Pesticide Biochemistry and Physiology 179 (2021) 104948

Contents lists available at ScienceDirect

Pesticide Biochemistry and Physiology

journal homepage: www.elsevier.com/locate/pest

Plant secondary metabolite, daphnetin reduces extracellular polysaccharides production and virulence factors of *Ralstonia solanacearum*

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ARTICLE INFO

Keywords:
Plant metabolite
Daphnetin
Extracellular polysaccharides
Ralstonia solanacearum
EpsB

ABSTRACT

Plants deploy a variety of secondary metabolites to fend off pathogen attack. Certain plants could accumulate coumarins in response to infection of bacteria, fungi, virus and oomycetes. Although coumarins are generally considered toxic to microbes, the exact mechanisms are often unknown. Here, we showed that a plant secondary metabolite daphnetin functions primarily by inhibiting *Ralstonia solanacearum* extracellular polysaccharides (EPS) production and biofilm formation *in vitro*, through suppressing genes expression of *xpsR*, *epsE*, *epsB* and *lexM*. Indeed, daphnetin significantly impaired virulence of *R. solanacearum* on tobacco plants. Transcriptional analysis suggested that daphnetin suppresses EPS synthesis cluster genes expression through transcriptional regulator XpsR. And daphnetin alter mainly virulence factors genes involved in type III secretion system, and type IV secretion system. *R. solanacearum* lacking EPS synthesis genes (*epsB* and *epsC*) that do not produce EPS, showed less virulence on tobacco plants. Molecular docking results indicated that the critical residues of domain in the binding pocket of the EpsB protein interact with daphnetin via conventional hydrogen bonding and hydrophobic interactions. Collectively, we found that daphnetin has potential as a novel virulence inhibitor of *R. solanacearum*, directly regulates EPS synthesis genes expression.

1. Introduction

Plants produce a large number of secondary metabolites, which sever as protectors in the fight against pathogenic microbes and animal pest (Mithofer and Boland, 2012; Wang et al., 2020). Some of these defense metabolites are conditionally exuded in plants under pathogen attacks and are known as phytoalexins (Ahuja et al., 2012; Dixon, 2001). Phytoalexins are structurally diverse and roughly separated into alkaloid, isoprenoid (trepenoid) and phenylpropanoid metabolites, produced by a range of crop plants from *Brassicaceae*, *Fabaceae*, *Solanaceae*, *Poaceae* and *Vitaceae* (Grosskinsky et al., 2012; Iriti and Faoro, 2009). Although an increasing number of phytoalexins and their biosynthetic pathways has been discovered, mechanisms of these defense metabolites conferring disease resistance is still largely unclearly. Mostly of defense metabolites function through their antimicrobial activity, and high concentration of compounds can inhibit or even kill microbes in *vitro* (Bai et al., 2016; Lee et al., 2020; Schmelz et al., 2011; Sun et al., 2014). For instance, camalexin shows toxicity activity on *Alternaria brassicicola* and *Botrytis cinerea* by inducing cell wall permeability and apoptosis (Joubert et al., 2011; Shlezinger et al., 2011). Scopoletin has been reported to inhibit *Phakopsora pachyrhizi* germination at concentrations from 100 μM to 500 μM (Beyer et al., 2019). Thus, whether natural plant metabolites contribute to disease resistance by inhibiting pathogen virulence remain largely unknown.

Coumarins are naturally secondary metabolites composed of fused benzene and α-pyrone rings via the phenylpropanoid pathway in several different plant species, and accumulate in response to infection by bacteria, fungi, virus and oomycetes (Barot et al., 2015; Stringlis et al., 2019). Recently studies have found that coumarins exhibit many biological activities, such as antibacterial, antifungal, anticoagulant, antioxidant, anticancer and anti-inflammatory activity (Barot et al., 2015). Young leaves of *Nicotiana attenuata* show higher resistance against *Alternaria alternata* than mature leaves, which is correlated with stronger induction of scopoletin (Sun et al., 2014). Coumarins scopolin, coniferin and syringing have been shown to be rapidly processed in the *Arabidopsis* roots upon infection by the oomycetes pathogen *Pythium sylvaticum*, giving rise to cell wall-fortifying lignin and antimicrobial scopoletin (Bednarek et al., 2005). Our previous studies found that certain hydroxycoumarins (umbelliferone, esculetin and daphnetin) have antibacterial activity against *R. solanacearum* (Yang et al., 2016).

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https://doi.org/10.1016/j.pestbp.2021.104948
Received 10 June 2021; Received in revised form 18 July 2021; Accepted 11 August 2021
Available online 14 August 2021

Daphnetin displays strongest antibacterial activity and significantly reduces virulence of *R. solanacearum* on tobacco with soil irrigated treatment (Yang et al., 2018). However, the mechanism of daphnetin against plant pathogen virulence factors still remains poorly understood.

Ralstonia solanacearum species-complex is the causal agent of bacterial wilt that infects more than 250 plant species, causing serious losses of many economically important crops such as tomato, potato, pepper, eggplant, tobacco and banana (Jiang et al., 2017; Mansfield et al., 2012). *R. solanacearum* invades host plants through root wounds, colonizes the xylem vessels and grows to high cell densities, resulting in host wilting and death (Genin, 2010; González and Allen, 2003). During the invasion process, *R. solanacearum* utilizes a large consortium of virulence factors to cause wilt disease on host plants, including secret plant cell-wall-degrading enzymes, extracellular polysaccharides (EPS), and lots of type III effectors (T3Es) (Coll and Valls, 2013; Liu et al., 2005; Poueymiro and Genin, 2009; Saile et al., 1997). The most important virulence factor of *R. solanacearum* is its high-molecular-mass EPS, which promotes rapid systemic colonization. Accumulation of EPS is largely responsible for vascular dysfunction that causes wilt symptoms in susceptible hosts (Genin and Denny, 2012). Screening for EPS inhibitors is considered as a potential method for controlling bacterial wilt. Raza et al has found that volatile compounds (VOCs) from biocontrol microbe *Bacillus amyloliquefaciens* (*B. amyloliquefaciens*) SQR-9 has significantly decreased EPS production of *R. solanacearum* (Raza et al., 2016a). Meanwhile, the VOCs of strain *B. amyloliquefaciens* T-5 significantly inhibited the motility traits, root colonization, biofilm formation, and *R. solanacearum* EPS (Raza et al., 2016b). Otherwise, 3-indolyacetonitrile and 6,7-dihydroxycoumarin increase the production of *R. solanacearum* EPS in culture (Tseng and Mila, 2017). Certain coumarins, umbelliferone suppresses the expression of T3SS regulatory factors and effectors genes and alters virulence of *R. solanacearum* on tobacco (Yang et al., 2017).

