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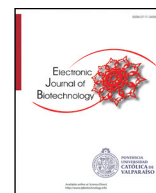
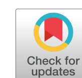


Research article

Fructosan form *Paenibacillus kribbensis* PS04 enhance disease resistance against *Rhizoctonia solani* and tobacco mosaic virus



Shu Canwei [1], Hu Xiaoyun [1], Nauman Ahmed, Wang Shiqi, Zhou Erxun, Liao Meide *

Integrative Microbiology Research Centre/National Engineering Research Center of Plant Space Breeding, College of Agriculture/Guangdong Province Key Laboratory of Microbial Signals and Disease Control/College of Agriculture, South China Agricultural University, Guangzhou, Guangdong 510642, China

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ABSTRACT

Background: Rice sheath blight (caused by *Rhizoctonia solani*) and tobacco mosaic virus are very important plant diseases, causing a huge loss in global crop production. *Paenibacillus kribbensis* PS04 is a broad-spectrum biocontrol agent, used for controlling these diseases. Previously, extracellular polysaccharides (EPS) from *P. kribbensis* PS04 had been purified and their structure was inferred to be fructosan. This study aimed to evaluate the effects of exogenous EPS treatment on plant–pathogen interactions.

Results: Plant defense genes such as phenylalanine ammonia-lyase, catalase, chitinase, allene oxide synthase, and PR1a proteins were significantly induced by exogenous EPS treatment. Moreover, subsequent challenge of EPS-pretreated plants with the pathogens (*R. solani* or tobacco mosaic virus) resulted in higher expression of defense-associated genes. Increased activities of defense-associated enzymes, total phenols, and flavonoids were also observed in EPS pretreated plants. The contents of malondialdehyde in plants, which act as indicator of lipid peroxidation, were reduced by EPS treatment.

Conclusions: This study comprehensively showed that EPS produced from *P. kribbensis* PS04 enhances disease resistance in plants by the activation of defense-associated genes as well as through the enhancement of activities of defense-related enzymes.

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1. Introduction

Plant diseases caused by plant pathogenic organisms result in about a 20% annual loss in global agricultural production [1]. Chemical microbicides are mainly used to control plant diseases, which cause serious environmental pollution and drug-resistance generation of pathogens and have very harmful effects on non-target organisms. In this scenario, biological control is a promising strategy to manage plant diseases. Biocontrol of plant diseases is the management of diseases using beneficial organisms or their metabolites as disease-controlling agents [2,3]. Biological control mechanisms include competition for nutrients and space, production of antibiotics and bacteriocins, detoxification and degradation of pathogen-produced toxins, and regulation of plant ethylene levels through the ACC deaminase enzyme and by the induction of systemic resistance in plants [2,4,5,6,7,8].

Plant-induced resistance is divided into induced systemic resistance (ISR) and systemic acquired resistance (SAR). ISR develops as a result of colonization of plant roots by biological control agents and is mediated by jasmonic acid or ethylene-sensitive (JA/ET) pathways. SAR develops locally or systemically in response to inducers and is mediated by salicylic acid (SA)-dependent pathways [9]. Plants can be induced to develop resistance against pathogens infection by treatment with a number of biotic inducers, such as fungi, bacteria, and viruses, as well as their metabolites, extracellular polysaccharides, glycoproteins, cell wall fragments, and toxins [10]. Extracellular polysaccharides (EPS) are high-molecular-weight polymers that are composed of sugar residues secreted by microorganisms, which can be divided into homo-polysaccharides, containing a single monosaccharide unit, and hetero-polysaccharides, having regular repeat units that are formed from two to eight monosaccharides [11,12,13,14]. Recently, progress has been made in study of the molecular structures and chemical compositions of EPS that possess biocontrol properties. The *Bacillus cereus* EPS, mainly composed of mannose, were purified and identified as a new microbe-

* Corresponding author.
E-mail address: liaomeide@scau.edu.cn (L. Meide).
[1] These authors contributed equally to this work.
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associated molecular pattern that can induce systemic resistance in *Arabidopsis* against *Pseudomonas syringae* pv. tomato DC3000. After treatment with *B. cereus* EPS in *Arabidopsis*, defense-associated enzymes, defense-related genes (*PR1*, *PR2*, and *PR5*), and mitogen-activated kinases cascade marker genes (*MPK6*) were significantly induced [14]. Little information has, however, been published about the structure of EPS of the biocontrol agent *Paenibacillus* spp. The genus *Paenibacillus* is characterized as rod-shaped gram-positive or gram-variable endospore-forming aerobic or facultatively anaerobic bacteria, which were originally included within the genus *Bacillus* and then reclassified as a separate genus in 1993 [15]. Many *Paenibacillus* isolates, such as *P. polymyxa*, *P. alvei*, *P. elgii*, and *P. lentimorbus*, have been obtained from a variety of environments. To date, many Paenibacillus isolates have been reported for their biocontrol potential against several plant pathogens [9,10,16,17].

Paenibacillus spp. produced a wide variety of different EPS with diverse physiological and biotechnological functions. In the previous study, we identified a *P. kribbensis* PS04 strain which showed high biocontrol effect against *R. solani* [18,19], and field application indicated that PS04 had a preventive effect on tobacco mosaic virus disease (data not shown). The EPS were purified from PS04 and identified as a single homogeneous component. The structure of EPS was inferred to be fructosan containing both β-(1,2) and β-(2,6) linkages. The degree of polymerization of EPS was seven by calculating the fructose/glucose ratio [20]. To the best of our knowledge, fructosans are the novel EPS in *P. kribbensis*. Therefore, here we raised a question: Can the purified fructosan EPS produced by PS04 induce plant resistance to pathogens? Interestingly, burdock fructooligosaccharide, a natural fructosan from *Arctium lappa*, was reported to induce plant resistance against tobacco mosaic virus in tobacco by inducing the levels of transcription of pathogenesis-related (PR) protein genes locally as well as systemically [21]. Sun et al. [22] reported that burdock fructooligosaccharide could also activate the SA-dependent pathway and increase the resistance of Kyoho grapes to *Botrytis cinerea*. Recently, Tarkowski et al. [23], by spraying inulin on mature lettuce leaves, showed that inulin-type fructans derived from burdock or chicory could reduce gray mold disease symptoms caused by *Botrytis cinereal*. Their study also indicated that the ethylene signaling pathway is needed for the enhanced defense response. These evidences indicate that fructosan plays a positive role in plant defense against plant pathogenic fungi and virus. Hence, this study is carried out to determine the effect of exogenous EPS from PS04 on the basal resistance of rice to *Rhizoctonia solani* and on the basal resistance of tobacco plants to tobacco mosaic virus (TMV). The expression profiles of several resistance-associated genes and the physiological and biochemical indexes were analyzed.

