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A Methyl Esterase 1 (PvMES1) Promotes the Salicylic Acid Pathway and Enhances Fusarium Wilt Resistance in Common Beans

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Abstract

Common bean (*Phaseolus vulgaris* L.) is an important food legume. Fusarium wilt caused by *Fusarium oxysporum* f. sp. *phaseoli* is one of the most serious soil-born diseases of common bean found throughout the world and affects the yield and quality of the crop. Few sources of Fusarium wilt resistance exist in legumes and most are of quantitative inheritance. In this study, we have identified a methyl salicylate esterase (MES), PvMES1, that contributes to plant defense response by regulating the salicylic acid (SA) mediated signaling pathway in response to Fusarium wilt in common beans. The result showed the role of PvMES1 in regulating SA levels in common bean and thus the SA signaling pathway and defense response mechanism in the plant. Overexpression of the *PvMES1* gene enhanced Fusarium wilt resistance; while silencing of the gene caused susceptibility to the diseases. RNA-seq analysis with these transiently modified plants showed that genes related to SA level changes included the following gene ontologies: a) interaction between host and pathogen; b) phenylpropanoid synthesis; and c) sugar metabolism as well as others. These key signal elements activated the defense response pathway in common bean to Fusarium wilt. Collectively, our findings indicate that PvMES1 plays a pivotal role in regulating SA biosynthesis and signaling, and increasing Fusarium wilt resistance in common bean, thus providing novel insight into the practical applications of both SA and MES genes and pathways they contribiute to for developing elite crop varieties with enhanced broad-spectrum resistance to this critical disease.

Introduction

Common bean (*Phaseolus vulgaris* L.) is major source of fiber, minerals, and protein to the diet with food calories and amino acids of legumes being second in importance only to cereals and animal sources (Blair et al. 2012&2016). Bean seeds are also nutraceutically important for humans (Suárez-Martínez et al. 2016). Common bean is a species that is plagued by many plant diseases especially those caused by fungal pathogens (Schwartz and Hall 2005). A major fungal disease of common bean is Fusarium wilt, avascular disease of great economic importance globally and in various crops, which in *P. vulgaris* is caused by *Fusarium oxysporum* f. sp. *phaseoli* (*Fop*). The pathogen has been found and identified in most of the bean-growing regions of the world and causes significant yield losse specially in wet highland environments or intensively cropped lands without adequate rotation (Buruchara and Camacho 2000).

Plants have evolved diverse mechanisms to resist the microorganism infestations. Plants triggers the activation of local defenses at the infection sites that lead to a secondary immune response termed systemic acquired resistance (SAR) in distal tissues (Fu and Dong 2013; Zhou and Zhang 2020). To date, a few molecules have been proposed as mobile signals leading to SAR (Dempsey and Klessig 2012; Klessig et al. 2018). Salicylic acid (SA) is considered as a classical phytohormone with critical roles in both local defense and SAR (Vlot et al. 2009; Zhang and Li 2019; Ding and Ding 2020). It demonstrated that SA accumulation is required for the establishment of resistance in both local and systemic tissues and exogenous SA was also indicated to play a role as an internal defense signal for plant immunity (Fu

and Dong 2013; Varanasi and Talati 2014). SA has also been shown to contribute to shaping the microbial populations associated with the plant roots and lead to enhanced plant fifitness in response to threats from pathogens (Lebeis et al. 2015). These have been shown to be the case in Fusarium wilt interactions with common bean as well (Xue et al. 2014). Thus, the roles of SA as a defense hormone have been greatly expanded.

Salicylic acid-binding protein 2 (SABP2) has an esterase activity that mediates hydrolysis of methyl salicylate (MeSA) to SA. It appears to be an essential enzyme for the SAR response in many plant species, working by releasing a long-distance signal required in phloem tissues that results in defense responses in infected plants of *Arabidopsis thaliana*, tobacco and potato. Transgenic tobacco plants with silenced *SABP2* gene that are infected with TMV are compromised in their ability to develop SAR (Kumar and Klessig 2003). By contrast, SA synthesized by SABP2 in tobacco plants is essential for transcomplementing the loss of SAR development in *SABP2*-silenced plants (Kumar et al. 2006). In *Arabidopsis*, three orthologs of tobacco *SABP2* expressed in *SABP2*-silenced tobacco plants complement for SAR deficiency (Vlot et al. 2008). The potato plants with suppressed activity of tobacco SABP2 ortholog (*StMES1*) compromised SAR against the challenge of *Phytophthora infestans* (Manosalva et al. 2010).

Our goal for this study was to determine the roles of SABP2 and MeSA in the SA mediated resistance pathway and defense mechanism of the common bean – Fusarium wilt pathosystem. Previously, we cloned seven orthologous genes of tobacco *NtSABP2* genes in common bean (Xue et al. 2018). However, it was not very clear whether the orthologs of SABP2 proteins in common bean functioned in defense responses. Therefore, in the current study, we analyzed one of the orthologs responsed to *Fop* infection most dramatically, named *Phaseolus vulgaris methyl esterase 1* (*PvMES1*), to assess its role in the SA mediated resistance signaling pathway against Fusarium wilt. After cloning of the entire gene, our specific objectives were to determine the biological/physiological significance of the *PvMES1* gene and transcript encoding product in the disease pathosystem by analyzing 1) *in vitro* esterase activity of the enzyme; 2) function when overexpressed; 3) results when gene silenced; and 4) transcriptome profiling of differentially expressed genes (DEGs) between control and *PvMES1* overexpressed/silenced plants.

