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The enzymatic hydrolysate of fucoidan from *Sargassum hemiphyllum* triggers immunity in plants

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ABSTRACT

Fucoidans are polysaccharides that consist predominantly of sulfated L-fucoses, from which, fucoidan oligosaccharides (FOSs) are prepared through different methods. Fucoidan has versatile physiological activities, like antiviral functions against SARS CoV-2 and bioactivitiy in enhancing immune responses. Although fucoidan or FOS has been widely used in mammals as functional foods and new drugs, its application in plants is still very limited. Moreover, whether fucoidan or its derived hydrolytic products can trigger immune responses in plants remained unknown. In this work, we demonstrate that the fucoidan enzymatic hydrolysate (FEH) prepared from *Sargassum hemiphyllum* triggers various immune responses, such as ROS production, MAPK activation, gene expression reprogramming, callose deposition, stomatal closure, and plant resistance to the bacterial strain *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000. Notably, FEH did not induce Arabidopsis root growth inhibition at the concentration used for triggering other immune responses. Our work suggests that EHF can potentially be used as a non-microbial elicitor in agricultural practices to protect plants from pathogen infection.

1. Introduction

Fucoidans from brown marine algae or marine invertebrates are polysaccharides that consist predominantly of sulfated L-fucoses, from which, fucoidan oligosaccharides (FOSs) are prepared mainly through physical degradation, chemical hydrolysis, and enzymatic preparation (Mulloy et al., 1994; Daniel et al., 1999; Jin et al., 2020; Zhu et al., 2021). It has been shown that fucoidan or its derived FOS has antioxidant activity (Wang et al., 2011; Zhao et al., 2011), anti-cancer activity (Choi et al., 2005; Yang et al., 2013), anticoagulant activity (Pereira et al., 1999; He et al., 2020), and other bioactivities in mammals. Notably, fucoidan/FOS also exhibits antiviral functions against SARS CoV-2 (Jin et al., 2020; Salih et al., 2021; Dwivedi et al., 2021) as well as activities in enhancing immune responses (Cho et al., 2014; Guo et al., 2021). Owing to the low viscosity, high solubility, versatile physiological activities, and great bioavailability, fucoidan has attracted considerable attention for its application as functional foods and new drugs (Zhu et al., 2021).

Plants live in an environment with a plethora of microbes including the phytopathogens that are threats to agricultural practices for many crops and vegetables. To survive such biotic stresses, plants have developed a sophisticated innate immune system to defend against pathogen invasion. Microbe-associated molecular patterns (MAMPs) or pathogen-associated molecular patterns (PAMPs) trigger immune responses in plants, a process known as pattern-triggered immunity (PTI). PTI acts the first line of the inducible host defense against a broad spectrum of phytopathogens (Jones and Dangl, 2006; Ge et al., 2022). MAMPs or PAMPs are recognized by the cognate cell surface-localized pattern recognition receptors (PRRs) in plants (Ge et al., 2022). FLS2 is one of the best-studied PRRs that perceives the bactrerial flagellin or

Abbreviations: FEH, fucoidan enzymatic hydrolysate; FOS, fucoidan oligosaccharides; PTI, pattern-triggered immunity; MAMP, microbe-associated molecular pattern; PAMP, pathogen-associated molecular pattern; MAPK, mitogen-activated protein kinase; ROS, reactive oxygen species; Pst, Pseudomonas syringaepv.tomato.

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its derived epitopic peptide, flg22 (Gómez-Gómez and Boller, 2000). MAMPs or PAMPs trigger immune responses including activation of mitogen-activated protein kinases (MAPKs), reactive oxygen species (ROS) burst, stomatal closure, induction of immune-responsive genes, callose deposition to reinforce the cell wall, and immunity to a wide range of pathogens (Dodds and Rathjen, 2010; Tang et al., 2017; Wang and Gou, 2021).

In the process of interaction between plants and phytopathogens, an array of structural components of pathogen cell walls or cell periphery and intracellular components of pathogens, such as glucan, chitin, flagellin, lipopolysaccharides (LPS), the N-terminal N-acylated elongation factor Tu (EF-Tu), and bacterial DNA can act as PAMPs or elicitors to trigger immune responses in plants (Abdul Malik et al., 2020; Ranf, 2017). Interestingly, there were also non-microbial elicitors that could induce defense in plants (Abdul Malik et al., 2020). The non-microbial elicitors can be isolated from algae and crustaceous materials and are similar to those from microbes, such as the chitin and chitosan isolated from algae or crustaceous materials as well as the linear hepta-β-glucoside laminarin produced by brown algae (Klarzynski et al., 2000; Iriti and Varoni, 2015; Chaliha et al., 2018; Abdul Malik et al., 2020).

