

# The Cotton Ghplp2 Positively Regulates Plant Defense against *Verticillium Dahliae* by Modulating Fatty Acid Accumulation and Jasmonic Acid Signaling Pathway

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## Research Article

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# Abstract

Patatin-like proteins (PLPs) have nonspecific lipid acyl hydrolyze (LAH) activity, which can hydrolyze membrane lipids into fatty acids and lysophospholipids. The vital role of PLPs in plant growth and abiotic stress has been well elucidated. However, the function of PLPs in plant defense response against pathogens is still poorly understood. Here, we isolated and identified a novel cotton (*Gossypium hirsutum*) patatin-like protein gene GhPLP2. *GhPLP2* expression was induced upon treatment with pathogens *Verticillium dahliae*, *Fusarium xysporum*, and signaling molecules jasmonic acid (JA), ethylene in cotton plants. Subcellular localization revealed that GhPLP2 was localized in the cell wall and plasma membrane. *GhPLP2*-silenced cotton plants showed reduced resistance to *V. dahliae* infection, while overexpression of *GhPLP2* in Arabidopsis enhanced the resistance to *V. dahliae*, with mild symptoms, decreased disease index, and fungal biomass. Hypersensitive response, callose deposition, and H<sub>2</sub>O<sub>2</sub> accumulation triggered by *V. dahliae* elicitor were reduced in silenced cotton plants. *GhPLP2*-transgenic Arabidopsis had more accumulation of JA and JA synthesis precursor linoleic acid (LA, 18:2) and  $\alpha$ -linolenic acid (ALA, 18:3) than control plants. Consistently, linoleic acid,  $\alpha$ -linolenic acid, and jasmonic acid have decreased in *GhPLP2*-silenced cotton plants. Further, the gene expression of the JA signaling pathway is up-regulated in transgenic Arabidopsis and down-regulated in silenced cotton plants, respectively. These results showed that *GhPLP2* is involved in plants' resistance to *V. dahliae* by maintaining fatty acid metabolism pools for JA biosynthesis and activation of the JA signaling pathway.

## Key Message

GhPLP2 can maintain fatty acids pool for endogenous JA biosynthesis and activation of JA signaling pathway, which enhances plants resistance against *Verticillium dahliae*.

## Introduction

In-plant, lipids play a critical role in cell membrane components, sustainable energy storage, and signaling transduction in response to biotic and abiotic stress (Li, et al. 2016; Lim, et al. 2017; Wang 2004). The accumulation of hydrolysis products via lipid acyl hydrolase (LAH) activity in lipid metabolism affects pathogenesis and resistance mechanisms in plant-microbe interactions (Shah 2005). As the key hydrolysis product of membrane lipids, fatty acids (FAs) participate in modulating the accumulation of endogenous nitric oxide, azelaic acid (AZA) biosynthesis contributing to systemic acquired resistance (SAR), reactive oxygen species (ROS) production, and the subsequent defense responses in plants (Mandal, et al. 2012; Wang, et al. 2014; Yu, et al. 2013). Oxidation and further conversions of polyunsaturated FAs by  $\alpha$ -dioxygenase ( $\alpha$ -DOX) and lipoxygenases (LOXs) generate various products collectively termed oxylipins (Savchenko, et al. 2014). Jasmonates (JAs) constitute a key member of oxylipins, many of which are involved in plant defense against pathogens and nematodes and herbivores (Campos, et al. 2014; Wasternack and Strnad 2016; Wasternack and Strnad 2017). Moreover, oxylipins were related to hypersensitive response (HR) initiation in the interactions between

plants with incompatible bacterial or fungal elicitors (Coll, et al. 2011; García-Marcos, et al. 2013; Sun, et al. 2014).

Patatin proteins constitute a significant family of LAHs with nonspecific LAH activity towards several membrane lipids, which was first identified and isolated as vacuolar storage proteins in potato tubers (Hendriks, et al. 1991; Scherer, et al. 2010). They generate free fatty acids and lysophospholipids by catalyzing the nonspecific hydrolysis of membranes lipids, including phospholipids, glycolipids, sulfolipids, mono- and diacylglycerols (Li and Wang 2014). Various functions of patatin proteins have been demonstrated in auxin-responsive, cellulose production, salt tolerance, drought response, and plant growth (Dong, et al. 2014; Li, et al. 2020; Li, et al. 2011; Yang, et al. 2012). More importantly, patatin and patatin-like proteins play an essential role in defense response to various pathogens. In Arabidopsis, patatin-related phospholipase I (AtPLAI) plays a role in maintaining basal jasmonic acid production and conferring resistance to *Botrytis cinerea* infection (Yang, et al. 2007). The Arabidopsis patatin-like protein 2 (AtPLP2) regulates plant cell death, oxylipin synthesis, and resistance to pathogens with distinct lifestyles (La Camera, et al. 2009). Overexpression of Arabidopsis patatin-related phospholipase IIIa (AtPLAIIIa) enhanced resistance to *turnip crinkle virus* (TCV), accompanied by higher salicylic acid/jasmonic acid ratio and increased expression of defense gene PR1 (Jang, et al. 2020). Silencing of pepper (*Capsicum annuum*) *CaPLP1* compromised the defense responses to avirulent *Xanthomonas campestris pv. vesicatoria* (Xcv) (Kim, et al. 2014). Several patatin-like proteins are involved in lipid peroxidation and jasmonic acid synthesis in grapevine *V. vinifera cv. Regent* (resistant cultivars) after infection with *Plasmopara viticola* (Laureano, et al. 2019). Furthermore, three patatin-like proteins (NtPAT1-3) were rapidly induced in tobacco during the HR response to the *Tobacco mosaic virus* (TMV) (Dhondt, et al. 2010). GhPat1 was reported co-expressed with GhLox1 in cotton plants during *Xanthomonas campestris*-mediated hypersensitive cell death (Cacas, et al. 2009).

Cotton (*Gossypium spp.*) is widely cultivated around the world because of the significant economic value of its textile industry (Gao, et al. 2013). However, the yield and quality of cotton can be seriously affected by *Verticillium wilt*, caused by the soil-borne pathogen *Verticillium dahliae* Kleb (Gong, et al. 2017). *V. dahliae* colonizes the plant through young roots, the sites of lateral root formation or puncture wounds to the xylem and leading to browning vasculature, defoliation, and wilting (Chen, et al. 2016; Shaban, et al. 2018). This pathogen can infect more than 200 dicotyledonous plants species and is particularly difficult to control by fungicides because the fungi reside in the woody vascular tissues (Fradin and Thomma 2006; Mo, et al. 2016). Moreover, the resting structures microsclerotia of *V. dahliae* can survive in the soil for a more extended period even without a host (Klosterman, et al. 2009). The cultivation of resistant varieties has proven to be an effective strategy for controlling *Verticillium wilt* of cotton. The identification and isolation of candidate resistance genes are essential for studying the mechanism of resistance to this disease (Gayoso, et al. 2010). Previous studies have reported a series of resistance genes in cotton plants and revealed the resistance mechanism against *V. dahliae* (Li, et al. 2019; Liu, et al. 2018; Wang, et al. 2020). However, the function of patatin proteins in cotton defense response against *V. dahliae* is largely unclear.

In this study, we report the identification and characterization of the *V. dahliae*-induced patatin-like protein gene *GhPLP2* in cotton (*Gossypium hirsutum*). The transcriptional expression patterns of *GhPLP2* were investigated in response to biotic and abiotic stress. Sequence analyses showed that GhPLP2 had conserved catalytic dyad residues, which is critical for the LAH activity of patatins. The recombinant GhPLP2 protein further confirmed its LAH activity *in vitro*. Reduced HR response during *V. dahliae* elicitor infection were observed in *GhPLP2*-silenced plants, suggesting the role of *GhPLP2* in HR-like cell death signaling. A potential role of *GhPLP2* in positively regulating plant resistance to *V. dahliae* was examined by overexpression in Arabidopsis and virus-induced gene silencing (VIGS) in cotton plants. Transgenic Arabidopsis plants exhibited higher accumulation of linoleic acid,  $\alpha$ -linolenic acid, and jasmonic acid, which were decreased in *GhPLP2*-silenced cotton plants. Besides, the expression of genes involved in the jasmonic acid synthesis pathway and defense response is positively correlated with the expression of *GhPLP2*. Together, we showed that *GhPLP2* positively regulates defense against *V. dahliae* by mediating fatty acids metabolism and activation of the JA signaling pathway.

## Materials And Methods

### Plant Growth and Culturing of *Verticillium dahliae*

Seeds of resistant cotton Zhongzhimian 2 (original strain no. GK44) were provided by the Cotton Research Institute, Chinese Academy of Agricultural Sciences (Henan, Anyang). The germinated seeds were cultured in nutrient soil and vermiculite (2:1, w/w) under a photoperiod of 16 h/8 h. After vernalization for three days at 4°C in Murashige-Skoog (MS) culture medium, both wild-type (Columbia-0) and transgenic Arabidopsis plants were grown in mixture of nutrient soil and vermiculite (1:1, w/w) under a controlled environment with 16-h light (25°C)/8-h dark (22°C). The aggressive defoliating isolate Vd991 of *V. dahliae* and *F. oxysporum* (strain AYP-1) were cultured on potato dextrose agar at 25°C for seven days and then inoculated in Czapek liquid medium. The experiments were performed using conidial suspensions of  $10^7$  conidia/mL.

### cDNA Isolation and Sequence Analysis of *GhPLP2*

Total RNA was extracted from *Gossypium hirsutum* 'Zhongzhimian 2' plants using the RNA extraction kit [BIOMED GENE TECHNOLOGY (Beijing) CO., LTD.], and cDNA was synthesized using the manufacturer's instructions with the FASTQuant cDNA RT Kit (TIANGEN Biotech Co., Ltd). The forward primer 5`-

ACGCGTCGACATGGAAAAAGT ACTGGAAAC-3` (*Sall*, *drestrictionsite*) and reverse' r5-

ATAGACTAGTTGGGCCAAGCTTGTGCA A-3` (Spe I, underlined restriction site) were designed to amplify the open reading frame (ORF) using the cDNA template. PCR product was transferred into the pMD18-T vector by manufacturer's protocols (Takara, Dalian, China), and the positive clone was sequenced.