2. Material and methods

2.1. Strains

P. kribbensis PS04 was preserved in CGMCC (China General Microbiological Culture Collection Center) with number 7996. *R. solani* AG1-IA GD118 strain, the causal agent of rice sheath blight and TMV, was stocked in our laboratory.

2.2. Polysaccharide preparation

The purification of EPS from *P. kribbensis* PS04 cells was carried out by reference to the method of Prakasha et al. [24] with some modifications. Briefly, 10 mL of *P. kribbensis* PS04 was inoculated into 100 mL cultural media (30 g sucrose, 3 g $NaNO_3$, 1 g $KH_2PO_4 \cdot 3H_2O$, 0.5 g KCl, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.01 g $FeSO_4 \cdot 7H_2O$, and 1 L ddH_2O) in a 500 mL flask and incubated at 37°C and 150 rpm for 48 h. The fermented liquid of *P. kribbensis* PS04 was heated at 100°C for 30 min and diluted with isopyknic ddH_2O and then placed in an ice bath for 30 min. The supernatant was obtained by centrifugation at 4°C and 8000 rpm for 15 min and precipitated with triple volume of pure ethanol for at least 6 h. The sediment was redissolved in ddH_2O and mixed with 1/5 volume of chloroform:butanol (5:1, v/v) by shaking vigorously. The mixture was placed in a stationary position, and the solvent layer including the protein was removed. The remaining water layer was extracted as described above until no protein existed in the interface between the solvent layer and the water layer and dialyzed (for example 5 L) for 24–48 h. Finally, pure EPS was obtained by freeze-drying the aqueous solution. Inulin, a kind of commercial fructosan derived from chicory (*Cichorium intybus*), was purchased from Guangzhou Zeyu Biotechnology Co., Ltd. and used as a control.

2.3. Plant materials and treatments

Ten pre-germinated seeds of rice cultivar 9311 were sown in a pot (80 cm × 40 cm × 15 cm) with sterilized nutrition soil for 3–4 weeks. The following treatments were included in the experiment: (CK) rice seedlings were sprayed with 50 mL ddH_2O; (T1) rice seedlings were sprayed with 50 mL 1.5 g/L EPS; (T2) rice seedlings were inoculated with *R. solani*; (T3) rice seedlings were sprayed with 50 mL 1.5 g/L EPS and then inoculated with *R. solani* two days later; (T4) rice seedlings were sprayed 50 mL 1.5 g/L inulin. The inoculation of *R. solani* was carried out by attaching two mycelia agar plugs to each individual sheath above the soil. Inoculated plants were placed in plastic containers and covered with a lid in a greenhouse at 28–30°C. Water was sprayed to maintain humidity higher than 90% to facilitate high disease incidence.

Tobacco seeds of *Nicotiana tabacum* cultivar K326 were sown in a pot (30 cm × 60 cm × 5 cm) and grown till 3–4 leaf stage under a light–dark cycle (16 h of light at 26°C, 8 h of dark at 18°C) with 50% humidity. After that, tobacco seedlings were transferred to another pot (8 cm × 8 cm × 4 cm) and grown to the nine-leaf stage. The following treatments were included in the experiment: (CK) tobacco seedlings were sprayed with 50 mL ddH_2O; (T1) tobacco seedlings were sprayed with 50 mL 1.5 g/L EPS; (T2) tobacco seedlings were inoculated with TMV; (T3) tobacco seedlings were sprayed with 50 mL 1.5 g/L EPS and then inoculated with TMV two days later; (T4) tobacco seedlings were sprayed 50 mL 1.5 g/L inulin. The inoculation method of TMV was as follows: 1 g of leaf tissue was macerated in 10 mL of 50 mM PBS buffer (pH 7.0) supplemented with 10 mM sodium sulfite on ice. The suspension was squeezed through two layers of muslin cloth, and the sap obtained was mechanically rubbed onto carborundum-dusted leaves tobacco seedlings. Three biological replicates were made for each treatment, and three technical replicates per biological replicate were made.

2.4. Real-time quantitative PCR (RT-qPCR)

The plant samples described above were harvested from each treatment at 0, 12, 24, 48, 72, 120, and 168 h post-inoculation (hpi). Total RNA was extracted by RNAiso Plus (TAKARA, Dalian, China) according to the manufacturer's instruction. A quantity of 1.0 μg of total RNA was used for reverse transcription using a PrimeScript™ RT reagent Kit with gDNA Eraser (TAKARA, Dalian, China) according to the manufacturer's instruction. RT-qPCR was performed using the Bio-Rad CFX96TM Real-Time PCR detection system (Bio-Rad, California, USA) and SYBR Premix Ex *Taq* II (Tli RNaseH Plus) (TAKARA, Dalian, China). The primers were listed in Table 1 and Table 2. The program contains a Hot-Start activation step at 95°C for 30 s, followed by 40 cycles of 95°C for 15 s, melting temperature (Tm) for 30 s, and 72°C for 30 s. The *Actin* gene was used as the reference gene respectively. Relative expression levels were analyzed by $2^{-\Delta\Delta Ct}$ method. Three biological and technical replicates were used for each time-point.

Table 1
Primers used for RT-qPCR in rice.