In order to better understand the mechanism of plant natural metabolite-daphnetin against *R. solanacearum* virulence factors. In this study, we investigated the effect of daphnetin on EPS production and biofilm formation of *R. solanacearum in vitro*. Then, we evaluated the effect of daphnetin on EPS transcriptional regulator genes and EPS biosynthesis genes expression using qRT-PCR. Furthermore, the effect of daphnetin on the bacterial population in stems and the suppression of disease index development on tobacco were investigated. Transcriptional analysis and molecular docking were used to reveal molecular mechanism of daphnetin against *R. solanacearum*.

2. Materials and methods

2.1. Bacterial strains, plant material and growth conditions

The *R. solanacearum* CQPS-1 (phylotype I, race 1, biovar 3) used in this study were provided by Laboratory of Natural Products Pesticides (Liu et al., 2017). Strain was grown in nutrient-rich medium (B medium) or M63 minimal medium at 30 °C (Yoshimochi et al., 2009).

The daphnetin used in the study were purchased from Shanghai Yuanye Bio-Technology Co., Ltd. (Shanghai, China), and the purity of compound (HPLC $\geq$ 98%) was validated by using High Performance Liquid Chromatography.

The plant used in this study was tobacco (*Nicotiana tabacum* Yunyan87). Six-week-old plants were used for inoculations. Plants experiments were conducted in climate room at 28 °C with a 14 h/10 h light/dark cycle.

2.2. Biofilm quantification

As previously reported, biofilm formation of *R. solanacearum* was performed in 96-well polystyrene plates (Yao and Allen, 2007). Briefly, *R. solanacearum* was inoculated in B medium overnight (OD_{600}=1.0), then added to medium supplemented with DMSO or different concentrations of daphnetin ranging from 5 mg L^{-1} to 25 mg L^{-1}. Then used 96-well polystyrene plates and added 200-μL bacterial suspension mixed with different concentration of daphnetin into each well. The plates were incubated without shaking for 24 h and 32 h at 30 °C. Biofilms of *R. solanacearum* was stained by crystal violet, dissolved in 95% ethanol and quantified by absorbance at 530 nm (OD_{530}). All assays were performed three independent experiments with six replications.

2.3. EPS quantifications

EPS quantification in M63 minimal medium supplemented with daphnetin was determined by measuring hexosamine content with the Elson Morgan Assay as described in previously study (Peyraud et al., 2016). Briefly, bacteria were grown in M63 minimal medium supplemented with L-Glutamate 20 mM for 1 day then centrifugated (5000x) during 15 min to remove EPS. Pellets were suspended in fresh M63 medium supplemented with different concentration of daphnetin treatment (5 mg L^{-1}, 10 mg L^{-1} and 25 mg L^{-1}), then diluted to final concentration with OD_{600}=0.2. After incubation for 12 h at 30 °C, *R. solanacearum* cells were filtrated (0.22 μM), and EPS production were precipitated in 2.5 mL tubes by adding 0.1 mM NaCl and 4 volumes of acetone, then the samples were stored at 4 °C overnight. Precipitated EPS were recovered in 200 μL H_2O and heated at 65 °C on heatblock. After discarded insoluble material, and hydrolysis of the sugar polymers was performed at 110 °C during 30 min with a heatblock. Quantification of hexosamine was determined by measuring absorbance at 530 nm (OD_{530}) and running standard curve of N-acetyl-galactosamine. All assays were performed three times.

2.4. Virulence assay

The drenching assay was used to evaluate virulence of *R. solanacearum* after daphnetin treatment. Six-week old unwounded tobacco plants (*Nicotiana tabacum* Yunyan 87) were pre-inoculated with 15 mL of 10 mg L^{-1} and 25 mg L^{-1} daphnetin without wounding the roots. After 24 h of pre-inoculation, individual plants were inoculated by pouring 15 mL of *R. solanacearum* suspension (1×10^8 CFU/mL) into the soil. All plants were placed in climate cabin at 28 °C with a 14 h/10 h light/dark cycle. Bacterial wilt symptoms were scored daily using a disease index scale from 0 to 4. Individual treatments contained 16 plants for each independent experiment, and the assay was repeated three times. The disease index was calculated as a weighted average.

2.5. Bacterial growth in planta

To measure the bacterial population in the tobacco stems, eight samples of each treatment were destructively harvested at the base of the stems 4, 7 and 12 d after inoculation with *R. solanacearum* as previously studies (Yang et al., 2018). The tissues were disinfected in 75% alcohol for 1 min, rinsed twice with sterile water, transferred into a 2.5 mL sterile centrifuge tube, and grinded with sterile glass beads on MP Biomedicals FastPrep (FastPrep-24™, M. P. Biomedicals, Santa Ana, California, USA). Next, the bacterial suspension was serially diluted, and 100 μL of suspension was plated on SMSA medium to quantify CFUs. Each treatment contained 24 plants, and the entire experiment was performed three times. The semi-selective SMSA medium used in the assay was previously described (Elphinstone et al., 1996).

2.6. RNA isolation and deep sequencing

R. solanacearum was overnight inoculated in rich B medium, then the bacterial suspension (10^8–10^9 CFU per mL) was centrifugation at 5000 rpm for 10 min, the bacteria were collected and diluted in M63 medium adjusted to with OD_{600} 0.2 and incubated on a shaker at 180 rpm and 28 °C for 4–5 h. Then bacterial cells were treated with daphnetin at the concentration with 25 mg L^{-1} for 1 h. The samples were harvested by

centrifugation at 5000 rpm for 10 min at 4 °C, the supernatants were removed, and the treated bacterial cells were collected and frozen in liquid nitrogen if RNA isolation was not conducted immediately. RNA was extracted by using TRNzol reagent according to the manufacturer's instructions (Tiangen Biotech Co. Ltd, Beijing, China) and then treated with RNase-free DNase I (Tiangen Biotech Co. Ltd, Beijing, China) to remove genomic DNA contaminations. RNA degradation and contamination were checked on 1% agarose gels and RNA concentration and purity were monitored using the Nanovue UV-Vs spectrophotometer (GE Healthcare Bio-Science, Uppsala, Sweden).

