germinate on other host plants.

There are several ASR control methods for soybean protection against *P. pachyrhizi*, including chemical control by fungicide application, growing ASR resistant soybean cultivars, and employing cultivation practices. Synthetic fungicides are the primary ASR disease control method. However, fungicide use can cause many problems such as environmental impacts (Maltby et al., 2009), increased production costs (Godoy et al., 2015), and the emergence of fungicide-resistant pathogens (Langenbach et al., 2016; Klosowski et al., 2018). Another major and effective control method is breeding or engineering of ASR resistant soybean cultivars. Analysis of soybean accessions disclosed six dominant *R* genes conferring resistance to a particular *P. pachyrhizi* race, and these loci were referred to as the *Rpp* 1–6 genes (Bromfield and Hartwig, 1980; McLean and Byth, 1980; Hartwig, 1986; Garcia et al., 2008; Li et al., 2012). However, none of the soybean accessions in the world show resistance to all *P. pachyrhizi* races (Monteros et al., 2007). Due to the limited resistance available in soybean cultivars, heterologous expression of resistance genes from other plant species in soybean has been investigated as an alternative source of ASR resistance. Kawashima et al. (2016) reported that soybean plants expressing *Cajanus cajan* Resistance against *Phakopsora pachyrhizi* 1 (*CcRpp1*) from pigeon pea (*Cajanus cajan*) showed full resistance against *P. pachyrhizi*. Conversely, identifying resistance traits from non-host plant species has become an intelligent approach. Uppalapati et al. (2012) screened *Medicago truncatula* *Tnt1* mutant lines and identified an inhibitor of rust germ tube differentiation 1 (*irg1*) mutant with reduced formation of pre-infection structures, including germ-tubes and appressoria. They demonstrated that the loss of abaxial epicuticular wax accumulation resulting in reduced surface hydrophobicity inhibited formation of pre-infection structures on the *irg1* mutant (Uppalapati et al., 2012). Furthermore, Ishiga et al. (2013) reported that gene expression related to pre-infection structure formation was activated on the hydrophobic surface of the *M. truncatula* wild-type, but not on the *irg1* mutant, based on *P. pachyrhizi* transcriptome analysis, suggesting that leaf surface hydrophobicity can trigger gene expression related to formation of pre-infection structures. Based on these previous studies, we hypothesized that modification of leaf surface hydrophobicity might be a useful strategy to confer resistance against *P. pachyrhizi*.

Cellulose is an organic polysaccharide consisting of a β -1,4 linked glucopyranose skeleton. Cellulose is an important structural component of plant primary cell walls and is essential in maintaining the plant structural phase. Due to the positive properties, cellulose has been investigated as an application in different research and development fields including energy, environmental, water, and biomedical related fields (Mondal, 2017). Cellulose nanofiber (CNF) can be produced from cellulose, which is one of the most abundant and renewable biomasses in

nature (Abe et al., 2007). Because CNF exhibits properties such as low weight, high aspect ratio, high strength, high stiffness, and large surface area, CNF potentially has wide areas of application. There are several CNF isolation methods, e.g., acid hydrolysis, enzymatic hydrolysis, and mechanical processes. The aqueous counter collision (ACC) method can make it possible to cleave interfacial interactions among cellulose molecules without any chemical modification (Kondo et al., 2014). Because of this characteristic, CNF made by the ACC method has higher thermal stability and crystallinity than chemically separated CNF. Both hydrophobic and hydrophilic sites co-exist in a cellulose molecule resulting in amphiphilic properties when CNF is derived from the ACC method. Kose et al. (2011) reported that coating with CNF derived from the ACC method could switch surface hydrophilic and hydrophobic properties, depending on substrate characteristics. They demonstrated that coating a filter paper and polyethylene with CNF changed the surface property into hydrophobic and hydrophilic, respectively (Kose et al., 2011). In addition, Halim et al. (2020) demonstrated that the contact angle of CNF prepared by the ACC method was smaller than that of CNF prepared by chemical treatment, suggesting that CNF made by the ACC method has higher wettability than CNF made by other methods. To investigate the potential application of CNF in agriculture, we examined whether coating with CNF protected soybean plants against *P. pachyrhizi*. We show that a specific CNF property can change soybean leaf surface hydrophobicity, resulting in reduced formation of pre-infection structures associated with reduced *P. pachyrhizi* infection.

MATERIALS AND METHODS

Plant Growth Conditions, Pathogen Inoculation Assay, and CNF Treatment

Susceptible soybean cultivar seeds (*Glycine max* cv. Enrei) were germinated in a growth chamber at 25/20°C with 16-h-light/8-h-dark cycle (100–150 $\mu\text{mol m}^{-2} \text{s}^{-1}$) for 3–4 weeks.

An isolate of the ASR pathogen *P. pachyrhizi* T1–2 (Yamaoka et al., 2014) was maintained on soybean leaves. Fresh urediniospores were collected and suspended in distilled water with 0.001% Tween 20 (FUJIFILM, Tokyo, Japan). The 3-week-old soybean plants were spray-inoculated with 1×10^5 spores/ml using a hand sprayer for uniform spore deposition. The inoculated plants were maintained in a chamber for 24 h with 90–95% humidity at 23°C in the dark. The plants were then transferred to a growth chamber (22/20°C with 16-h-light/8-h-dark cycle) and incubated further to allow symptom development. To quantify ASR lesion number on CNF-treated plants, soybean leaves were spray-inoculated with *P. pachyrhizi*. At 10 days after inoculation, photographs were taken, and lesions were counted to calculate the lesion number per cm^2 . Lesions were counted from 54 random fields on three independent leaves.

Cellulose nanofiber (marketed as nanoforest®) was supplied through the courtesy of Chuetsu Pulp & Paper (Takaoka, Japan). CNF suspension was adjusted to a concentration of 0.1% (v/v) in water including 0.02% Tween 20 before treatment. Both adaxial and abaxial sides of soybean leaves were spray-treated with 0.1%

CNF till runoff and then the treated soybean plants were dried at room temperature for 3–4 h before inoculation. Scopoletin (TCI, Tokyo, Japan) was pre-solved as 500 mM stock solutions in dimethyl sulfoxide (DMSO; FUJIFILM) and diluted to 500 μ M in *P. pachyrhizi* spore suspensions.

Quantification of Pre-infection Structures Formation

To quantify the formation of pre-infection structures including germ-tubes and appressoria on control, CNF-, and scopoletin-treated plants, soybean leaves were spray-inoculated with *P. pachyrhizi* 1×10^5 spores/ml. At 6 h after inoculation, the leaves were observed with an Olympus BX51 fluorescence microscope after Calcofluor White (Sigma-Aldrich, St. Louis, MO, United States) staining and photographed. The germ-tubes forming differentiated appressoria were counted as appressoria. The differentiated germ-tubes without appressoria that grew on the leaf surface were also counted from at least 100 urediniospores on three independent leaves.