Gene	GenBank No.	Primers	Product size
PAL	EF576408	5′-TTCCCGCTCTACCGCTTCGT-3′ 5′-GCTCGCCGTTCCACTCCTTG-3′	163 bp
CatA	EF371902	5′-CGTCATCGTCCGCTTCTCCACCGTC-3′ 5′-AAGTTTGTTGCCGAGGAGGTCCCAGT-3′	126 bp
Rcht	AB016497	5′-AGATAAACAAGGCGACTTCTCCAC-3′ 5′-CGCCGTCATCCAGAACCAG-3′	179 bp
PR1a	AJ278436	5′- CGTCTTCATCACCTGCAACTACTC -3′ 5′- CATGCATAAACACGTAGCATAGCA − 3′	132 bp
AOS	AY062258	5′- CAATACGTGTACTGGTCGAATGG − 3′ 5′- AAGGTGTCGTACCGGAGGAA − 3′	134 bp
Actin	AB047313	5′- GAGCGGGAAATTGTGAGGGA-3′ 5′- GGAAACGCTCAGCACCAATG-3′	154 bp

2.5. Defense-associated enzymes activity assays

The plant samples described above were collected from each treatment at 0, 1, 3, 5, 7, and 9 d post inoculation (dpi). A quantity of 0.5 g fresh sample was added to 2 mL of 50 mM phosphate buffer (pH 5.5), ground into homogenate in an ice bath, and centrifuged at 12,000 *g* for 20 min at 4°C. The supernatant, referred to as the crude enzyme extract, was used to assay the activity of enzymes.

For Peroxidase (POD) activity determination, the reaction mixture was made by adding 168 μL guaiacol to 300 mL 0.1 M phosphate buffer, followed by heating until guaiacol was dissolved. After the mixture was cooled, it was added with 114 μL 30% H_2O_2 and placed in a fridge. 0.1 mL crude enzyme extract was mixed with 5.9 mL reaction buffer and the absorbance of mixture was measured at 470 nm. One unit of POD was defined as a 0.01 decrease of OD 470/min/g.

Catalase (CAT) activity was determined by monitoring the decomposition of H_2O_2 at 240 nm. One mL crude enzyme was mixed with 2 mL 50 mM Tris–HCl (pH 7.0) and 3 mL dd H_2O and incubated at 25°C for 5 min. The mixture was added with 200 μL 200 mM H_2O_2 and the absorbance was measured at 240 nm. One unit of CAT was defined as a 0.1 decrease of OD 240/min/g.

Superoxide dismutase (SOD) activity was assayed by measuring its ability to inhibit the photochemical reduction of nitro blue tetrazolium (NBT). The reaction mixture contained 0.6 mL 130 mM methionine, 0.6 mL 375 μM NBT, 0.6 mL 100 μM EDTA, 0.6 mL 10 μM riboflavin, and 1.0 mL crude enzyme extract. The reaction was exposed to a 4000 Lx white light incubator for 15–20 min at 25°C and measured at 560 nm. One unit of SOD activity was defined as the amount of enzyme per fresh mass sample causing 50% inhibition of the photochemical reduction of NBT.

To measure the content of malondialdehyde, 0.2 g sample was homogenized in 2 mL of 10% trichloroacetic acid and centrifuged at 4°C and 12,000 *g* for 15 min. A measure of 2 mL supernatant was mixed with 2 mL of 0.60% (w/v) thiobarbituric acid (TBA). The mixture was incubated in boiling water for 15 min and was stopped at 0°C immediately. The supernatant was used to measure the absorbance at 450, 532, and 600 nm. The MDA concentration was calculated according to the formula:

$$\mathrm{MDA}(\mu\mathrm{mol/g \cdot Fw}) = [6.452 \times (\mathrm{OD532}-\mathrm{OD600})-0.56 \times \mathrm{OD450}] \times \mathrm{Vt})\ /\mathrm{Fw}$$

where Vt was the total volume of the extract solution and Fw was the fresh weight of the sample.

For total phenols and flavonoids determination, 0.2 g fresh plant sample was homogenized in 5 mL pre-cold 1% hydrochloric acid-methanol solution and extracted at 4°C for 4 h. The mixture was centrifuged at 4°C, 12,000 *g* for 15 min, and the supernatants were used to measure the absorbance at 280 nm and 325 nm, respectively. The total phenols and flavonoids concentration were calculated as $OD_{280}\ g^{-1}$Fw and $OD_{325}\ g^{-1}$Fw, respectively, where Fw was the fresh weight of the sample.

2.6. Statistical analysis

Each value is the resultant mean of three replicates. The vertical bars represent standard errors of the mean. Statistical significances of the means were determined by Duncan's multiple range test (DMRT), with significance at $p < 0.05$, using SPSS 13.0 software.

3. Results

3.1. Effects of EPS on the expression of resistance-related genes in rice plant

The time frames of relative gene expression of *PAL*, *CatA*, *Rcht*, *AOS*, and *PR1a* were shown in Fig. 1. These genes encode for phenylalanine ammonia-lyase, catalase, chitinase, allene oxide synthase, and PR1a protein, respectively. EPS treatment (T1) had shown a rapid increase in expression of *PAL* which peaked at 12 hpi and then gradually decreased until 120 hpi. While only RS treatment (T2) had gradually upregulated transcript level which was maximized at 48 hpi. A rapidly increased and peaked expression level of *PAL* was observed at 24 hpi by EPS + RS treatment (T3) (Fig. 1 A).

A gradually upregulated *CatA* gene expression was observed by EPS treatment (T1) until 24 hpi and then decreased gradually till 120 hpi. In RS treatment (T2), the expression levels of *CAT* gradually increased until 48 hpi and then decreased after that. EPS + RS treatment (T3) had shown a significantly upregulated expression level at 24 hpi which was the highest expression level observed of *CatA*. After 24 hpi, however, its expression level gradually decreased (Fig. 1B).

The expression level of *Rcht* gene was upregulated until 24 hpi and then decreased until 168 hpi by EPS treatment (T1). It was observed that *Rcht* gene was sharply upregulated by EPS + RS treatment (T3) and peaked at 48 hpi with the highest expression level of 4.72-fold than that of the CK. By RS treatment (T2), however, transcript level reached its maximum level at 120 hpi with 4.21-fold change relative to the CK (Fig. 1C).