The theoretical isoelectric point (pI) and molecular mass were calculated with ProtParam (<http://web.expasy.org/protparam/>). Multiple amino acid sequence alignment was performed with

Clustal Omega (<http://www.ebi.ac.uk/Tools/msa/clustalo/>), and the multiple alignment file was shaded with BoxShade ([http://www.ch.embnet.org/software/BOX\\_form.html](http://www.ch.embnet.org/software/BOX_form.html)). The phylogenetic tree was constructed with the neighbor-joining method using MEGA 7 with bootstrap values from 1000 replicates indicated at the nodes, and motifs were annotated using MEME (<http://meme-suite.org/tools/meme>) (Bailey and Elkan 1994; Kumar, et al. 2016). The homology model of GhPLP2 was generated using SWISS-MODEL, and three-dimensional models were analyzed and visualized using EzMol, Version<sup>1.20</sup>(Bordoli, et al. 2009; Reynolds, et al. 2018). The crystal structure of SeMet Patatin (1oxw.1.B) was selected as a template to predict the theoretical model.

### Analysis of *GhPLP2* Expression Pattern

For abiotic stresses and hormone treatments, two-week-old cotton seedlings were gently uprooted and replanted in Hoagland medium containing 2.5% (w/v) PEG6000, 100  $\mu$ M JA, 2 mM ethrel respectively (Champion, et al. 2009; Pei, et al. 2019). For pathogen treatment, seedling roots were inoculated with *V. dahliae* and *F. oxysporum* conidial suspension for 5 min and then transplanted into sterile soil. Control samples were treated with sterile water. The inoculated samples were collected at appropriate time points for RNA extraction. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) for *GhPLP2* gene was conducted using SYBR Premix Ex Taq (Tli RNaseH Plus) (Takara Bio, Dalian, China) on an ABI 7500 thermocycler (Applied Biosystems, Foster City, CA, United States). The expression levels of *GhPLP2* genes were normalized to *GhUBQ7* (DQ116441), and relative gene expression was calculated using the  $2^{-\Delta\Delta Ct}$  method (Liu, et al. 2018; Livak and Schmittgen 2001). Data were analyzed in Origin 8 and presented as the mean  $\pm$  standard error (SE) of three independent experiments. The primer sequences were listed in Supplementary Table S1.

### Expression and Purification of Recombinant GhPLP2 Protein

GhPLP2 was cloned into the 6 $\times$  His-Tagged protein expression vector pET-22b(+) (Novagen). The primers were listed in Supplementary Table S1. The recombinant vector was expressed in *Escherichia coli* BL21 (DE3). Single colonies were cultured at 37°C in LB broth containing 100  $\mu$ g/mL ampicillin when OD<sub>600</sub> was reaching 0.6 induced with 0.4 mM IPTG for another 10 h at 22°C with 200r/min. Protein was purified using 6 $\times$ His-Tagged Protein Purification Kit (CW BIO) following manufacturer's instructions and detected using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Purified protein concentration was determined using the Bradford Protein Assay Kit (Takara Bio, Dalian, China).

### GhPLP2 Enzyme Activity Assay Using p-Nitrophenyl palmitate and Phospholipids As Substrates

Lipid acyl hydrolase (LAH) activity assay of GhPLP2 protein was determined using artificial substrate p-Nitrophenyl palmitate (p-NPP) and phospholipids (Camera, et al. 2010; Hong, et al. 2008). Briefly, the reaction mixture comprised 200 $\mu$ L of Tris-HCl (pH 8.0), 10 mM CaCl<sub>2</sub>, 0.05% (v/v) Triton X-100 and 2 mM p-Nitrophenyl palmitate, 25  $\mu$ g purified protein were added and incubated at 37 °C for 30 min. Then 700  $\mu$ L absolute ethanol was added and centrifuged for 2 min at 7000r/min. The absorbance of p-nitrophenol (NP) liberated from p-Nitrophenyl palmitate was measured at 405 nm.

Phospholipases substrates were 1,2-distearoyl-sn-glycerol-3-phosphoethanolamine, 1,2-distearoyl-sn-glycerol-3-phosphocholine, or L- $\alpha$ -phosphatidylglycerol (Aladdin ®, China). The reaction mixture comprised 50 mM Tris-HCl (pH 8.0), 10 mM CaCl<sub>2</sub>, 0.05% (v/v) Triton X-100, 500  $\mu$ g substrate and 10  $\mu$ g purified protein in a final volume of 600  $\mu$ l. Reactions were performed at 37 °C for 1 h and termination by adding 500  $\mu$ l chloroform/methanol (2:1, v/v). The product fatty acids released from the substrates were separated and analyzed according to the method described before (Camera, et al. 2010). Free fatty acids are methylated in 1 mL 0.5 mol/L KOH-methanol at 60°C for 2 h. Then 1 mL hexane containing 0.01% butylated hydroxytoluene (BHT) and 0.1 mg methyl nonadecanoate was added. After shaking and standing for stratification, the supernatant containing fatty acid methyl esters (FAMES) was separated. Quantitation of individual FAMES was analyzed by gas chromatography (GC) equipped with an Agilent column (USA) (30 m by 0.25 mm, 0.25  $\mu$ m film) and a flame ionization detector (FID).

### Generation of Transgenic Plants

The PCR product with Sal I/Spe I restriction site was inserted into a modified pCAMBIA 1300 vector harboring a hygromycin phosphor-transferase (hptII) gene and the green fluorescent protein (GFP) (Wang, et al. 2011). The construct was introduced into *Agrobacterium tumefaciens* strain GV3101 by freeze-thaw method, and transformation of Arabidopsis was performed by the floral dip method (Clough and Bent 1998). Transgenic Arabidopsis seeds were screened on MS plates containing 25 mg/mL hygromycin B and T3 homozygous lines displaying 100% hygromycin resistance were used for further experiments.

### Construction of VIGS Vectors and *Agrobacterium*-mediated VIGS

The VIGS transient expression methods were followed by Gao (Gao, et al. 2011). The silenced fragments of *GhCLA1* (Chloroplastos alterados 1) and *GhPLP2* were amplified from cotton cDNA and inserted into the *TRV:00* vector to generate the *TRV: GhCLA1* and *TRV: GhPLP2* vectors. Then plasmids of *TRV: GhCLA1* and *TRV: GhPLP2* were transformed into *A. tumefaciens* strain GV3101 by heat shock (Dong, et al. 2007). *TRV:GhCLA1* plants were used as positive controls. Two weeks after infiltration, when *GhCLA1*-silenced plants showed clear signs of albinism in leaves, the efficiency of *GhPLP2* was evaluated by RT-PCR. *GhUBQ7* was amplified as internal control with primers qUBQ-F/R. The primers used in vector construction were listed in Supplementary Table S1

### *V. dahliae* Inoculation and Disease Investigation

Two weeks after VIGS, when true leaves of *GhCLA1*-silenced cotton plants showed clear signs of albinism, inoculation with *V. dahliae* (10<sup>7</sup> conidia/mL) was performed previously reported (Liu, et al. 2018). Plant disease index (DI) was monitored as the following formula according to Wang (Wang, et al. 2020):  $DI = [\sum(n \times \text{the number of seedlings at level } n) / (4 \times \text{the number of total seedlings})] \times 100$ , n denotes disease level, cotton seedlings were divided into five levels based on their disease severity after *V. dahliae* inoculation (level 0, 1, 2, 3, 4). For Arabidopsis plants, four-week-old *GhPLP2*-transgenic and wild-type (WT) Arabidopsis plants were inoculated with *V. dahliae* spores as previously described (Gao, et al. 2013).

The controls were dipped in sterilized water. Disease index and symptom classification were performed as previously report (Pei, et al. 2019). Data were collected from three independent replicates ( $n \geq 30$ ).

*V. dahliae* recovery assay was conducted at 21 d after infection (Fradin, et al. 2009). Stem section above the cotyledons was taken from inoculated cotton plants, six slices were transferred onto potato dextrose agar supplemented with kanamycin (50 mg/L) after surface sterilized. *V. dahliae* biomass was quantified following a previously described protocol (Ellendorff, et al. 2009). Inoculated cotton and Arabidopsis plants were harvested at 21 days and 14 days, respectively. DNA was extracted from 100 mg of the fine powder. *V. dahliae* biomass was determined by qPCR using fungus-specific ITS1-F and *V.dahliae*-specific ST-Ve1-R primers (Fradin, et al. 2011). The *GhUBQ*-F/R and *AtEF1 $\alpha$* -F/R primers were used as reference genes. All primers were listed in Supplementary Table 1.

#### Analysis of HR In Cotton Induced by PevD1 Elicitor From *V. dahliae*

Primers are designed to obtain PevD1 fragment from *V.dahliae* (Supplementary Table S1) and cloned into the pET-28a(+) vector (Novagen). The recombinant vector was expressed in *E. coli* BL21 (DE3), and protein was induced with 0.1 mM IPTG for 10 h at 22°C with 200r/min. Protein purification and concentration assay performed as described above. The treatment of PevD1 elicitor was performed as Li (Li, et al. 2019). Expression of HR marker genes *GhHSR203* and *GhHIN1* triggered by PevD1 were detected in cotton leaves at 24h after infection. Callose deposition was stained by 0.1% aniline blue as previously (Wang, et al. 2018). Images were observed using FLUOVIEW FV1000 confocal laser scanning microscope (OLYMPUS, Tokyo, Japan). The level of H<sub>2</sub>O<sub>2</sub> was measured by freshly FOX reagent [Fe(NH<sub>4</sub>)<sub>2</sub>(SO<sub>4</sub>)<sub>2</sub>(250 mM), xylenol orange (100  $\mu$ M), sorbitol (100  $\mu$ M), H<sub>2</sub>SO<sub>4</sub> (25 mM), 1% ethanol] according to Pei (Pei, et al. 2019).