The formation of pre-infection structures on borosilicate glass slides and polyethylene tape with or without CNF treatment was quantified after dropping *P. pachyrhizi* spores (2×10^5 /ml). Six hours after inoculation, pre-infection structures were observed with a Nikon ECLIPSE 80i phase contrast microscope. The germ-tubes forming differentiated appressoria were counted as appressoria. The differentiated germ-tubes without appressoria that grew on the leaf surface were also counted from at least 500 urediniospores on three independent leaves.

Contact Angle Measurement on Soybean Leaves and Polyethylene Tapes

The surface hydrophobicity on the CNF-treated leaves, borosilicate glass slides, and polyethylene tapes were investigated based on contact angle measurement using an automatic contact angle meter DM-31 (Kyowa Interface Science, Niiza, Japan). The contact angle was measured by dropping 2 μ l of water from a syringe attached to the DM-31 automatic contact angle meter. The contact angle was measured on the adaxial and abaxial leaf surfaces, and polyethylene tapes with or without 0.1% CNF treatments. The contact angle was analyzed using the multi-functional integrated analysis software FAMAS (Kyowa Interface Science).

RNA-Spray-Induced Gene Silencing of Chitin synthases

Double-stranded RNA (dsRNA) of *green fluorescent protein (GFP)*, and *chitin synthase (CHS)* were synthesized using the *in vitro* Transcription T7 Kit (TaKaRa, Otsu, Japan). Briefly, we designed three primer sets to amplify *P. pachyrhizi CHS5-1* fragments (Supplementary Figure 1 and Supplementary Table 1). After RT-PCR amplification, fragments were purified and used as templates for *in vitro* transcription. The products of RNA transcripts were confirmed by gel electrophoresis (Supplementary Figure 1) and quantified by NanoDrop (Thermo Fisher Scientific, Waltham, MA, United States). We

equally mixed three dsRNA fragments and used for spray-induced gene silencing (SIGS) assay on polyethylene tape. The formation of pre-infection structures and expression levels of *CHSs* were quantified after dropping 1×10^5 /ml of *P. pachyrhizi* spores containing 10 ng/ml dsRNA on polyethylene tape. Six hours after inoculation, pre-infection structures were observed with a Nikon ECLIPSE 80i phase contrast microscope.

Quantitative RT-PCR Analyses

For urediniospores attachment assay, 4-week-old soybean leaves covered with or without 0.1% CNF were spray-inoculated with *P. pachyrhizi* 1×10^5 spores/ml. The inoculated leaves were immediately fixed, and total RNA was extracted from the leaf areas and purified using RNAiso Plus (TaKaRa). To investigate the SIGS efficacy, expression levels of *CHSs* were quantified after dropping 1×10^5 /ml of *P. pachyrhizi* spores containing 10 ng/ml dsRNA on polyethylene tape. Six hours after inoculation, total RNA was purified using RNAiso Plus. To investigate the gene expression profiles of *P. pachyrhizi CHSs* during infection, 4-week-old soybean leaves were spray-inoculated with *P. pachyrhizi* 1×10^5 spores/ml and incubated in darkness overnight, and then transferred to a growth chamber (22/20°C with a 16-h-light/8-h-dark cycle). At 2, 4, 6, 12, and 24 h after inoculation, total RNA was extracted from the inoculated leaf areas and purified using RNAiso Plus. For gene expression profiles of *P. pachyrhizi CHSs* and soybean defense-related genes, 4-week-old soybean leaves covered with or without 0.1% CNF were spray-inoculated with *P. pachyrhizi* 1×10^5 spores/ml and incubated in darkness overnight, and then transferred to a growth chamber (22/20°C with a 16-h-light/8-h-dark cycle). At 6, 12, and 24 h after inoculation, total RNA was extracted from the inoculated leaf areas and purified using RNAiso Plus according to the manufacturer's protocol.

Two micrograms of total RNA were treated with gDNA Remover (TOYOBO, Osaka, Japan) to eliminate genomic DNA, and the DNase-treated RNA was reverse transcribed using the ReverTra Ace qPCR RT Master Mix (TOYOBO). The cDNA (1:10) was then used for quantitative RT-PCR using the primers shown in Supplementary Table 1 with THUNDERBIRD SYBR qPCR Mix (TOYOBO) on a Thermal Cycler Dice Real Time System (TaKaRa). *P. pachyrhizi ubiquitin 5 (PpUBQ5)* and soybean *ubiquitin 3 (GmUBQ3)* were used to compare urediniospores attachment on soybean leaves. *P. pachyrhizi elongation factor 1 α (PpEF1 α)* and *PpUBQ5* were used to normalize *P. pachyrhizi* gene expression. Soybean *GmEF1 α* and *GmUBQ3* were used as internal controls to normalize soybean gene expression.

RESULTS

Covering Soybean Leaves With CNF Confers Resistance Against *P. pachyrhizi*

To investigate the potential application of CNF in agriculture, especially disease resistance against pathogens, we first treated soybean leaves with CNF. Four hours after spraying with 0.1% CNF, we challenged soybean leaves with *P. pachyrhizi* and

observed lesion formation including uredinia at 10 days after inoculation. CNF-treated leaves showed reduced lesion area compared to control leaves (**Figure 1A**). CNF-treated leaves showed significantly reduced lesion number compared to control leaves (**Figure 1B**). These results indicate that covering soybean leaves with CNF confers resistance against *P. pachyrhizi*. Next, we investigated urediniospores attachment on control and CNF-treated leaves by quantifying the relative ratio of *ubiquitin* gene transcripts in soybean and *P. pachyrhizi*. As shown in **Figure 1C**, we found no significant difference in the relative ratio of ubiquitin transcripts between control and CNF-treated leaves, indicating that urediniospores were uniformly sprayed on control and CNF-treated leaves.

CNF Converts Leaf Surface Properties From Hydrophobic to Hydrophilic

Cellulose nanofiber has amphipathic properties, and thus can convert material surface properties from hydrophobic to hydrophilic, and *vice versa* (Kose et al., 2011). To confirm whether CNF-treatment can convert soybean leaf surface properties from hydrophobic to hydrophilic, we quantified the differences in surface hydrophobicity by measuring the contact angle at the interface of a liquid (water) drop with the leaf surface. A greater contact angle ($>90^\circ$) is indicative of poor wetting or hydrophobicity. Interestingly, significant differences in the contact angle were observed between control and CNF-treated adaxial leaf surfaces (**Figures 1D,E**). The adaxial leaf surface of control leaves exhibited an average contact angle of 128° , whereas CNF-treated leaves showed a dramatic decrease in the contact angle (around 90°), which is indicative of a hydrophilic surface (**Figure 1E**). Similarly, significant differences in the contact angle were observed between control and CNF-treated abaxial leaf surfaces (**Figures 1D,E**). The abaxial leaf surface of control leaves exhibited an average contact angle of 127° , whereas CNF-treated leaves showed a dramatic decrease in contact angle (around 70° ; **Figure 1E**). These results clearly indicate that CNF-treatments can convert leaf surface properties from hydrophobic to hydrophilic.