Materials And Methods

Materials and growth conditions

Common bean genotypes BRB130 (Fusarium wilt susceptible) and CAAS260205 (Fusarium wilt resistant) were obtained from the Institute of Crop Sciences of the Chinese Academy of Agricultural Sciences (CAAS) in Beijing, China (Xue et al. 2015). Ten-day-old greenhouse grown plants were used for the varying treatments at the fully-expanded, ‘cotyledonary’ leaf, seedling, growth stage. Treatments included 1) control with no applications; 2) infection by an aggressive *F. oxysporum* f. sp. *phaseoli* isolate, FOP-DM01 (*Fop*) at a density of 5.0×10^6 colony forming units, cfu g^{-1} at 12 d after inoculation with the bean pod mottle virus (BPMV)-derived vectors or leaf extracts challenged by vectors; or 3) spraying of the

leaves for five consecutive days with 2 mM salicylic acid (SA) diluted in Tween 20 (0.02 % v/v) using previous described methods (Xue et al. 2014). The three plants for each treatment and each time point were fertilized every week from then on. The plants were grown in a greenhouse maintained at approximately 22–28°C. All treatments were grown in the same greenhouse with normal daylight with supplemental lighting for a total of 14 days. Root tissues were harvested at 0, 24, 48, 72, 96 and 120 hours post inoculation (hpi) in the case of *Fop* treatment and with an additional 6 hours collection for SA treatment.

RNA extraction and quantitative real-time PCR analysis

Total RNAs were extracted from root samples of the treatments described above using TRIzol reagent followed by DNAse treatment (Tiangen, China). These were quantified by Nanodrop 2000 spectrophotometer and qualified by running in a gel electrophoresis unit. A total of 200 ng of RNA was then used to synthesize cDNA and subsequently used as a template for PCR and quantitative real-time PCR (qRT-PCR) analysis using a reverse transcription kit (Tiangen) according to manufacturer's protocol. Expression analysis was performed using a qTOWER 2.2 Real-time PCR system (Analytikjena, Germany) with three biological replicates and the actin gene as an internal control to standardize the data (Chen et al. 2009). Threshold values (CT) were used to quantify relative gene expression followed by the comparative $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001). Primers used in this study are listed in Table S2.

PvMES1 **gene cloning and bioinformatic analysis**

Gene-specific primers (MES-F1/MES-R1) were used to amplify the full-length gene both from a cDNA source and for the full-length genomic (gDNA) copy of the *PvMES* gene from common bean, *P. vulgaris*. The cDNA and gDNA amplified fragments were cloned into a pMD18-T vector and sequenced. The PCR products were sequenced with an ABI3730xl DNA analyzer using standard techniques and consumables by the Sanger method. Sequence homology and gene identity was determined using BLAST software (Altschul et al. 1990) and the nucleotide database at NCBI (www.ncbi.nlm.nih.gov). Other MES protein sequences from different plants were downloaded from Genbank and compared with the PvMES1 sequence. Bioinformatic analysis of *PvMES1* gene included the following aspects: number of amino acids (NA), molecular weight (MW), and isoelectric points (pI) predicted using the protein/peptide characterization tool ProtParam provided on the ExPASy (Expert Protein Analysis System) proteomics server of the Swiss Institute of Bioinformatics (http://www.expasy.org/tools/protparam.html). The signal peptide and subcellular localization were predicted by iPSORT (http://ipsort.hgc.jp/), and WoLF PSORT (https://www.genscript.com/wolf-psort.html) programs, respectively. The three-dimensional structure of the protein was constructed using the Swiss-model Workspace Package (http://swissmodel.expasy.org/) and confirmed with PyMol software (http://www.pymol.org).

Protein expression and activity assay

The *PvMES1* gene was also cloned into an expression vector, pET28A, and this construct was used to transform *E.coli* BL21(DE3) for protein expression. The resulting 6×His-tagged recombinant protein was

purified using Ni Sepharose^{TM6} Fast flow columns (GE Healthcare) following the manufacturer's instructions. The purified protein concentration was determined using the BCA protein assay kit (Pierce, Rockford, IL). MeSA esterase activity was measured by a two-step radiochemical esterase assay using 0.5μg of purified PvMES1 transcripts encoding product incubated with MeSA as the substrate (Forouhar et al. 2005).

SA quantification

To evaluate MeSA conversion to SA, all root samples were extracted and quantified, then analyzed automatically on a Wakopak LC 2010A reversed-phase HPLC with C18 analytical column (PTH 250×4.6 mm) from Wakosil (Wako Inc., Osaka, Japan) using our previously described method (Xue et al. 2014).

Fungal quantification

For quantification of bean root pathogen colonization, total genomic DNA was extracted directly from 500 mg of fresh plant root tissues using a Tiangen Plant Genomic DNA kit (Tiangen Inc., Beijing, China) as described by the manufacturer. The primer pair Q*Fop*A/Q*Fop*B reported by Xue et al. (2014) was used for amplification and quantification of pathogen DNA, and the reaction conditions were adjusted experimentally to optimize the PCR protocol.

Measurement of pathogen defense-related factors

A series of pathogen induced pathways were measured based on activity of key enzymes: Phenylalanineammonialyase (PAL) activity was assayed following an established method (Assis et al. 2001), with some modifications; Peroxidase (POD) activity was assayed according to a previous method as well (Do et al. 2003); Superoxide dismutase (SOD) activity and malondialdehyde (MDA) content were assayed following the method (Zhang et al. 2019). Total hydrogen peroxide (H_2O_2) content was measured according to Sagisaka (1976), with a few modifications: namely, absorbance of the supernatant was measured at 480 nm after 12,000 rpm spin of the reaction mixture in an E5804 centrifuge (Eppendorf, Germany).