#### Fatty Acid Profile Analysis and Hormone Quantitation

Four-week-old Arabidopsis and two-week *GhPLP21*-silenced cotton plants after VIGS were used to analyze the fatty acid profile and hormone quantification. Hormone quantitation was detected as previously reported (Li, et al. 2020). Fresh samples were ground into fine powder in liquid nitrogen. 100 mg powder was extracted with 1 mL of 80% methanol-water (containing 1% formic acid) using ultrasound at 4°C for 10 min and centrifuged at 4°C at 12,000 rpm for 10 min. The supernatant was transferred to a 2mL centrifuge tube containing 50mg primary secondary amine (CNW Technologies GmbH, Germany), dried with nitrogen, and added 100  $\mu$ L 80% methanol-water (containing 1% formic acid). The samples were analyzed by Agilent 6410B Triple Quadrupole HPLC – MS/MS (Agilent Technologies, USA), which is equipped with an HPLC reverse phase C18 column (Athena C18-WP 2.1  $\times$  50 mm, 3  $\mu$ m). The flow rate was 0.3 mL/min. Methanol and ultrapure water were used as mobile phases A and B, respectively. The isocratic elution was carried out for 5 min at a 65(A):35(B) ratio. MS was performed using the multiple reaction monitoring modes and negative electrospray ionization. All the parameters were performed according to Li (Li, et al. 2020).

For fatty acids profile analysis, plant samples were dried to a constant weight under 90 °C, and 50 mg of dry powder was weighed. Methyl esterification of fatty acids and analysis were performed as described above. GC parameters were as follows: 180°C for 10 min followed by a ramp to 190°C at 1°C min<sup>-1</sup>, holding 190°C for 3 min and then heating up to 220°C with a gradient at 4°C min<sup>-1</sup>, final temperature was maintained for 3 min.

## Gene Expression Profiling of Jasmonic Acid Signaling Pathway In Arabidopsis and Cotton Plants

Total RNA was obtained from non-inoculated plants with an RNA extraction kit [BIOMED GENE TECHNOLOGY (Beijing) CO., LTD.]. The synthesis of cDNA and qRT-PCR assays was conducted as mentioned above. *AtEF1a* and *GhUBQ7* were employed as internal standards genes in Arabidopsis and cotton, respectively. Relative gene expression was calculated using the  $2^{-\Delta\Delta C_t}$  method. Data were presented as the mean  $\pm$  standard error (SE) of three independent experiments. The primer sequences are listed in Supplementary Table S1.

## Data analysis

Data are obtained from three independent replicates per treatment and presented as mean  $\pm$  standard error. Significant differences between groups were analyzed by ANOVA using statistical software IBM SPSS statistics 20 followed by Student's t-test. Asterisks indicates a significant difference compared with control (\*P < 0.05, \*\*P < 0.01).

## Results

### Identification and sequence analysis of *GhPLP2*

The full-length *GhPLP2* cDNA consists of a 68 bp 5'untranslated region (5' UTR), 138 bp 3' UTR, and 1218 bp open reading frame (ORF) encoding a 405 amino acid protein with a theoretical pI of 8.21 and molecular weight of 44.44 kDa. GhPLP2 protein contains the conserved serine hydrolase motif GX SXG at residues 63–67 and a conserved aspartic acid (D) residue at 214 within the patatin domain (residues 21–221) (Fig.S1). The catalytic dyad of serine and aspartic acid residues is critical for the LAH activity of patatin (Rietz, et al. 2010; Rydel, et al. 2003). The homology modeling result shows active sites composed of Ser-Asp catalytic dyad responsible for its LAH activity (Fig.S2). GhPLP2 has no transmembrane domain or signal peptide. The phylogenetic analysis and multiple alignments were generated with plant patatin homologs previously reported. The phylogenetic analysis showed patatins were divided into three groups and GhPLP2 located in group II containing NtPat1(Cacas, et al. 2005), GhPat1 (Cacas, et al. 2009), CaPLP1(Kim, et al. 2014), and AtPLP2 (La Camera, et al. 2009) (Fig. 1a). These genes have been reported to be involved in disease resistance and pathogen-mediated hypersensitive cell death. Consistent with the phylogenetic analysis result, the patatin homologs were divided into three groups based on the conserved motif (Fig. 1b). Group II proteins have the canonical S-D dyad esterase motif constituted by GxSxG and DGG/A in patatin catalytic centers. Also, Group II proteins contain a conserved

anion binding element DGGGxxG and proline motif APP. GhPLP2 was classified as a group II protein with the conserved catalytic dyad residues.

### Lipid acyl hydrolase (LAH) activity assay of GhPLP2

The LAH activity of patatin proteins can hydrolysis membrane lipids into free fatty acids and lysophosphatidic acid, leading to a series of signaling pathways involved in growth development and defense response (Ryu 2004). To further understand the role of GhPLP2 protein, the LAH activity of the recombinant GhPLP2 protein was detected. The recombinant protein of GhPLP2 was produced and purified from *E. coli* BL21 (DE3). GhPLP2 protein was induced with 0.4 mM IPTG at 22 °C for 2h (Fig. 2a). GhPLP2 fusion protein was purified using Ni columns, and the elution fractions were identified by SDS-PAGE (Fig. 2b). When p-NPP was used as the substrate, the His-GhPLP2 protein exhibited high LAH activity ( $97 \text{ nmol min}^{-1} \text{ mg}^{-1}$ ); however, the His alone was no enzyme activity (Fig. 2c). Besides, when phospholipids phosphocholine (PC), phosphoethanolamine (PE), phosphatidylglycerol (PG) were used as substrates, the release of free fatty acids was quantified. Purified GhPLP2 protein released fatty acids from these phospholipids and exhibited variant enzyme activities in different phospholipids substrates (Fig. 2d). The results indicate that GhPLP2 functions as a lipid acyl hydrolase to release free fatty acids in plants.

### Subcellular Localization of GhPLP2 protein

To determine the subcellular localization of GhPLP2 protein, we examined the root tissues of GhPLP2-transgenic Arabidopsis with GFP by Confocal Laser Scanning Microscopy (Fig. 3). The result indicated that GhPLP2 was located in the cell wall or the plasma-membrane (Fig. 3d-f). To further clarify the location of GhPLP2, the seedlings were treated with 0.8 M mannitol for 10 min. After plasmolysis, the result revealed that GhPLP2 was distributed in both cell wall and plasma membrane (Fig. 3g-i)..

### Induction of *GhPLP2* by Various Stresses

Expression of the *GhPLP2* gene in cotton was examined by qRT-PCR. *GhPLP2* was most abundant in the cotton root (Fig. 4a), which was the first physical barrier in cotton plants against *V. dahliae* infection. The change of *GhPLP2* expression in response to various stresses was investigated. The expression of *GhPLP2* was upregulated in response to infection with *V. dahliae* or *F. oxysporum*. *GhPLP2* expression was significantly increased at 0.5 h, 1 d, 5 d, and 7 d after inoculation with *V.dahliae* (Fig. 4b). With the infection of *F.oxysporum*, *GhPLP2* expression upregulated at 0.5 h, 3 d, 5 d, and 7 d (Fig. 4c). We further investigated the expression of *GhPLP2* after treatment with the defense-related signaling molecules JA and ethylene (ET). *GhPLP2* expression was extremely higher at 3 h, and another peak appeared at 24 h after JA treatment (Fig. 4d). In contrast, ET induced *GhPLP2* expression increased at 0.5 h, with a maximum level observed at 3 h (Fig. 4e). The treatment with 2.5% (w/v) PEG 6000 markedly increased gene expression at 1h and returned to normal level at 24 h (Fig. 4f).

### Enhanced disease susceptibility of *GhPLP2*-silenced cotton Plants to *V. dahliae* Infection

The *GhPLP2* gene was silenced in cotton plants to clarify its function by virus-induced gene silencing (VIGS) (Gao, et al. 2013). The cotton gene *GhCLA1*, which is involved in chloroplast development, was used as a positive control (Gao, et al. 2011). To assess the gene silencing efficiency, the expression of *GhPLP2* was monitored by semi-quantitative RT-PCR and qRT-PCR in *TRV:00* and *TRV: GhPLP2* cotton true leaves after two weeks of VIGS (Fig. 5g and Fig. S3). The results indicated that *GhPLP2* expression was effectively reduced in *GhPLP2*-silenced cotton plants.

To verify the function of *GhPLP2* in the interactions between cotton with *V. dahliae*, cotton plants were inoculated with *V. dahliae*. After inoculation, typical disease symptoms appeared at ten days in *GhPLP2*-silenced cotton. At 14 days, leaf chlorosis and necrosis were more severe in *GhPLP2*-silenced plants (Fig. 5a,d). The plant disease index of silenced plants was higher than that of control plants (Fig. 5h). At 21 days, dark and necrotic vascular bundles of the dissected stems were more apparent in *GhPLP2*-silenced plants (Fig. 5b,e). The fungal renewal cultivation showed that the disease conditions of *GhPLP2*-silenced plants were more serious compared with controls (Fig. 5c,f). Besides, the fungal biomass of the stems from the *GhPLP2*-silenced plants was higher than the controls determined by qRT-PCR analysis (Fig. 5i). These results suggest that the silencing of *GhPLP2* attenuates cotton resistance to *V. dahliae*.

Silencing of *GhPLP2* compromises the HR symptom triggered by *V. dahliae* elicitor PevD1

Patatin-like proteins have been shown to participate in hypersensitive response (HR) during avirulent *Xanthomonas campestris* pv. *Vesicatoria* infection in pepper plants and response to *Tobacco mosaic virus* (TMV) in tobacco plants. Therefore, *GhPLP2* may regulate resistance to *V. dahliae* by mediating HR induced by *V. dahliae* elicitors. To test our hypothesis, we expressed and purified an elicitor PevD1 from *V. dahliae* (Fig. S4), which triggered HR and resistance responses in cotton, Arabidopsis, and tobacco (Bu, et al. 2013; Liu, et al. 2016; Wang, et al. 2012). The HR-like symptom was initiated at 24h after injection with PevD1 elicitor, while HR-like cell death was significantly reduced in silenced leaves (Fig. 6a). Expression of HR marker genes *GhHIN1* and *GhHSR203* and callose deposition triggered by PevD1 were compromised in *GhPLP2*-silenced leaves at 24h after infection (Fig. 6b-d). Also, the silencing of *GhPLP2* significantly compromised the induction of H<sub>2</sub>O<sub>2</sub> by PevD1 (Fig. 6e).