Covering Soybean Leaves With CNF Suppresses Formation of *P. pachyrhizi* Pre-infection Structures

Since CNF-treatments suppressed the lesion number, we next investigated the formation of pre-infection structures including germ-tubes and appressoria on CNF-treated leaves. In control leaves, around 90% of urediniospores germinated, and ~75% formed appressoria on adaxial and abaxial leaves (**Figures 1E,G**). In CNF-treated leaves, around 90% of urediniospores germinated, and interestingly ~50 and ~30% of them formed appressoria on adaxial and abaxial leaves, respectively (**Figures 1E,G**). Scopoletin is known to protect soybean from soybean rust by suppressing the formation of pre-infection structures (Beyer et al., 2019). Thus, we also investigated the scopoletin application effect. Consistent with a previous study, in scopoletin-treated leaves, ~70 and ~60% of urediniospores germinated, and ~40 and ~30%

of them formed appressoria on adaxial and abaxial leaves, respectively (**Figures 1E,G**). These results suggest that covering soybean leaves with CNF suppresses formation of pre-infection structures, including germ-tubes and appressoria.

Hydrophobicity With CNF Suppresses Formation of *P. pachyrhizi* Pre-infection Structures

Since CNF-treatments converted leaf surface properties from hydrophobic to hydrophilic, and suppressed the formation of pre-infection structures, we next investigated the effect of CNF treatment on hydrophobic polyethylene tape. The hydrophilic borosilicate glass slide exhibited an average contact angle of 16.8° , whereas the hydrophobic polyethylene tape showed an average contact angle of 115.1° (**Figures 2A,B**). Interestingly, CNF-treated polyethylene tape showed a dramatic decrease in contact angle (around 75°), which is indicative of a hydrophilic surface (**Figures 2A,B**). On control polyethylene tape, around 90% of urediniospores germinated, and ~50% formed appressoria on hydrophobic surfaces (**Figure 2C**). On CNF-treated polyethylene tape, around 90% of urediniospores germinated, and interestingly ~20% of them formed appressoria (**Figure 2C**). We also investigated the scopoletin application effect, since scopoletin is known to suppress the formation of pre-infection structures (Beyer et al., 2019). Scopoletin suppressed urediniospore germination (**Figure 2C**). These results suggest that covering hydrophobic surfaces with CNF suppresses formation of appressoria, which resulted from conversion of surface properties from hydrophobic to hydrophilic.

Phakopsora pachyrhizi Chitin Synthases Are Required for Formation of Pre-infection Structures

Ishiga et al. (2013) reported that gene expression related to formation of pre-infection structures was induced on the hydrophobic surface based on *P. pachyrhizi* transcriptome analysis. CHSs are key enzymes in the biosynthesis of the fungal cell wall structural component, chitin. Since Ishiga et al. (2013) demonstrated that *P. pachyrhizi* CHS expression was induced on the hydrophobic leaf surface, we next tested the expression profiles of *P. pachyrhizi* CHS genes in soybean leaves. Except for *CHS2-1* and *CHS3-3*, all CHS gene transcripts were significantly induced within 2 h after soybean leaf inoculation (**Figure 3A** and **Supplementary Figure 2**), suggesting CHSs may be involved in the formation of pre-infection structures, including germ-tubes and appressoria.

To investigate *P. pachyrhizi* CHSs function on pre-infection structures formation, we performed RNA-SIGS targeting CHS genes. We designed dsRNA to target all CHS genes, and checked these gene expression levels on a hydrophobic polyethylene surface with or without CHS dsRNA for 6 h. As expected, all CHS transcripts were significantly suppressed by treatment with CHS dsRNA (**Supplementary Figure 3**). We next investigated the effect of CHS dsRNA on pre-infection structures formation. On control polyethylene tape with GFP dsRNA treatment, around 90% of urediniospores germinated, and ~50% of them