Carbohydrate, phenolics and lignin analysis

Samples of approximately 0.5 g of roots each were subject to extraction for 30 min with 10 ml of an ethanol/watermixture (80:20, v/v) used as a high temperature (80°C) extraction solvent. The solution was filtered and evaporated to dryness under nitrogen, with an aliquot of the filtrate redissolved in 0.5 ml of 0.1 mM ethylenediaminetetraacetic acid calcium disodiumsalt (CaEDTA) solution and filtered through a 0.45 μm Milipore filter before being used for carbohydrate analysis. Glucose, fructose and sucrose were estimated enzymatically as described (Morkunas et al. 2005), and starch was measured by the method (Fredeen et al. 1989). Total phenolic and lignin content was measured as previously described (Velioglu et al. 1998; Syros et al. 2004).

Preparation of gene silencing and overexpression constructs, infectious clones from transcription

An *in planta* gene silencing vector was prepared using a 501-bp DNA fragment amplified from the *PvMES1* cDNA clone using sequence-specific primers (MESvigs-F/R) with the *BamH*I and *Cla*I restriction sites. Simultaneously, a *PvMES1* overexpression vector for the same BPMV system was made using the open reading frame (ORF) fragment amplified from the *PvMES1* cDNA clone by the primer pair OEMES-F/R with the same restriction enzymes. The amplified PCR products were gel purified and digested with *BamH*I and *Cla*I and subcloned into pG7R2V previously digested with the same restriction enzymes resulting in silencing and overexpression vectors, pG7R2V-501 and pG7R2V-*PvMES1*, respectively. The empty vector plasmid pG7R2V was digested by *Msc*I and then set aside to use as a control in both inoculations. All vectors were further verified by Sanger sequencing.

In vitro transcription was conducted with the plasmid constructs above using RiboMAX™ Large Scale RNA Production Systems Kit (Promega, Madison, WI, USA) following the manufacturer's instructions. Yield and integrity of the transcripts were analyzed by electrophoresis on a 1.0% agarose gel.

BPMV-derived vectors used for inoculation

The pG7R2V-501 and pG7R2V-*PvMES1* RNAs were separately expressed and used as RNA2 transcripts in combination with RNA1 from the infectious BPMV viral strain Kentucky-Hopkins (K-Hop). These combinations were used for inoculation of common bean seedlings planted in a greenhouse in Shenyang, Liaoning province at Liaoning Academy of Agricultural Sciences (LAAS). Inoculation was done on the primary leaves by rubbing with a sponge/carborundum. However, specially for gene overexpression analysis, leaf extracts prepared from plants infected with empty vector and pG7R2V-*PvMES1* vector by the method above were used to treat the primary leaves of ten-days-old plants as control and treatment (Díaz Camino et al. 2011). The wild type plants were also used as controls of both analysis.

Transcriptome sequencing

RNA-seq experiments for transcriptome sequencing were designed with the infected plants described above, consisting of infection of ten-day-old seedlings in all cases. Treatments consisted of seedlings infected with pG7R2V-*PvMES1*(overexpression) labeled by O-*PvMES1* and with pG7R2V-501 (gene silencing) labeled by S-*PvMES1*. The seedlings treated with leaf extracts from plants infected with empty vector were used as the control of O-*PvMES1* named by E-BPMV1, and plants treated with the empty pG7R2V vector were used as the control of S-*PvMES1* named by E-BPMV2. The wild type plants were also used as controls for both treatments. Root tissues were collected at 12d (0 d post inoculation with *Fop*) and 15 d (3 d post inoculation with *Fop*) after inoculation by the BPMV-derived vectors. Each treatment and control was designed with three biological replicates (8 seedlings each). The libraries were made with indices for identification, then sequenced using an IlluminaHiSeq 2500 at BioMarker Technologies.

Bioinformatics data analysis

The statistical analysis of the RNA-seq data was performed by means of two-way ANOVA for the factors of genotype (empty BPMV control versus *PvMES1* overexpression versus *PvMES1* silencing) and

pathogen infection (non-infected control versus 5.0×10^6 cfu g^{-1} *Fop* inoculation). In detail, this involved cleaning reads and mapping them to the genome after examining sequence quality and removing low-quality sequence using Tophat v. 2.0.1. We compared our sequences to those of the common bean genome (*Phaseolus vulgaris* L, 2n = 22) from Phytozome v12.1 (https://phytozome.jgi.doe.gov/pz/portal.html). The transcriptome assemblies were performed for each library by Cufflinks (Trapnellet al., 2012). To estimate the read count of each gene in each library, the mapping results were filtered to maintain only uniquely mapped reads before being being piped into Cuffdiff (http://cole-trapnelllab.github.io/cufflinks/). The fragments per kilobase of exon per millionfragments mapped (FPKM) value was calculated using the internal script of the count table based on Cuffdiff's output. DESeq was used to detect DEGs according to Anders and Huber (2010). The thresholds to identify DEGs and the corrected *P*-values from this method accounted for multiple tests by using the key factor, which was based on a false discovery rate (FDR) < 0.01 and fold change >2 or < 0.5. The assembled sequences were compared with the nonredundant database (NR, Deng et al. 2006) from NCBI, the Swiss-Prot database (Apweiler et al. 2004), the GO database (Ashburner et al. 2000), the Clusters of Orthologous Group database (COG, Tatusov et al. 2000) and the Kyoto Encyclopedia of Genes and Genomes database (KEGG, Kanehisa et al. 2004). Fold changes in the expression levels between samples were used as the criteria in the screening process.