Rice seedlings treated with EPS (T1) had shown a gradual and significant upregulated expression level of *AOS* mRNA at 48 hpi which was 18.33 times higher than that of CK. The expression of *AOS* gene by EPS treatment (T1), however, was then sharply decreased after 72 hpi until 120 hpi. RS (T2) and EPS + RS treatment (T3) had also significantly upregulated expression levels on their peaks at 72 hpi with a maximal intensity of approximately 22.20-fold and 26.08-fold, respectively, as compared to CK plants (Fig. 1D).

A slight increase in expression level of *PR1a* gene was observed at 12–24 hpi by only EPS treatment (T1). By only RS treatment (T2), the expression level of *PR1a* gene showed the highest at 48 hpi and then decreased later. Interestingly, *PR1a* gene significantly upregulated at 12 hpi and attained the highest level at 24 hpi as a result of EPS + RS treatment (T3). Moreover, this was also three times higher than

Table 2
Primers used for RT-qPCR in tobacco.

Gene	GenBank No.	Primers	Product size
N	EF091690	5′-CCATCGTACGCTTGAACAAA-3′ 5′-TTGCAACAGGTGAGATCCAG-3′	127 bp
PR1a	X12737	5′-TGGATGCCCATAACACAGC-3′ 5′-AATCGCCACTTCCCTCAG-3′	168 bp
PR1b	X66942	5′-GATGTGGGTTGATGAGAAGC-3′ 5′-CTCCAATTACCAGGTGGATC-3′	183 bp
NPR1	AF480488	5′-ACATCAGCGGAAGCAGTAG-3′ 5′-GTCGGCGAAGTAGTCAAAC-3′	161 bp
MAPK	AF325168	5′-TCACAACGGTGAAATCCAAG-3′ 5′-TCGGGTTAGATCCGAAAGAG-3′	100 bp
Actin	U60495	5′-ATGCCTATGTGGGTGACGAAG-3′ 5′-TCTGTTGGCCTTAGGGTTGAG-3′	197 bp

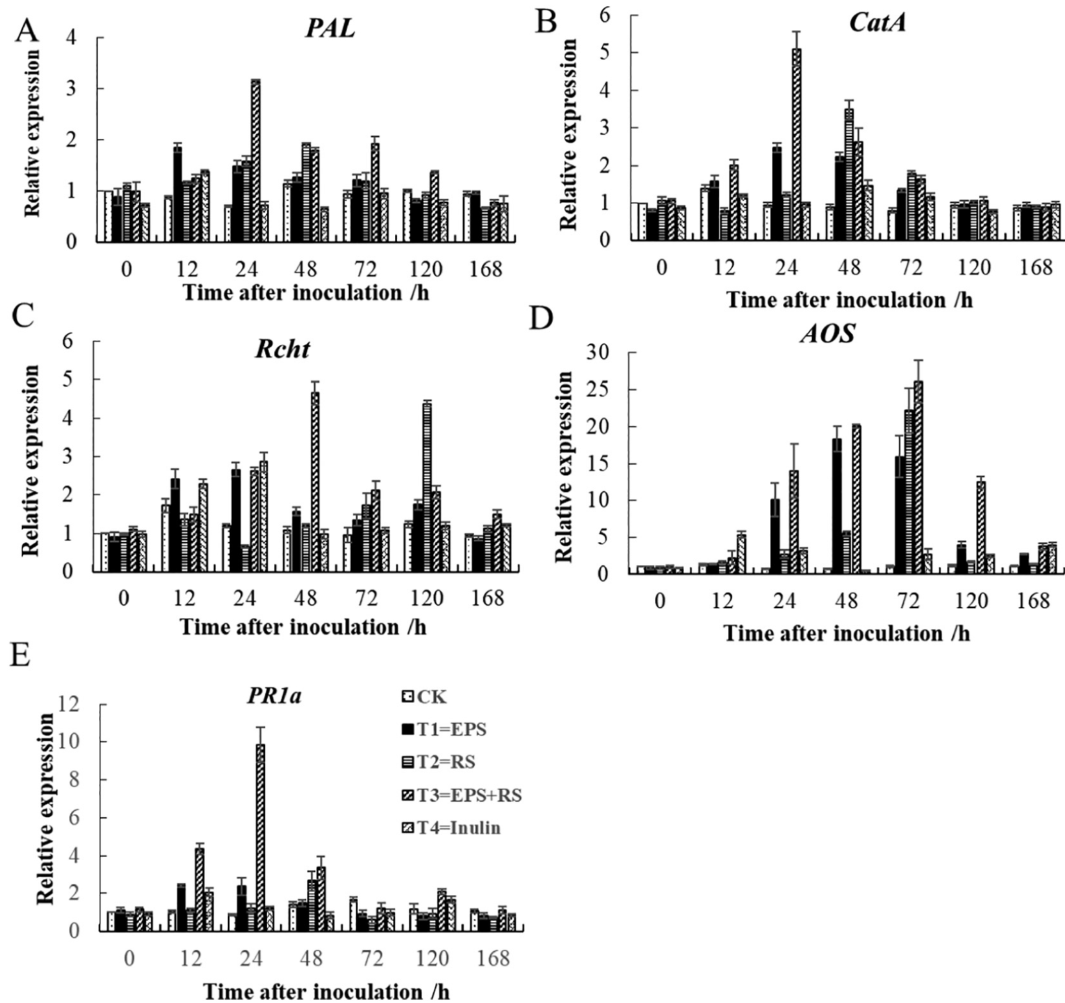

Fig. 1. Differential expression of resistance-related genes in rice. Leaves of rice were harvested after indicated time intervals for extracting total RNA. Gene expression levels were determined by qRT-PCR. (A–E) Time course of expression of PAL, CatA, Rcht, AOS, and PR1a genes in the rice leaves treated with ddH_2O (CK), *Paenibacillus kribbensis* PS04 EPS treatment (T1), inoculated with *Rhizoctonia solani* AG1-IA alone (T2), EPS pretreated plants inoculated with *R. solani* AG1-IA (T3), and inulin treatment plants (T4). The expression values of the individual genes were normalized using *Actin* gene as an internal standard. Data represents the average values of at least three biological replicates, each repeated in duplicate in the same run. All experiments were performed three times, and similar results were obtained.

maximal expression level obtained from RS treatment (T2) at 48 hpi. Overall, it was observed that EPS + RS treatment (T3) resulted in higher expression levels in all studied genes. However, the treatment of inulin (T4), which is a natural plant-derived fructosan, showed no significant induction of resistance-related genes overall.