Overexpression of *GhPLP2* in Arabidopsis confers enhanced resistance to *V.dahliae*

To further evaluate the role of *GhPLP2* in cotton response to *V. dahliae*, we examined the resistance of wild-type and transgenic Arabidopsis seedlings under *V.dahliae* infection. Three homozygous transgenic (T3 generation) lines with the highest expression levels of *GhPLP2* (L1, L4, and L8) were selected for further experiments (Fig. S5).

Four-week-old transgenic and wild-type (WT) plants were infected with *V.dahliae* spores by the root dipping method. After inoculation, WT plants showed more serious wilt, yellowish, and necrosis than the transgenic plants at 14 dpi (Fig. 7a); this was consistent with the disease index investigation (Fig. 7b). The fungal biomass of WT plants was remarkably higher compared to the transgenic as determined by

qRT-PCR analysis (Fig. 7c). These results indicate that overexpression of *GhPLP2* in Arabidopsis plants confers enhanced resistance to *V.dahliae*.

### *GhPLP2* involved in free fatty acid accumulation and JA Signaling Pathway

Free fatty acids (FFAs) are one of the products of patatin-like proteins by the nonspecific lipid acyl hydrolase (LAH) activity (Rivas and Heitz 2014). Oxidation and further conversions of polyunsaturated FAs with lipoxygenases (LOXs) may generate the JA production (Fu, et al. 2015; Sun, et al. 2014; Walley, et al. 2013). The endogenous LAH activity of crude proteins from different genotype plants was detected using p-nitrophenyl palmitate (pNPP) according to Lin (Lin, et al. 2011). LAH activity of *GhPLP2*-transgenic Arabidopsis lines was higher than that of WT plants and decreased in *TRV: GhPLP2* cotton plants (Fig. S6). To assess the metabolic differences between different genotype plants, fatty acid profile, basal JA accumulation, and gene expression were determined in non-inoculated plants (Fig. 8). Compared with WT plants, fatty acid profile analysis display that the level of polyunsaturated fatty acid linoleic acid (LA, 18:2) and  $\alpha$ -linolenic acid (ALA, 18:3) has increased in *GhPLP2*-overexpression Arabidopsis (Fig. 8a). The basal level of JA in transgenic Arabidopsis plants was also higher than in WT plants (Fig. 8b). Expression of genes involved in JA biosynthesis pathways such as LOX1, LOX2, AOS, OPR3, and JA-responsive marker PDF1.2 in transgenic Arabidopsis was induced compared with the WT plants (Fig. 8c). On the contrary, *GhPLP2*-silenced cotton plants has decreased accumulation of linoleic acid and  $\alpha$ -linolenic acid and the basal accumulation of JA was also compromised in *TRV: GhPLP2* cotton plants (Fig. 8d,e). Expression levels of LOX2, AOS, OPR3 and PDF1.2 associated with the JA signaling pathway were downregulated in *TRV: GhPLP2* cotton plants (Fig. 8f). The results showed that *GhPLP2* contributes to fatty acid accumulation and is involved in JA signaling pathway.

## Discussion

Patatin-like proteins (PLPs) are a significant family of lipases with lipid acyl hydrolase(LAH) activity and play an essential role in lipid metabolism during plant defense immunity (Rivas and Heitz 2014). In the present study, we identified a novel patatin-like protein gene *GhPLP2* from cotton and demonstrated its role in fatty acid metabolism and JA signaling pathway conferring cotton resistance against *V.dahliae*.

Patatin-related proteins have a conserved S-D catalytic dyad constituted by a serine of esterase motif (GxSxG) and a central aspartic acid, which is essential for the LAH activity (Rydell, et al. 2003). These residues were also identified in human Ca<sup>2+</sup>-independent phospholipase A2 (iPLA2s) and many microorganism proteins; their function was confirmed by previous studies (Burke and Dennis 2009; Heller, et al. 2018; Mansfeld 2009). Same as other patatin proteins in plants, GhPLP2 has a canonical esterase motif GxSxG and a conserved S-D catalytic dyad for its lipid acyl hydrolase activity (Fig. 1b). The enzyme activity assay *in vitro* showed that the fusion protein has LAH activity which for hydrolyzing phospholipids as previously described (Holk, et al. 2002). It suggested that GhPLP2 has functional LAH activity enabling it to release fatty acids from membrane lipids. Bioinformatic analysis and subcellular localization experiments showed that GhPLP2 localized in both cell wall and plasma membrane (Fig. 3),

which was consistent with the localization of several PLPs (Holk, et al. 2002; Kim, et al. 2014). The localization on the plasma membrane suggests that GhPLP2 may affect plant functions through the regulation of lipid signaling by hydrolysis of membrane lipids.

Many studies have suggested that PLPs with soluble LAH activity might be necessary for plant response to various environmental stresses such as drought, wounding, or pathogen infection (Matos and Pham-Thi 2009; Rivas and Heitz 2014). PLP proteins in tobacco, Arabidopsis, pepper, and grape show distinct disease resistance in different host-pathogen interactions (Cacas, et al. 2005; Kim, et al. 2014; La Camera, et al. 2009; Laureano, et al. 2019; Yang, et al. 2007). Further analysis is necessary to clarify the role of PLPs in the interaction between different pathogens and plants. In this work, the *GhPLP2* gene was strongly induced at the early stage after infection with *V.dahliae* (Fig. 4b), followed by a decrease and reached another peak at 7d with the about 7-fold change. It suggests a crucial role for *GhPLP2* in the interaction of cotton plants with *V.dahliae*. To further determine the role of the *GhPLP2* gene in plant defense against *V.dahliae*, ectopic overexpression of the *GhPLP2* in Arabidopsis and knockdown of *GhPLP2* expression by VIGS in cotton plants were employed. Silencing of *GhPLP2* in cotton plants enhanced the susceptibility to *V.dahliae* infection (Fig. 5), and overexpression of *GhPLP2* in Arabidopsis plants enhanced their disease resistance with mild disease symptoms (Fig. 7). Thus, these results indicated that *GhPLP2* played a positive role in maintaining the resistance of the plant to *V.dahliae* infection.

HR is one of the defense mechanisms that confer plant ability to inhibit pathogen infection and further induce a rapid defense response (Kombrink and Schmelzer 2001; Zhang, et al. 2017). Growing evidence showed that PLPs play an essential role in cell death execution in HR in plants. In Arabidopsis, *AtPLP2* is an executor of plant cell death by providing fatty acid precursors for specific oxylipins' biosynthesis (La Camera, et al. 2009). *CaPLP1* regulates the production of phospholipid-derived molecules, which leading to the HR-like cell death during the incompatible interaction of pepper plants with avirulent *Xcv* (Kim, et al. 2014). The effector protein PevD1 from *V.dahliae* could induce typical HR-like necrosis in tobacco and trigger innate immunity in cotton plants (Bu, et al. 2013; Wang, et al. 2012). In our study, GhPLP2 silencing has reduced HR phenotype and expression of the HR marker gene during induction of *V. dahliae* elicitor PevD1 in cotton leaves. The plant immune responses, including H<sub>2</sub>O<sub>2</sub> accumulation and callose deposition, were damaged in silenced leaves (Fig. 6). It suggests that GhPLP2 is required for resistance (R) gene-mediated disease resistance in cotton plants. However, the mechanism of GhPLP2 involved in the HR response induced by the *V. dahliae* elicitor is still unclear.

Fatty acids are not only major membrane components of the cell, but they also directly or indirectly participate in a series of immune responses in plants (Lim, et al. 2017; Walley, et al. 2013). Significantly, the 18C fatty acids play an essential role in plant disease resistance. The hydrolysis of 18C fatty acids at C9 double bond generated dicarboxylic acid azelaic acid (AZA), an inducer of SAR. Pathogen infection induces the accumulation of 18:1, 18:2, and 18:3 free FAs, and exogenous application of 18:1 or 18:2 induces AZA biosynthesis and SAR in plants (Yu, et al. 2013). Furthermore, previous studies confirmed that linoleic acid (18:2) and  $\alpha$ -linolenic acid (18:3) are precursors for JA biosynthesis (Banilas, et al. 2007;

Hung and Kao 1998; Ishiguro, et al. 2001; Li, et al. 2003). Oxygenation by 13-lipoxygenases (13-LOXs), followed by sequential action of allene oxide synthase (AOS), allene oxide cyclase (AOC), cis-OPDA reductase 3 (OPR3) and eventually resulting in the synthesis of less active (-)-JA (Gupta, et al. 2020). In cotton plants, cytochrome P450 CYP82D (SSN) competes for C18 fatty acids substrates with LOXs. SSN inhibition leads to free fatty acid entering LOXs metabolism for JA synthesis, thereby enhancing JA levels and disease resistance to *V.dahliae* (Sun, et al. 2014). More and more evidence showed that JA signaling positively regulates the cotton defense to *V.dahliae* infection (He, et al. 2018; Tan, et al. 2015).