RNA concentrations were assessed using Qubit® RNA Assay Kit in Qubit® 2.0 Flurometer (Life Technologies, CA, United States) and Nano 6000 Assay Kit of the Bioanalyzer 2100 system (Agilent Technologies, CA, United States). The quality standard of RNA samples including minimum RNA integrity number (RIN) of 7, absorbance values A260/280 in the range 1.8–2.0 and A260/230 over 1.8. Libraries construction and RNA-Seq were performed by the Shenzhen Hengchuan (Shenzhen, China). RNA-Seq libraries were generated using NEBNext® Ultra ™ RNA library Prep Kit for Illumina (NEB, United States) following manufacturer's recommendations. After synthesis first strand cDNA and second strand cDNA, the samples were sequenced on the Agilent Bioanalyzer 2100 system. The length of the reads was around 150 bp. Quality control of the RNA-Seq raw data was performed using FastQC (Ewels et al., 2016). The reference genome of *R. solanacearum* CQPS-1 were downloaded from GenBank (NZ_CP016914.1) (Liu et al., 2017). The raw data were filtered by discarding low-quality sequences and removing adaptor sequences. Read mapping of the reference genome was using HITAT2 (Kim et al., 2015). Differentially expressed genes (DEGs) of daphnetin treatment were performed using the DEGSeq2 (Love et al., 2014). To extract genes with differentially expression changes, the cutoff of q-value < 0.01 and $|\log_2$ Fold change$| > 2$ was applied (Nobori et al., 2018).

2.7. Quantitative real-time RT-PCR

cDNA was synthesized using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. The primers for the tested genes were synthesized by BGI Technologies (Shenzhen, Guangzhou, China). All quantitative real-time PCR (qRT-PCR) analyses were carried out in 96-well plates in a 20 μl reaction system with C1000 Thermal Cycler (Bio-Rad, Hercules, CA, USA). Three technical replicate reactions were used for each sample. Normalized gene expression was calculated by Bio-Rad CFX and SerC was used as reference gene (Monteiro et al., 2012). The mean values of all experiments were averaged with SD, and the statistical significance between the daphnetin treatment and CK treatment was evaluated by Student's *t* test analysis.

2.8. Molecular docking

Molecular docking was performed using AutoDock 4.2 as previously described (Zhou et al., 2021). The three-dimensional (3D) model of EpsB and its binding pocket were generated by the I-TASSER server (http://zhanglab.ccmb.med.umich.edu/I-TASSER/) (Yang et al., 2015). The 3D model of the ligands and their energy minimization were established by ChemOffice 2004. The model results were analysed by Discovery Studio Visualizer 4.5 (Accelrys Software Inc., San Diego, CA, USA) (Patil et al., 2010).

2.9. Statistical analysis

The data were analysed with the SPSS 17.0 statistical software program (SPSS Inc. Chicago, IL) using ANOVA and Student's *t*-test under significance levels of 0.05 and 0.01 (P-value < 0.05 and P-value < 0.01)

3. Results

3.1. The effect of daphnetin on EPS production and biofilm formation of *Ralstonia solanacearum*

As showed in Fig. 1A, the wild-type (WT) strain CQPS-1 form fluidal and pink centered colonies, and daphnetin treated bacteria form red, less mucoid colonies. Then, we evaluated the effect of daphnetin on EPS production and biofilm formation of *R. solanacearum*. As showed in Fig. 1B, daphnetin significantly suppressed EPS production, with a 2.4-fold decrease under 25 mg L^{-1} concentration. Furthermore, the

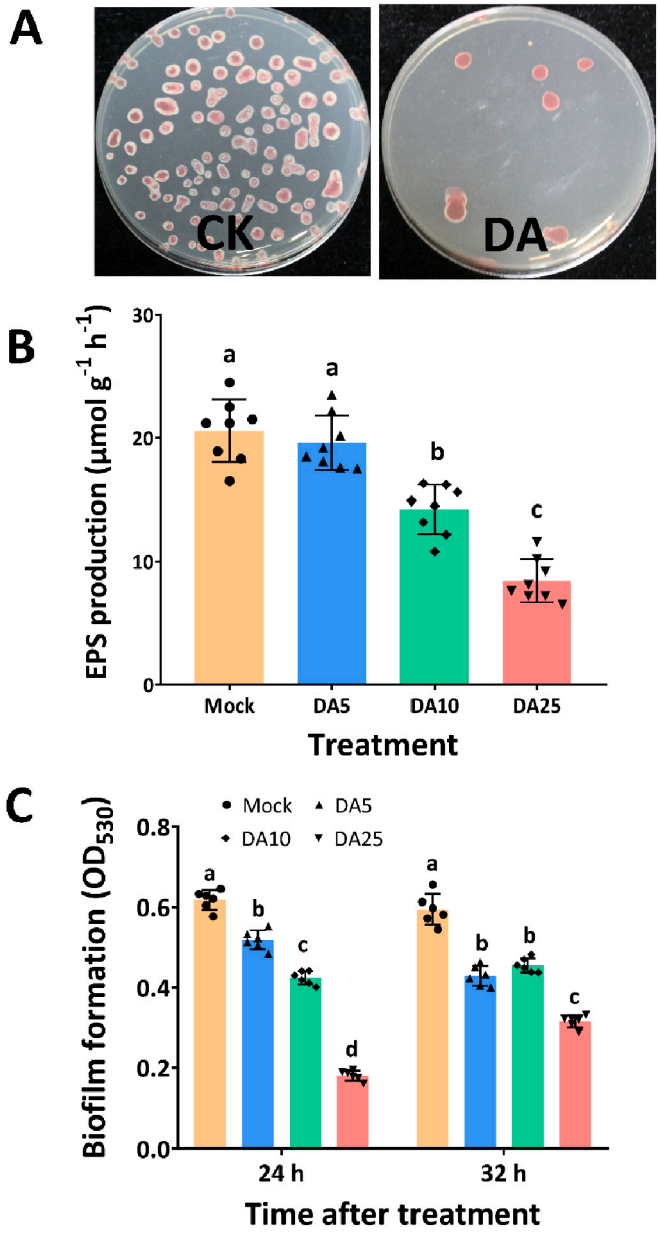

Fig. 1. Daphnetin reduses extracellular polysaccharide (EPS) and biofilm formation of *Ralstonia solanacearum in vitro*. (A) Bacteria colony morphology changed by daphnetin in the plate after inoculation for 48 h. (B) Effect of daphnetin on extracellular polysaccharide production of *R. solanacearum*. (C) The effect of daphnetin on the biofilm formation of *R. solanacearum* after 24 h and 32 h incubation. Data shown are the means of independent experiments with a least three replicates. Each bar represents the mean ± SE of three replications. Different letters indicate significant differences between different treatments according to one-way ANOVA with Duncan's test ($P < 0.05$).

inhibition activity of daphnetin on biofilm formation of *R. solanacearum* was concentration-dependent. 10 mg L^{-1} and 25 mg L^{-1} daphnetin significantly reduced biofilm formation with 1.56-fold and 1.90-fold (Fig. 1C).

