GbCYP86A1-1 from Gossypium barbadense positively regulates defence against Verticillium dahliae by cell wall modification and activation of immune pathways

Guilin Wang1, Jun Xu1, Lechen Li1, Zhan Guo1, Qingxin Si1, Guozhong Zhu1, Xinyu Wang2 and Wangzhen Guo1

1State Key Laboratory of Crop Genetics & Germplasm Enhancement, Nanjing Agricultural University, Nanjing, Jiangsu Province, China
2College of Life Sciences, Nanjing Agricultural University, Nanjing, Jiangsu Province, China

Summary
Suberin acts as stress-induced antipathogen barrier in the root cell wall. CYP86A1 encodes cytochrome P450 fatty acid ω-hydroxylase, which has been reported to be a key enzyme for suberin biosynthesis; however, its role in resistance to fungi and the mechanisms related to immune responses remain unknown. Here, we identified a disease resistance-related gene, GbCYP86A1-1, from Gossypium barbadense cv. Hai7124. There were three homologs of GbCYP86A1-1 in cotton, which are specifically expressed in roots and induced by Verticillium dahliae. Among them, GbCYP86A1-1 contributed the most significantly to resistance. Silencing of GbCYP86A1-1 in Hai7124 resulted in severely compromised resistance to V. dahliae, while heterologous overexpression of GbCYP86A1-1 in Arabidopsis improved tolerance. Tissue sections showed that the roots of GbCYP86A1-1 transgenic Arabidopsis had more suberin accumulation and significantly higher C16-C18 fatty acid content than control. Transcriptome analysis revealed that overexpression of GbCYP86A1-1 not only affected lipid biosynthesis in roots, but also activated the disease-resistant immune pathway; genes encoding the receptor-like kinases (RLKs), receptor-like proteins (RLPs), hormone-related transcription factors, and pathogenesis-related protein genes (PRs) were more highly expressed in the GbCYP86A1-1 transgenic line than control. Furthermore, we found that when comparing V. dahliae -inoculated and noninoculated plants, few differential genes related to disease immunity were detected in the GbCYP86A1-1 transgenic line; however, a large number of resistance genes were activated in the control. This study highlights the role of GbCYP86A1-1 in the defence against fungi and its underlying molecular immune mechanisms in this process.

Introduction
Verticillium dahliae is a soil-borne fungal pathogen that can infect up to 200 plant species and poses a destructive threat to crops (Fradin and Thomma, 2006). Signals from plant roots trigger the life cycle of V. dahliae, beginning with the germination of microsclerotia (Klimes et al., 2015). The germinated hyphae penetrate the epidermal cells of the roots and then colonize the xylem vessels (Zhao et al., 2014). After successful colonization, the fungus continues to grow along the vascular tissues and produce conidia and microsclerotia, which block water transport and xylem vessels, eventually leading to plant wilting or defoliation (Klimes et al., 2015). Due to the limited number of resistance genes, low effective control measures and few high resistance germplasm resources to cope with V. dahliae, it is difficult to control once plants are infected with the disease (Klosterman et al., 2009).

The most important factor in preventing the invasion of V. dahliae from roots is the epidermal cell wall, which provides a mechanical and chemical barrier as the first line of defence, including a suberin lamella, thick cuticle and synthesis of phenolic compounds (Schreiber, 2010). In addition, plants activate a complex series of immune pathways to delay or block further expansion of the pathogen inside the cell (Jones and Dangl, 2006). Pattern recognition receptors (PRRs) on the surface of the plasma membrane sense pathogen-associated molecular patterns (PAMPs) and damage-related molecular patterns (DAMPs), which could cause the response of internal defence-associated phytohormones, activation of pathogenesis-related genes (PRs) and production of reactive oxygen species (ROS) (Alvarez and Vasquez, 2017; Bacte et al., 2018). PRRs are either surface-localized receptor-like kinases (RLKs) or receptor-like proteins (RLPs) containing various ligand-binding ecto-domains that perceive PAMPs or DAMPs (Gómez-Gómez and Boller, 2000; Zipfel, 2014). A typical DAMP is represented by oligogalacturononides (OGs), which are the main component of cell wall pectin. OGs can be released by polygalacturonase-inhibiting proteins (PGIPs) and pectate lyases in the early stage of pathogen infection (Benedetti et al., 2015; Brutus et al., 2010). As a powerful dynamic barrier, plant cell walls can trigger a variety of changes in response to pathogen attack. Some of these are well described, but many remain obscure (Malinovský et al., 2014; Nüesch, 2012).

Plants have special phenolic ester-based protection system that is accompanied by various metabolic and structural adaptations, including the formation of cell wall modifications, such as the above-ground cuticles and the wide distributed suberin in root (Kolattukudy, 1980; Pollard et al., 2008). Suberin is a glyceroipid–phenolic polymer with a sturdy structure and the chemical properties of a hydrophobic polymer, which make the root resistant to pathogen infection. Histochemical studies have
demonstrated that environmental stress can enhance the suberin's production (Franke and Schreiber, 2007). For example, treatment with *V. dahliae* or stress-responsive hormones induces suberin accumulation in tomato cell wall (Robb et al., 1991). When the tuber of potato is damaged, it can induce the suberization of peridermis and increase the resistance to the *V. dahliae* (Lulai, 2005). Thangavel et al. (2016) screened potato somatic cells that were resistant to *Streptomyces* spp. toxin and found that there was higher expression of genes associated with suberin biosynthesis, indicating the important role of suberin in the resistance of potato to pathogens. In addition, cell wall suberization can also increase the resistance of *Glycine max* to *Phytophthora sojae*, thus avoiding decay of roots and stems (Thomas et al., 2007). In *Arabidopsis*, it has also been found that the invasion of pathogens induces cell wall suberization (Franke et al., 2005). Therefore, the suberized cell walls are more conducive to the defence against pathogen infection than nonmodified cell walls composed of carbohydrates (Franke and Schreiber, 2007).