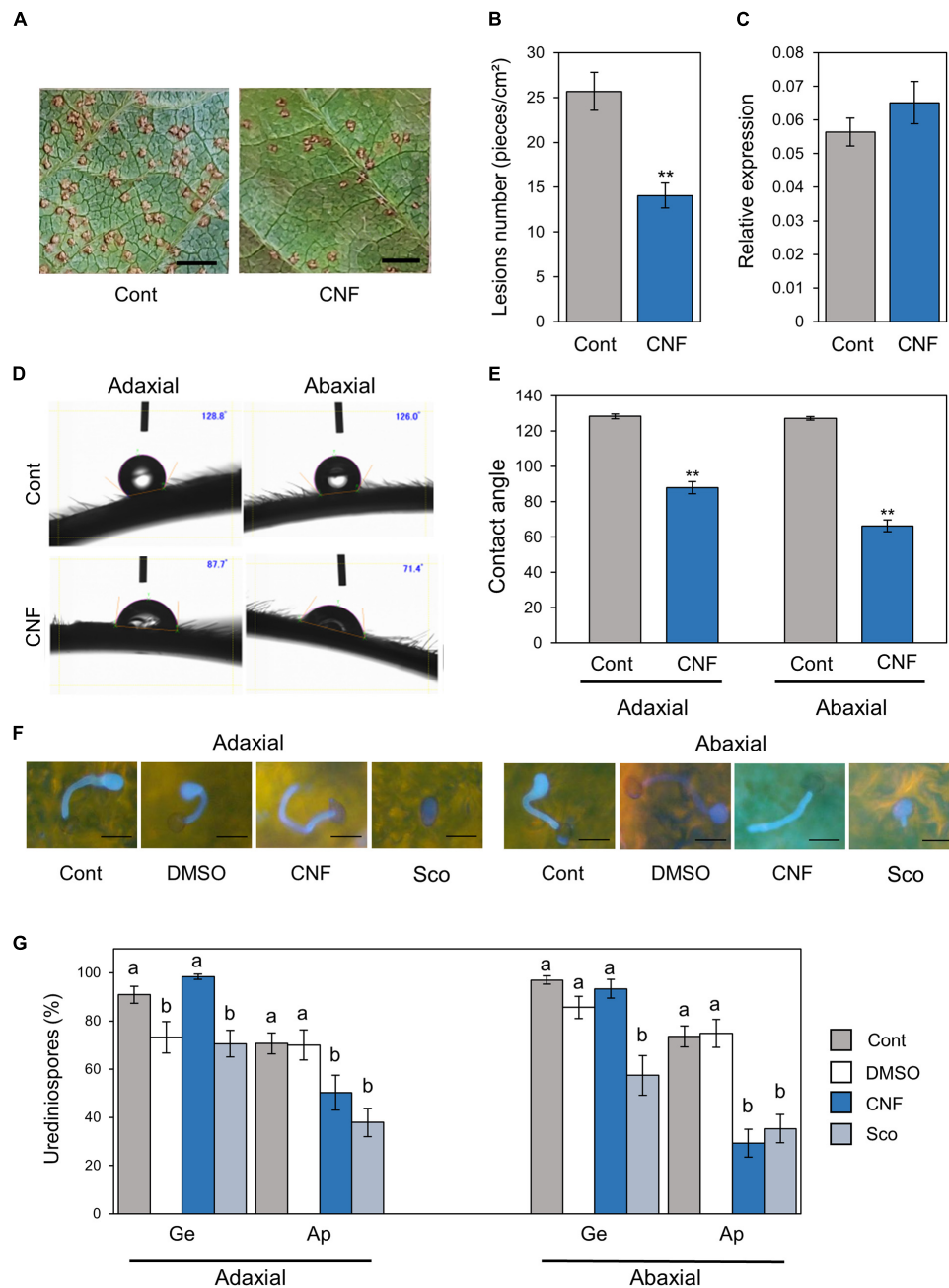
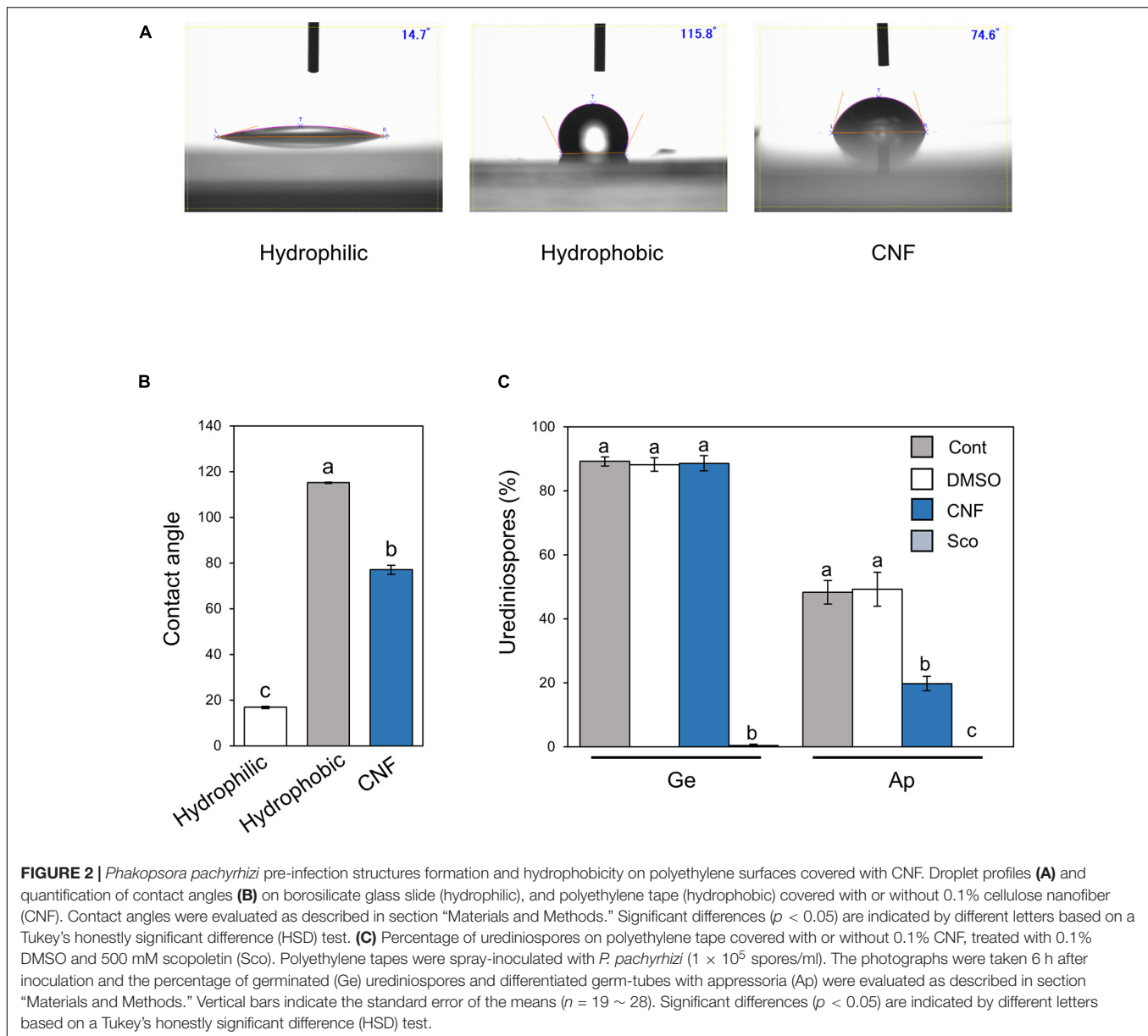


FIGURE 1 | *Phakopsora pachyrhizi* lesion formation, pre-infection structures formation, and hydrophobicity on CNF-treated soybean leaves. Disease lesions **(A)** and lesion numbers **(B)** resulting from *P. pachyrhizi* infection on the abaxial leaf surface of control, and leaves covered with 0.1% cellulose nanofiber (CNF). Soybean plants were spray-inoculated with *P. pachyrhizi* (1×10^5 spores/ml). Photographs were taken 10 days after inoculation. Bars indicate 0.2 cm. Lesion numbers were counted to calculate lesion number per cm^2 . Vertical bars indicate the standard error of the means ($n = 54$). Asterisks indicate a significant difference between control and CNF-treatments in a *t*-test (** $p < 0.01$). **(C)** Urediniospore attachment quantification on the leaf surface of control and leaves covered with 0.1% CNF derived. Soybean plants were spray-inoculated with *P. pachyrhizi* (1×10^5 spores/ml) and immediately total RNAs including soybean and *P. pachyrhizi* were purified. Relative expression of soybean *ubiquitin 3* (*GmUBQ3*) and *P. pachyrhizi* *ubiquitin 5* (*PpUBQ5*) were evaluated using RT-qPCR. Vertical bars indicate the standard error of the means ($n = 4$). Droplet profiles **(D)** and quantification of contact angles **(E)** on the adaxial and abaxial leaf surface of control, and leaves covered with 0.1% CNF derived. Contact angles were evaluated as described in section “Materials and Methods”. Vertical bars indicate the standard error of the means ($n = 60$). Asterisks indicate a significant difference between control and CNF-treatments in a *t*-test (** $p < 0.01$). *P. pachyrhizi* pre-infection structure formation **(F)** and percentage of urediniospores **(G)** on the adaxial and abaxial surfaces of control, and leaves covered with 0.1% CNF, treated with 0.1% DMSO and 500 mM scopoletin (Sco). Soybean plants were spray-inoculated with *P. pachyrhizi* (1×10^5 spores/ml). The pre-infection structures were stained with Calcofluor White and photographs were taken 6 h after inoculation. Bars indicate 50 μm . The percentage of germinated (Ge) urediniospores and differentiated germ-tubes with appressoria (Ap) were evaluated as described in section “Materials and Methods.” Vertical bars indicate the standard error of the means ($n = 21$). Significant differences ($p < 0.05$) are indicated by different letters based on a Tukey’s honestly significant difference (HSD) test.



formed appressoria on the hydrophobic surface (Figures 3B,C). Interestingly, with *CHS* dsRNA treatment, around ~60% of urediniospores germinated, and less than 5% of them formed appressoria (Figures 3B,C). These results clearly indicate that *P. pachyrhizi* CHSs are required for formation of pre-infection structures, including germ-tubes and appressoria.

