

Enhanced phenylpropanoid metabolism underlies resistance to *Fusarium oxysporum* vasinfectum f. sp. race 4 infection in the cotton cultivar Pima-S6 (*Gossypium barbadense* L.)

Jonathan Ojeda-Rivera

Texas Tech University

Mauricio Ulloa

USDA-ARS, CSRL

Hector Rogelio Najera-Gonzalez

Texas Tech University

Philip A. Roberts

University of California, Riverside

Ricardo Chavez Montes

Texas Tech University

Luis Herrera-Estrella

Texas Tech University

Damar Lopez-Arredondo (✉ Damar.Lopez-Arredondo@ttu.edu)

Texas Tech University

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Abstract

Background

Fusarium oxysporum f. sp. *vasinfectum* (FOV) race 4 (FOV4) is a highly pathogenic soil-borne fungus responsible for Fusarium wilt in cotton (*Gossypium* spp.) and represents a continuing threat to cotton production in the southwest states of the U.S., including California, New Mexico, and Texas. Even though the Pima (*G. barbadense* L.) cotton gene pool shows more susceptibility to this pathogen than Upland (*G. hirsutum* L.) cotton, fortunately, some Pima cultivars present resistance to FOV4 infection, like Pima-S6. To gain insights into the mechanism underlying FOV4 resistance, we performed comparative transcriptional and metabolomic profiling of FOV4-resistant Pima-S6 and FOV4-susceptible Pima S-7 and Pima 3–79 cotton.

Results

Comparative analysis of transcriptional FOV4 infection responses on these genotypes indicated a differential response between resistant Pima-S6 and susceptible Pima S-7 and 3–79. GO-enriched analysis found clusters related to various biological processes, including transmembrane transport, glycoside hydrolysis, metabolism of small molecules, and phenylpropanoid metabolism. Arabidopsis ortholog genes comparison with categories significantly enriched in Pima-S6 resulted in multiple categories clustered to similar biological processes such as the metabolism of small molecules, regulation of enzymatic activity, diterpenoid biosynthetic processes, and phytohormone signaling-related processes, being phenylpropanoid biosynthesis and metabolism highly enriched. These results correlated with the accumulation of secondary metabolites in Pima-S6 roots, specifically esculetin, a coumarin that inhibits the growth of *Fusarium*. Transcriptomic profiling enabled the identification of candidate genes potentially involved in the resistance mechanism of FOV4-resistance in protecting the Pima-S6 cotton cultivar from this pathogenic fungus. Some genes of this pathway were found to be in chromosome-QTL regions linked with resistance to FOV4.

Conclusions

Our results highlight an essential role for the phenylpropanoid synthesis pathway in FOV4 resistance and create opportunities for gene discovery and SNP development for marker-associated selection (MAS) for breeding for FOV4 resistance.

Background

Fusarium oxysporum f.sp. *vasinfectum* (FOV) is a pathogenic soil-borne fungus responsible for Fusarium wilt in cotton (*Gossypium* spp.). For over a decade, the FOV race 4 (FOV4) strain has adversely impacted cotton production areas in the U.S., causing plant wilt and death [1, 2, 3, 4, 5]. Upland (*G. hirsutum* L.) and

Pima (*G. barbadense* L.) cotton account for around 95.5 % and 4.5, respectively, of total fiber production in the U.S., and Pima cotton represents a valuable genetic source of essential traits like fiber quality and disease resistance [3, 6, 7, 8]. Pima fiber is highly valued in the cotton industry because its extra-long, strong, and fine fibers are best suited to produce high-quality fabrics. Previous studies have shown that Pima cotton cultivars are more susceptible to FOV4 than Upland, and Pima S-7 and PIMA 3–79 have been used as susceptible checks in many of these studies [1, 3, 5, 9]. Fortunately, some Pima cultivars, such as Pima S-6, identified in 2004, present resistance to FOV4 infection [1, 10]. Pima S-6 has been subjected to several cycles of evaluation and selection under naturally FOV4-infested fields to increase its uniformity and resistance level. This new source, renamed Pima-S6, has been incorporated into cotton breeding programs to combat the highly pathogenic FOV4 in the U.S. [6, 11, 12].

Previous QTL and GWAS studies in interspecific mapping populations (Upland x Pima) and Upland cotton have provided insights into the genetic basis of FOV4 resistance in this crop [3, 4, 9, 13, 14]. Some studies have linked specific *G. barbadense* chromosomes to FOV4 resistance [3, 4]. However, the genes underlying FOV4 resistance in Pima cotton have yet to be found and characterized. While these studies establish that FOV4 resistance is present in specific cultivars of Pima cotton, the underlying functional mechanisms for disease resistance are unknown. Because Pima-S6 is resistant to FOV4 and Pima S-7 and Pima 3–79 are not, these genotypes are suitable models to perform transcriptional profiling and determine the differences in gene expression in response to FOV4 infection.

In this work, we performed comparative transcriptional and metabolomic profiling of FOV4-resistant Pima-S6 and FOV4-susceptible Pima S-7 and Pima 3–79 cotton accession to gain insights into the mechanism underlying FOV4 resistance. Transcriptomic profiling enabled the identification of candidate genes potentially involved in the resistance mechanism of FOV4, protecting the Pima-S6 cotton cultivar from this pathogenic fungus. Our results highlight an essential role for the phenylpropanoid synthesis pathway, which represents a source of key metabolites for plant development and environmental adaptation [15], in the resistance to FOV4 present in Pima-S6.