Results

Cloning and characterization of the PvMES1 gene

Previous study indicated seven SABP2's putative orthologs were found and designated as PvMES1-PvMES7 in common bean, all with strong sequence homology to NtSABP2 (Table S1). The members of the *PvMES* gene family can be divided into three subfamilies according to the genetic relationship with the *NtSABP2* (Figure S1). The expression level of seven *PvMES* genes in susceptible varieties BRB130 and resistant varieties CAAS260205 infected by *Fop* was detected by qRT-PCR (Xue et al. 2018). The results showed that the expression of *PvMES1* was significantly upregulated at 3 dpi, with most significant increase of 5.6 folds in CAAS260205. Thus *PvMES1* gene was selected for this study to continued all our work. The genetic locus for *PvMES1* was found at Phvul.003G248200.1 located from bp position 48,561,200 to 48,564,505 on Chromosome 3, with 59.2% sequence identity with *NtSABP2* (Table S1).

The *PvMES1* gene was found to contain three exons and two introns (Fig. 1a). The original cDNA clone indicated that the mature mRNA sequence contained a single ORF of 834 bp encoding a 277-aa extracellularly secreted protein with a calculated molecular mass of 30.99 kDa and a putative 22 amino acid signal peptide highlighted in red (Fig. 1b). The protein also contained a catalytic triad (Ser102, His227, and Asp255) that formed part of the active site pocket (Fig. 1c, highlighted in orange and red), also highly conserved among other NtSABP2 orthologs from *Arabidopsis* (AtMESs, Vlot et al. 2008) and potato (StMES1, Manosalva et al. 2010). Finally, PvMES1 contained 14 of the 15 aa identified in

NtSABP2 that interact with SA (Fig. 1b and c, highlighted in yellow and orange), suggested that these two proteins have similar biological functions and biochemical properties.

A full-length gene with a sequence length of 973bp was cloned from the common bean genotype CAAS260205 infected by *Fop* using RT-PCR method and sequenced (Figure S2). Comparison of the 973bp sequence with non-redundant (nr) sequences in the GenBank nucleotide database indicated that the cDNA fragment isolated was a member of *SABP2* gene family, named and deposited in the database as *PvMES1* (Genbank accession no. MT471345). The 3265 bp full-length genomic (gDNA) fragment was also obtained which contained a 3182 bp gene sequence (Figure S2).

Phenotypic and PvMES1 gene expression differences under Fop infection

To further analyze the biological role of the *PvMES1* gene we evaluated its expression in contrasting Fusarium wilt susceptible and resistant genotypes. Notable phenotypic differences between BRB130 and CAAS260205 symptomatology were found when infected with *Fop* (Figure S3). We followed this phenotypic characterization with a time course study to evaluate the expression of *PvMES1* under *Fop* infection and SA treatments using qRT-PCR and relative expression compared to controls. We found that the transcription of *PvMES1* in the infected but resistant CAAS260205 roots was strongly and rapidly induced by *Fop* infection especially at 48 and 72 hpi (Fig. 1d). In response to SA treatment, *PvMES1* expression was repressed over the period from 24 to 72 hpi in both genotypes and then gradually recovered to the original expression level when CAAS26025 had higher SA induced expression of the gene (Fig. 1e).

PvMES1 displays SA-inhibited MeSA esterase activity

To determine the biochemical properties of PvMES1, its corresponding full-length cDNA was expressed in *Escherichia coli*, the protein affinity purified, and analyzed for MeSA esterase activity. The results showed that the C-terminal His×6-tagged PvMES1 transcript encoding product was successfully expressed and found isolated to an inclusion body. Dissolved in an 8 M urea solution, it electrophoresed well and was estimated to have a molecular weight of 30 kDa (Fig. 1f). Purified *PvMES1* transcript product readily converted MeSA to SA. Simultaneously, we also tested the transcript product in a SA-binding assay and found that *PvMES1* transcript encoding product reproducibly bound SA, and that the esterase activity was dramatically inhibited by SA when an LC_{50} reached approximately 6.2 μM (Fig. 1g).

Overexpression and silencing of PvMES1 gene in roots mediated by BPMV-based method

The transcript product mixture of recombinant the pG7R2V constructs (pG7R2V-*PvMES1* for overexpression and pG7R2V-501 for silencing, respectively) and pGHopR1 containing RNA1 from BPMV were inoculated as infective virus onto the fully expanded unifoliate leaves of plants which would be later infected by *Fop* at 12 dpi after inoculation with the virus. The difference in disease phenotype between the wild type (WT) plants, E-BPMV1 controls and *PvMES1*-overexpressed plants was even more noticeable at 4 weeks post inoculation with *Fop* (Fig. 2a, upper portion). Similarily, the typical stem

browning and leaf yellowing phenotype of *PvMES1*-silenced plants appeared earlier than WT and its E-BPMV2 control, even at 2 weeks after *Fop* infection, *PvMES1*-silenced plants almostly withered away, while Fusarium wilt symptoms just occurred on the control seedlings of WT and E-BPMV2 obviously (Fig. 2a, lower portion). Disease scores were significantly different ($P < 0.05$) in treatments versus controls (Fig. 2b).