Previous studies have shown that PLPs mediates plant immune responses through regulating FA metabolism and JA signaling pathway. In tobacco, the patatin-like protein was rapidly induced preceding JA accumulation in response to the *Tobacco mosaic virus (TMV)* (Dhondt, et al. 2010). AtPLAI plays a critical role in maintaining the homeostatic pool of free FA and basal JA, which increase resistance to *B. cinerea* (Yang, et al. 2007). Overexpression of a patatin-like protein gene OSAG78 in Arabidopsis, increased linoleic acid and linolenic acid amount and induced expression levels of the JA-related defense genes PDF 1.2 and PR4 (Lin, et al. 2011). In this study, *GhPLP2* expression was induced upon treatment with exogenous JA (Fig. 4d), suggesting that *GhPLP2* is likely to involve in JA-dependent defense pathways. Given the LAH activity of GhPLP2 protein *in vivo* and *in vitro*, we analyzed the accumulation of FA and its derivative JA. The data showed that accumulation of C18 FA linoleic acid,  $\alpha$ -linolenic acid, and JA are positively correlated with the expression of *GhPLP2* (Fig. 8). Meanwhile, transcript levels of LOX2, AOS, OPR3 in JA biosynthesis pathway and JA-responsive marker gene PDF1.2 were increased in overexpressed Arabidopsis plants and decreased in *GhPLP2*-silenced cotton plants. These results suggested that increased JA concentration and activation of the JA signal pathway may be explained by the elevated accumulation of linoleic acid and  $\alpha$ -linolenic acid released by the LAH activity of GhPLP2 protein. Taken together, we propose a model of *GhPLP2* involved in plant basal resistance against *V.dahliae* (Fig. 9). *GhPLP2* contributes to the build-up of C18 FA pools, including linoleic acid and  $\alpha$ -linolenic acid, which are precursors for the JA synthesis. The accumulation of linoleic acid and  $\alpha$ -linolenic acid leading to the activation of JA synthesis pathway and JA signaling pathway in plants without pathogen infection, thereby enhancing the plant's disease resistance against *V.dahliae* infection.

## Conclusions

Our study provides evidence that patatin-like protein GhPLP2 with LAH activity plays a positive role in enhancing plant disease resistance. Hypersensitive response, callose deposition, and H<sub>2</sub>O<sub>2</sub> accumulation induced by *V. dahlia* elicitor were compromised in *GhPLP2*-silenced cotton plants. Silencing of *GhPLP2* in cotton plants attenuated resistance against *V. dahlia*, and overexpression of *GhPLP2* in arabidopsis enhanced resistance against *V. dahliae* infection. The resistance mechanism of GhPLP2 was exerted by releasing jasmonic acid synthetic precursor linoleic acid and  $\alpha$ -linolenic acid via its LAH activity, following by accumulation of JA and activation of JA signaling pathway. These results stressed some clues for the mechanism of *GhPLP2* in plant defense response and highlighted the potential application of *GhPLP2* in genetic engineering for the disease resistance of cotton germplasm.

# Declarations

## Compliance with ethical standards

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

**Author Contribution statement** YH, FL and YZ conceived and designed the study. YZ conducted most of the experiments and wrote the manuscript. XH, LG, and ZG provided technical assistance to YZ. YP and YJ provided analysis tools. XG contributed reagents and materials. All authors reviewed and approved the final manuscript.

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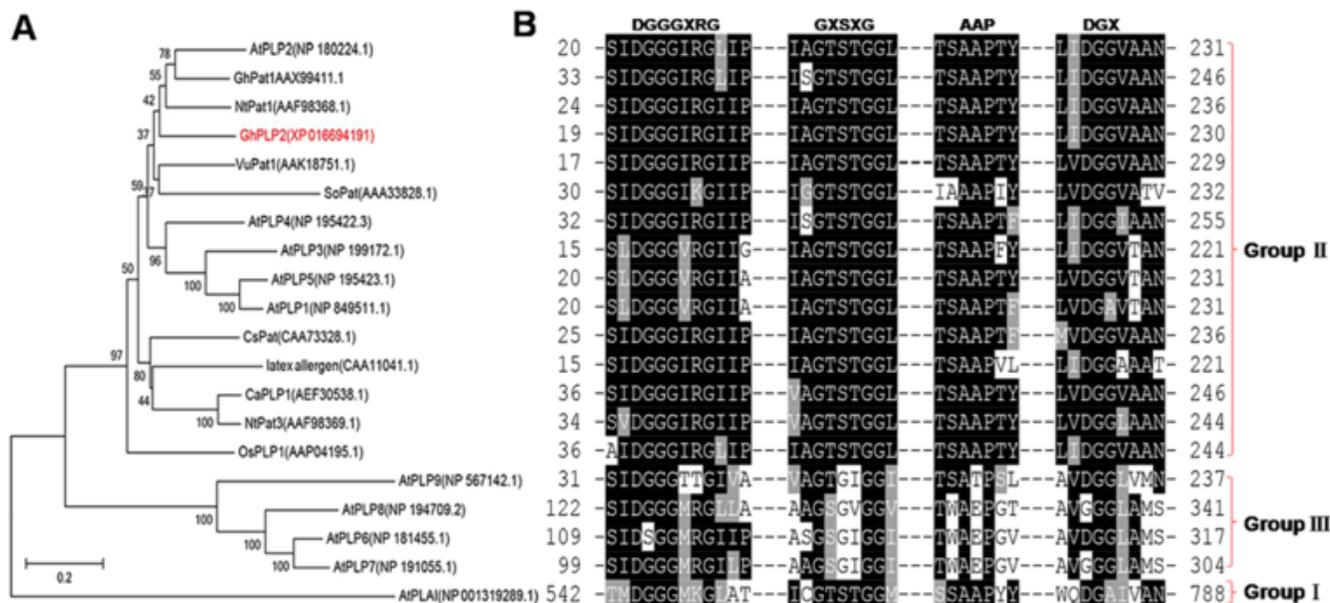
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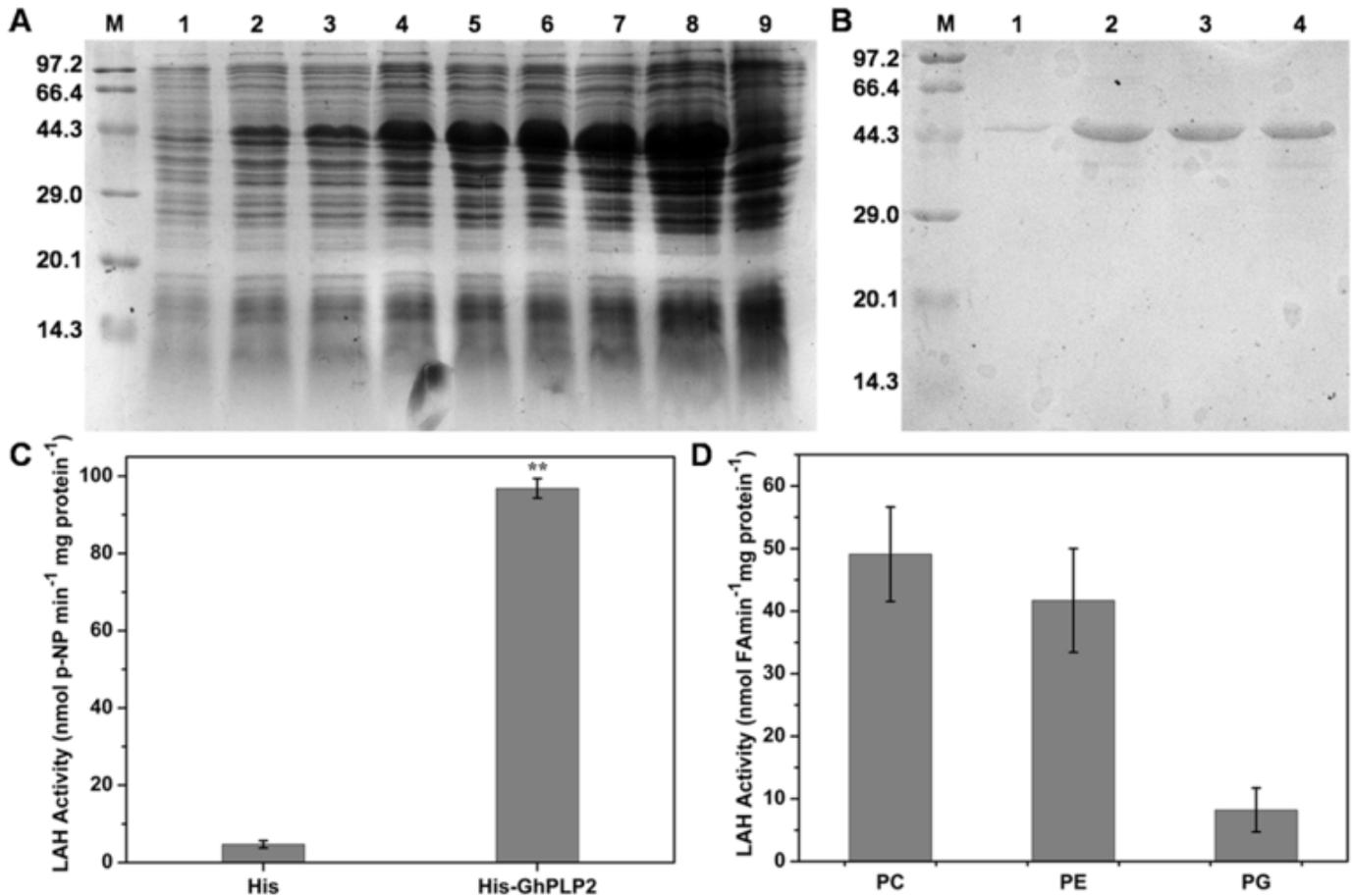
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## Figures