3.2. The effect of daphnetin on expression of EPS associated genes

In order to confirm this finding, the mRNA levels of EPS associated transcription regulators supplemented with DMSO or daphnetin treatment were measured. The results showed that the expression of *xpsR* was significantly suppressed supplemented with daphnetin treatment (Fig. 2). However, daphnetin had no effect on expression of other upstream regulators (*phcA*, *vsrC*, *vsrD* and *epsR*). Additionally, the expression of EPS biosynthesis cluster genes such as *eps*E and *epsB* were down regulated by daphnetin treatment.

3.3. Daphnetin reduces the virulence of Ralstonia solanacearum in tobacco plants

Based on the suppression activity of daphnetin on *R. solanacearum* biofilm formation, EPS production and expression of EPS transcription regulators, the effect of daphnetin on tobacco bacterial wilt disease progress was evaluated. As shown in Fig. 3A, compared with mock, daphnetin altered the disease progress of bacterial wilt ($P < 0.05$) in the presence of 25 mg L^{-1}. Moreover, 25 mg L^{-1} concentration of daphnetin significantly reduced the population of *R. solanacearum* in the base stem of the tobacco plants. Compared with the mock treatment, the population of tobacco stems supplemented with 25 mg L^{-1} daphnetin was significantly reduced by 15.43-fold, 14.86-fold, and 38.33-fold at 4 d, 7 d and 10 d after inoculation, respectively (Fig. 3B).

3.4. Daphnetin suppresses the expression of most EPS biosynthesis cluster genes

To determine the mechanism of daphnetin on *R. solanacearum* virulence factors, RNA-Seq was performed to examine the expression of EPS associated genes in the presence and absence of daphnetin. The EPS biosynthesis cluster genes were significantly suppressed by daphnetin

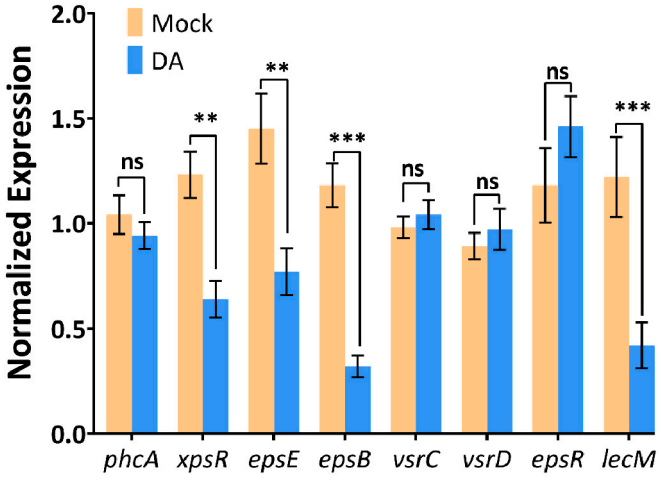

Fig. 2. The effect of daphnetin on the expression of EPS production associated genes. The expression of EPS pathway genes measure in M63 medium supplemented with DMSO or 25 mg L^{-1} daphnetin is using quantitate real-time PCR (qRT-PCR). *SerC* was used as the reference gene to normalize the gene expression using the ΔΔCq method. Each bar represents the mean ± SE of three replications. Asterisks indicate significant differences between daphnetin and control treatment according to Student's *t*-test (* indicates $P < 0.05$, ** indicates $P < 0.01$, *** indicates $P < 0.001$).

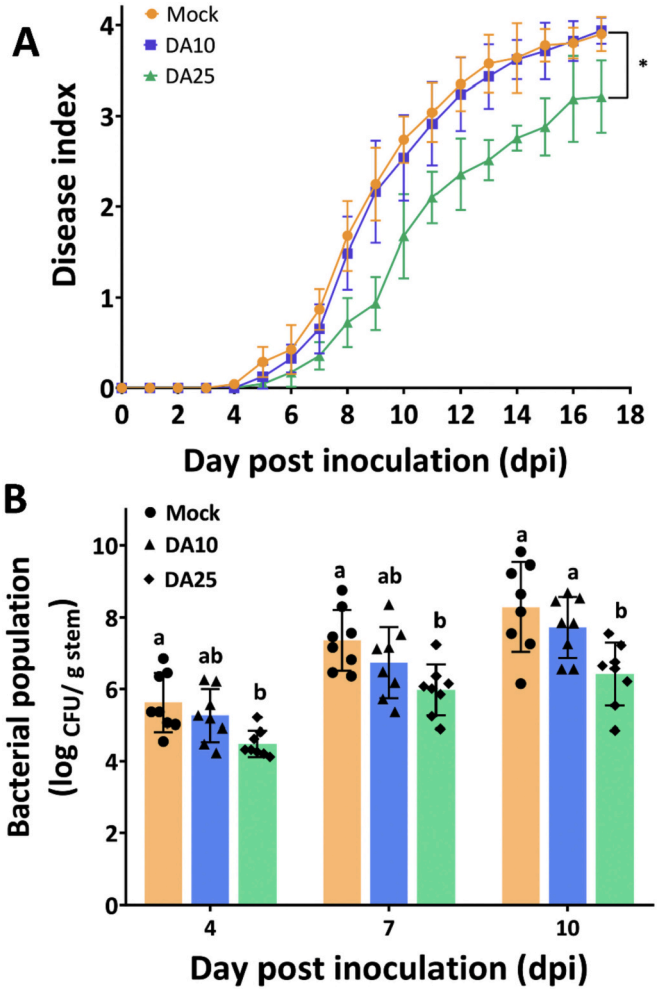

Fig. 3. Daphnetin reduces virulence of *R. solanacearum* on tobacco plants. (A) The disease index of tobacco bacterial wilt pre-treated with 10 and 25 mg L^{-1} daphnetin. After 24 h of application, the *R. solanacearum* suspension was poured around the plant soil. (B) Daphnetin significantly reduced the bacterial population of *R. solanacearum* in the tobacco stem tissues. Each bar represents the mean ± SE of eight replicates. Different letters indicate significant differences between different treatments according to one-way ANOVA with Duncan's test ($P < 0.05$).

treatment, including *epsA*, *epsP*, *epsB*, *epsC*, *wecC*, *epsE* and *epsF* (Table 1). Compared with CK treatment, the mRNA levels of EPS biosynthesis genes were decreased 5.5–14.3-fold under daphnetin treatment. Furthermore, other genes coding for EPS acyl transferase protein such as *Rsp1013*, *Rsp1012* and *Rsp1007* were down-regulated 4.2–7.1-fold change. The transcriptional level of *xpsR* was significantly decreased 8.3-fold change under DA treatment (Table 1). However, daphnetin didn't affect expression of other EPS control transcription regulators, like *phcA*, *vsrC*, *vsrD* and *epsR*. The RT-PCR and RNA-Seq result indicated that daphnetin might inhibit expression of EPS biosynthesis genes through controlling transcription regulator *XpsR*.