Soybean Defense-Related Gene Expression Analysis

Nanofibers such as chitin nanofibers induce plant immune responses by activating defense-related gene expression (Egusa et al., 2015). Therefore, one could argue that the CNF-induced resistance phenotype in soybean plants may result from defense response activation, rather than from the direct effects of CNF treatments against *P. pachyrhizi*. To rule out this possibility,

we investigated the expression profiles of the defense marker *PR* genes and defense-related genes, including phenylpropanoid and isoflavonoid pathways leading to phytoalexin production. Except for *chalcone reductase* (*CHR*) and *isoflavone reductase* (*IFR*), all defense marker *PR* genes and defense-related genes were clearly induced within 6 h of *P. pachyrhizi* inoculation, and these transcripts reached high levels at 12 h (Figure 4 and Supplementary Figure 4). Interestingly, the transcript levels of defense marker *PR* genes and defense-related genes were significantly less at 6 h on CNF-treated soybean leaves compared to control leaves (Figure 4 and Supplementary Figure 4), suggesting that CNF treatment does not induce *PR* and defense-related genes. These results confirmed that the resistance phenotype against *P. pachyrhizi* on CNF-treated soybean leaves is a direct effect of CNF treatment.

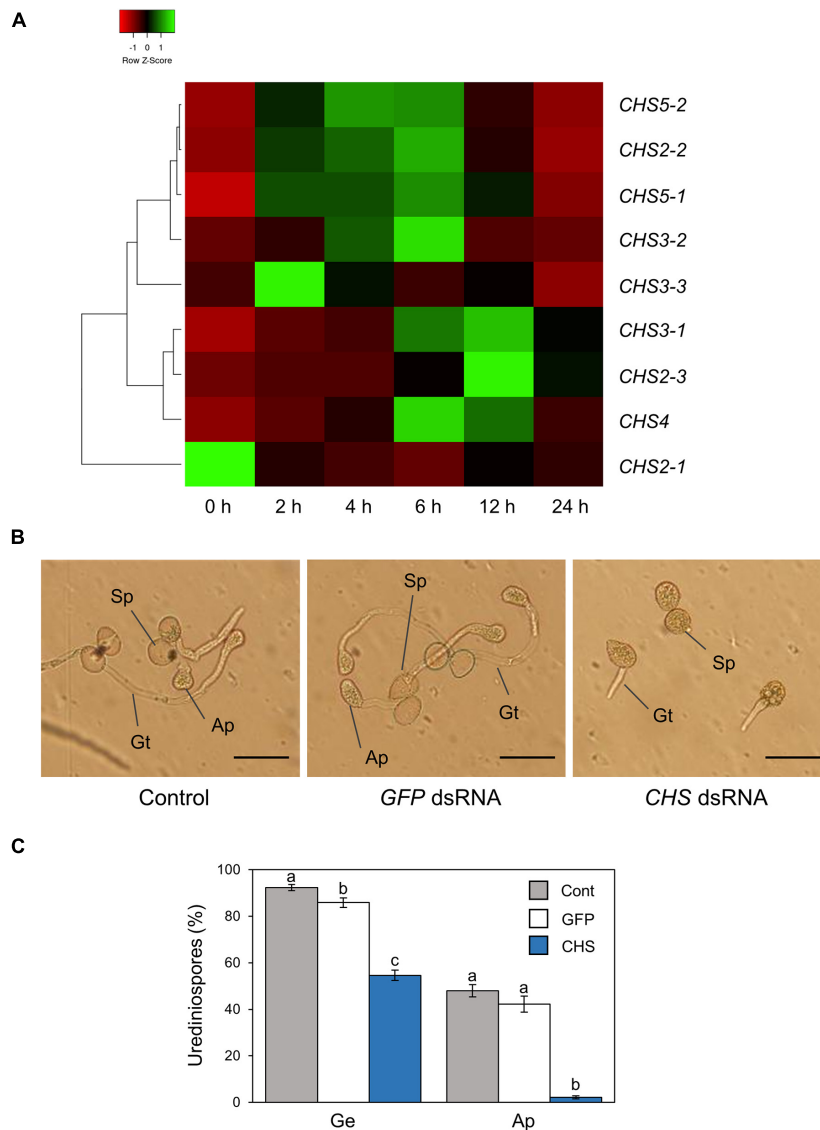
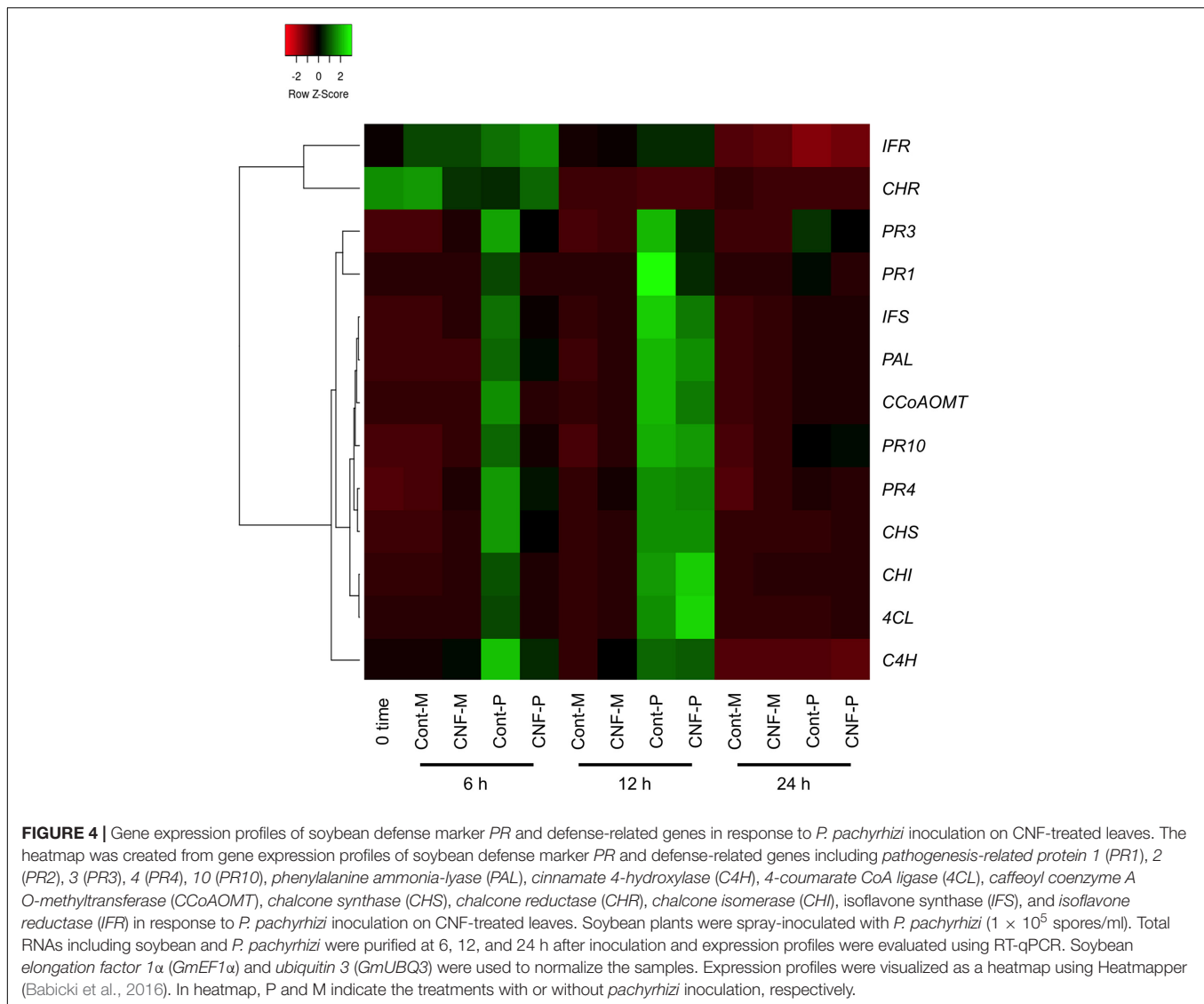