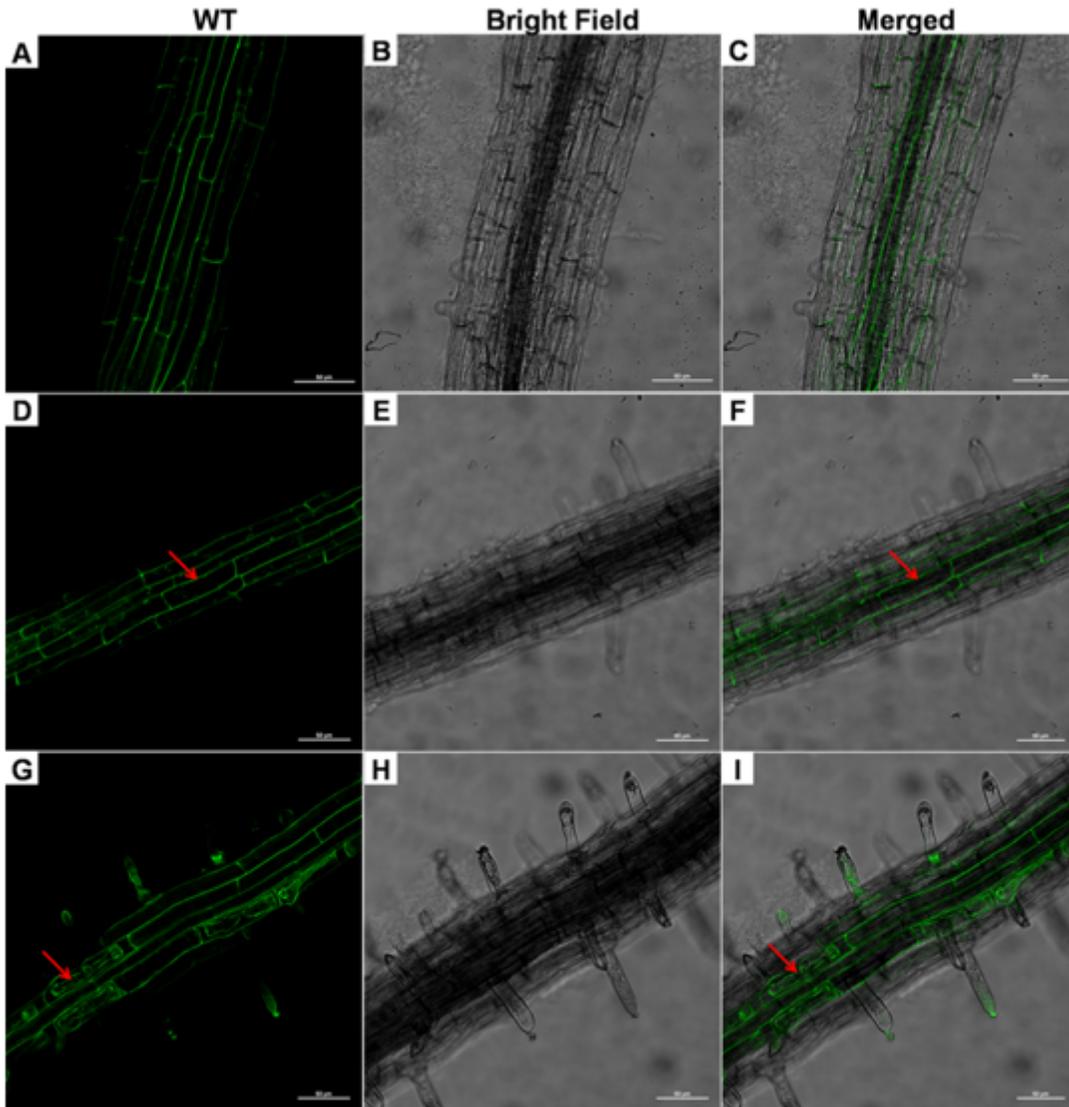


Sequence analyses of GhPLP2 and other patatin-like proteins. (a) Phylogenetic analysis of GhPLP2 and other plant patatin-like proteins. At1g61850 (AtPLAI) was used as the outgroup. The numbers on the tree represent bootstrap scores. (b) Alignment of conserved motifs of patatin-like proteins. Esterase box GxSxG and residues DGX constitute S-D catalytic dyad. Anion binding element: DGGGxxG and proline motif: APP



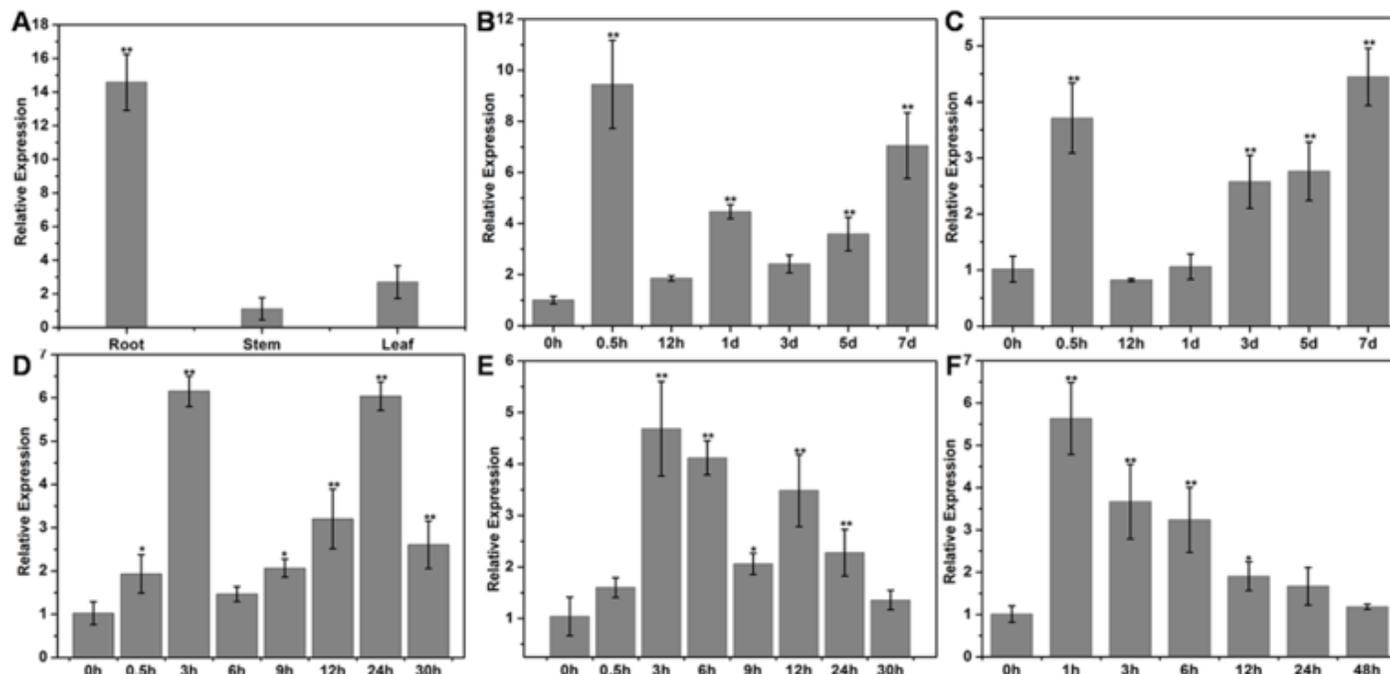
**Figure 2**

Induction and purification of His-GhPLP2 protein and LAH activity assay. (a) Induced proteins were analyzed by SDS-PAGE. M: molecular mass markers (kDa); 1 uninduced crude bacterial protein extract; 2-9 IPTG-induced crude bacterial protein extract at 22°C with different time points. (b) GhPLP2 fusion proteins purified on a Ni-column. 1-4 a series of elution protein. The red arrow indicates GhPLP2 fusion proteins. (c) p-nitrophenol (NP) liberated from p-Nitrophenyl palmitate by lipid acyl hydrolase activity of GhPLP2 protein. Asterisks indicate a significant difference compared with His alone (\*\*P < 0.01, Student's t-test). (d) Fatty acid was released by lipid acyl hydrolase activity of GhPLP2 protein when PC, PE, and PG were used as substrates. Data were obtained from three replicates and presented as mean ± standard error



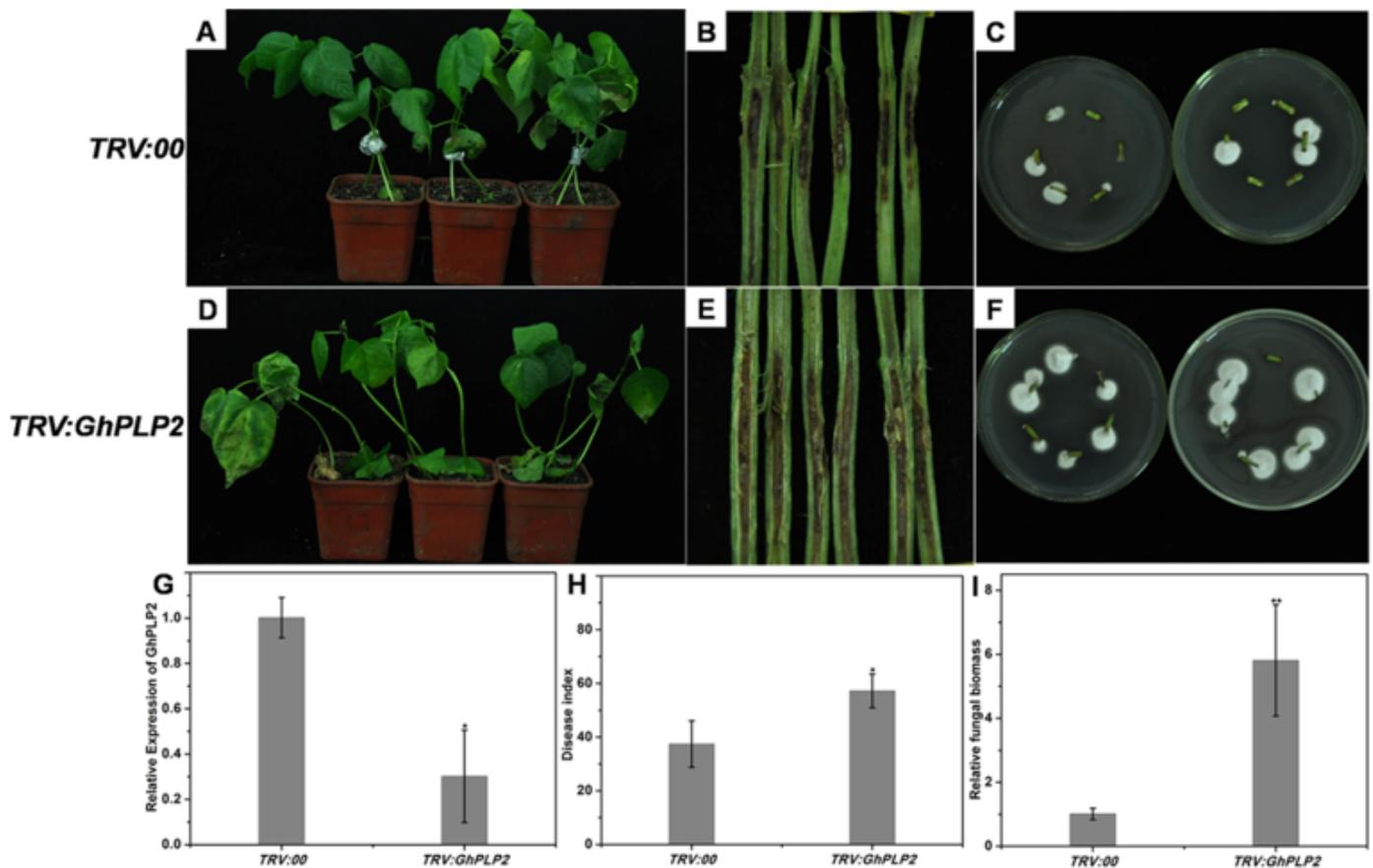
**Figure 3**

Subcellular localization of GhPLP2-GFP fusion protein in transgenic Arabidopsis. (a-c) Super1300::GFP empty vector as a control. (d-f) GhPLP2-GFP fusion protein. (g-i) GhPLP2-GFP fusion protein after plasmolysis. Scale bar represents 50  $\mu\text{m}$