3.5. The effect of daphnetin on expression of virulence-associated genes

The results showed that *R. solanacearum* transcriptome signatures influenced by daphnetin, including up-regulation of 312 genes and down-regulation of 346 genes (Fig. 4A). There were 302 genes encoded cellular components, a total of 143 genes were related to molecular functions and 179 genes were related to biological process. In cellular

Table 1
Daphnetin significantly reduced the transcriptional expression of genes involved in EPS biosynthesis and transcriptional regulator in *R. solanacearum*.

CQPS locus_tag	GMI1000 locus	gene name	Fold Change (DA-vs-CK)	q value	Description
BC350_RS23900	*RSp1020*	*epsA*	-8.11	1.77E-33	EPS I polysaccharide export outer membrane protein EpsA
BC350_RS23905	*RSp1019*	*epsP*	-5.49	1.58E-22	Low molecular weight protein-tyrosine- phosphatase
BC350_RS23910	*RSp1018*	*epsB*	-9.39	4.39E-57	EPS I polysaccharide export transmembrane protein/polysaccharide biosynthesis tyrosine autokinase
BC350_RS23915	*RSp1017*	*epsC*	-9.37	2.49E-42	udp-n-acetylglucosamine 2-epimerase protein
BC350_RS23920	*RSp1016*	*wecC*	-8.78	1.24E-42	UDP-N-acetyl-D-mannosamine dehydrogenase
BC350_RS23925	*RSp1015*	*epsE*	-13.35	1.30E-84	EPS I polysaccharide export inner membrane transmembrane protein
BC350_RS23930	*RSp1014*	*epsF*	-14.34	6.35E-83	EPS I polysaccharide export inner membrane transmembrane protein
BC350_RS23935	*RSp1013*	-	-7.12	5.54E-46	Putative EPS I polysaccharide biosynthesis protein, acyl transferase protein
BC350_RS23940	*RSp1012*	-	-6.59	1.85E-23	Putative EPS I polysaccharide biosynthesis protein, acyl transferase protein
BC350_RS23945	*RSp1011*	-	-5.37	3.18E-48	Putative EPS I polysaccharide biosynthesis protein, dehydrogenase
BC350_RS23950	*RSp1010*	-	-5.70	2.73E-47	Putative EPS I polysaccharide biosynthesis protein, Heparinase II/III-like
BC350_RS23955	*RSp1009*	-	-5.50	3.84E-41	Glycosyltransferase
BC350_RS23965	*RSp1007*	-	-4.18	4.07E-25	Putative acetyl transferase protein
BC350_RS23985	*RSp1003*	*xpsR*	-8.27	1.63E-26	Transcription regulator XpsR transcription regulator protein

component category, integral component of membrane, membrane, plasma membrane, bacterial-type flagellum basal body and ribosome were enriched. In molecular function, structural constituent of ribosome, transporter activity, rRNA binding and nucleic acid binding were enriched. There were more biological process categories were enriched, such as transport, translation, transmembrane transport, lipid metabolic process, fatty acid biosynthetic and metabolic process (Fig. 4B). Daphnetin down-regulated expression of T3SS regulators through *prhA*, *prhI*, *prhR*, *prhJ* and *hrpG*. Furthermore, expression of type III effectors *ripX*, *ripF1-1*, *ripN* and *ripAB* was down regulated. Otherwise, expression of type VI secretion genes such as *tssD*, *tssE*, *tssH*, *tssM*, *tssF*, *Rsp1139*, *Rsp0746*, *Rsp0761*, *Rsp0745* and *Rsp0629* were down-regulated supplemented with daphnetin (Fig. 4C).

Furthermore, many of the genes encode for stress response proteins were differentially expressed in the presence of daphnetin. Eleven genes coding for putative drug efflux pumps were up-regulated following daphnetin exposure. The notably high expression levels of putative drug efflux pump gene *Rsp0817* and *Rsp0819* (increased 115-fold and 288-fold by daphnetin) suggest that this pump could export daphnetin. Oxidative stress genes, such as *katGb*, *coxM*, *Rsp0993*, *Rsc2493* and *dnaK* were also induced by daphnetin. Siderophore biosynthesis genes (*Rsp0414*, *Rsp0415*, *Rsp0416*, *Rsp0417*, *Rsp0418*, *Rsp0419*, *Rsp0421*, *Rsp0422*, *Rsp0423* and *Rsp0424*) were induced by daphnetin. Certain translation genes were repressed by daphnetin treatment.

3.6. EPS biosynthesis genes are necessary for virulence of *R. solanacearum* on tobacco

We constructed three EPS biosynthesis genes mutants of *R. solanacearum* CQPS-1, the colony morphology of the Δ*eps*- mutants (*epsB*, *epsC* and *wecC*) were different from the wild type, formed red and small colonies with less mucoid. The Δ*eps*- mutants grew similarly with the WT in liquid medium (Fig. S1). Compared with WT, Δ*eps*- mutants exhibited biofilm formation that was significantly reduced by 1.5–2-fold ($p < 0.01$) (Fig. 5A). However, Δ*eps*- mutants induced swimming activity in *R. solanacearum* (Fig. 5C). The EPS content was reduced in Δ*eps*- mutants. Compared with the WT, the *epsB*, *epsC* and *wecC* mutants exhibited EPS content that reduced by approximately about 84.45%, 91.93% and 89.84%, respectively (Fig. 5B). Furthermore, we observed that the Δ*eps*- mutants (*epsB*, *epsC* and *wecC*) significantly decreased the pathogenicity of *R. solanacearum* on tobacco compared to WT, especially the *epsB*- and *epsC*- mutants, which showed nonpathogenicity on tobacco (Fig. 5D).