3.2. Effects of PS04 polysaccharide on the expression of resistance-related genes in tobacco plant

A thorough study of expression of *PR1a*, *PR1b*, *NPR1*, *MAPK*, and *N* genes encoding PR1a protein, PR1b protein, Non-expresser of Pathogenesis Related Gene 1, Mitogen-Activated Protein Kinase, and TMV resistance protein N, respectively, was done. Their relative expression levels were shown in Fig. 2. It showed that, as a result of EPS treatment (T1) in tobacco, *PR1a* gene expression was upregulated until 24 hpi and then decreased gradually. A rapid increase in expression level of *PR1a* was observed by EPS + TMV (T3) treatment which was peaked at 24 hpi and then sharply declined. Moreover, both EPS and EPS + TMV treatment (T1 and T3) have shown a maximal intensity of approximately 16.05-fold and 52.63-fold, respectively, at 24 hpi, compared to the CK (Fig. 2A).

PR1b gene was sharply upregulated after 12 hpi in tobacco plants by EPS treatment (T1) and resulted in an increase of 9.66-fold change, as compared to CK expression level at 24 hpi, and then prominently declined at 48 hpi. The expression level of *PR1b* gene peaked at 24 hpi with an increase of 11.51-fold relative to the CK by EPS + TMV treatment, and the gene expression level declined until 168 hpi (T3). Only TMV treatment (T2) resulted in an early rise in transcript level of *PR1b* in tobacco seedlings at 12 hpi with 5.56-fold increase compared to the CK, and then declined gradually (Fig. 2B).

Only EPS treatment (T1) resulted in a maximum of relative expression level of *NPR1* at 24 hpi in tobacco plants with a 3.80-fold change, and then the expression level of *NPR1* gradually decreased at later time points of the experiment. *NPR1* expression was slowly upregulated in response to only TMV treatment (T2) and reached its maximum level at 72 hpi with a 3.56-fold and then had a 2.40-fold decline at 120 hpi compared to the control group. At 24 hpi, a peaked expression level of *NPR1* was observed as a result of EPS + TMV treatment (T3) with a 4.84-fold increase relative to the control group (Fig. 2C).

The expression of *MAPK* peaked at 24 hpi with EPS + TMV treatment (T3) causing a 6.32-fold change compared with the control group. However, the expression of *MAPK* reached its maximum at 48 hpi with a 78-fold increase relative to the control group by only EPS treatment (T1). By only TMV treatment (T2), the transcriptional level of *MAPK* increased gradually until 72 hpi where it was 3.95-fold