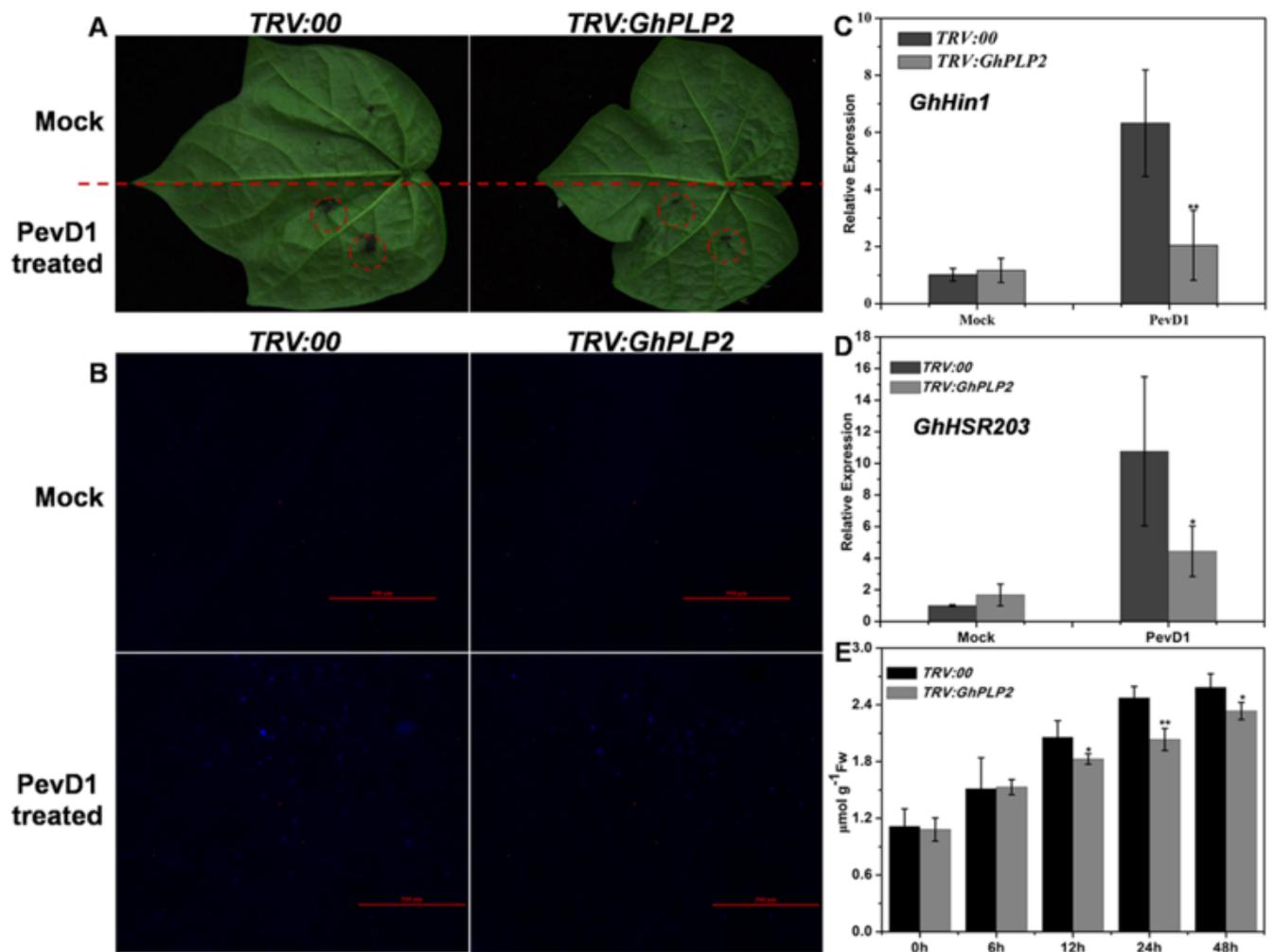
**Figure 4**

Expression patterns of GhPLP2 under different stress in cotton. (a) The expression pattern of GhPLP2 in different tissues. (b,c) GhPLP2 expression after inoculation with *V.dahliae* and *F.oxysporum*, respectively. (d-f) GhPLP2 expression after treatment with 100 μM JA (d), 2 mM ethrel (e) or 2.5% (w/v) PEG6000 (f). Data were obtained from three independent biological replicates and presented as mean ± standard error. Asterisks indicate a significant difference compared with control (\*P < 0.05, \*\*P < 0.01, Student's t-test)



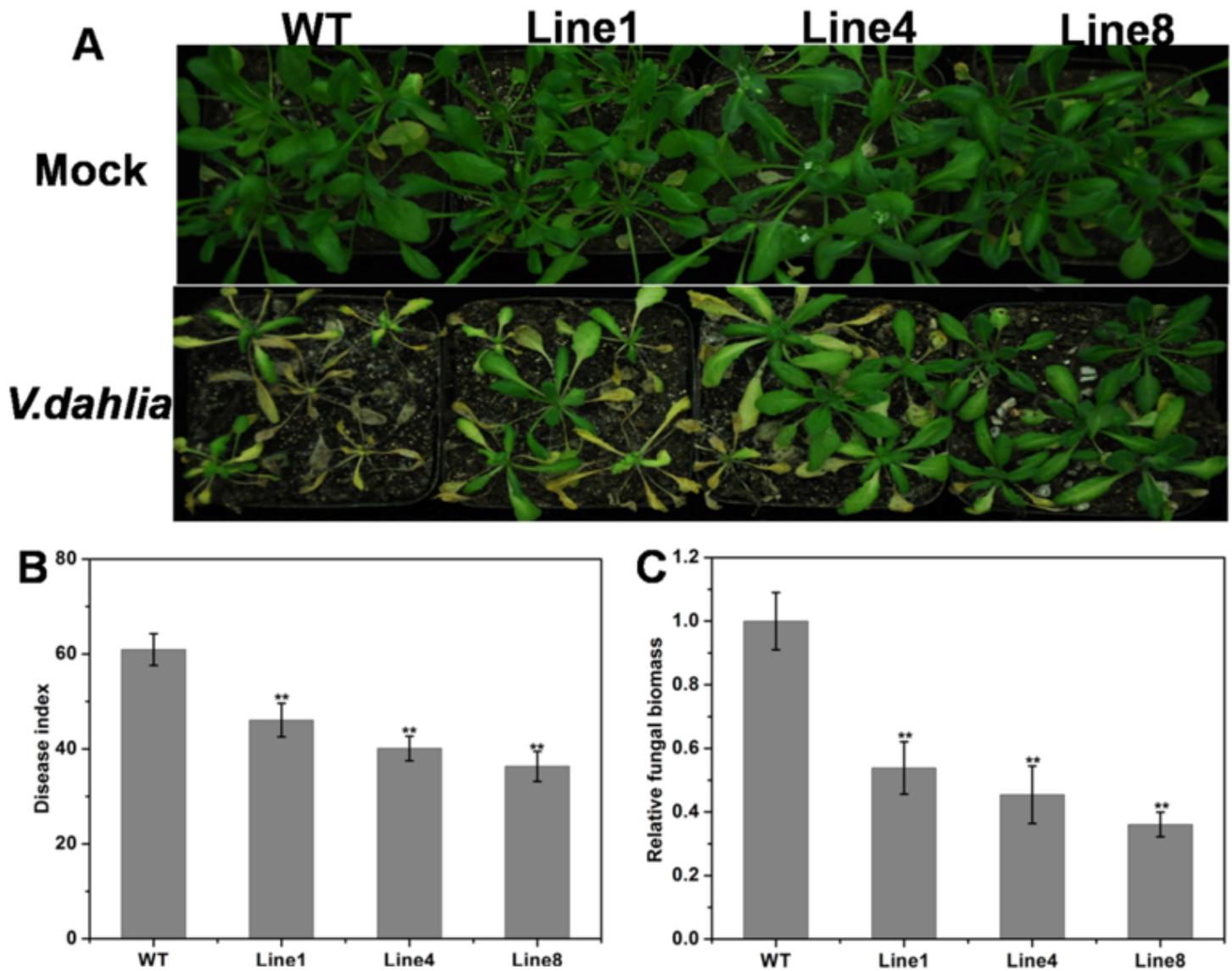
**Figure 5**

Attenuated resistance of GhPLP2-silenced cotton plants to *V.dahliae*. Disease phenotypes of the control (a) and GhPLP2-silenced cotton plants (d) inoculated with *V.dahliae* at 14days. Vascular browning in the stem of the control (b) and GhPLP2-silenced cotton plants (e) infected by *V.dahliae* at 21 days. The fungal renewal cultivation of the control (c) and GhPLP2-silenced cotton plants (f) infected by *V.dahliae* at 21 days. (g) VIGS efficiency of GhPLP2 was evaluated by qRT-PCR after 2 weeks. Data were obtained from three independent biological replicates. Asterisks indicate a significant difference compared with TRV:00 (\* $P < 0.05$ , Student's t-test). (h) Disease index of control and GhPLP2-silenced cotton plants inoculated with *V.dahliae* at 14days. (i) Relative fungal biomass of the control and GhPLP2-silenced cotton plants infected by *V.dahliae* at 21 days. Data represent the means  $\pm$  standard error of three independent repeats. Asterisks indicate significant difference compared with TRV:00 (\*\* $P < 0.01$ , Student's t-test)