Although fucoidan is widely used in the animal field, its application in plants is still very limited. It was shown that fucoidan induced the local and systemic resistance against tobacco mosaic virus in tobacco (Klarzynski et al., 2003). However, whether fucoidan or its derived hydrolytic products can trigger immune responses in plants remained unknown. In this work, we demonstrate that the fucoidan enzymatic hydrolysate (FEH) prepared from *Sargassum hemiphyllum* induces an array of immune responses, such as MAPK activation, ROS burst, immune-responsive gene expression, callose deposition, stomatal closure, and plant immunity to the bacterial strain *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000. Interestingly, FEH did not induce Arabidopsis root growth inhibition at the concentration used for triggering other immune responses. Our work suggests that EHF can be used as a potential elicitor in agricultural practices to induce immune responses in plants.

2. Material and methods

2.1. Source of fucoidan and preparation of fucoidan enzymatic hydrolysate

Fucoidan was extracted from *Sargassum hemiphyllum*. First, ddH_2O was added to *S. hemiphyllum* with a ratio of about 50:1 (w/w), and then was heated up to 80 °C for up to 8 h. After centrifugation, the supernatant was kept and the recombinant fucoidanase was added for polysaccharide hydrolysis and to prepare fucoidan enzymatic hydrolysate (FEH). The reaction mixture was incubated with the temperature increasing from 20 °C to 50 °C progressively for 5 h and then was deproteinized by heating at 95 °C for 30 min. After cooling down, the precipitate was removed by centrifugation, then the membrane separation technology is applied to ultrafiltrate and purify FEH (Li et al., 2019). The freeze-dried powder was made from the FEH solution. Using these preparation approaches, the FEH powder contained about 16.0% fucose, 17.1% galactose, and 26.7% sulfate. The FEH was sterilized by autoclaving at 121 °C for the following physiological assays.

2.2. Plant material and growth conditions

For pathogen assays, Arabidopsis (*Arabidopsis thaliana,* the Col-0 ecotype) plants were grown in soil in a growth room, under 70 μE m^{-2} s^{-1} light (white fluorescent bulbs), with a 12-h photoperiod, and at 22 °C with 60% relative humidity for 4 weeks. For other assays, Arabidopsis seeds were surface sterilized [with 50% (v/v) bleach for 5 min] and washed four times with sterile water. Then the sterile seeds were germinated and grown on 1/2 MS plates supplemented with 0.5% (w/v) sucrose. The plates were put in the same growth room as above mentioned after they were kept for 2 d in the dark at 4 °C to break dormancy.

2.3. Reverse transcription (RT)-quantitative real-time polymerase chain reaction (qPCR)

Seven-day-old Arabidopsis seedlings were incubated in sterile water overnight in a 6-well plate; then the water was replaced with 400 μg/ml FEH solution, and the seedlings were treated with FEH for the indicated times. Total RNA was extracted from whole Arabidopsis seedlings using TRIzol (Invitrogen) following the manufacturer's instructions. cDNA was synthesized using 1 μg of DNase I-treated total RNA with a reverse transcription kit (Invitrogen). Then real-time PCR was performed using a SYBR Green PCR kit (Takara), on a Bio-Rad CFX-96 Real-Time PCR system. The expression levels of immune responsive genes were normalized to that of *GAPC* that was used as a reference gene.

2.4. MAPK assays

Seven-day-old Arabidopsis seedlings grown on plates with 1/2 MS medium were transferred into sterile water and were kept overnight in a 6-well plate; the water was replaced with 400 μg/ml FEH solution, and the seedlings were treated with FEH for the indicated times. Total plant proteins were isolated from whole seedlings and the MAPK activation was examined by immunoblotting with anti-pErk1/2 antibodies (1/5000 dilution, Cell Signaling Technology, USA). GAPDH was examined as a loading control with anti-GAPDH antibodies (1/3000 dilution, Proteintech Group, USA).

2.5. Callose deposition

Seven-day-old Arabidopsis seedlings were incubated in sterile water overnight in a 6-well plate. The water was replaced with 400 μg/mL FEH solution, and the seedlings were treated with FEH for 24 h. Callose deposition in Arabidopsis leaves was analyzed as previously described (Gómez-Gómez and Boller, 2000). Callose deposits were counted using the ImageJ 1.42q software (http://rsb.info.nih.gov/ij/).