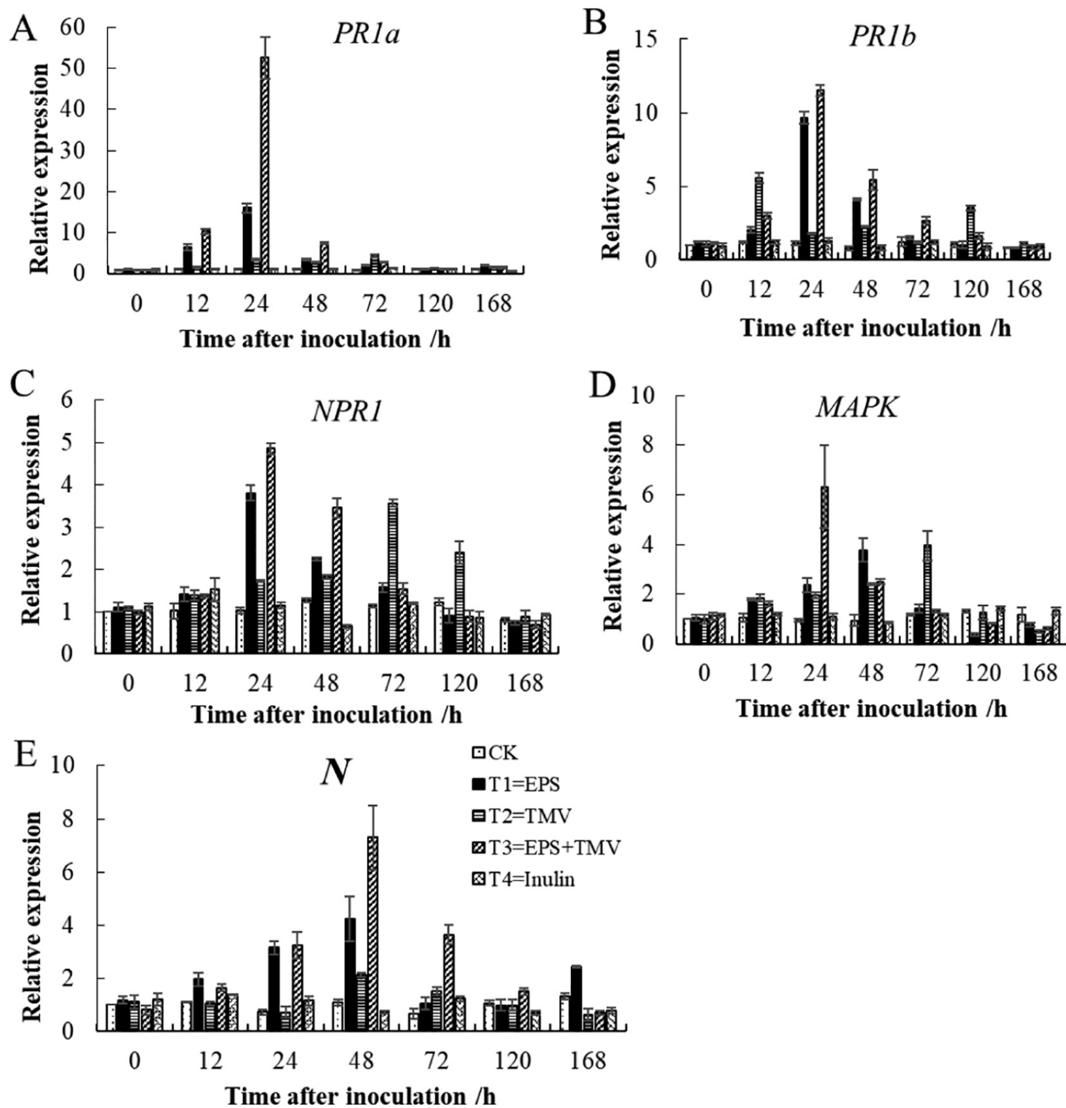

Fig. 2. Differential expression of resistance-related genes in tobacco. Leaves of tobacco were harvested at the indicated time intervals for extracting total RNA. Gene expression levels were determined by qRT-PCR. (A–E) Time course of expression of PR1a, PR1b, NPR1, *MAPK*, and *N* genes in the leaves of tobacco treated with ddH_2O (CK), *Paenibacillus kribbensis* PS04 EPS (T1), inoculated with TMV (T2), EPS pretreated plants inoculated with TMV (T3), and inulin (T4). The expression values of the individual genes were normalized using *Actin* gene as an internal standard. This data represents the average values of at least three biological replicates, each repeated twice in the same run.

compared to the control group. It was observed that the highest level of expression was obtained rapidly as a result of EPS + TMV treatment (T3) at 24 hpi, while in the case of only TMV (T2) or EPS treatment (T1), a gradual increased pattern of expression was observed (Fig. 2D).

A gradual increase in upregulated *N* gene in tobacco plants was observed which attained its maximum level by only EPS treatment (T1) at 48 hpi having a maximal intensity 4.25 times higher than that of the control group. Later, the expression of *N* gene decreased until 120 hpi and then slightly increased at 168 hpi. Gradual increase and peak expression levels of *N* gene were observed by EPS + TMV treatment (T3) at 48 hpi with maximal intensity 7.29 times higher than that of the control plants group. Then, the expression level of *N* gene decreased at later time points of the experiment (Fig. 2E). Inulin treatment (T4) showed a persistent low expression of resistance-related genes during the whole experiment.

3.3. Effects of PS04 polysaccharide on the expression of defense-related proteins in rice and tobacco

A comprehensive study of the activities of POD (peroxidase), SOD (superoxide dismutase), and CAT (catalase) was done where their activities were measured by different treatments at different time intervals. Rice seedlings have shown slightly increased and peaked POD activity at 5 dpi by EPS treatment only, having a maximal activity of 1.80-fold compared to the control group. Moreover, POD activity was increased 1.66-fold by EPS + RS treatment (T3) at the same time (Fig. 3 A). At 3 dpi, an increasing trend of SOD activity was observed in rice seedlings treated with EPS (T1), which reached its peak at 5 dpi and then subsequently decreased. By EPS + RS treatment (T3), SOD activity was also increased gradually and peaked at 5 dpi and subsequently declined (Fig. 3 B). CAT activity increased significantly in EPS treated (T1) rice seedlings and reached its maximum level at 3 dpi with a 1.61-fold expression level as compared to the control group. EPS + RS (T3) treatment in rice plants showed a maximum level of 2.10-fold CAT activity relative to the control group at 5 dpi. RS treatment (T2) in plants resulted in a slight increase in CAT activity which reached its maximum value at 5 dpi and then subsequently decreased. (Fig. 3 C).

In tobacco seedlings, as a result of EPS treatment (T1), POD activity was sharply increased and peaked with a maximal expression level of 2.66-fold at 5 dpi. Similarly, POD activity was increased to 1.75-fold at 5 dpi as result of EPS + TMV treatment (T3). However, the activity of

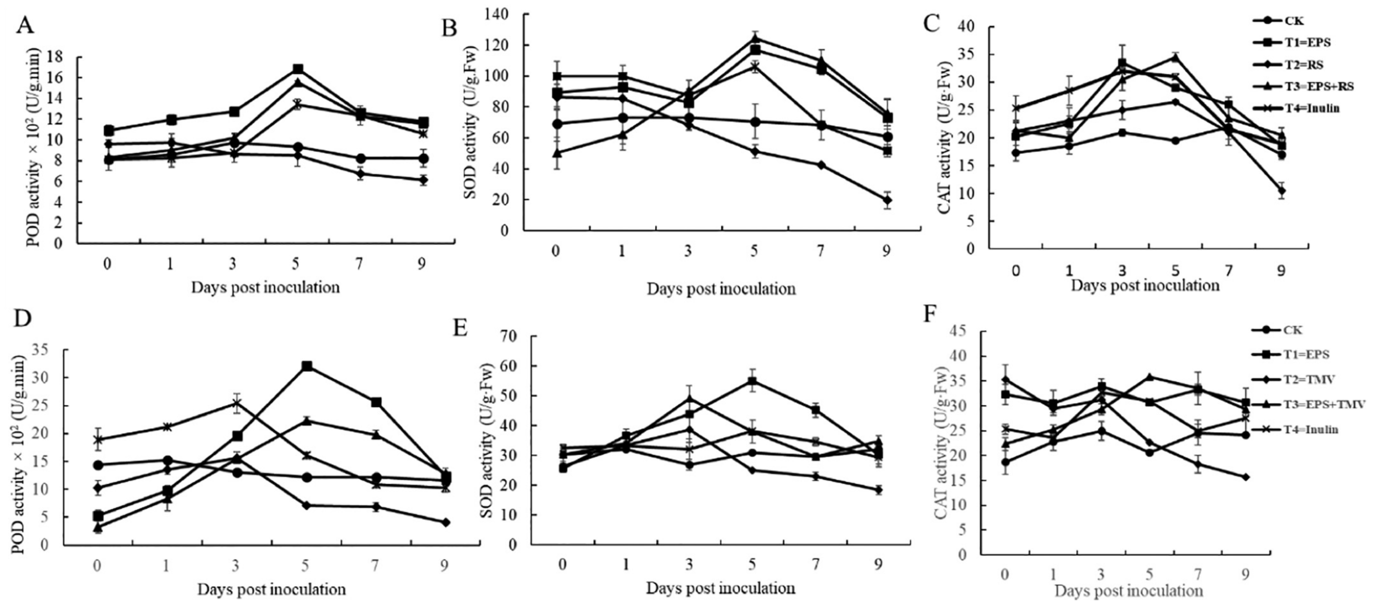

Fig. 3. Time course of content of peroxidase activity (POD), superoxide dismutase activity (SOD), and catalase activity (CAT) in the leaves of rice (A–C) and tobacco (D–E). CK: plants treated with ddH_2O; T1: Plants treated with only *Paenibacillus kribbensis* PS04 EPS; T2: Plants treated with only *Rhizoctonia solani* AG1-IA or TMV; T3: EPS pretreated plants inoculated with *R. solani* AG1-IA or TMV; T4: Plants treated with inulin. Data represent the average values of at least three biological replicates, each repeated in duplicate in the same run.