FIGURE 3 | Gene expression profiles and functional analysis of *P. pachyrhizi* chitin synthase genes. **(A)** The heatmap created from gene expression profiles of *P. pachyrhizi* chitin synthases, including *CHS2-1*, *CHS2-2*, *CHS2-3*, *CHS3-1*, *CHS3-2*, *CHS3-3*, *CHS4*, *CHS5-1*, and *CHS5-2* on soybean leaves. Soybean plants were spray-inoculated with *P. pachyrhizi* (1×10^5 spores/ml). Total RNAs including soybean and *P. pachyrhizi* was purified at 0, 2, 4, 6, 12, and 24 h after inoculation, and expression profiles were evaluated using RT-qPCR. *P. pachyrhizi* elongation factor and ubiquitin 5 were used to normalize the samples. Expression profiles were visualized as a heatmap using Heatmapper (Babicki et al., 2016). *P. pachyrhizi* pre-infection structure formation **(B)** and percentage of urediniospores **(C)** on polyethylene tapes treated with *GFP* double-stranded RNA (dsRNA) and *chitin synthase* (*CHS*) dsRNA. Polyethylene tapes were spray-inoculated with *P. pachyrhizi* (1×10^5 spores/ml). The photographs were taken 6 h after inoculation. Bars indicate 50 μ m. The percentage of germinated (Ge) urediniospores and differentiated germ-tubes with appressoria (Ap) were evaluated as described in section “Materials and Methods.” Vertical bars indicate the standard error of the means ($n = 46 \sim 47$). Significant differences ($p < 0.05$) are indicated by different letters based on a Tukey’s honestly significant difference (HSD) test.

Covering Soybean Leaves With CNF Changes Gene Expression Profiles Related to Formation of Pre-infection Structures

Phakopsora pachyrhizi CHSs are required for formation of pre-infection structures (Figure 3). We next investigated gene expression profiles of CHSs in control and CNF-treated leaves at 6, 12, and 24 h after *P. pachyrhizi* inoculation.

Except for *CHS2-1* and *CHS3-3*, all *CHS*s gene transcripts were clearly induced within 6 h in control soybean leaves (Figure 5). However, the expression of these genes was clearly suppressed in CNF-treated leaves (Figure 5), indicating that covering soybean leaves with CNF changes gene expression profiles of CHSs. Together, these results suggest that CNF-treatments suppress the expression of *CHS*s, resulting in reduced chitin biosynthesis activity in the *P. pachyrhizi* cell wall.



DISCUSSION

We investigated the potential application of CNF in agriculture, especially disease protection, and found that CNF-treated soybean leaves conferred resistance against the rust pathogen *P. pachyrhizi* (Figures 1A,B). CNF-treatments convert soybean leaf surface properties from hydrophobic to hydrophilic (Figures 1D,E), resulting in suppression of *P. pachyrhizi* *CHS* genes involved in the formation of pre-infection structures, including germ-tubes and appressoria (Figure 5) associated with reduced appressoria formation (Figures 1F,G). These results provide new insights into CNF application on *P. pachyrhizi* disease management strategies.

Cellulose nanofiber-treatments conferred soybean resistance against *P. pachyrhizi* associated with reduced lesion formation (Figures 1A,B). The application of chitin nanofibers for plant protection against pathogens has been investigated. Egusa et al. (2015) reported that chitin nanofibers effectively

reduced fungal and bacterial pathogen infections in *Arabidopsis thaliana* by activating plant defense responses, including reactive oxygen species (ROS) production and defense-related gene expression. Furthermore, chitin nanofiber treatment can reduce the occurrence of Fusarium wilt disease in tomato plants (Egusa et al., 2019). These results suggest that chitin nanofibers activate plant immunity, resulting in reduced pathogen infection. However, we showed no CNF elicitor activity based on defense gene expression profiles (Figure 4 and Supplementary Figure 4). Although there is no similarity to the mechanism by which nanofibers, including cellulose and chitin, function to protect plants against pathogens, both nanofibers will be able to provide eco-friendly disease control strategies in sustainable agriculture.