2.6. Transcriptome sequencing

Total RNA for transcriptome sequencing was extracted as described above. Library preparation, sequencing, and data analysis were performed by Novogene and was described previously (Zou et al., 2018). Differential expression analysis of seedlings treated with 400 FEH and water for three biological replicates was performed using the DESeq R package (1.18.0). The differentially expressed genes were defined for those with $|\log_2 FC|>2$, and a significant false discovery rate (FDR) < 0.01.

2.7. Pathogen infection assay

The pathogen infection assay was performed as previously described (Liu et al., 2022). The *Pseudomonas syringae* pv. *tomato* (*Pst*) strain DC3000 was cultured overnight at 28 °C in liquid King's B (KB) medium supplemented with 50 μg mL^{-1} rifampicin. True leaves of 5-week-old plants were infiltrated with 400 μg/mL FEH or 1.5 μM flg22 for 24 h using a needless syringe, then the leaves were infiltrated with 5×10^8 colony forming unit (cfu) mL^{-1} bacterial solution also using a needleless syringe. Two days after infiltration, leaf discs were ground and 10-fold serial dilutions of the bacteria solution were prepared. Bacteria were cultured on KB plates with 50 μg mL^{-1} rifampicin for 3 days, and bacterial CFUs were counted.

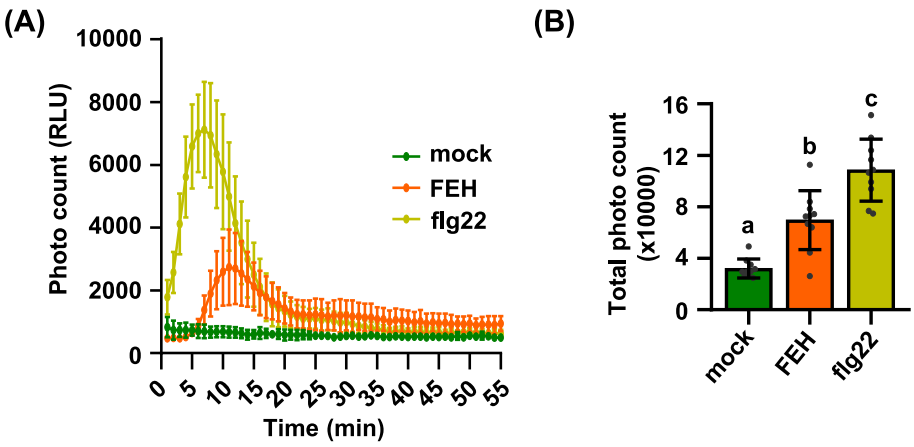

Fig. 1. FEH induces ROS burst in Arabidopsis. (A) Leaf discs from 4-week-old plants were treated with 400 μg/mL FEH or 200 nM flg22, and RLUs (relative light units, representing the relative amounts of H_2O_2) were immediately measured. Data are shown as means ± SD (n = 10, n means leaf disc number). (B) The total ROS production within 55 min after the FEH or flg22 treatment. Values are mean RLUs ± SD (n = 10 leaf discs). Different letters denote significance difference (one-way ANOVA, $P < 0.05$).

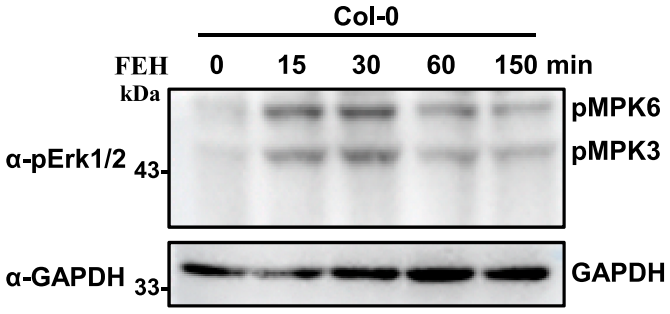

Fig. 2. FEH induces MPK3/6 activation in Arabidopsis.
Seven-day-old seedlings were treated with 400 μg/mL of FEH for 0, 15, 30, 60, and 150 min. MAPK activation was detected by immunoblotting with anti-pErk1/2 antibodies. GAPDH was detected by immunoblotting as the loading control.