POD decreased gradually and reached the same level as the control until 9 dpi (Fig. 3 D). SOD activity was sharply increased in EPS-treated tobacco seedlings (T1), causing a 1.44-fold increase at 5 dpi relative to untreated tobaccos plants. SOD activity was also induced when tobaccos subjected to EPS + TMV treatment (T3) having a maximal activity of 1.61-fold at 3 dpi (Fig. 3 E). A decreasing trend of SOD activity was observed in both T1 and T3, where it reached the level of the control group until 9 dpi. However, it was observed that CAT activity remained at a relatively low level during the time of the experiment (Fig. 3 F).

3.4. Effects of PS04 polysaccharide on the expression of malondialdehyde (MAD), total phenols, and bioflavonoids in rice and tobacco

For a more detailed study, lipid peroxidation damage in plants was tested by measuring the activity of MDA (malondialdehyde). It was observed that RS treatment (T2) in rice had increased the MDA level from 1 dpi until its maximum level at 7 dpi. Interestingly, we observed a decrease in MDA level by EPS + RS treatment (T3) in rice (Fig. 4A), which indicated that the application of EPS reduced the production of MDA in plants. Moreover, a similar trend of MDA

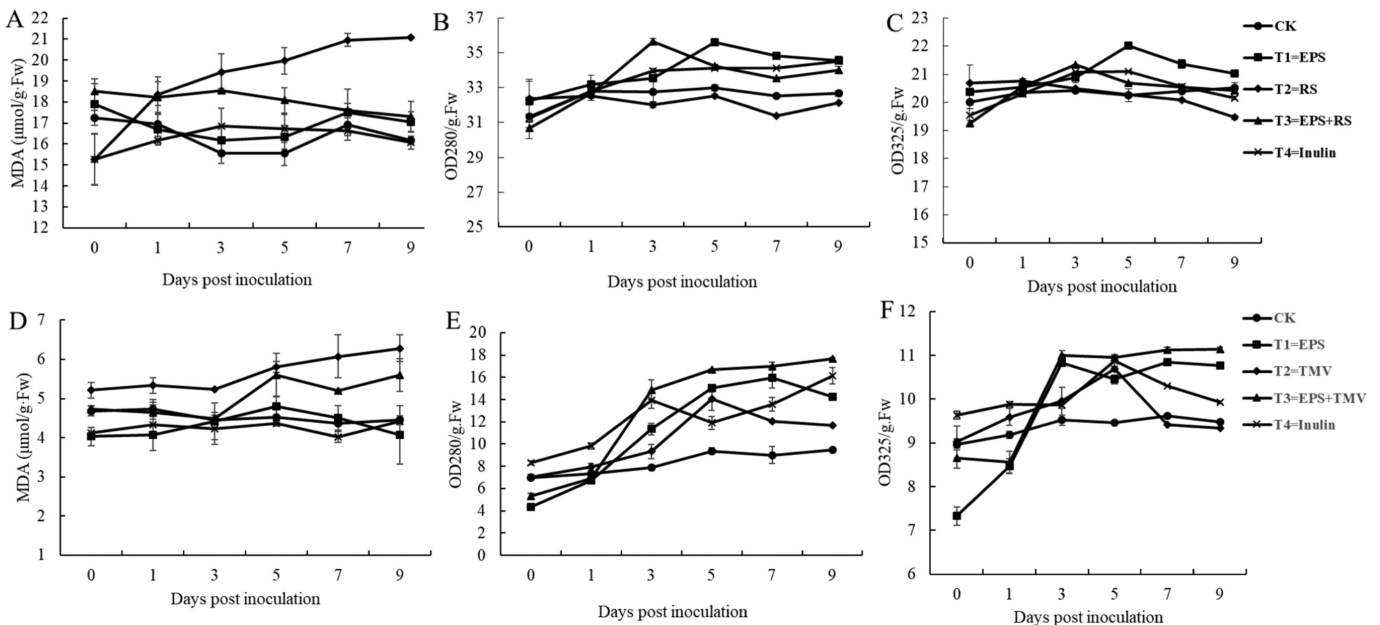

Fig. 4. Time course of content of malondialdehyde (MAD), total phenols, and bioflavonoids in the leaves of rice (A–C) and tobacco (D–E). CK: plants treated with ddH_2O; T1: plants treated with *Paenibacillus kribbensis* PS04 EPS; T2: plants treated with *Rhizoctonia solani* AG1-IA or TMV; T3: EPS pretreated plants inoculated with *R. solani* AG1-IA or TMV; T4: plants treated with inulin. Data represent the average values of at least three biological replicates, each repeated in duplicate in the same run.

content was observed in tobacco seedlings (Fig. 4 D). Further, it was noted that plants treated with only EPS or inulin, as well as the control, showed persistently low MDA activity during the whole experiment duration.