CYP86 is a subfamily of the cytochrome P450 (CYP) family that is mainly involved in the hydroxylation of fatty acyl-CoA ω sites to form ω-hydroxy acids, some of which are oxidized to ω,ω-dicarboxylic acids (Wercz-Reichhart and Feyereisen, 2000), and further involved in the biosynthesis of protective biopolymers such as cutin and suberin (Kandel et al., 2006). *AtCYP86A2, AtCYP86A4* and *AtCYP86A8* are three homologous genes with high similarity that play a role in the biosynthesis of extracellular lipids and are involved in the hydroxylation of long-chain fatty acids. Previous studies have indicated that these genes are involved in the process of cell wall cuticle modification, and the certain cutin-related fatty acids synthesized by *AtCYP86A2* may inhibit bacterial type III gene expression (Wellens et al., 2001; Xiao et al., 2004). *AtCYP86A1*, which is involved in the hydroxylation of C16-C18 long-chain fatty acids, was the first fatty acid ω-hydroxylase identified in plants (Benveniste et al., 1998). Several studies have identified that *AtCYP86A1* is a key enzyme for aliphatic root suberin biosynthesis. *Arabidopsis* CYP86A1 mutants showed a significant decrease in C16 and C18 suberin monomer content in roots, and the total aliphatic suberin content decreased by 60%. Simultaneously, CYP86A1 has been localized to the endoplasmic reticulum (ER) of root endodermal cells, indicating that suberin monomer biosynthesis takes place in this subcellular compartment before intermediates are exported in the cell wall (Höfer et al., 2008). After RNA interference with *StCYP86A1*, the content of C16 and C18 ω-hydroxy acid and ω,ω-dicarboxylic acid decreased by about 70–90% (Serra et al., 2009). CYP86B1 is clustered in a different class to CYP86A1. As a very long-chain fatty acid hydroxylase, CYP86B1 participates in the hydroxylation of C22-C24 very long-chain fatty acids (Compagnon et al., 2009). Compared to wild type (WT), knockout of *AtCYP86B1* led to almost complete lack of C22 and C24 corresponding ω,ω-dicarboxylic acids and ω-hydroxy acids in root and seed coat aliphatic suberin (Molina et al., 2009). Overall, CYP86 genes encoding fatty acid hydroxylase are inextricably linked to protective biopolymer biosynthesis (Duan and Schuler, 2005; Höfer et al., 2008).

As one of the world’s major cash crops, cotton is the most important source of textile fibre and seed oil, but damage from *V. dahliae* seriously affects the yield and quality (Daayf et al., 2005; H. oler., 2008). Overexpression of *GbCYP86A1* not only improved structural resistance in roots through greater accumulation of suberin, but also activated the disease resistance-related immune pathways. This is the first report that *GbCYP86A1* plays an important role in *V. dahliae* resistance, and we also provide effective gene resources for the development of *Verticillium* wilt-resistant cultivars through cotton breeding programmes.

### Results

#### Identification and expression patterns of the CYP86 genes in *Gossypium*

Based on P450 domain and whole-genome sequence of *Gossypium raimondii*, we identified 373 GrCYPs. Through phylogenetic analysis with 245 AtCYPs in *Arabidopsis* (Xu et al., 2015), ten GbCYP86 genes were further identified (Figure S1). Following homology alignment analysis, we identified 10, 19 and 19 CYP86s in *G. arboreum, G. hirsutum* acc. TM-1 and *G. barbadense* cv. Hai7124, respectively, and carried out their systematic naming based on the corresponding homology in *Arabidopsis* (Data S1). GrCYP86s can be divided into three classes (A, B, and C) (Figure S2a). There was more than 80% homology between homologous genes of the same class, with the exception of *GrCYP86B1* (Figure S2b; Table S1). Only three genes, *GrCYP86A7-2, GrCYP86B1-1* and *GrCYP86B1-2*, had introns (Figure S2c). All GrCYP86s had a P450 domain, including the helix K domain, helix I domain and heme-binding domain (Figure S3), and a transmembrane domain at the N terminus, with the exception of *GbCYP86A7* (Figure S2d).

Transcriptome data from TM-1 vegetative tissues (root, stem and leaf), floral tissues (petal, stamen and pistil), ovule tissues and fibre tissues at different developmental stages showed that GhCYP86s had diverse developmental and spatial regulation in various tissues, with similar expression patterns in the homologous genes (Figure 1a; Data S2). The GhCYP86A1 homologs were specifically expressed in roots. GhCYP86A8 homologs were predominantly expressed in reproductive organs. GhCYP86A7 homologs were expressed in stem, leaf, petal and 10 dpf fibre tissues. GhCYP86B1-1 was highly expressed in all tissues, but GhCYP86B1-2 was only expressed in floral and 25 dpf fibre tissues. GhCYP86C1 was not expressed in most tissues. These findings indicate that CYP86s have conserved structures but diverse functions.
With detectable expression levels in root tissues following transcriptome analysis, the responses of CYP86A1-1, CYP86A1-2, CYP86A1-3 and CYP86B1-1 were further investigated after V. dahliae inoculation in cotton. Overall, the expression of these CYP86s was higher in G. barbadense cv. Hai7124 than G. hirsutum cv. Junmian 1. In particular, the expression of CYP86A1-1, CYP86A1-2 and CYP86A1-3 was significantly increased after inoculation in these two plants, especially in Hai7124 after 144 h (Figure 1b). Therefore, these three genes are related to V. dahliae resistance.

Functional characterization of GbCYP86A1s in V. dahliae resistance through virus-induced gene silencing (VIGS)

To elucidate the role of GbCYP86A1s in cotton defence against V. dahliae, we used VIGS to specifically silence one of the three GbCYP86A1 genes (marked as TRV2: GbCYP86A1-1, TRV2: GbCYP86A1-2 and TRV2: GbCYP86A1-3) and silenced simultaneously the three genes by selecting their conserved regions (TRV2: H3091) in Hai7124, with TRV: 00 as mock treatment and TRV: GbCLA1 as positive control. As expected, the cotton leaves showed obvious photobleaching phenotype two weeks after agroinfiltration with TRV: GbCLA1 (Figure S4a). Further, the cotton seedlings infiltrated with different constructs were sampled for RNA isolation and qRT–PCR analysis. The expression of GbCYP86A1-1, GbCYP86A1-2 and GbCYP86A1-3 was significantly reduced in the separately silenced plants compared with mock-treated plants, and the expression of three genes in the cosuppression plants by TRV2: H3091 was also significantly reduced (P < 0.01) (Figure 2a). Off-target silencing of other homologs showed that the three homologs had no obvious influence each other in the each specifically silenced plants (Figure S4b).