2.8. ROS burst assay

ROS production was measured as described previously (Liu et al., 2022). The third or fourth pair of true leaves from 4-week-old Arabidopsis plants grown on soil were punched into leaf discs (4 mm in diameter). Then the leaf discs were incubated in sterile water overnight, and were then transferred in ROS measurement solution [50 μM luminol (Sigma), 10 μg mL^{-1} horseradish peroxidase (Sigma), and 400 μg/mL FEH]. Luminescence was captured using a GLOMAX 96 microplate luminometer (Promega). The ROS production values are represented by Relative Light Units (RLU).

2.9. Stomatal aperture measurement

Stomatal aperture was measured as described previously (Liu et al., 2022). Five-week-old plants were kept under the light for 2 h to make sure that most of the stomata were opened. The leaves were incubated in 400 μg/mL FEH solution for 1 h and the stomata were observed under a light microscope (Leica). The stomatal aperture was measured using Image J software (NIH, Bethesda, MD, USA).

3. Results and discussion

3.1. FEH induces ROS burst, MAPK activation, and the expression of immune responsive genes

ROS production and MAPK activation are early events in immune signaling (Asai et al., 2002; Tang et al., 2017). We found that ROS production was elicited after 5 min of FEH treatment and increased until 11 min, and then it gradually declined (Fig. 1A). Overall, the ROS burst triggered by FEH was weaker and slower than that by flg22 (Fig. 1A and B).

Meanwhile, MAPK3/6 activation in Arabidopsis was induced after 15 min of FEH treatment. Moreover, MAPK3/6 activation lasted until 150 min of FEH treatment (Fig. 2). On the other hand, the expression of several immune-responsive genes *NHL10*, *OXI1*, and *WRKY30* was induced after 30 min of FEH treatment, and then it was reduced gradually, as analyzed by RT-qPCR (Fig. 3). These results suggest that FEH treatment can induce the early immune responses.

3.2. FEH induces stomatal closure in Arabidopsis

Upon immune activation, stomata are closed to prevent the entry of

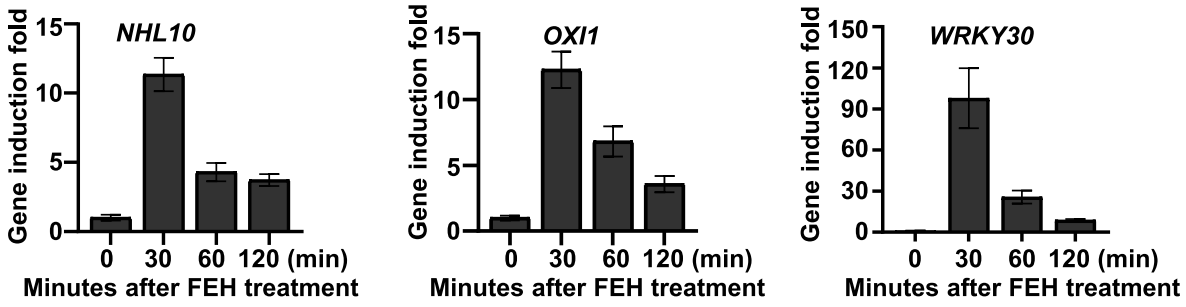

Fig. 3. FEH induces the expression of the immune-responsive genes in Arabidopsis.
Seven-day-old Arabidopsis seedlings were treated with 400 μg/mL FEH for 0, 0.5, 1, and 2 h. The expression of immune-responsive genes *NHL10*, *OXI1*, and *WRKY30* was analyzed by RT-qPCR, and transcript levels were normalized to that of *GAPC*. Values are means ± SD of three technical repeats. The experiments were repeated three times with similar results.