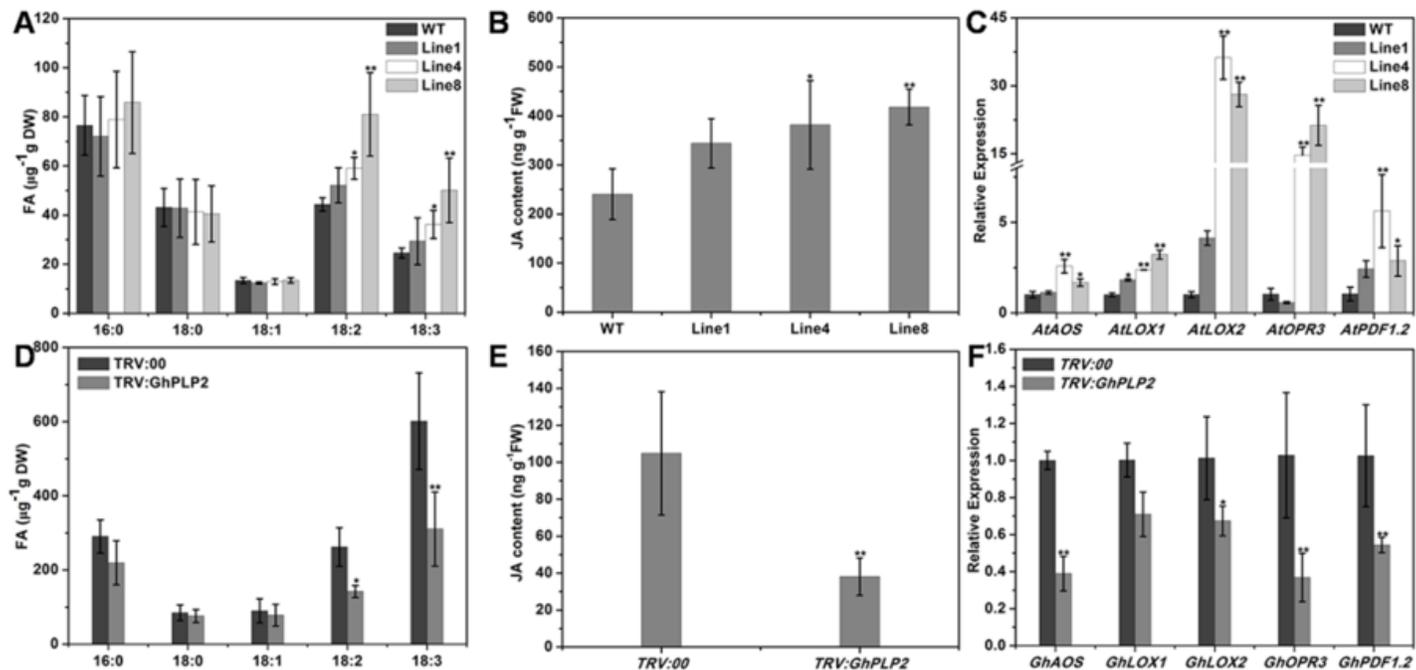
**Figure 6**

GhPLP2 mediated HR triggered by *V. dahliae* elicitor PevD1. (a) HR phenotypes developed on leaves after elicitor PevD1 injection. (b) Callose deposition on the infected leaves at 24 h after injection. Similar results were obtained in three independent experiments. Scale bar represents 500 μm. (c,d) Expression of HR marker genes GhHIN1 and GhHSR203. (e) Detection of H<sub>2</sub>O<sub>2</sub> in leaves during injection with PevD1. Data were obtained from independent biological replicates. Asterisks indicate significant difference, as determined by Student's t-test (\*P < 0.05, \*\*P < 0.01).



**Figure 7**

Increased resistance to *V.dahliae* in GhPLP2-overexpressed Arabidopsis. (a) Arabidopsis plants were inoculated with sterile water and *V.dahliae* by root dipping, respectively. Images were taken at 14 dpi. (b–c) Disease index (b) and fungal biomass (c) of Arabidopsis plants at 14 dpi after inoculation. Data represent the means  $\pm$  standard error of three independent repeats. Asterisks indicate a significant difference compared with WT. (\*\* $P < 0.01$ , Student's t-test)



**Figure 8**

Fatty acids profile, JA accumulation, and gene expression in Arabidopsis and cotton plants. (a) Fatty acids profile analyses of Arabidopsis plants. (b) JA accumulation in Arabidopsis plants. (c) The relative expression of genes in non-inoculated WT and GhPLP2-transgenic Arabidopsis plants. *AtEF1 $\alpha$*  was used as a standard internal gene. (d) Fatty acids profile analyses of cotton plants. (e) JA accumulation in cotton plants. (f) The relative expression of genes in non-inoculated TRV:00 and GhPLP2-silenced cotton plants. GhUBQ was used as a standard internal gene. Data represent the means  $\pm$  standard error of three independent repeats. Asterisks indicate a significant difference compared with WT or TRV:00, respectively. (\* $P < 0.05$ , \*\* $P < 0.01$ , Student's t-test)

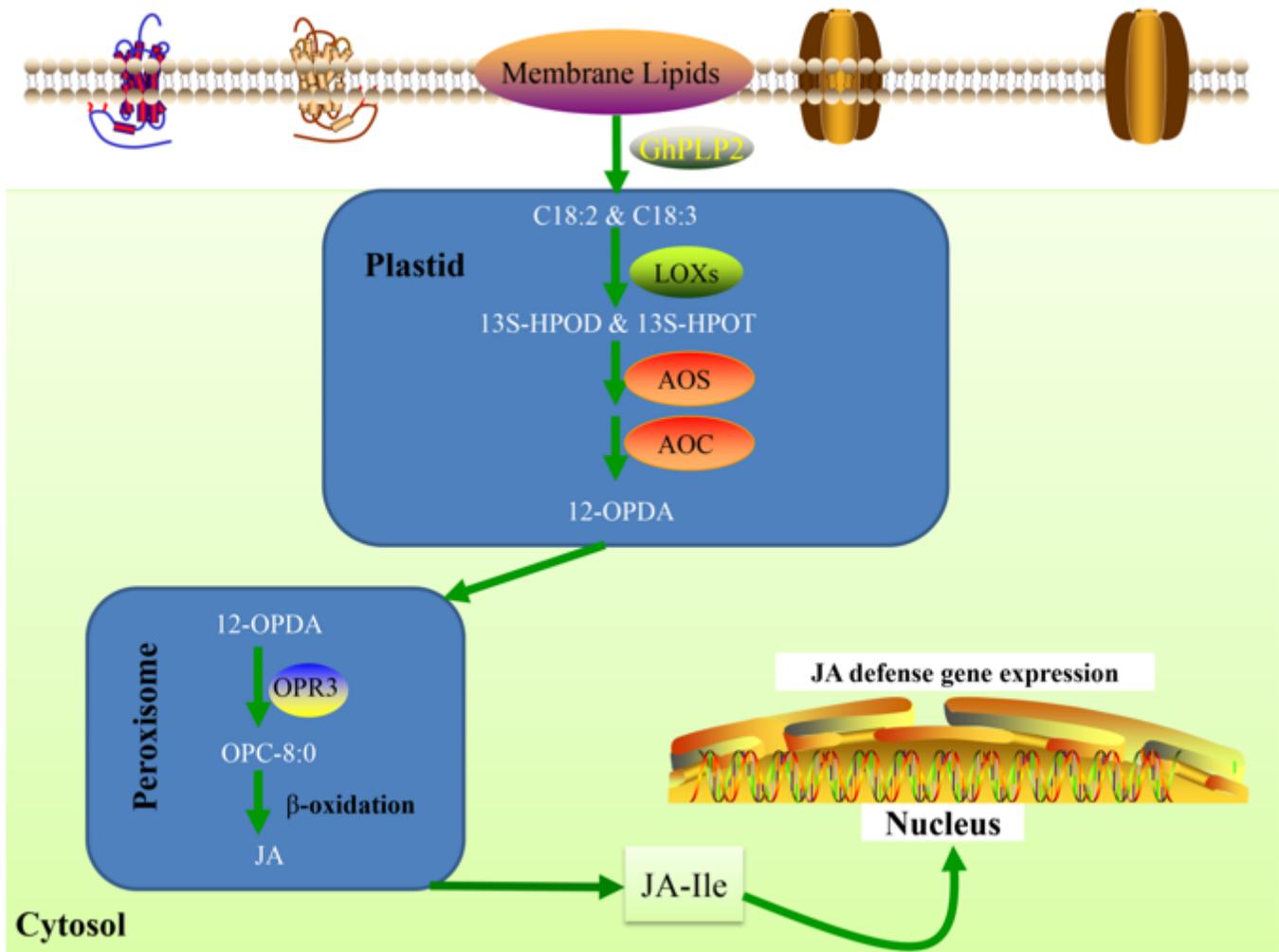


Figure 9

Model of GhPLP2 involvement in plant resistance against *V.dahliae*. LOXs: 13-lipoxygenase; 13S-HPOD: 13S-hydroperoxylinolenic acid; 13S-HPOT: 13S-hydroperoxylinolenic acid; AOS: allene oxide synthase; AOC: allene oxide cyclase; 12-OPDA: 12-oxophytodienoic acid; OPR3-OPDA reductase3; OPC-8:0: 3-oxo-2-(cis-20-pentenyl)-cyclopentane-1-octanoic acid;

## Supplementary Files

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