3.7. Molecular docking

To examine the interaction between daphnetin and EpsB, molecular docking was used to analysis the binding mode of daphnetin within the binding pocket of EpsB. As shown in Table S3, the binding energy of daphnetin was -4.25 kcal mol^{-1}, which indicates that daphnetin can be considered a specific ligand of EpsB protein. The binding modes and orientations of daphnetin with EpsB were showed in Fig. 6(D,E). Four key amino acids (LEU222, ARG227, ALA285, and GLU286) were interacted with daphnetin via conventional hydrogen bonding and hydrophobic interactions in the binding pocket of EpsB (Fig. 6F). The hydrogen atoms of the hydroxyls at positions 8 of the benzene ring form a conventional hydrogen bond (3.07 and 1.97) with ALA285. In addition, the acidic residues ALA213, ASP225, SER226, GLU283, GLU284, VAL290, and LEU289 interact with daphnetin via Van der Waals interactions in the binding pocket of EpsB.

4. Discussion

Coumarins are produced via the phenylpropanoid pathway and accumulated in plant tissues, responded to infection of a diversity of pathogens and play dual roles in plant defense due to the antimicrobial activity and plant defense signaling (Gnonlonfin et al., 2012; Stringlis et al., 2019). Further, coumarins play role in the interaction of plant and soil-bored pathogen (Stringlis et al., 2018). Although daphnetin is bactericidal at high concentrations (Yang et al., 2016), its concentrations are much lower in the tobacco plant (Tsaballa et al., 2020). As a landmark discovery, advances in the study of bacterial virulence factors have provided evidence that EPS is one of the main pathogenicity determinants in *R. solanacearum*. In this study, we demonstrated that

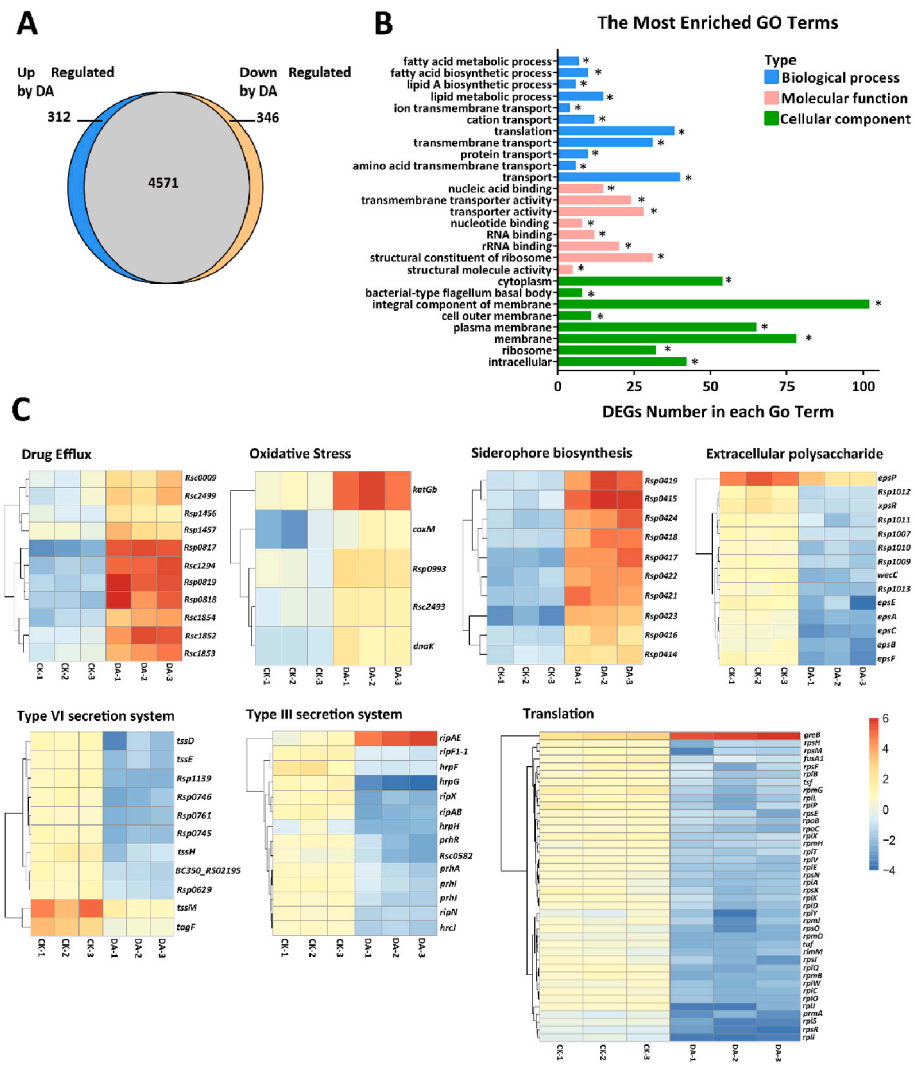

Fig. 4. Daphnetin modulates *R. solanacearum* virulence-associated gene expression. (A) A proportional Venn diagram of differential expression genes supplemented with daphnetin treatment. (B) The most enriched GO terms for the DEGs. The blue columns refer to 'biological process' terms, the red columns refer to 'molecular function' terms and the green columns refer to 'cellular component' terms. The numbers following the columns show the number of DEGs in each term. (C) Heatmaps of differentially expression genes supplemented with daphnetin. The DEGs associated with "drug efflux", "oxidative stress", "siderophore biosynthesis", "extracellular polysaccharide", "type IV secretion system", "type III secretion system" and "translation". Different color of genes represents different expression, red indicates upregulated genes, and blue indicates downregulated genes.

plants indeed possess secondary metabolites that inhibit virulence function of pathogenic microbes. Plant-derived compound daphnetin that are inhibitory to EPS production and suppressed transcription of EPS biosynthesis genes. Furthermore, daphnetin reduced the virulence of *R. solanacearum* on tobacco plants.