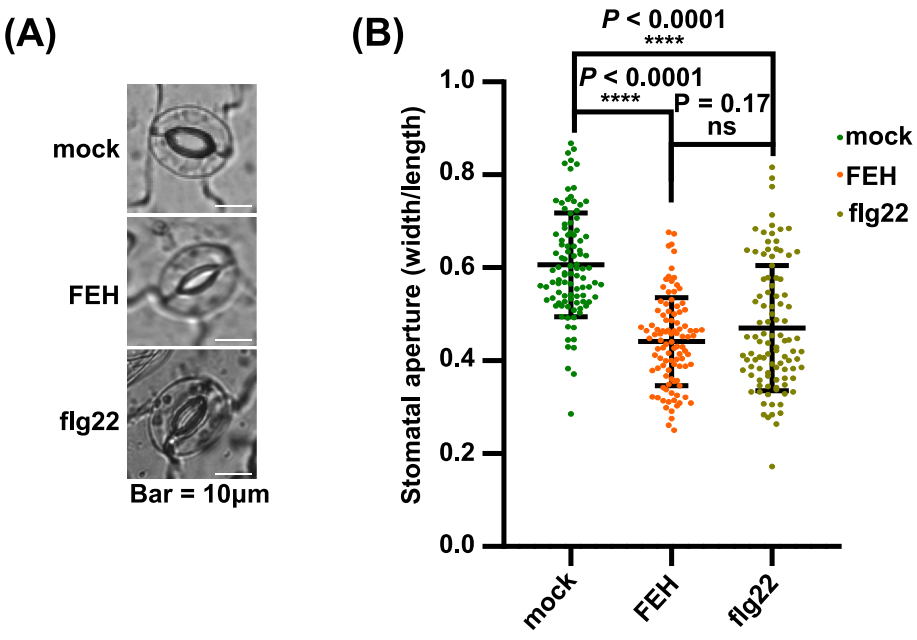

Fig. 4. FEH induces stomatal closure in Arabidopsis. Leaves from 5-week-old Arabidopsis were treated with 400 μg/mL FEH or 10 μM flg22 for 1 h. (A) The images of stomata upon FEH or flg22 treatment, Bar = 10 μm; (B) FEH induces stomatal closure. The stomatal aperture was measured using ImageJ software. Each data point represents a single stoma, values are means ± SD for n = 99 stomata from three biological repeats. Statistical significance compared with the control was determined by one-way ANOVA with multiple comparisons: ****P < 0.0001, ns: not significant.

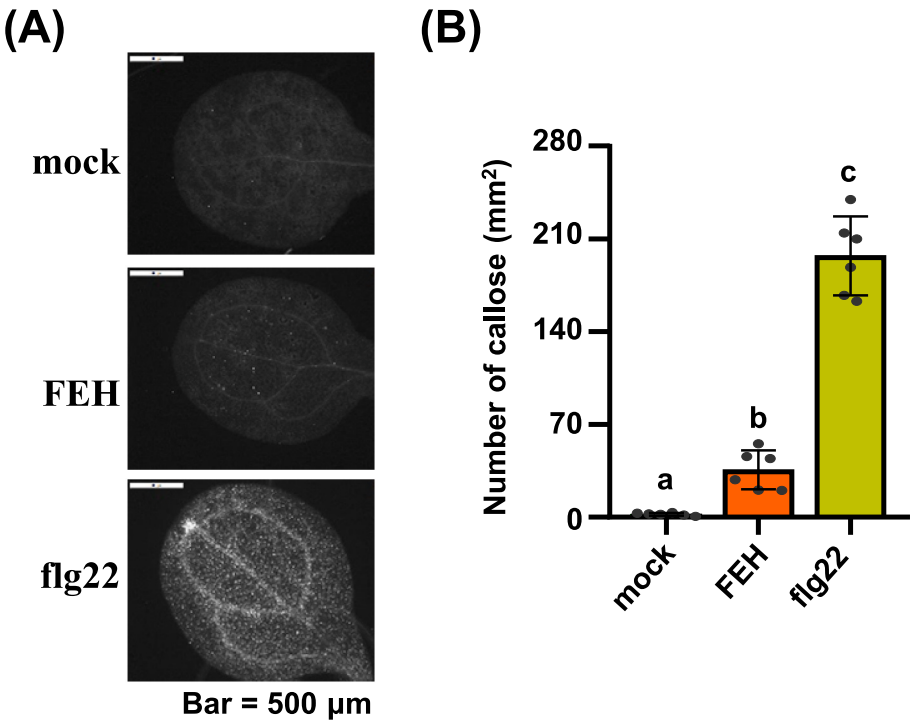

Fig. 5. FEH induces callose deposition in Arabidopsis. Seven-day-old Col-0 seedlings were treated with 400 μg/mL FEH or 2 μM flg22 for 24 h. (A) The images of cllose deposited, Bar = 500 μm. (B) Quantification of FEH-induced callose deposition. Data are shown as means ± SD (n = 6 leaves from three biological replicates). Different letters denote significance difference (one-way ANOVA, P < 0.05).

bacterial pathogens, meanwhile the callose is deposited to reinforce the cell wall for blocking pathogen infection (Melotto et al., 2006; Zou et al., 2018). We found that, just like the flg22 treatment, FEH also induced stomatal closure (Fig. 4A and B). Moreover, callose deposition was also induced by the FEH treatment, although to a less extent than that induced by flg22 treatment (Fig. 5A and B).