Formation of appressoria was significantly suppressed in CNF-treated leaves compared to control leaves (Figures 1F,G). Consistent with our results, Uppalapati et al. (2012) reported the reduced formation of pre-infection structures on a *M. truncatula* *irg1* mutant, in which the epicuticular waxes were completely

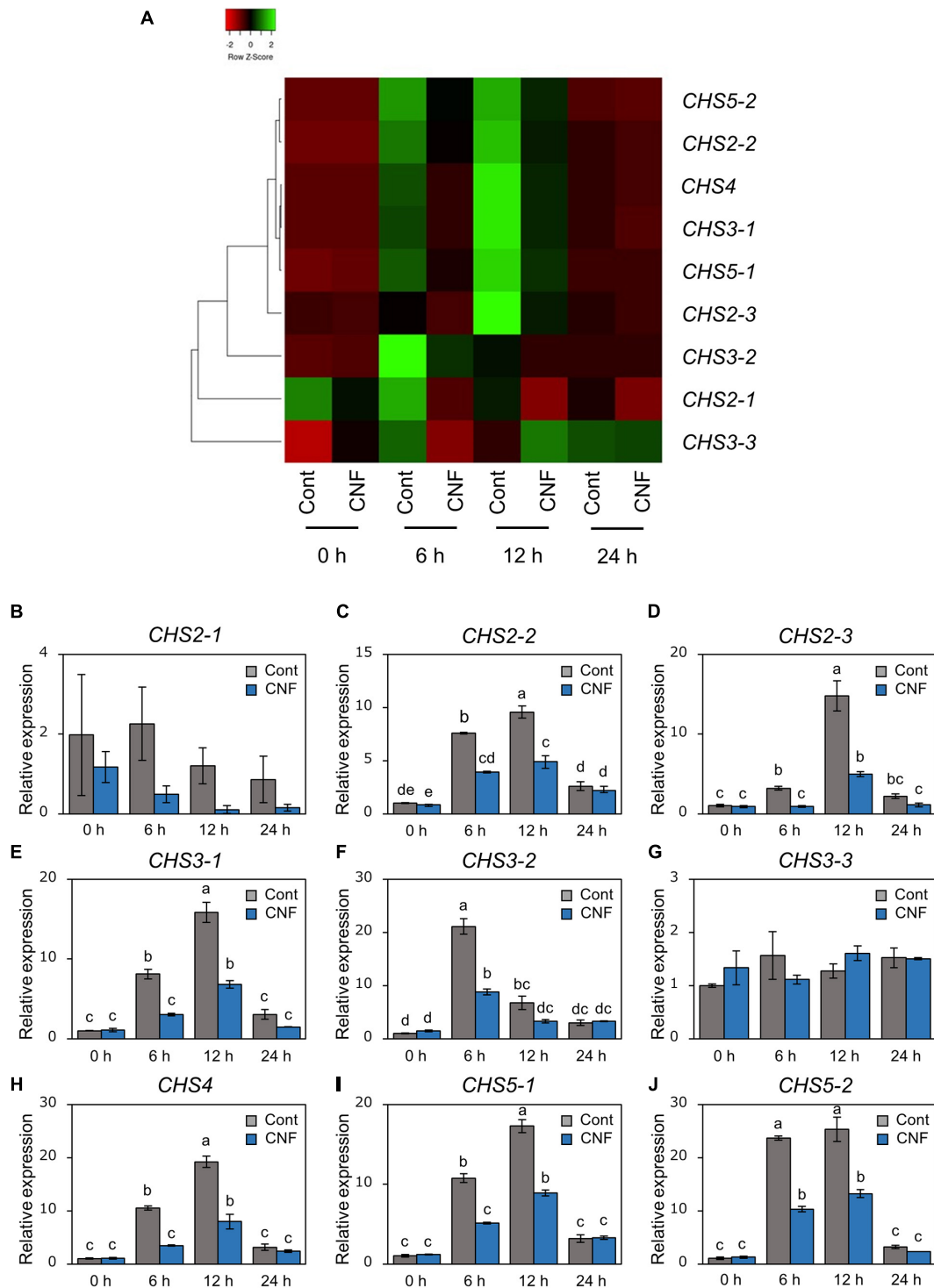
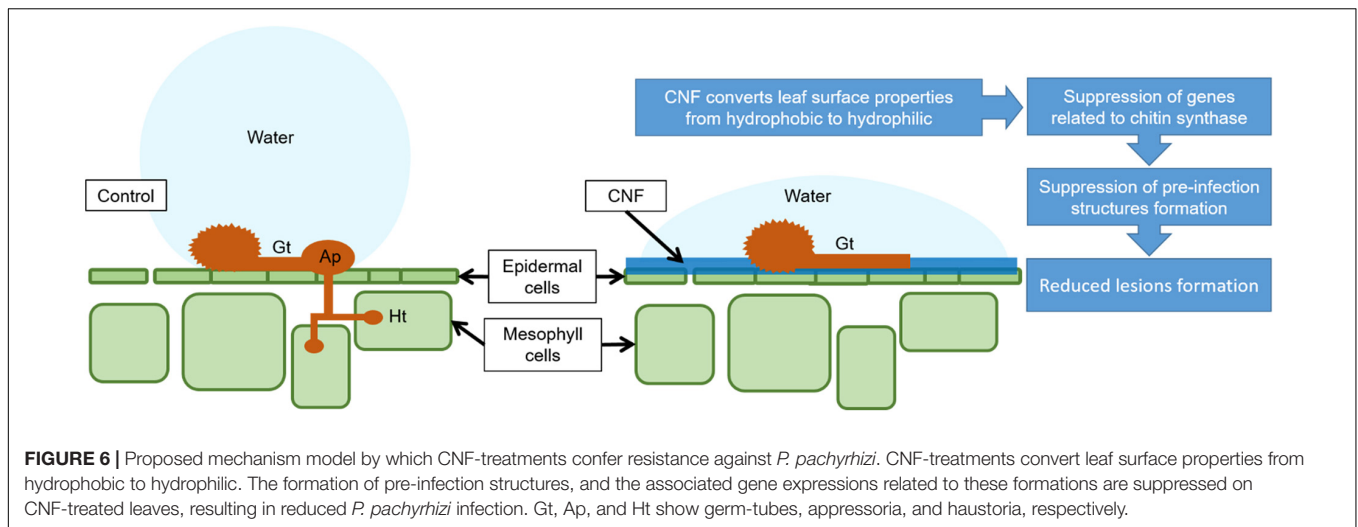


FIGURE 5 | Gene expression profiles of *P. pachyrhizi* chitin synthase genes on CNF-treated soybean leaves. **(A)** The heatmap was created from gene expression profiles of *P. pachyrhizi* chitin synthases, including *CHS2-1*, *CHS2-2*, *CHS2-3*, *CHS3-1*, *CHS3-2*, *CHS3-3*, *CHS4*, *CHS5-1*, and *CHS5-2* on soybean leaves covered with or without 0.1% CNF. Soybean plants were spray-inoculated with *P. pachyrhizi* (1×10^5 spores/ml). Total RNAs including soybean and *P. pachyrhizi* were purified at 0, 6, 12, and 24 h after inoculation, and expression profiles were evaluated using RT-qPCR. *P. pachyrhizi* elongation factor 1 α (*PpEF1 α*) and ubiquitin 5 (*PpUBQ5*) were used to normalize the samples. Expression profiles were visualized as a heatmap using Heatmapper (Babicki et al., 2016). Gene expression profiles of *P. pachyrhizi* chitin synthases, including *CHS2-1* **(B)**, *CHS2-2* **(C)**, *CHS2-3* **(D)**, *CHS3-1* **(E)**, *CHS3-2* **(F)**, *CHS3-3* **(G)**, *CHS4* **(H)**, *CHS5-1* **(I)**, and *CHS5-2* **(J)** on soybean leaves covered with or without 0.1% cellulose nanofiber (CNF). Vertical bars indicate the standard error of the means ($n = 4$). Significant differences ($p < 0.05$) are indicated by different letters based on a Tukey's honestly significant difference (HSD) test.



defective and the surface property was changed to hydrophilic. These results indicate that properties such as hydrophobicity are important to form *P. pachyrhizi* pre-infection structures during early infection stages. The importance of hydrophobicity and/or epicuticular waxes on the formation of germ-tubes and appressoria has also been reported for other fungal pathogens (Mendoza-Mendoza et al., 2009; Hansjakob et al., 2010; Weidenbach et al., 2014). Further characterization of the mechanisms by which fungal pathogens recognize plant surface properties and initiate infection behavior will be needed to develop effective and sustainable disease control methods.