With Hai7124 and Junmian 1 plants as resistant and susceptible controls to V. dahliae, respectively (Figure S4c), after V. dahliae strain V991 inoculation, the cotton seedlings of the silenced plants, particularly those containing TRV2: GbCYP86A1-1, TRV2: GbCYP86A1-2 and TRV2: GbCYP86A1-3, exhibited more wilting, etiolated and even abscission of leaves than control, especially in GbCYP86A1-1- and H3091-silenced plants (Figure 2b). We used at least 30 plants per treatment to calculate the disease index and found that the disease index of TRV2: 00 control plants was approximately 61.5% at 30 days after inoculation. However, the disease index in the GbCYP86A1-1-silenced and H3091-silenced plants reached 85.4% and 89.6%, respectively. In addition, the disease index of GbCYP86A1-2-silenced plants and GbCYP86A1-3-silenced plants was 77.1% and 74.0%, respectively (Figure 2c; Table S2). The stereomicroscope was used to visually observe the accumulation of invading V. dahliae in vascular tissues; GbCYP86A1-1-silenced and H3091-silenced plants showed more wilting, etiolated and even abscission of leaves than control, especially in GbCYP86A1-1- and H3091-silenced plants (Figure 2b). We used at least 30 plants per treatment to calculate the disease index and found that the disease index of TRV2: 00 control plants was approximately 61.5% at 30 days after inoculation. However, the disease index in the GbCYP86A1-1-silenced and H3091-silenced plants reached 85.4% and 89.6%, respectively. In addition, the disease index of GbCYP86A1-2-silenced plants and GbCYP86A1-3-silenced plants was 77.1% and 74.0%, respectively (Figure 2c; Table S2). The stereomicroscope was used to visually observe the accumulation of invading V. dahliae in vascular tissues; GbCYP86A1-1-silenced and H3091-silenced plants showed more wilting, etiolated and even abscission of leaves than control, especially in GbCYP86A1-1- and H3091-silenced plants (Figure 2b). We used at least 30 plants per treatment to calculate the disease index and found that the disease index of TRV2: 00 control plants was approximately 61.5% at 30 days after inoculation. However, the disease index in the GbCYP86A1-1-silenced and H3091-silenced plants reached 85.4% and 89.6%, respectively. In addition, the disease index of GbCYP86A1-2-silenced plants and GbCYP86A1-3-silenced plants was 77.1% and 74.0%, respectively (Figure 2c; Table S2). The stereomicroscope was used to visually observe the accumulation of invading V. dahliae in vascular tissues; GbCYP86A1-1-silenced and H3091-silenced plants showed more wilting, etiolated and even abscission of leaves than control, especially in GbCYP86A1-1- and H3091-silenced plants (Figure 2b). We used at least 30 plants per treatment to calculate the disease index and found that the disease index of TRV2: 00 control plants was approximately 61.5% at 30 days after inoculation. However, the disease index in the GbCYP86A1-1-silenced and H3091-silenced plants reached 85.4% and 89.6%, respectively. In addition, the disease index of GbCYP86A1-2-silenced plants and GbCYP86A1-3-silenced plants was 77.1% and 74.0%, respectively (Figure 2c; Table S2). The stereomicroscope was used to visually observe the accumulation of invading V. dahliae in vascular tissues; GbCYP86A1-1-silenced and H3091-silenced plants showed more wilting, etiolated and even abscission of leaves than control, especially in GbCYP86A1-1- and H3091-silenced plants (Figure 2b). We used at least 30 plants per treatment to calculate the disease index and found that the disease index of TRV2: 00 control plants was approximately 61.5% at 30 days after inoculation. However, the disease index in the GbCYP86A1-1-silenced and H3091-silenced plants reached 85.4% and 89.6%, respectively. In addition, the disease index of GbCYP86A1-2-silenced plants and GbCYP86A1-3-silenced plants was 77.1% and 74.0%, respectively (Figure 2c; Table S2). The stereomicroscope was used to visually observe the accumulation of invading V. dahliae in vascular tissues; GbCYP86A1-1-silenced and H3091-silenced plants showed more wilting, etiolated and even abscission of leaves than control, especially in GbCYP86A1-1- and H3091-silenced plants (Figure 2b).
Figure 2  Silencing of GbCYP86A1-1, GbCYP86A1-2, GbCYP86A1-3 and H3091 in the resistant cultivar G. barbadense cv. Hai7124 leads to increased susceptibility to Verticillium dahliae infection. Gene-specific DNA fragment for specifically silencing one of the three GbCYP86A1s and conserved region of these three genes (named as H3091) were selected as the target, respectively. (a) Verification of GbCYP86A1-1, GbCYP86A1-2 and GbCYP86A1-3 silencing by qRT–PCR in different VIGS plants. Asterisks indicate statistically significant differences, as determined by Student’s t-test (*** P < 0.01). (b) Disease symptoms of GbCYP86A1-1, GbCYP86A1-2, GbCYP86A1-3 and H3091 silenced cotton plants at 20, 25 and 30 days after V. dahliae inoculation. (c) Disease progression curves in GbCYP86A1-1, GbCYP86A1-2, GbCYP86A1-3 and H3091 silenced cotton plants after V. dahliae inoculation. Each biological repeat contains at least 30 seedlings. Error bars represent the standard deviation of three biological replicates. (d) Vascular discoloration in GbCYP86A1-1, GbCYP86A1-2, GbCYP86A1-3 and H3091 silenced cotton plants compared with the controls (Hai7124 and TRV:00) after inoculation with V991 and Hai7124 without inoculation (H2O). Photographs were taken by stereoscope (Olympus MVX10, Tokyo, Japan) at 15 dpi.
silenced plants were more severely affected than control plants (Figure 2d). These findings suggest that after silencing GbCYP86A1s, the disease resistance of the plants was weakened to varying degrees. Knocking down GbCYP86A1-1 led to a phenotype that was more susceptible to V. dahliae infection compared with other two homologs.

Chromosome location, phylogenetic analysis and subcellular localization of GbCYP86A1s

Chromosome location showed that CYP86A1s distributed on different chromosomes with good collinearity in G. raimondii and G. barbadense (Figure S5). Phylogenetic analysis of GbCYP86A1 orthologs from 12 species showed that CYP86A1s are widely found in different species, and CYP86A1-1, CYP86A1-2 and CYP86A1-3 can be clearly distinguished into three branches in cotton, with higher homology between CYP86A1-2 and CYP86A1-3. Besides Gossypium, the CYP86A1 genes in cotton have the closest homology to cacao and poplar, and more than 75% with Arabidopsis, indicating that CYP86A1 may have similar functions in different plants (Figure 3a). Subcellular localization showed that all three GbCYP86A1 proteins were present in ER (Figure 3b), which is consistent with their function in the suberin biosynthesis pathway.

Functional identification in V. dahliae resistance by overexpressing GbCYP86A1s in Arabidopsis

We generated transgenic Arabidopsis lines that heterologously expressed GbCYP86A1-1, GbCYP86A1-2 and GbCYP86A1-3, respectively. For each construct, more than ten independent transgenic lines were obtained, and six well-grown strains from each homozygous T3 line were used for genomic and transcript level identification (Figure 4a). Further, two transgenic lines with high expression were chosen for further analysis.