3.3. FEH induces resistance to the bacterial pathogen in Arabidopsis

It is known that flg22 induces plant resistance to the bacterial pathogen *Pseudomonas syringae* pv. *tomato* (*Pst*) DC3000 (Zipfel et al., 2004). Consistently, we also found that the flg22-pretreated Arabidopsis leaves were more resistant than the H_2O-pretreated plants as measured by bacterial multiplication at 2 days post infiltration. Similarly, the pretreatment with FEH also resulted in decreased bacterial growth in Arabidopsis leaves (Fig. 6A). To exclude the possibility that FEH may directly affect the bacterial multiplication, we monitored the growth of *Pst* DC3000 on the solid medium containing 400μg/mL FEH. The results showed that bacterial colonies on FEH medium were comparable to those in the control (Fig. 6B). These results suggest that FEH induces plant immunity to pathogens.

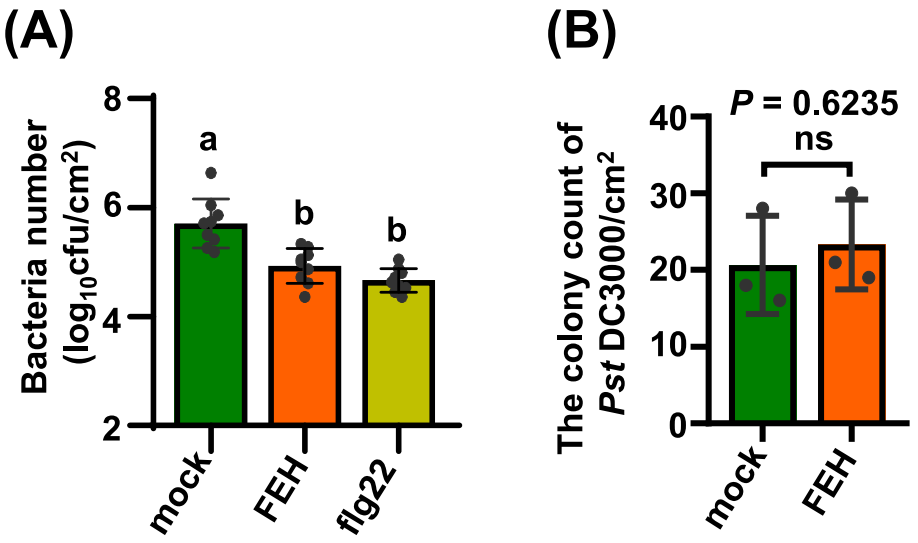

Fig. 6. FEH induces immunity to bacterial pathogens in Arabidopsis. (A) FEH induces immunity to *Pst* DC3000 in Arabidopsis. Five-week-old Col-0 leaves were pretreated with 400 μg/mL FEH or 1.5 μM flg22 for 24 h, and then were infiltrated with *Pst.* DC3000. Two days after inoculation, the bacterial growth was evaluated as colony-forming units per cm^2 of leaf area (cfu/cm^2). Data are shown as means ± SD (n = 9 leaves from three biological replicates). Different letters denote significance difference (one-way ANOVA, $P < 0.05$). (B) FEH does not directly affect the growth of *pst* DC3000. *pst* DC3000 was cultured on KB medium with or without 400 μg/mL FEH, the bacterial colonies were counted. Values means ± SD of three biological replicates. Statistical significance compared with the control was determined by student's *t*-test, ns: not significant.