We demonstrated that ASR was suppressed by CNF made from the ACC method (Figures 1A,B). Various preparation methods have been proposed including mechanical and chemical processes (Tsuji et al., 2021). CNF made from the ACC method has both hydrophobic and hydrophilic sites resulting in amphiphilic properties (Kondo et al., 2014). Halim et al. (2020) demonstrated that CNF made from the ACC method was more hydrophilic than that made from the chemical method based on the contact angle measurements. Therefore, it is necessary to investigate whether the same protective effect is observed not only for the CNF made from the ACC method but also for the CNF treatment made from other methods.

Cellulose nanofiber-treatments suppressed *P. pachyrhizi* *CHS5* expression related to chitin formation, which are associated with reduced formation of pre-infection structures (Figures 1F,G, 5). CHSs are important in cell wall formation in most filamentous fungi (Takeshita et al., 2005; Lenardon et al., 2010). Treitschke et al. (2010) reported that an *Ustilago maydis* *CHS5* mutant $\Delta msc1$ showed reduced virulence associated with abnormal hyphal morphology. Madrid et al. (2003) also demonstrated that *CHS5* in *Fusarium oxysporum*, a causal agent of tomato vascular wilt, has a crucial role in virulence and mediates the tomato protective response. A *F. oxysporum* *CHS5* mutant could not infect tomato, and exhibited abnormal morphologies such as hyphal swelling, due to changes in the cell wall properties (Madrid et al., 2003). These results suggest that *CHS5* gene deficiency or mutation causes morphological abnormalities in fungal cell

wall formation, leading to virulence suppression. Together, it is tempting to speculate that suppression of *P. pachyrhizi* *CHS5* in CNF-treated leaves may result in changes in the cell wall properties of *P. pachyrhizi* pre-infection structures. Further characterization of CHSs, especially *CHS5* based on dsRNA-mediated silencing such as SIGS and host-induced gene silencing (HIGS), in conjunction with analysis of *P. pachyrhizi* cell wall properties on CNF-treated leaves, will be necessary to understand CHSs molecular function during formation of pre-infection structures.

We demonstrated that CNF-treatments suppressed ASR caused by *P. pachyrhizi*, one of the most important soybean diseases (Figures 1A,B) associated with reduced formation of pre-infection structures (Figures 1F,G). Because numerous rust and filamentous fungal pathogens form pre-infection structures during early infection stages, these results imply that CNF might be an additional disease management tool to prevent crop diseases against these pathogens. However, we tested the ability of CNF to protect plants against an obligate biotrophic pathogen, but not other pathogen types, including hemibiotrophs and necrotrophs. Therefore, further characterization of CNF effects on disease suppression not only against fungal pathogens, but also against bacterial pathogens will be needed.

Our results demonstrated that SIGS targeting *P. pachyrhizi* *CHSs* functioned successfully in reducing pre-infection structures formation on hydrophobic polyethylene surfaces (Figures 3B,C and Supplementary Figure 3). SIGS is a technology that promotes silencing by spraying the target dsRNA on the plant surface. Therefore, it is possible to silence a specific phytopathogen gene and protect the plant without the need for plant gene recombination (Cagliari et al., 2019; Wytinck et al., 2020). Hu et al. (2020) demonstrated that SIGS targeting *P. pachyrhizi* genes encoding an acetyl-CoA acyltransferase, a 40S ribosomal protein S16, and glycine cleavage system H protein reduced pustule numbers over 70%. SIGS against filamentous fungi threatening agronomically important crops has also been studied, including head blight caused by *Fusarium graminearum* and gray mold caused by *Botrytis cinerea*

(Koch et al., 2016; Wang et al., 2016; Nerva et al., 2020). Koch et al. (2016) demonstrated that SIGS targeting *F. graminearum* cytochrome P450 genes, which are required for fungal ergosterol biosynthesis, successfully inhibited fungal growth in barley. Although further precise studies for SIGS targeting *P. pachyrhizi* virulence genes will be needed, SIGS is a powerful tool to develop sustainable disease management strategies.

Expression profiles of soybean leaves revealed that gene transcripts related to the phenylpropanoid and isoflavonoid pathways were upregulated within 6 h of *P. pachyrhizi* inoculation, and reached high levels at 12 h (Figure 4 and Supplementary Figure 4). Consistent with our results, previous studies reported that the expression of these genes was upregulated within 12 h after *P. pachyrhizi* inoculation (Schneider et al., 2011; Ishiga et al., 2015; Hossain et al., 2018). We demonstrated that the transcript levels of defense marker *PR* genes and defense-related genes were significantly less at 6 h in CNF-treated soybean leaves compared to control leaves (Figure 4 and Supplementary Figure 4). Further, appressoria formation was significantly reduced in CNF-treated leaves compared to controls (Figures 1E,G). Therefore, it is tempting to speculate that reduced transcripts of defense marker *PR* genes and defense-related genes in CNF-treated leaves is the result of the decreased penetration rate associated with reduced appressoria formation. However, since the transcripts of defense marker *PR* genes and defense-related genes were suppressed even though *P. pachyrhizi* infection was not completely prevented in CNF-treated soybean leaves, further investigation of CNF effects on plant defense responses will be necessary.

In summary, CNF-treatments confer resistance against *P. pachyrhizi*, a causal agent of ASR. Moreover, CNF-treatments can change leaf surface hydrophobicity, resulting in *CHSs* gene suppression related to *CHS*, which is associated with reduced formation of pre-infection structures including *P. pachyrhizi* germ-tubes and appressoria (Figure 6). Since CNF is an abundant and renewable biomass in nature, CNF application for plant

protection will provide a new avenue into eco-friendly and sustainable disease management.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

HS, NSa, and YI contributed to conception and design of the study and performed the statistical analysis. HS, YY, NSa, TI, NSh, GU, VN, EY, and YI performed the experiments. HS wrote the first draft of the manuscript. NSa, YY, and YI wrote sections of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fpls.2021.726565/full#supplementary-material>

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