All the transgenic and WT plants were cultured for four weeks under the same conditions, and no distinct phenotypic differences were observed in growth and development (Figure S6). After V991 inoculation, a more resistant phenotype could be observed in GbCYP86A1-1 transgenic line, with less wilting, chlorosis, early senescence and necrosis present. Although the disease resistance of the GbCYP86A1-2 transgenic line was also improved compared to WT, it was lower than that in the GbCYP86A1-1 transgenic line. In addition, the disease resistance of the GbCYP86A1-3 transgenic line was not significantly different to WT plants (Figure 4b). We used at least 30 plants per treatment to calculate the disease index. Two weeks after infection, the disease index of WT plants reached 72.5%; however, the two GbCYP86A1-1 transgenic lines were only 30% and 14%, respectively (Figure 4c; Table S3). Fungal biomass analysis confirmed that the degree of fungal invasion in different tissues was altered in the transgenic plants; there was significantly fewer fungal biomass accumulated in the tissues of the GbCYP86A1-1 and GbCYP86A1-2 transgenic line, especially in GbCYP86A1-1 transgenic line. The fungal biomass of the GbCYP86A1-3 transgenic line was not significantly different to WT (Figure 4d). A recovery assay to examine the degree of colonization of V. dahliae in infected stems also showed that only a small number of colonies were present in the stems of GbCYP86A1-1 and GbCYP86A1-2 transgenic lines compared to WT and GbCYP86A1-3 transgenic lines. The GbCYP86A1-1 transgenic line, in particular, showed a significantly enhanced resistance to V. dahliae (Figure 4e).

GbCYP86A1-1 contributes to greater suberin accumulation in cell walls and hinders the invasion of V. dahliae in Arabidopsis roots

Based on these results, we individually selected a transgenic line with the greatest resistance phenotype for each construct, GbCYP86A1-1 from OE2 and GbCYP86A1-2 and GbCYP86A1-3 from OE1, for further analysis. No significant difference in root length, root cell length and root hair length in seedlings of the three GbCYP86A1-overexpressing transgenic Arabidopsis was detected (Figure S7). To detect changes in the suberin composition of roots at maturity, we sampled a cross section, 2 cm from the base of the main root (Figure 5a). The sections were stained with the lipophilic dye Sudan 7B and observed, and we found that the cell wall of the outer layer showed greater lipid content in GbCYP86A1-1 transgenic line compared with other two transgenic and WT plants, as the red colour was deeper and more obvious (Figure 5b). To assess the metabolic differences between the WT and transgenic plants, fatty acid content was measured. Interestingly, the relative content of C16-C18 fatty acids in GbCYP86A1-1 transgenic line was significantly higher than in the WT (P < 0.01). However, the other two transgenic lines did not show any difference, with the exception of higher levels of C18:1 fatty acids in the GbCYP86A1-2 transgenic line (Figure 5c). These results indicate that GbCYP86A1-1 affects lipid content in peridermal cell walls and fatty acid metabolism in roots, with highly suberized cell walls. In addition, we performed freehand sections and fungal biomass analysis of the root tissue after three days of V991 treatment. Compared with WT and the other two transgenic lines, very little invasion of black mycelia was detected and the relative fungal biomass was also very low in the GbCYP86A1-1 transgenic line (Figure 5d; Figure S8). Taken together, the suberized modification of the cell wall in the GbCYP86A1-1 transgenic line inhibited the ability of V. dahliae to invade and multiply.

Molecular investigation of the increased disease resistance in the overexpressing GbCYP86A1-1 transgenic plants

To obtain insights into the molecular basis for the increased resistance of the GbCYP86A1-1 transgenic line upon V991 infection, we sampled the roots of the GbCYP86A1-1 transgenic plants treated with water and V991 for three days, with WT as a control. Comparative transcriptome analysis between transgenic and WT plants under water treatment showed a total of 481 differential expression genes (q < 0.05 and a fold change > 1.5) (Data S3). With 57 unknown proteins or pseudogenes removed, 222 genes were found to be up-regulated and 202 genes down-regulated. Gene ontology (GO) analysis showed that a subset of the genes were further enriched (Figure 6a; Table S4), including those involved in response to stress (FDR = 5.00E-15), response to stimulus (FDR = 5.30E-13), response to biotic stimulus (FDR = 4.10E-08), defence response (FDR = 4.10E-08), secondary metabolic process (FDR = 5.10E-07), systemic acquired resistance (FDR = 2.90E-04), external encapsulating structure (FDR = 6.40E-04), response to chitin (FDR = 1.50E-03) and lipid transport (FDR = 2.70E-03). These overrepresented genes indicated that structural resistance and disease-resistant immune pathways were initiated in roots of the GbCYP86A1-1 transgenic line.

Lipid transfer proteins (LTPs) and ATP-binding cassette (ABC) transporters are important candidate proteins involved in the
Overexpression of GbCYP86A1-1 enhances the resistance to *Verticillium dahliae*.

Figure 3  Characterization of GbCYP86A1 genes. (a) Phylogenetic trees of CYP86A1s from *G. raimondii* (Gr), *G. hirsutum* (Gh), *G. barbadense* (Gb), *Theobroma cacao* (Tc), *Populus trichocarpa* (Pt), *Vitis vinifera* (Vv), *Nicotiana attenuata* (Na), *Artemisia annua* (Aa), *Helianthus annuus* (Ha), *Glycine max* (Gm), *Vigna angularis* (Va), *Arabidopsis thaliana* (At), *Oryza sativa* (Os) and *Zea mays* (Zm) plants. The neighbour-joining tree was constructed using the MEGA5.1 program (http://www.megasoftware.net/). Identity value was relative to GbCYP86A1-1 and was calculated using software DNAMAN (http://www.lynnnon.com). (b) Localization of GbCYP86A1s in tobacco epidermal cells by GFP fusion. HDEL: DsRed (red) is an endoplasmic reticulum (ER) marker. The results show that GbCYP86A1s colocalize with the ER marker. Bars = 50 μm.
transmembrane transport of suberin components (Vishwanath et al., 2015). In this study, a number of genes encoding LTPs, ABCs and some AAA-ATPase family proteins related to ATPase activity had higher expression levels in GbCYP86A1-1 transgenic line compared to WT. Further, genes involved in phenylpropanoid metabolism or the synthesis of suberin were also enriched. For example, several P450 superfamily genes, β-ketoacyl-CoA synthases (KCS) genes, and genes involved in flavonoid biosynthesis had significantly higher expression in the GbCYP86A1-1 transgenic line (Table S5). These results indicate that genes involved in secondary metabolic processes and biosynthesis of suberin were highly induced in GbCYP86A1-1 transgenic line.

Changes in cell wall structure or metabolism cause the hydrolysis of pectin and release of oligosaccharides (OGs). OGs released from the plant cell wall as DAMPs can be recognized by receptor RLKs or RLPs, thereby activating downstream immune responses including changes in phytohormones and expression of PRs (Vallarino and Osorio, 2012). Genes encoding pectin lyase-like superfamily protein and PGIP2, RLKs and RLPs, PRs, ethylene (ET) and salicylic acid (SA) synthesis-related genes, as well as hormone-responsive transcription factors, such as ERFs and WRKYs, were significantly up-regulated in the GbCYP86A1-1 transgenic line, while some jasmonate (JA)-related genes were significantly down-regulated (Table S5). These integrated results indicate that GbCYP86A1-1 affects the immune pathways, activates PR expression and enhances plant disease resistance.