The qRT-PCR results indicated that fungal pathogen DNA provided evidence for the level of *Fop* infection and was significantly lower ($P < 0.05$) in roots of *PvMES1*-overexpressed plants compared to control roots at 18 dpi, but higher in the *PvMES1*-silenced plant roots than the control (Fig. 2c). Gene expression analysis showed levels in the *PvMES1*-overexpressed plants were significantly higher than control at 6 dpi with the BPMV vector and continued to increase up to a 13.6-fold difference at 18 dpi by BPMV (6 dpi by *Fop*) (Fig. 2d, upper portion). The *PvMES1*-silenced plants had lower gene expression every day of measurement during dual fungal and viral infection, which reduced with 0.51-fold difference compared to control at the final time point (Fig. 2d, lower portion). Similar patterns were seen with MeSA esterase activity and SA accumulation. The MeSA esterase activity of *PvMES1*-overexpressed plants increased dramatically during the infection with BPMV, especially inoculated with *Fop* pathogen, reaching a maximum of 0.43 nmol/min/μg at 18 dpi with BPMV (Fig. 2e, upper portion), and only 0.024 nmol/min/μg in the silenced plants at the same time point (Fig. 2e, lower portion). Correspondingly, SA accumulation in *PvMES1*-overexpressed plants constantly increased with BPMV inoculation, especially after infection by *Fop*, which almost double that of control at 18 dpi with BPMV (Fig. 2f, upper portion). In *PvMES1*-silenced plants, the SA level significantly decreased sharply declined after 12 dpi with BPMV (Fig. 2f, lower portion). These results strengthened the hypothesis that *PvMES1* gene played a vital role in the resistance against Fusarium wilt through the level regulation of the active free SA in common beans.

Transcriptome profiling of PvMES1-regulated genes in Fop infected bean roots

To comprehensively understand the effects of *PvMES1* on the resistance to Fusarium wilt in common beans, we performed RNA-seq experiments and compared the transcriptome profiles: 1) *PvMES1*-overexpressed DEGs versus *Fop* infected DEGs versus both *PvMES1*-overexpressed and *Fop* infected DEGs (Fig. 3a); 2) *PvMES1*-silenced DEGs versus *Fop* infected DEGs versus both *PvMES1*-silenced and *Fop* infected DEGs (Fig. 4a). The transcriptome sequencing results of WT plants have no significant difference from E-BPMV1 and E-BPMV2 (Data not shown), thus we respectively selected E-BPMV1 and E-BPMV2 as controls of gene overexpression and silencing for the transcriptome analysis. The numbers of total reads, mapped reads, and the percentage of mapped reads in each replicate are shown in Figure S4. Principal component analyses showed that both *Fop* attack and *PvMES1*-regulation profoundly influenced the transcriptome results (Figure S5).

The number of DEGs was 1123 in O-*PvMES1*(-*Fop*) versus E-BPMV1 (-*Fop*), with 506 upand 617 downregulated genes, while the DEGs in E-BPMV1 (+ *Fop*) versus E-BPMV1 (-*Fop*) included 2072 with 798 upregulated and 1274 downregulated genes (Fig. 3b; Data S1). Comparing O-*PvMES1* (+ *Fop*) versus E-BPMV1(-*Fop*) resulted in 2330 DEGs (663 up and 1667 down-regulated genes) of which 1463 (386 up

and 1077 down) were affected by the pathogen. Many of these pathogen-induced genes (70.6%) were also regulated by *PvMES1* in the gene overexpression experiment (Fig. 3b). In the interaction, 589 DEGs (173 up and 416 down-regulated) were affected by *PvMES1* overexpression. Among the upregulated pathogen-induced DEGs, 93.3% (360 of 386) of the genes were upregulated by both pathogen and PvMES1. Almost all the genes downregulated by pathogen (1074 of 1077; 99.7%) were also decreased by *PvMES1* (Fig. 3c, d).

In the gene silencing comparision, theb number of DEGs was 955 in S-*PvMES1* (-*Fop*) versus E-BPMV2 (-*Fop*), with 222 upregulated and 733 downregulated genes. The DEGs in E-BPMV2 (+ *Fop*) versus E-BPMV2 (-*Fop*) were 2400 (737 up and 1663 down-regulated). In the comparison of S-*PvMES1* (+ *Fop*) versus E-BPMV2 (-*Fop*) there were 2468 DEGs of which 918 were upregulated and 1550 were downregulated (Fig. 4b; Data S2). Among the *PvMES1*-silenced and pathogen-induced genes, 1557 were regulated by the fungal pathogen, 64.9% of the total; and among these 487 were upregulated and 1070 were downregulated. Interestingly, 99.8% (486 of 487) of the genes upregulated by the fungal pathogen were also upregulated by *PvMES1*. Conversely, 97.8% (1046 of 1070) of the genes that were down-regulated by the fungal pathogen were also down-regulated by *PvMES1* (Fig. 4c,d).

GO analysis indicated that genes regulated by *Fop* and *PvMES1* are enriched in three categories of biological process, cellular component and molecular function (Figure S6). KEGG analysis of the genes in the interaction term indicated that DEGs induced by both affects are enriched in pathways related to phenylpropanoid biosynthesis, starch and sucrose metabolism, plant hormone signal transduction, plant-pathogen interaction confirming that *PvMES1* plays a key role in orchestrating the transcription of pathogen-induced genes (Figure S7).