The transcriptional level of eps operon coding for biosynthetic enzymes for EPS, controlled by a complex network that including transcriptional activator XpsR, three distinct two-component regulatory systems (VsrAD, VsrBC and PhcSR), and a lysR-type regulator PhcA (Huang et al., 1998). When sufficient signals were accumulated and activated PhcA that triggering the expression of *xpsR*, which directly control the expression of eps cluster genes (Huang et al., 1995). To production of EPS I, the mainly contain of EPS, requires the transcriptional expression of 18 kb *eps* gene cluster that include *eps*(*epsAPBCDEF*) (Huang and Schell, 1995). EPS I apparently is not required for root invasion or growth in planta, but is required for wilting and killing plant (Denny et al., 1990). Recent study implicated that the expression of 97.2% of the positively QS-regulated genes was down-regulated in the *epsB*-deleted mutant. Furthermore, expression of 98% of the negatively QS-regulated genes was up-regulated in Δ*epsB*. These results demonstrated that major exopolysaccharide, EPS I, is associated with the feedback loop in the quorum sensing of *R. solanacearum* strain OE1-1 (Hayashi et al., 2019). Our primary study has proven that hydroxycoumarins such as umbelliferone and daphnetin display inhibition activity against expression of T3SS regulators hrpG of *R. solanacearum* (Yang et al., 2016; Yang et al., 2017). Since hrpG is global regulator that controls virulence and metabolism in response to environmental conditions, daphnetin might change the *R. solanacearum* phenotype. In this study, we found that daphnetin significantly suppresses EPS production and biofilm formation, qRT-PCR and RNA-Seq analysis indicated that daphnetin inhibits EPS production by suppressing expression of key transcriptional factor *xpsR*, without affect other regulator expression (*phcA*, *vsrC*, *vsrD*, and *phcS/phcR*). Furthermore, the expression of EPS biosynthesis clusters genes (*epsA*, *epsP*, *epsB*, *epsC*, *wecC*, *epsE* and *epsF*) were suppressed by daphnetin treatment.

Due to the key role of EPS in *R. solanacearum* pathogenicity, screening for inhibitor of EPS production might be potential strategies for controlling bacterial wilt. *Aspergillus flavus* volatiles were demonstrated to suppress EPS production of *R. solanacearum* (Spraker et al., 2014). Recently discovered *bacillus* FZB42 and LSSC22 volatiles inhibit bacterial growth and significantly suppress transcriptional expression of *R. solanacearum* EPS associated genes (*epsI*, *epsB*, *epsD*, and *epsP*) (Tahir et al., 2017). *β*-hydroxypalmitate methyl ester hydrolase (*β*HPMEH) purified from betaproteobacterium *Ideonella sp. 0-0013* displays high hydrolyzing activity towards the ester bond of 3-OH PAME and eliminates the 3-OH PAME activity, resulting suppression of *in vitro* production of the *R. solanacearum* EPS (Shinohara et al., 2007). In this study, these results suggest that daphnetin reduces the virulence of *R. solanacearum* in tobacco plant via suppressing biofilm formation, EPS production and the expression EPS associated genes. Recent study

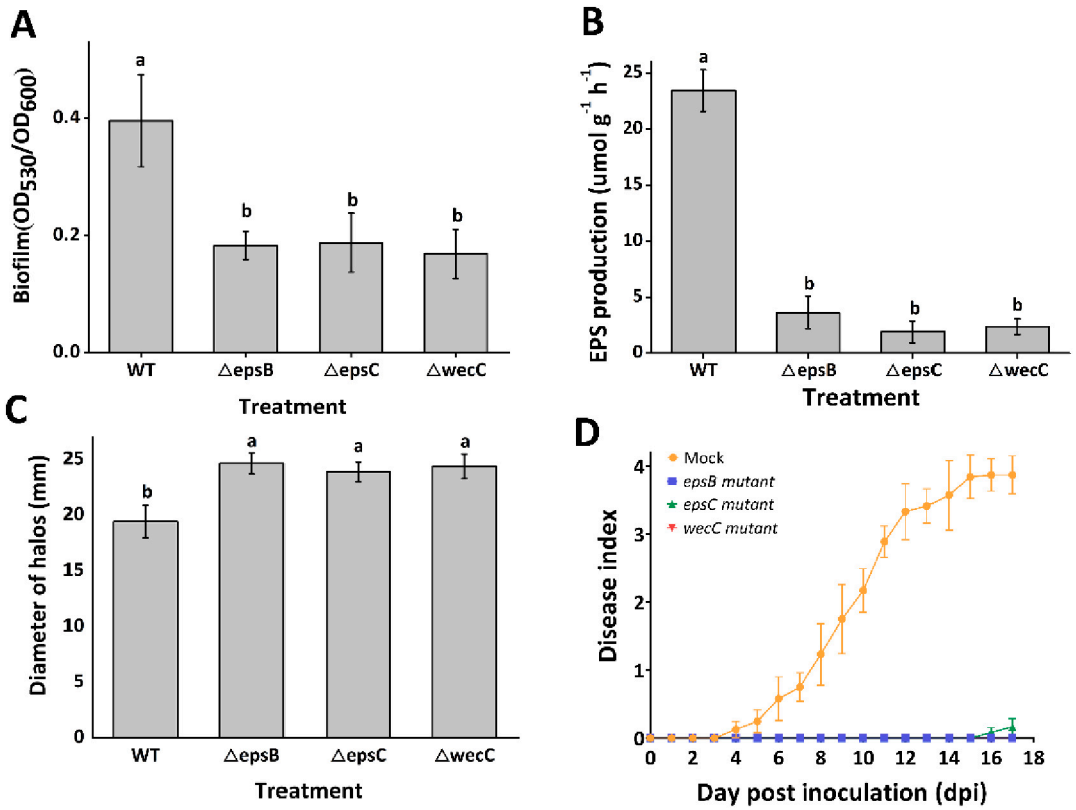

Fig. 5. Extracellular polysaccharide biosynthesis genes are necessary for biofilm formation and virulence on tobacco plants. (A) Biofilm formation of *R. solanacearum* in polystyrene microtiter plates. Bacterial suspension were inoculated in rich medium and kept at 30 °C for 24 h without shaking. Biofilm formation was measured by OD530 after stained with crystal violet. (B) Effect of eps mutants on the EPS production in liquid medium. (C) Swimming motility of *R. solanacearum* in minimal medium. The diameter of swimming halo was measured after 48 h cultivation at 30 °C. (D) The disease index of wild-type (CQPS-1) and *eps* mutant on tobacco plants. Each bar represents the mean ± SE of eight replicates. Different letters indicate significant differences between different treatments according to one-way ANOVA with Duncan's test ($P < 0.05$).