We further compared the differentially expressed genes in the roots of GbCYP86A1-1 transgenic and WT plants with inoculated and noninoculated V. dahliae treatment. Interestingly, the number of differential genes showed significant differences. In detail, 2,541 differential genes were found in the GbCYP86A1-1 transgenic line, 277 of which had a 1.5-fold change (Data S4). These differential genes were mainly involved in biological processes related to the response to a variety of abiotic factors, as well as growth or metabolism, such as response to temperature stimulus, response to chemical and flavonoid biosynthetic process. In contrast, 3,616 differential genes were found in WT, and 1,515 of these had a 1.5-fold change (Data S5). In addition to the response to abiotic factors, growth or metabolism, genes mainly involved in disease-related biological processes were enriched, including those involved in the response to biotic stimuli, defence response, response to fungus, ethylene-activated signalling pathway, and response to salicylic acid (Figure 6b; Tables S6 and S7). WT roots were attacked by V. dahliae and severely damaged internally. In response, more genes were activated, including a large number of genes related to growth or metabolism and resistance genes, in order to prevent further propagation of V. dahliae. This might lead to a trade-off between growth and resistance in plants and show the susceptible phenotype in the WT.

To confirm the RNA-seq expression data and its reliability, a total of 30 transcripts of secondary metabolism-related genes, RLKs and RLPs, phytohormone-related transcription factors and PRs, were selected for qRT–PCR analysis. The qPCR results were highly correlated with RNA-seq analysis, with the fitting degree above 85% (Figures S9 and S10).

GbCYP86A1-1 knockdown affects the expression of PRs in the roots of G. barbadense cv Hai7124

Transcriptome analysis showed that PRs were highly expressed in GbCYP86A1-1 transgenic Arabidopsis. We analysed the expression patterns of PRs in Hai7124 and Junmian 1 after treatment with V991. Eight genes, PR1, PR2, PR3, PR4, PR5, PR6, PR9 and PR16 were significantly induced in the two cotton cultivars, suggesting their important roles against V. dahliae infection in cotton (Figure 7a). To determine whether silencing GbCYP86A1-1 affected downstream defence-related genes in Hai7124, we further analysed the expression of these PRs in the GbCYP86A1-1-silenced plants. Compared with that in Hai7124 and TRV: 00 control plants, the expression of GbPR1, GbPR2, GbPR4, GbPR5, GbP16 was significantly lower in GbCYP86A1-1-silenced plants (Figure 7b), indicating that down-regulation of GbCYP86A1-1 in cotton affects the expression of PRs.

Overall, overexpressing GbCYP86A1-1 not only promotes lipid metabolism and transport in roots, leading to the cell wall suberization, but also activates disease-resistant immune pathways, which prevents V. dahliae infection of roots and improves plant disease resistance.

Discussion

GbCYP86A1-1 is involved in the accumulation of suberin in the roots

Plant roots are protected by specialized lipid-derived cell wall modifications, such as suberin. The hydrophobic barrier formed by the deposition of suberin is important in controlling the transport of water and nutrients, as well as limiting the invasion of pathogens and toxic compounds in root tissue (Frankle and Schreiber, 2007). In this study, several pieces of evidence support the important role of GbCYP86A1-1 in root suberization.
Overexpression of GbCYP86A1-1 enhances the resistance to *Verticillium dahliae*. 

(a) 

![Graph showing relative expression of GbCYP86A1-1, GbCYP86A1-2, and GbCYP86A1-3 in different genotypes.](image)

(b) 

![Comparison of plant growth under different conditions.](image)

(c) 

![Graph showing disease resistance in different genotypes after 2 weeks of inoculation.](image)

(d) 

![Bar charts showing relative fungal biomass in roots, stems, and leaves of different genotypes.](image)

(e) 

![Images showing fungal growth inhibition in different genotypes.](image)
It has been demonstrated in *Arabidopsis* and potato that CYP86A1 mutation results in the destruction of suberin lamellae in roots (Molina et al., 2009; Serra et al., 2009). In this study, using Sudan 7B staining methods (Höfer et al., 2008), we demonstrated that the cell wall of the periderm cells had obvious accumulation of suberin in GbCYP86A1-1 transgenic *Arabidopsis* roots. In addition, the relative content of C16-C18 long-chain fatty acids increased significantly in transgenic *Arabidopsis* roots, indicating sufficient precursors for lipid synthesis and metabolism. 

RNA-seq sequencing of GbCYP86A1-1 transgenic *Arabidopsis* further showed that GbCYP86A1-1 affected the biosynthesis and transport of root lipids and affected many transporter proteins in this process, which eventually led to the suberization of the cell wall. In the past decade, great progress has been made in the study of the biosynthesis of suberin monomers. A number of key enzymes and proteins have been discovered in *Arabidopsis* and potato, and the related composition, structure, distribution and biosynthesis of suberin have been reported (Beisson et al., 2012). Biosynthesis of suberin monomers is carried out in the ER and involves the hydroxylation of fatty acids, extension of fatty acid precursors, activation of fatty acids to fatty acyl-CoA thioesters and reduction of fatty acyl chains to fatty alcohols, various acylations largely involving glycerol, phenolic compound incorporation and polymerization of monomers (Pollard et al., 2008, Vishwanath et al., 2015). Here, we further confirmed that GbCYP86A1-1 is located on the ER. Although comparative transcriptome analysis did not enrich significantly the differential expression genes related to C16 and C18 fatty acid biosynthesis, many genes, such as LTPs and ABCs, which encodes proteins involved in the transmembrane transport of suberin components, were up-regulated in overexpressing GbCYP86A1-1 transgenic line. It has been found that RCN1/OsABCG5 in *Oryza sativa* is involved in the suberization of the hypodermis (Shiono et al., 2014). ABCG1 is also involved in the formation of suberin in the periderm of potato tubers (Landgraf et al., 2014). AtABCG2, AtABCG6 and AtABCG20 are involved in the formation of suberin lamellae in the endodermis of roots and seed coats in *Arabidopsis*, and the suberin structure, composition and properties were changed in roots of the corresponding triple mutant, abc2/abc6/abc20 (Yadav et al., 2014). Recent studies have shown that the nonspecific lipid transfer protein AtLtpI-4 is required for the formation of suberin in *Arabidopsis* crown gall tumours, and the AtLtpI-4 loss-of-function mutant had a significant decrease in the content of suberin components (C18:0) (Deeken et al., 2016). AAA family genes have the same ATPase activity as the ABC transporters, and they are involved in the transport of matter in...
Figure 6  RNA-seq reveals that GbCYP86A1-1 overexpression affects lipid-related pathways and immunity-related pathways in root of Arabidopsis. (a) GO enrichment analysis of the differentially expressed genes in roots of GbCYP86A1-1 transgenic line and the WT control. The RNAs isolated from roots of three individual plants were used for RNA-seq. P value of 0.05 adjusted by false discovery rate (FDR). Rich factor: Percentage of enriched genes comparing with background in corresponding GO term. (b) The number of differentially expressed genes between GbCYP86A1-1 transgenic Arabidopsis plants and WT after challenged with Verticillium dahliae for 3 days. GO terms were separately shown by enrichment analysis. P value of 0.05 adjusted by false discovery rate (FDR).
Figure 7  Pathogenesis-related (PR) gene expression assays. (a) The relative expression of PR genes in resistant cultivar Hai7124 and susceptible cultivar Junmian1 roots after inoculation with *Verticillium dahliae* strain V991. The statistical analyses were performed by comparing expression levels at different time points after *V. dahliae* infection to 0 h without inoculation. (b) The effects of *GbCYP86A1-1* silencing on PR genes expression in Hai7124. Error bars show the standard deviation of three biological replicates. Statistical analyses were performed using Student’s t-test (*P < 0.05, **P < 0.01).
the cell’s inner membrane. These are also likely to be important candidate genes for regulating lipid transport (Yedidi et al., 2017). In addition, several highly expressed secondary metabolic process-related genes have also been found, such as KCS is mainly involved in the extension of suberin precursors (Lee et al., 2009). The genes encoding GDSL-like lipase catalyse in vitro transesterification of monoacylglycerol precursors (Yeats et al., 2014). Some cytochrome P450 genes such as CYP86B1 and flavonoid synthesis-related genes are significantly expressed in transgenic lines, and this may be related to the complexity of plant secondary metabolism (Nesi et al., 2000). Taken together, GbCYP86A1-1 is involved in the synthesis of suberin in roots and up-regulates many genes involved in secondary metabolic processes of suberin.