Phenylpropanoid pathway-related genes upregulation promotes lignin biosynthesis

A subset of genes participating in multiple branches of the phenylpropanoid pathway, including lignins, flavonoids, and cinnamaldehyde, were identified based on RNA-seq analysis (Fig. 5a). All of the enzymes indicated with red boxes were significantly upregulated by the enhanced SA biosynthesis induced by *PvMES1* overexpression. When *PvMES1* was silenced, the DEGs in red boxes are those downregulated both before and after *Fop* inoculation. Enzymes such as 4-coumarate–CoA ligase, cinnamoyl-CoA reductase, mannitol dehydrogenase and peroxidase were upregulated in O-*PvMES1* but downregulated in S-*PvMES1* versus control with or without *Fop* inoculation shown in a heatmap with $\log_2$ FPKM (Fig. 5b; Data S3). The result was further verificated by qRT-PCR analysis (Fig. 5c). Pathogen defense-related indices such as PAL, SOD, POD activity, H_2O_2 contents, total phenolic and lignin contents were significantly higher in the *PvMES1*-overexpressed, and lower in the *PvMES1*-silenced plants compared to the controls after 72 h post inoculation with *Fop*, but MDA contrastly changed (Figure S8).

Sugar metabolism pathway modulating defense response to Fusarium wilt

Several branches of amino sugar and nucleotide sugar metabolism, starch and sucrose metabolism pathways showed DEGs regulated by *PvMES1* expression changes. Those which were significantly upregulated by SA enhancement were marked with the red boxes while downregulated genes were marked in blue boxes (Fig. 6a,b). The production of sugar molecular such as glucose, fructose, sucrose and their phosphorylated derivatives as defense signalling factors were indicated to be affected by the pathosystem and promoted by the upregulation of relevant enzymes. Cellulose and pectinas the essential components for cell wall reinforcement upon fungal infection were also regulated by *PvMES1* expression degree change. Digital transcriptome expression analysis of sugar metabolism DEGs were shown in a heatmap with $\log_2$ FPKM values (Fig. 6c; Data S4). The qRT-PCR results showed that ten key genes in the sugar metabolism pathway were significantly upregulated by overexpressing *PvMES1* and downregulated by silencing *PvMES1* (Fig. 6d). We also analyzed sugar content in the roots of controls and treatment plants infected by *Fop*. Glucose, fructose, sucrose and starch concentration in the roots of beans were increased by *PvMES1* overexpression, but declined during *PvMES1* silencing compared with each corresponding control after 24 h post inoculation with *Fop* (Figure S9).

Expression of hormone signaling-related genes

Selected hormone signaling-related genes encoding receptors and response factors, identified from KEGG pathway analysis, were shown to be affected by *PvMES1* levels (Fig. 7a, Data S5). SA response factors TGA and PR-1 were upregulated in *Fop*-inoculated roots of common bean by *PvMES1* overexpression and downregulated by *PvMES1* silencing, These results indicated the change of SA level had a closed relationship with *PvMES1* expression degree. Meanwhile, JAZ and MYC2 from jasmonic acid (JA) mediated signaling, and AUX1, AUX/IAA from auxin mediated signaling were significantly repressed by *PvMES1* overexpression and induced to high expression levels by *PvMES1* silencing with or without *Fop*, whereas, ARF and GH3 were activated. The response factors from other selected plant hormone have no obvious connection with the defense response to *Fop* mediated by SA in common beans. The expression levels of the selected hormone signaling-related genes were analyzed by qRT-PCR (Fig. 7b).

Interaction mechanism regulating the oxidative burst and calcium transporting

To investigate whether the *PvMES1* had a role in oxidative burst or calcium transport based *Fop* resistance in common bean, we evaluated genes from the host-pathogen interaction signaling pathway found in KEGG (Fig. 8a, Data S6). The result showed that a calcium-dependent protein kinase (Phvul.001G135300) was upregulated by *PvMES1* overexpression, but downregulated in *PvMES1*-silenced plants after *Fop* infection, We also did see the upregulations of a pair of respiratory burst oxidases (Phvul.002G293700, Phvul.006G090200) with *PvMES1* gene overexpression. In contrast, two cyclic nucleotide-gated ion channels (Phvul.008G036200, Phvul.010G113100), calmodulin (Phvul.001G155400) and calcium-binding protein (Phvul.001G095600) were repressed in O-*PvMES1*, and activated significantly in S-*PvMES1* post inoculation with *Fop*. The expression level of selected DEGs in

the interaction pathway were identified through the heatmap analysis based on log_2FPKM values (Fig. 8b).

Activation of a PvMES1-mediated defense signaling pathway in common bean confers resistance to Fusarium wilt

To better understand how *PvMES1*-mediated *Fop* resistance is regulated, we performed RNA-seq to examine the expression of 17,037 genes in the controls, *PvMES1*-overexpressed and -silenced plants (Fig. 9a; Figure S7; Data S7). Further comparisons of the RNA-seq data of O-*PvMES1*, S-*PvMES1* and control plants at 72 hpi (FDR-adjusted *P* value < 0.01) revealed 1,450 DEGs. Compared to the control E-BPMV1, 228 upregulated and 550 downregulated genes were identified in O-*PvMES1*, whereas 590 upregulated and 442 downregulated genes were identified in S-*PvMES1* compared with control E-BPMV2. Inaddition, 116 genes were upregulated in O-*PvMES1*, but downregulated in S-*PvMES1*, and 145 genes were downregulated in O-*PvMES1*, but upregulated in S-*PvMES1* after infection with *Fop* (Fig. 9b). Therefore, these 261 genes were regulated in an opposite manner in the overexpression and silencing lines of *PvMES1*. Selected DEGs involved in the signaling transduction, transcription factors, defense metabolism and sugar transportion were regulated in *PvMES1*-mediated resistance of common beans towards *Fop* (Fig. 9c-f).