Oxidative stress is an important phenomenon during plant–pathogen interactions. Due to their significant impact on oxidative stress, we tested total phenols and bioflavonoids activities and found some supporting results. In EPS-treated rice seedlings (T1), the activity of total phenols gradually increased and peaked at 3 dpi which was 1.09-fold relative to the control. Similarly, total phenol activity was increased 1.01-fold in EPS + RS (T3) treated rice seedlings at 5 dpi (Fig. 4 B). However, the activity of total phenols was decreased subsequently but maintained at a higher level as compared to the control during the experiment time frame. It was observed that the activity of total phenols was significantly increased and remained at the highest level after 5 dpi, having maximal activity of 1.98-fold as compared to the control, in EPS + TMV treatment (T3) in tobacco seedlings. Similarly, an increasing trend of activity was observed in EPS treated tobacco seedlings (T1), resulting in a 1.87-fold increased level at 7 dpi relative to the control. However, a slight increased activity was seen in tobaccos only inoculated with TMV (T2) (Fig. 4 E).

By EPS treatment (T1) in rice, the activity of bioflavonoids gradually increased and peaked at 5 dpi with a maximal activity of 1.09-fold change and then subsequently decreased. However, the activity of bioflavonoids in other treatments remained at a relatively low level. Bioflavonoids activity increased significantly in EPS + TMV (T3) treated tobacco seedlings at 1 dpi as compared to the control. At 3 dpi, the activity is about 1.19 times higher than that of the control, which afterwards eventually reached a plateau. A similar trend was observed when tobacco seedlings were treated with EPS (T1). In the case of tobacco seedlings treated with TMV (T2) or inulin (T4), bioflavonoids activity increased gradually and reached its highest value at 5 dpi and then subsequently decreased. However, bioflavonoids activity in the control plants remained at relatively low levels during the course of the experiment (Fig. 4 F).

4. Discussion

Elicitors, also known as inducers, are signal metabolites, which are recognized by plant cells. They help to trigger plant defenses. Elicitors can be generated by the activation of degrading enzymes by the plants themselves or by production from pathogens or biocontrol agents [25]. At present, several elicitor molecules have been characterized which include a wide group of compounds such as peptides, glycolipids, and oligosaccharides [26,27,28]. Previously, our laboratory authors found a new biocontrol agent, *P. kribbensis* PS04, which could control rice sheath blight and tobacco mosaic disease effectively. Naturally, EPS were separated from *P. kribbensis* PS04 and identified as fructosan. This study had shown that treatment of PS04 EPS had significantly increased the transcript levels of plant resistance-related genes and the contents of defense-related proteins, total phenols, and flavonoids. It reduced the content of malondialdehyde in both rice and tobacco. To the best of our knowledge, this is the first study presenting the function of EPS which is a fructosan from *Paenibacillus* that induces disease resistance.

It has been reported that SA regulates the expression of genes for acidic PR proteins and induces defense responses against biotrophic pathogens, whereas JA/ET regulates the expression of genes for basic PR proteins and activates the defense response against necrotrophic pathogens [29]. A cross talk exists between SA and the JA/ET signal pathway [10] which is mediated by node genes that are involved in the signal transduction network [30,31,32]. Among these node genes, *NPR1* gene not only positively regulates SA pathway in the nucleus, but also coordinates SA and the JA/ET signal pathway in the cytoplasm [14]. In this study, the expression of two genes (*PAL* and *PR1a*) which involved in SA pathway and an *AOS* gene involved in JA/ET pathway were significantly induced in rice seedlings pretreated with EPS. The expression of these three genes showed a higher trend when rice seedlings were pretreated with EPS and subsequently challenged with a notorious necrotrophic fungus *R. solani*, which is a virulent strain that causes rice sheath blight disease. In tobacco seedlings, two genes (*PR1a* and *PR1b*) involved in SA pathway and the *NPR1* gene were also significantly activated by pretreatment with EPS and inoculation with TMV. These results strongly indicate that PS04 EPS activated both SA and JA/ET pathways in plants and served as an elicitor to activate resistance-related genes. Our finding was consistent with previous studies that EPS produced by *B. cereus* could simultaneously activate SA and JA/ET signaling pathways [14]. Lee et al. [10] also reported that volatile organic compound elicitors of *P. polymyxa* E681 prime the transcription of SA and the JA/ET signal pathway genes to protect plants against *Pseudomonas syringae*.

The induction of defense-related enzymes by different biocontrol agents has been reported in many plants and is correlated to increased disease resistance and reduced disease severity. Defense-related enzymes include peroxidases (POD), superoxide dismutase (SOD), and catalase (CAT) as they play important roles in reactive oxygen species (ROS) homeostasis. POD is involved in lignification of host plant cells and is considered as a key enzyme related to the defense reaction against pathogen infections. The superoxide radical is efficiently converted to H_2O_2 by the action of SOD, while H_2O_2 is destroyed primarily by CAT [33,34]. Our study showed that EPS from *P. kribbensis* PS04 induced the activities of POD, SOD, and CAT at 3–5 dpi, indicating that disease resistance in both rice and tobacco is enhanced by EPS treatment. These results were supported by Kumar et al. [35], who reported that priming of tobacco with *P. lentimorbus* B-30488 resulted in accumulation of defense-related enzymes in response to pathogen infection.

During the stress or suffering from pathogen infection, plant cells accumulate ROS resulting in cell membrane lipid peroxidation and metabolic disorders. In plants, MDA is one of the most frequently used indicators of lipid peroxidation [36]. Our results showed that MDA content in rice and tobacco was gradually increased after inoculation with *R. solani* AG1-IA or TMV and that the injury in plants increased in a prolonged infection period. After EPS treatment, the content of MDA in rice and tobacco was significantly reduced as compared to those inoculated with pathogen only. These indicated that EPS reduce membrane lipid peroxidation in plants when suffering from pathogen infection. Total phenols and bioflavonoids are involved in the protection against oxidative stress. Our finding showed that the content of total phenols and bioflavonoids in both rice and tobacco were increased when treated with EPS, indicating that EPS promote the synthesis of total phenols and bioflavonoids and eliminate reactive oxygen species.

In conclusion, our study showed that EPS from *P. kribbensis* PS04, which is a fructosan, significantly induce plant resistance against *R. solani* AG1-IA and tobacco mosaic virus (TMV). EPS from PS04 activate the expression of defense genes associated with SA and JA/ET pathways and induce antioxidant enzyme activities in plants. EPS form PS04 can be used as an elicitor for the control of rice sheath blight disease and TMV disease.

Conflict of interest

No conflicts of interest have been declared.

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