**Cell wall suberization in roots could enhance plant resistance to pathogen**

Suberization of the cell wall creates a strong barrier that can make it difficult for pathogens to penetrate and colonize the roots (Andersen et al., 2011; Franke and Schreiber, 2007). In-depth study of suberin biosynthesis may help develop agricultural crops with broad pathogen resistance. Despite extensive evidence on the requirement of CYP86A1 for suberin synthesis (Höfer et al., 2008; Molina et al., 2009), and the expression of CYP86A genes may be affected by some hormones or environmental factors (Duan and Schuler, 2005). However, there have been no reports on its potential role in pathogen resistance. The present analyses show that there are three GbCYP86A1 homologs in G. barbadense. GbCYP86A1-1 has more three amino acids than GbCYP86A1-2 and GbCYP86A1-3 with some amino acid differences among the three homologs. In addition, the GbCYP86A1s are specifically expressed in roots and significantly induced by V. dahliae with higher expression in disease-resistant cultivar Hai7124 than disease-susceptible Jumman 1, implying the potential relationship between suberin and disease resistance involving GbCYP86A1s. Among them, GbCYP86A1-1 contributed the most significantly to resistance. Silencing of GbCYP86A1-1 in Hai7124 led to increased susceptibility, while GbCYP86A1-1 overexpression conferred enhanced tolerance and increased suberin in cell walls. In addition, sectional observation also demonstrated that overexpression of GbCYP86A1-1 affected the infection of V. dahliae in roots.

Another interesting finding is that there were few differentially expressed genes in the roots of GbCYP86A1-1 transgenic plants compared with WT when treated with V. dahliae. In addition, few differentially expressed genes were enriched for disease-related pathways. However, the WT roots showed more susceptibility to invasion and destruction by pathogens, with a large amount of genes expressed differentially in order to maintain normal growth and prevent further spread of pathogens. These results provide compelling evidence that GbCYP86A1-1 enhances plant disease resistance by promoting accumulation of root suberin and regulating the trade-off between growth and resistance in plants.

**Overexpression of GbCYP86A1-1 activates the disease-resistant immune pathways**

In addition to increasing the structural resistance of cell wall, plants can also activate intracellular complex disease resistance immune pathways to prevent further propagation of pathogens (Jones and Dangl, 2006). Here, we present a new discovery that the suberization of the cell wall boosts the activation of the immune pathway. Plant cell walls are highly heterogeneous extracellular structures that mainly include pectin, cellulose and hemicellulose (Braidwood et al., 2014). This structure will be affected by the increase in lipid polymer content in the lamellae of suberized cell walls (Schreiber, 2010). Pectin, as the most complex polysaccharide, constitutes the middle lamella of the cell wall and is mainly composed of polygalacturonan (PG) (Caffall and Mohnen, 2009). OGS can be released from the cell wall as DAMPs by pectin lyase and PG’s partial hydrolysis and further activate plant immune responses (Benedetti et al., 2015). In addition, PGPs can interact with PGs and lead to the accumulation of OGS (Mattei et al., 2005). Here, through RNA-seq analysis, we have found that many pectin lyase-like superfamily proteins (polygalacturonase activity) and PGIP2 expression are activated in GbCYP86A1-1 transgenic Arabidopsis, which may be associated with changes in the cell wall and lead to the release of OGS. OGS as signalling molecules can be recognized by RLKs or RLPs in the membrane system to activate pathogen-associated immune responses of plants. A typical example is plant wall-associated kinase 1 (WAK1) as a receptor for OGS, which rapidly activates the immune system of plants (Gramenga et al., 2016). In this study, the expression levels of many RLKs and RLPs were significantly higher in GbCYP86A1-1 transgenic Arabidopsis, such as WAK1, RLK1 and RLP38, compared to WT.

The activation of the immune pathways is mainly reflected by the response of disease-resistant phytohormones and the expression of PRs (Koornneef and Pieterse, 2008). Compared to the WT, the expression of some genes associated with ET and SA was up-regulated in GbCYP86A1-1 transgenic plants, while JA-related gene expression was down-regulated. Among them, the expression of ERFs and WRKYs were significantly up-regulated. ERFs are not only involved in ethylene-mediated disease resistance, but also bind to the PR promoter element GCC box and regulate PR expression, so they are also known as pathogenesis-related transcription factors (Büttner and Singh, 1997; Guttenberg and Reuber, 2004). In addition, WRKYs have been recognized as key transcription factors for SA responses and they promote the expression of downstream PRs (Verk et al., 2008). PRs can be induced by biotic stresses and in many cases are the marker genes responding to the defence-associated phytohormones such as ET, SA, JA (Pieterse et al., 2009). For example, PR1 (CAP) superfamily proteins are cysteine-rich secretory proteins that have been frequently used as marker genes for systemic acquired resistance in plants (Lee et al., 2015). The PR2 family encodes β-1,3-glucanase, a PR2 that may play an important role in Puccinia triticina defence response (Mauch et al., 1988). The PR5 family encodes permatins, osmotins, zeamatin and thaumatin-like proteins, and PR5 is the marker gene of the ET signalling pathway in Brachypodium distachyon (Kouzai et al., 2016). Transgenic Arabidopsis expressing PR5 of Ocimum basilicum confers tolerance to fungal pathogens (Misra et al., 2016). In this study, we found many highly expressed PRs in GbCYP86A1-1 transgenic Arabidopsis. In addition, we also detected significantly down-regulated PR expression in GbCYP86A1-1-silenced plants. Taken together, GbCYP86A1-1 is involved in activating downstream immune pathways to enhance resistance to V. dahliae.