Discussion

Our major results were to find that SA and the newly characterized gene producing it, *PvMES1*, played key roles in the interaction between *Fop*, an important fungal pathogen of the common bean and its host plant. PvMES1 displayed SA-inhibitable MeSA esterase activity *in vitro* similar to NtSABP2 and StMES1, AtMES1, AtMES4, AtMES9. The effective gene overexpression and silencing methods based on BPMV-derived vector were utilized to identify the biological function of *PvMES1* gene. Thus, it proved that MeSA and SA participated in a key pathway for defense signaling in common bean.

SA is a signaling molecule naturally found in plants and shown to be involved in various plant defense-related actions against infection by various pathogens. White and his colleague firstly reported that salicylates as disease resistance-inducing chemicals (White 1979; Antoniw and White 1980). The methylated form of SA (MeSA) can be found in various plants, its role as an alternative inactive storage form of free SA (Shulaevet al. 1997; Kawano et al. 1998). Forouhar et al. (2005) have reported a successful demonstration that SA-binding protein isolated from tobacco, designated as SABP2 (SA-binding protein 2) has strong esterase activity with MeSA as substrate. Therefore, release of free SA from MeSA by SABP2 contributed to rapid SA increase in the systemic tissues of TMV-infected tobacco plants for SAR development (Park et al. 2007). In this study, we describe the identification of *PvMES1* from common bean based on homology with SABP2 and demonstrate that it shares similar biochemical properties with its orthologs from tobacco, *Arabidopsis* and potato. Expression of *PvMES1*, in the same way was induced by pathogen infection and repressed by SA, the *PvMES1* transcript product exhibits esterase activity toward MeSA, and this activity is feedback inhibited by SA (Fig. 1). This suggesed that

PvMES1 could perform a similar defense function as SABP2 in the SA-mediated defense pathway in common bean. Furthermore, the overexpression of *PvMES1* led to increased *Fop* resistance and *PvMES1*-silenced plants enhanced the susceptibility and compromised SAR dramatically (Fig. 2). We combined *PvMES1* regulation and pathogen infection assays to reveal that SA-responsive genes and pathogen induced genes in common beans by RNA-seq, and it indicated that PvMES1 plays a pivotal role in plant resistance to the Fusarium wilt pathogen (Fig. 3,4). Through increasing SA levels, PvMES1 regulates the expression of genes from the pathway of phenylpropanoid biosynthesis, sugar metabolism, SA signaling and interaction between *Fop* and common bean.

Phenylpropanoids are the important and necessary precursors of numerous structural polymers and defense compounds (such as phytoalexins and lignin) in plants, and thus serve as the core mediators of crosstalk between developmental and defense-related pathways (Alessandra et al. 2010). The genes identified by RNA-seq in this study focused on the main branches of the phenylpropanoid pathway with an emphasis on the synthesis and deposition of lignin, and also leading to the synthesis of flavonoid and cinnamaldehyde (Fig. 5).

Previous studies have demonstrated that the phenylpropanoid pathway leading to lignin synthesis and deposition from the oxidative coupling of three monolignols named p-hydroxyphenyl, guaiacyl and syringyl as a physical barrier is activated because lignification and reinforcement of cell walls are important processes in plants during fungal penetration (Smit and Dubery 1997; Bhuiyan et al. 2009; Naoumkina et al. 2010). Particularly, the lignins has been reported to play critical roles in the responses to *F. oxysporum* in flax with the enhancement of MeSA level (Aleksandra et al. 2017). Some of pathogen defense-related indices measured in this study, such as PAL and POD, belonged to enzymes involved in the core and entrypathway of the phenylpropanoid pathway and biosynthesis of lignin (Marjamaa et al. 2009; Huang et al. 2010). In addition, phenylpropanoid pathway allows plants to produce various secondary metabolites in defense response to infection including flavonoids (isoflavonoids in particular) as phytoalexinsin the legume specise (Andersen and Markham 2006; Bednarek and Osbourn 2009; Naoumkina et al. 2010) and the cinnamaldehyde which affected the morphogenesis and growth of the pathogen fungus (Xing et al. 2014). Overall, it indicated that change of phenylpropanoid pathway mediated by *PvMES1* will provide new insights into the control of Fusarium wilt and disease resistance development in common beans.

Sugars act as a regulatory factor affecting almost all processes of growth and development in plants (Wind et al. 2010; Stokes et al. 2013). It indicated that sugars constitute the primary substrate providing energy and structural material for defense responses in plants, while they may also act as signal molecules in activating PTI and ETI, interacting with the hormonal signaling network regulating the plant immune system (Bolouri Moghaddam and Van den Eden 2012; Rolland et al. 2002). In most fungal pathogen-plant systems, the high product level of sugar metabolism such as sucrose, glucose, fructose etc. in plant tissues enhances plant resistance (Morkunas and Ratajczak 2014). Gupta et al. (2010) demonstrated the enhanced expression of sugar cleaving enzymes led to an incompatible interaction between chickpea and *Fusarium oxysporum* f.sp *ciceri* suggesting a vital role of sugars in defense

against Fusarium wilt. It has also been reported that sucrose, glucose and fructose can play an important role in resistance to fungal pathogens through stimulation of phenylpropanoid metabolism (Forlani 2010; Morkunas et al. 2011; Gibertia et al. 2012).