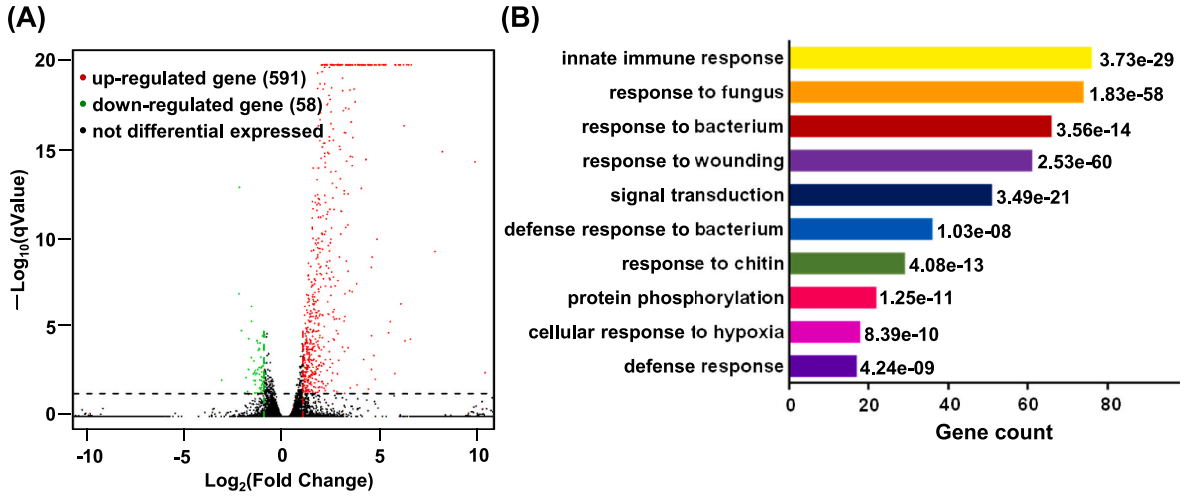

Fig. 7. Analysis of differentially expressed genes after FEH treatment. (A) Volcano plot of differentially expressed genes after FEH treatment. Seven-day-old Arabidopsis seedlings were treated with 400 μg/mL FEH for 30 min. RNA was isolated and RNA-seq analysis was performed. The differentially expressed genes relative to the control were defined for those with $|\log_2 FC|>2$, and a significant false discovery rate (FDR) < 0.01. The FPKM value for each dot was the mean of three biological replicates. Red indicates up-regulated transcripts, and green down-regulated transcripts. (B) GO analysis of genes upregulated by 400 μg/mL FEH. The top 10 biology process (BP) terms were shown. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.4. *Transcriptomic analyses of genes differentially expressed after FEH treatment*

To further investigate the activity of FEH in triggering immune responses in plants, we performed RNA sequencing (RNA-seq) analysis on Arabidopsis seedlings (Col-0 ecotype) exposed to 400 μg/mL FEH for 30 min. The FEH treatment resulted in 651 up- and 102 down-regulated genes ($|\log_2 FC|>2$, FDR<0.01) (Fig. 7A). Moreover, the results of transcriptome analysis were further confirmed by RT-qPCR assays (Supplemental Figs. 1A and B). The results of gene ontology (GO) analysis demonstrate that the up-regulated genes by FEH are involved in response to chitin/bacteria/fungus/wounding, defense response, innate immune response, protein phosphorylation, and signal transduction (Fig. 7B). These results suggest that FEH treatment induces plant innate immunity, and the FEH-induced gene expression reprogramming is likely shared by plants under other biotic stresses as well.

3.5. *The inhibition of Arabidopsis root growth by FEH*

PTI is always accompanied with plant growth inhibition as evident by root growth retardation (Gómez-Gómez and Boller, 2000). To investigate whether FEH treatment also induces plant growth inhibition, we examined the root length of Arabidopsis seedlings grown on the 1/2 MS medium containing different concentrations of FEH for 10 days. The results showed that the growth of Arabidopsis, as monitored by root length, was almost not inhibited at low concentrations of FEH (200 μg/mL) and slightly inhibited by FEH at the concentration of 600 μg/mL (Fig. 8A–D). Moreover, when high concentrations of FEH (higher than 1000 μg/mL) was applied, the root growth was inhibited by FEH in a dose-dependent manner (Supplemental Figs. 2A and B). These results suggest that low concentrations of FEH can be used as a potential elicitor in agricultural practices to induce immune responses in plants but without too much growth penalty.

We investigated the activities of the enzymatic hydrolysate of *S. hemiphyllum* fucoidan in triggering immune responses in Arabidopsis. Our work demonstrate that FEH induces immunity to bacterial pathogen *pst* DC3000. FEH is derived from natural resources and is environment-friendly, therefore FEH can potentially be used as a non-microbial, pollution-free, and green elicitor in agricultural practices. Identification of FEH receptor and signaling components in plants will be pursued