Based on these, we propose a model of GbCYP86A1-1 involvement in plant resistance against V. dahliae (Figure 8). GbCYP86A1-1 proteins were localized to the ER and are likely involved in the suberin biosynthesis. Overexpression of GbCYP86A1-1 led to the synthesis of more fatty acids in the plastid, which were activated into fatty acyl-CoAs in the ER. Fatty
acyl-CoA is oxidized by GbCYP86A1-1 to α-hydroxy fatty acids and α,ω-dicarboxylic acids, and this promotes the synthesis of suberin, which is then modified by series of suberin biosynthetic enzymes to form monoacylglycerol esters (Yang et al., 2012;). The suberin monomers cross the plasma membrane under the effect of the transport proteins and accumulate in the cell wall. Pectin is the main component of cell wall. Due to cell wall suberization, pectin may be hydrolysed by pectin lyase and release small molecules of OGs. OGs can be detected by members of the receptor protein kinase family or receptor protein family and are responsible for the constitutive activation of pathogen-related defence responses in plant cells, including initiation of hormone signal transduction and defence gene expression. ERFs and WRKYs are located in the nucleus, in addition to responding to hormones, they can also regulate the expression of disease-related proteins, further preventing the spread of pathogens. This study presents the novel discovery that GbCYP86A1-1 plays important roles in cell wall modification and activation of immune pathways. We are developing the stable GbCYP86A1-1 overexpressing and RNAi transgenic upland cotton lines, which will help to thoroughly verify the function of GbCYP86A1-1 in cotton. Hopefully, combining GbCYP86A1-1 overexpressing cotton lines with traditional breeding techniques will effectively improve the durable disease resistance in cotton.

**Experimental procedures**

**Plant materials and treatments**

The expression of CYP86s was analysed in G. barbadense cv. Hai7124 and G. hirsutum cv. Junmian 1 following different treatments. Seedlings were grown in the same controlled environment chamber under the following conditions: 16-h light/8-h dark cycle at 28°C for 2 weeks. Wild-type Arabidopsis (Col-0) and transgenic Arabidopsis were grown in a controlled environmental chamber maintained at 23/21°C (day/night) conditions.
Seedlings of Hai7124 and Junmian 1, which showed resistance and susceptibility to V. dahliae, respectively, were inoculated with the fungal pathogen (V991) using the dip-inoculation method (Wang et al., 2011). V991, a highly aggressive and defoliating strain of V. dahliae obtained from our lab, was cultured on potato dextrose agar medium (PAD) at 24°C for 4–5 days and then transferred to Czapek’s medium for incubation at 25°C for 5 days (Gao et al., 2013). Subsequently, we used deionized water to adjust the concentration to 1 × 10^7 conidia/mL for inoculation of the seedlings. Seedling roots were harvested at 0, 24, 48, 96 and 144 h after V991 treatment for use.

RNA isolation and expression pattern analysis
Total RNA was isolated from roots using the CTAB-acidic phenolic method, and the RNA samples were reverse-transcribed into cDNA using the HiScript Q RT SuperMix for qPCR (+gDNA wiper) (Vazyme). Gene-specific primers for qRT–PCR analysis were designed using Beacon Designer 7.0. Cotton histone 3 (AF024716) and Arabidopsis Ubq5 (At3g62250) were individually used as reference genes. All primer information is shown in Table S8. Real-time PCR were performed on a Roche 480 PCR System using AcceQ SYBR Green Master (Vazyme).

G. hirsutum acc. TM-1 high-throughput RNA-sequencing data from our laboratory (Zhang et al., 2015) were used to analyse the expression patterns of GhCYP86s in different tissues. The log2 (FPKM) formula was used to calculate their expression. A heat map was generated with Multi Experiment Viewer v.4.9 (http://en.bio-soft.net/chip/MeV.html).

Cloning of the CYP86A1s in Hai7124
Gene-specific primers were designed using Primer 5.0 software to amplify the CYP86A1s with complete ORFs (Table S8). High-fidelity ExTaq DNA Polymerase (TaKaRa) was used in standard PCR reactions. All PCR products were cloned into pMD19-T Vectors (TaKaRa) and transformed into strains DH5α. At least six clones per gene were randomly selected and sequenced.

VIGS experiments
TRV2 vectors were used for VIGS analysis. The 3’ UTR region-specific sequences were selected to construct TRV2: GbCYP86A1-1, TRV2: GbCYP86A1-2 and TRV2: GbCYP86A1-3 vectors, and the homologous segments were selected to construct the TRV2: H3091 vector. GbCLA1 (Cloroplastos alterados 1) was used to construct a TRV2: GbCLA1 vector. These vectors were transformed into A. tumefaciens strain GV3101. The internal transcribed spacer (ITS) region of ribosomal DNA was targeted using the fungus-specific ITS1-F primer in combination with the V. dahliae-specific reverse primer STVe1-R. Primers for AtUbq5 were used as endogenous plant controls (Gutiérrez et al. 2008). Real-time PCR was carried out on genomic DNA (Santhanam et al., 2013).

To analyse the V. dahliae infection rate by recovery assay, stem sections 2 cm from the base were surface sterilized in 70% ethanol and rinsed with sterile water after V991 treatment for a week. The stem segments were placed on the PDA supplemented with chloramphenicol (34 mg/L) and cultured for 3 d at 25°C and then photographed (Gong et al., 2018).

Subcellular localization of GbCYP86A1s
The ORFs of GbCYP86A1-1, GbCYP86A1-2 and GbCYP86A1-3 were fused to GFP in the pBin-GFP4 expression vector (Liu et al., 2014). The three vectors were transiently expressed in N. benthamiana leaf cells via the A. tumefaciens infiltration method. The binary vectors were transiently co-expressed in N. benthamiana leaves with RFP-HDEL via agroinfiltration (Waadt and Kudla, 2008). Fluorescence signals were detected using a confocal laser scanning microscope (Zeiss, LSM710) 3 d after infiltration.