Sucrose also functions as a signaling molecule (Wind et al. 2010). Sucrose responsive element is reported to influence the expression of WRKY transcription factor during defense (Sun et al. 2003). Sucrose synthase is also related to cellulose synthesis, it can provide UDP-glucose as an essential substrate for cell wall development (Fujii et al. 2010). As well known, the cellulose and pectin are the essential components made of plant cell wall, which constitutes the first barrier to microbial invasion and processes associated with its reorganization are among the early events occurring in plant defense (Albrecht et al. 2008). Trehalose is also a well-known disaccharide with signaling functions in plant growth and development, as well as defense responses, and its derivativetrehalose 6-phosphate has a dual function as a signal and homeostatic regulator of sucrose levels in plants (Paul et al. 2008; Delatte et al. 2011). It has been shown to partially induce resistance against powdery mildew (*Blumeriagraminis* f. sp. *tritici*) in wheat by the activation of phenylalanine ammonia-lyase and peroxidase genes (Reignault et al. 2001; Muchembled et al. 2006). However, sugar production alone is insufficient to control signal transduction. This role is performed by the sugar transporters. It revealed that control of sugar uptake, managed by regulation of a host sugar transporter, is a defense strategy deployed against microbial infection in *Arabidopsis* (Yamada et al. 2016).

Before our study, not much information existed on the expression of these sugar molecules and transportersin common beans especially as related to disease infection. Our study demonstrated that sugars helped in generating alarm signals and efficiently transmitted them throughout with the aid of sugar transporters and played the key role in the cellulose and pectin synthesis. Therefore, the activation of SA inducible genes in the metabolism and transport of sugar molecules enhanced the defense signaling and the cell wall reinforcement in response to *Fop* infection in common bean (Fig. 6; Fig. 9f; Figure S8).

Phytohormones such as abscisic acid, jasmonic acid, ethylene, salicylic acid, auxin, gibberellin, cytokinein, and brassinosteroids appeared to play other critical roles in the complex signalling cascades and were integrated into current models of defence responses (Bari and Jones 2009). Xue et al. (2014) has reported that SA applied to foliar tissue is capable of enhancing the systemic acquired resistance of common bean roots to infection by *Fop* through the induction of endogenous free SA accumulation. In this study, SA response factors TGA and PR-1 were induced by *PvMES1* activation following the endogenous SA accumulation (Fig. 7). Otherwise, the use of mutants and transgenic plants of *Arabidopsis* indicated that SA was involved in the sugar dependent activation of these PR protein-coding genes (Thibaud et al. 2004).

For auxin signaling, the previous study indicated that overexpression of *GH3.5* conferred enhanced SA accumulation, pathogen resistance, and defense gene expression (Park et al. 2007; Zhang et al. 2007; Westfall et al. 2016). Similar with *GH3.5* from subfamily , mutations in *GH3.12* as a member from

subfamily □, also reduced SA accumulation and pathogen resistance in *Arabidopsis*, that indicated *GH3.12* likely acts upstream of SA in defense signaling (Lee et al. 2007; Nobuta et al. 2007). However we saw no role for these transcription factors.

To date, a variety of possible receptors and corresponding signaling mechanisms has been proposed for the modes of SA signal transduction in plants. Du et al. (2009) observed that binding of Ca^{2+}/CaM to the *Arabidopsis* transcription factor SR1 repressed expression of EDS1 and suppressed SA accumulation and defense. In addition, Chen et al. (1993) have proposed the involvement of ROS as key signals that relay SA signals in plants. An oxidative burst through *F. oxysporum* infection led to ROS overproduction in the *in vitro* cultured embryo axes of yellow lupin, and the processes are much more intensive when treated with exogenous sucrose, the defense signaling molecule shown above (Morkunas et al. 2013). These studies opened the door for SA studies regarding calcium signaling and oxidative bursts. In this study, it indicated that resistance enhancement in common bean to *Fop* through suppressing calcium-mediated signaling and activating ROS accumulation as mediators by SA induction (Fig. 8).

In summary, PvMES1 plays a pivotal role in regulating the SA inducible resistance to Fusarium wilt in common bean (Fig. 10). The pathway of phenylpropanoid synthesis and sugar metabolism regulated by PvMES1 are demonstrated to be important for the enhancement of defense response. SA and other elements as key signals derived from the phenylpropanoid synthesis, sugar metabolismand interaction activated the resistance pathway based on the signaling transduction, transcription factors, defense metabolism in common bean towards Fusarium wilt (Fig. 9c-e). The current understanding of the interactions between SA signaling and plant pathogen attack mainly comes from studies in *Arabidopsis* and tomato, and different resistance mechanisms provide an angle to understand the infection process and the defense response of *F. oxysporum*in the root systerm of common beans. Collectively, our study provides insights into the roles of SA in regulating defense responses, indicating a central role for *PvMES1* in common bean against *Fop.* The significant practical applications for engineering disease resistance and support to the broad-spectrum resistance in common bean by manipulating SA levels and thereby increasing SAR development remains to be discussed.

Declarations

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Author contributions statement

Renfeng Xue and Matthew W. Blair planned the research. Renfeng Xue, Ming Feng and Jian Chen performed most of the important experiments. Renfeng Xue analyzed the sequencing data. Ming Feng and Renfeng Xue generated the constructs and treated plants. Matthew W. Blair provided some vectors and Weide Ge grew the plants. Ming Feng and Jian Chen contributed to analyze the testing data. Renfeng Xue and Matthew W. Blair wrote the article.

Availability of data and material

Data supporting the current study can be obtained by contacting the corresponding author (xuerf82@hotmail.com).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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