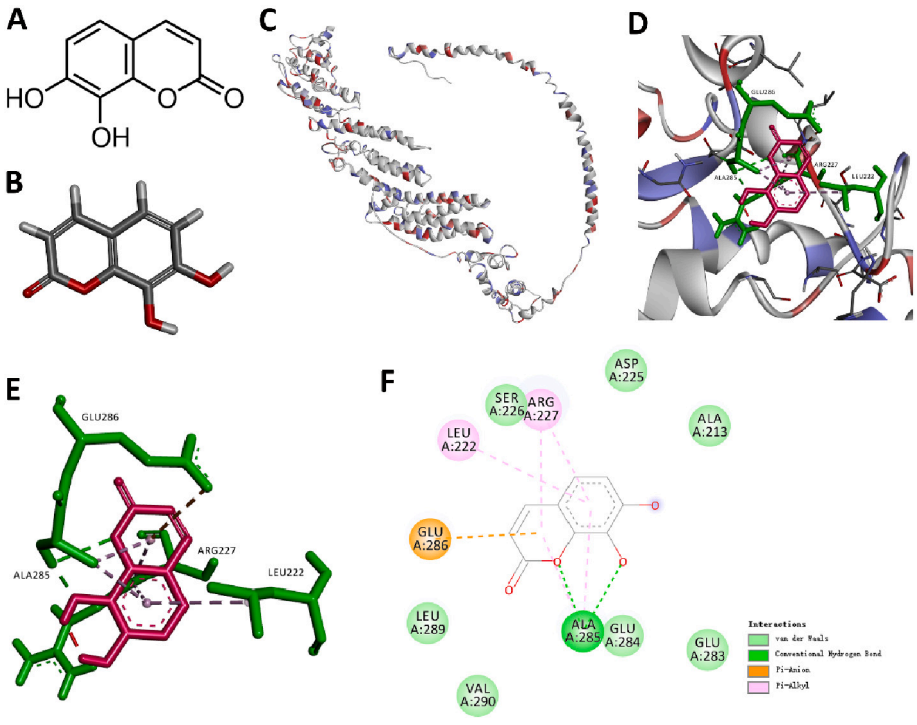

Fig. 6. Molecular docking of EpsB protein to daphnetin. (A) Chemical structural formula of daphnetin. (B) The cartoon representation of daphnetin. Red regions represent oxygen atoms; gray region indicate carbon atoms. (C) Homology modeling 3D-structure of EpsB. (D) Best conformation of daphnetin docked to binding pocket of EpsB. (E) The recognized binding modes and molecular interactions of daphnetin in the active site of EpsB. (F) The two-dimensional interactions scheme of daphnetin to EpsB.

implicated that major exopolysaccharide, EPS I, is associated with the feedback loop in the quorum sensing of *R. solanacearum* strain OE1-1 (Hayashi et al., 2019). Interestingly, *epsB* plays key role in quorum sensing of *R. solanacearum*. Recent study found that 3-hydroxy-8-phenyl-octanoate inhibited quorum sensing and biofilm formation (Yoshihara et al., 2020). Then, we found that *epsB* is required for EPS production, biofilm formation of *R. solanacearum* and *epsB* mutant showed non-pathogenicity on tobacco plants (Fig. 5). Molecular docking and homology modeling are novel and effective approaches to characterize conformation protein-ligand interaction patterns (Zhou et al., 2021). Our docking results indicated that the critical residues of domain in the binding pocket of the EpsB protein, such as LEU222, ARG227, ALA285, and GLU286, interact with daphnetin via conventional hydrogen bonding and hydrophobic interactions (Fig. 6). These results indicated that daphnetin might suppressed the quorum sensing by inhibiting the expression of *epsB*, and then affect the other virulence associated genes.

Certain plant phenolic compounds (salicylic acid, p-coumaric acid, trans-4-hydroxycinnamohydroxamic acid, 4-methoxy-cinnamic acid, benzoic acid, trans-2-phenylcyclopropane-1-carboxylic acid, N-(4-methoxycinnamyl) phthalimide) were determined as type III secretion system (T3SS) inhibitor of plant pathogens (Fan et al., 2017; Khokhani et al., 2013; Li et al., 2015; Li et al., 2009; Puigvert et al., 2018). Coumarins are naturally secondary metabolites via the phenylpropanoid pathway in several different plant species (Stringlis et al., 2019). In the previous study, we found hydroxycoumarins suppressed virulence of *R. solanacearum* on tobacco plants, and umbelliferone was demonstrated as a T3SS inhibitor and showed control effect of tobacco bacterial wilt supplemented pre-application treatment (Yang et al., 2017; Yang et al., 2018). In our study, the global transcriptional response of *R. solanacearum* to daphnetin indicated that exposure to this chemical is stressful to the pathogen, consistent with our previously observation that a higher daphnetin concentration (64 mg L^{-1}) inhibits growth of *R. solanacearum* (Yang et al., 2016). *R. solanacearum* manipulated a variety of virulence factors to develop bacterial wilt process when invade to plant tissues, such as EPS, flagella, biofilm, T3SS and type IV pili (Genin and Denny, 2012). In this study, daphnetin repressed diverse genes encoding translational machinery and ribosomal, and daphnetin induced expression of oxidative stress genes, drug efflux pomp genes, siderophore biosynthesis genes. Furthermore, exposure to daphnetin, *R. solanacearum* cells displayed reduced expression of virulence genes encoding type III secretion system, type IV secretion system, and extracellular polysaccharide biosynthesis enzymes.

5. Conclusions

In conclusion, daphnetin significantly reduced EPS production and biofilm formation through inhibiting the expression of EPS biosynthesis genes (*epsA*, *epsP*, *epsB*, *epsC*, *wecC*, *epsE* and *epsF)* and transcriptional regulator factor *xpsR*, resulted delaying tobacco bacterial wilt disease progress and reducing bacterial density in the stem. RNA-Seq results indicated daphnetin alter the transcriptional expression of *R. solanacearum* via significantly suppressed EPS biosynthesis cluster genes, T3SS and T4SS. Finally, the Δ*epsB* mutant displayed key role for EPS production and virulence of *R. solanacearum* on tobacco plants. The critical residues of domain in the binding pocket of the EpsB protein interacted with daphnetin via conventional hydrogen bonding and hydrophobic interactions. This study suggests that daphnetin is a very promising plant metabolite for the development of a virulence factor inhibitor for controlling bacterial wilt.

Author contributions

WD, LY conceived and designed the experiments. LY, ZW, SL, RX, QX performed the experiments. LY, ZW, RX analyzed the data. LY, WD wrote the paper.

Declaration of Competing Interest

We declare that no competing interests exist.

Acknowledgement

The research was supported by the key project of the China National Tobacco Corporation (110201901042), the National Natural Science Foundation of China (31972288), the Chongqing Special Postdoctoral Science Foundation (Xm2020096).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.pestbp.2021.104948.

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