Identification of transgenic GbCYP86A1s Arabidopsis plant resistance
The full-length sequences of the GbCYP86A1-1, GbCYP86A1-2 and GbCYP86A1-3 were inserted into the pBI121 vector with the 35S promoter. The overexpression vectors were transferred into GV3101 to transform Arabidopsis using the floral dip method. Pure lines were screened in a growth chamber. DNA and RNA were extracted from roots of transgenic lines for detection of positive plants.

For Arabidopsis inoculations, 4-week-old seedling roots were rinsed in water and incubated in V. dahliae conidial suspension (1 × 10^7 conidia/mL) for 90 seconds, and the plants were then replanted in fresh soil. Severity of disease symptoms was recorded using an index ranging from 0 (healthy plant) to 4 (dead plant) for the disease index calculation (Fradin et al., 2011).

For biomass quantification in planta, various tissues were collected for DNA extraction after ten days of treatment with V991. The internal transcribed spacer (ITS) region of ribosomal DNA was targeted using the fungus-specific ITS1-F primer in combination with the V. dahliae-specific reverse primer STVe1-R. Primers for AtUbq5 were used as endogenous plant controls (Santhanam et al., 2013).

To analyse the V. dahliae infection rate by recovery assay, stem sections 2 cm from the base were surface sterilized in 70% ethanol and rinsed with sterile water after V991 treatment for a week. The stem segments were placed on the PDA supplemented with chloramphenicol (34 mg/L) and cultured for 3 d at 25°C and then photographed (Gong et al., 2018).

Cellular observation of roots section
Arabidopsis plants were grown in vermiculite for four weeks, about 2 cm base of the main root were sampled for cellular observation based on methods described previously (Höfer et al., 2008). Samples were dehydrated in ethanol and embedded with resin, stem sections (2 μm thickness) were cut using a Reichert Ultracut microtome for further analysis (Shang et al., 2015). Sections were stained with Sudan red 7B and microscopically observed (Olympus BX53) as described previously (Höfer et al., 2008).

Freehand sections were prepared from the same location in roots after V991 treatment for 3 days. Samples were fixed in the groove of the carrot, sliced with thin blade and microscopically observed (Olympus BX53).

Lipid analysis
The 2.244 g potassium hydroxide was added to 100 mL methanol solution to prepare methyl esterification reagent. WT and transgenic line roots were ground to powder, and 0.02 g of sample was placed in 1 mL n-hexane for total lipid extraction while 2 μg of dotriacontane was added as internal standard. The

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The cell wall of Arabidopsis thaliana was treated with V991 or water. Total RNA was isolated from the treated Arabidopsis thaliana and the output of enrichment was determined using HTSeq (Anders and Huber, 2010) and imported into Arabidopsis genome. The number of reads aligning to the Arabidopsis genome was determined using HTSeq (Anders and Huber, 2010) and imported into R statistical software where differential expression analysis was accomplished using the DESeq with a cut-off of 0.05 q value and a fold change of > 1.5. GO analysis of the differentially expressed genes in the biological process was conducted using the AgriGO software (Du et al., 2010). The background was constituted by the whole annotated gene sequence of Arabidopsis and the output of enrichment needed FDR < 0.05. All samples contained three biological repeats.

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Competing interests

The authors declared that they had no competing interests.

Authors’ contributions

WG conceived the original screening and research plans; GW and JX performed most of the experiments; ZG, LL, GZ and QS provided technical assistance; WG and JW designed the experiments and analysed the data; WG, GW and JW conceived the project and wrote the article with contributions of all the authors; WG and GW supervised and complemented the writing.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Figure S1** Phylogenetic relationship of CYP86 subfamily genes in Arabidopsis and G. raimondii.

**Figure S2** Phylogenetic classification and structural analysis of CYP86 genes in G. raimondii.

**Figure S3** Amino acid sequence alignment of GrCYP86 subfamily genes.

**Figure S4** Verification of VIGS silencing system and phenotype of cotton seedlings upon V. dahliae inoculation.

**Figure S5** Chromosomal distribution of CYP86 genes in G. raimondii and G. barbadense.

**Figure S6** Phenotype observation of the above-ground part of transgenic Arabidopsis lines.

**Figure S7** Roots morphology of Arabidopsis plants overexpressing GbCYP86A1-1 in seedling stage.

**Figure S8** qPCR analysis of fungal biomass in different transgenic and the WT Arabidopsis roots after three days of V991 infection.

**Figure S9** Correlation of fold change analyzed by RNA-seq data with results obtained from qRT-PCR.

**Figure S10** Expression patterns of DEGs between WT and GbCYP86A1-1 transgenic Arabidopsis line related to secondary metabolic processes, RLKs or RLPs, phytohormones-related transcription factors and PRs.

**Table S1** Comparison of homology between CYP86 family genes in G. raimondii.

**Table S2** Disease index of the TRV: GbCYP86A1-1, TRV: GbCYP86A1-1-2, TRV: GbCYP86A1-3 and TRV: H3091 after V. dahliae inoculation.

**Table S3** Disease index of the transgenic Arabidopsis lines overexpressing GbCYP86A1-1, GbCYP86A1-1-2, GbCYP86A1-1-3 and WT after V. dahliae inoculation.

**Table S4** Genes ontology (GO) analysis involved in biological processes from differentially expressed genes by comparing GbCYP86A1-1 transgenic line with WT roots.

**Table S5** Differentially expressed genes by comparing GbCYP86A1-1 transgenic line with WT roots.

**Table S6** Genes ontology (GO) analysis involved in biological processes from differentially expressed genes by comparing GbCYP86A1-1 transgenic line with WT roots.

**Table S7** Genes ontology (GO) analysis involved in biological processes from differentially expressed genes by comparing isolated and non-inoculated V. dahliae in GbCYP86A1-1 transgenic line.

**Table S8** Genes ontology (GO) analysis involved in biological processes from differentially expressed genes by comparing isolated and non-inoculated V. dahliae in WT.

**Table S9** Information on PCR primers used in this study.

**Data S1** Identification of CYP86 genes in G. raimondii and their phylogenetic relationship in Arabidopsis thaliana, G. arboreum, G. hirsutum and G. barbadense, respectively.

**Data S2** Expression profiles of CYP86 genes in G. hirsutum acc. TM-1.

**Data S3** Information on 481 differential expression genes (q < 0.05 and a fold change > 1.5) by transcriptome comparative analysis between transgenic plants and WT.

**Data S4** Information on 277 differential expression genes (q < 0.05 and a fold change > 1.5) by transcriptome comparative analysis between inoculated and non-inoculated V. dahliae treatment in GbCYP86A1-1 transgenic line.

**Data S5** Information on 1515 differential expression genes (q < 0.05 and a fold change > 1.5) by transcriptome comparative analysis between inoculated and non-inoculated V. dahliae treatment in WT.

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