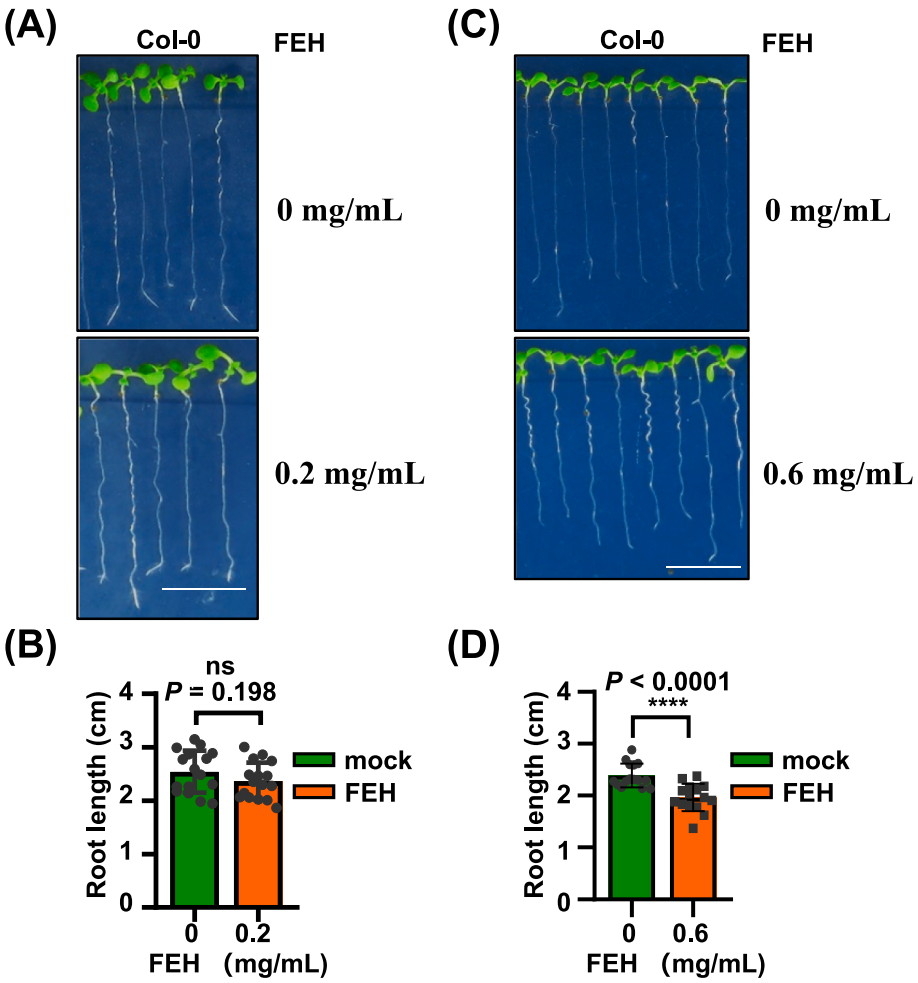

Fig. 8. Low concentrations of FEH does not inhibit Arabidopsis root growth. (A) and (C) Growth of the primary roots of 7-day-old Col-0 seedlings. Arabidopsis seedlings were grown on 1/2 MS medium containing 200 μg/mL or 600 μg/mL FEH for 10 days. Bar = 1 cm. (B) and (D) Quantification of root length in (A) and (C). The date shown are means ± SD. Statistical significance compared with the control was determined by student's *t*-test, ****$P < 0.0001$, ns: non significant.

in the future.

4. Conclusion

We found that the enzymatic hydrolysate of *S. hemiphyllum* fucoidan triggered early immune responses including ROS burst, MAPK activation, and the expression of immune responsive genes. Moreover, RNA-seq analaysis demonstrate that the up-regulated genes by FEH are involved in response to chitin/bacteria/fungus/wounding, defense response, and innate immune response. Furthermore, FEH also induces stomatal closure, callose deposition, and resistance to the bacterial pathogen *Pst* DC3000. Taken together, our work indicates that the enzymatic hydrolysate of fucoidan from *S. hemiphyllum* triggers immunity in plants.

CRediT authorship contribution statement

Ranran Wang: Formal analysis, Performing most immune response analysis. **Jiaojiao Bai:** Formal analysis, writing the manuscript. **Guofu Yan:** Preparation of fucoidan enzymatic hydrolysate. **Zejun Xiao:** Formal analysis, Performing RT-qPCR for analyzing immune gene expression. **Kexin Chen:** Formal analysis, Performing RT-qPCR for analyzing immune gene expression. **Kaikai Li:** FEH purification. **Jie Tang:** Conceiving the project and investigation. **Dongping Lu:** Conceiving the project and writing the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

We thank Science and Spectrum R&D Center in Qingdao, China and Shanghai Shidande Biotechnology Co., Ltd for chemical analysis of the enzymatic hydrolysate of fucoidan used in this work.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jplph.2023.153967.

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