Results

FOV4-resistant Pima-S6 presents a specific transcriptional response to FOV4 infection

To characterize the transcriptional response to Fusarium wilt disease caused by FOV4 in Pima cotton, we performed RNA-seq analysis of plant roots grown under control and FOV4-infected conditions. Three Pima cotton cultivars were studied, one FOV4-resistant cultivar, Pima -S6, and two FOV4-susceptible cultivars, Pima S-7 and Pima 3–79 (see Materials and Methods). We performed three biological replicates of each experiment to generate 18 RNA-seq libraries. Alignment statistics and library names are listed in Supplementary Table 1.

To get insights into the molecular mechanisms underlying FOV4 resistance, we first performed a comparative analysis of the transcriptional response of all FOV4-susceptible (Pima S-7 and Pima 3–79) and resistant (Pima-S6) cultivars (Fig. 1). We performed sample relationship analysis to determine whether the samples clustered by the tested genotypes and treatments. RNA-seq libraries were observed to cluster together in response to both genotype and treatment conditions (Supplementary Fig. 1). Susceptible Pima 3–79 and Pima S-7 cultivars present similar expression profiles in response to FOV4 infection as they cluster closely in the sample relationship analyses, while Pima-S6 sample libraries cluster more closely and separated from those of Pima 3–79 and Pima S-7 in response to FOV4 treatments (Supplementary Fig. 1). This result indicates a differential response to FOV4 infection in Pima-S6 compared to Pima S-7 and Pima 3–79.

To further analyze the differences in response to FOV4 infection among the tested cultivars, we performed a comparative transcriptomic response analysis to determine which genes are commonly regulated in response to FOV4 treatment (Fig. 1a). We determined that 6657 and 7393 genes were upregulated and downregulated in Pima 3–79 in response to FOV4 treatment (Fig. 1a) and that up to ~ 67% of this gene set is specific to this cultivar. In the case of Pima S-7, we detected the upregulation and downregulation of 3977 and 5028 genes, respectively, in response to FOV4 treatment (Fig. 1a), of which ~ 44% was specific to this cultivar. Lastly, for the case of Pima-S6, we detected that 1586 and 2820 genes were upregulated and downregulated, respectively, in response to FOV4 treatment (Fig. 1a), of which ~ 79% of this gene set is specific to Pima -S6. Genes commonly upregulated and downregulated in susceptible cultivars Pima S-7 and Pima 3–79 are 2318 and 1928, respectively, which correspond to a ~ 47% of the Pima S-7 transcriptional response to FOV4 (~ 30% of the Pima 3–79 response), indicating that a relatively large number of genes of the response to FOV4 is commonly regulated in susceptible cultivars. In contrast, Pima-S6 and Pima S-7 had only 352 upregulated and 212 downregulated genes in common, corresponding to ~ 6% and ~ 12% of the FOV-4 responsive genes in Pima S-7 and Pima-S6, respectively. Considering the three genotypes, 105 genes were upregulated and 114 downregulated in response to FOV4; this number equals less than 4% of the global transcriptional response for all the genotypes. The transcriptomic landscape of the response to FOV4 (Fig. 1b) agrees with the observed differences in gene expression between susceptible and resistant cultivars. It also indicates differences in the magnitude of the fold change in gene expression. Overall, our data showed that susceptible genotypes share a more significant portion of their transcriptional response to FOV4 among themselves than with Pima-S6, and that the transcriptional response of the resistant cultivar is specific and different from that of the susceptible cultivars.

Gene Ontology (GO)-enriched categories in Pima-S6 in response to FOV4 relate to phenylpropanoid metabolism

To get biological insights into the FOV4-resistance mechanism(s) that are transcriptionally activated in Pima-S6 in response to FOV4 treatment, we performed differential Gene Ontology (GO) enrichment analysis (Fig. 2). The complete list of GO-enriched categories, P-values, and gene identifiers is included in Supplementary Dataset 1. First, we determined the GO categories enriched in the gene sets that are

transcriptionally activated in Pima 3–79, Pima-S6, and Pima S-7, respectively, in response to FOV4 treatment (Fig. 2a). We then focused our analysis on categories significantly enriched in Pima-S6 but in the susceptible genotypes (Fig. 2b). The identified GO categories presented in Fig. 2b are related to a variety of biological processes including transmembrane transport (GO:0042626, GO:0005372), glycoside hydrolysis (GO:0016161, GO:0102229, GO:0000272, GO:0004565), metabolism of small molecules and amino acids (GO:0006520, GO:0004471), phospholipid metabolism (GO:0009395), phytohormone metabolism (GO:0010102, GO:0009850, GO:0009690, GO:0051213) and phenylpropanoid metabolism (GO:0046417, GO:00168411). Because the Arabidopsis genome is more functionally characterized than the cotton genome, we searched for Arabidopsis orthologs of the genes included in these GO categories to get more insights into their possible roles in FOV4 resistance (Fig. 2c, Supplementary Dataset 1). GO enrichment and KEGG pathway analysis [16] of Arabidopsis ortholog genes (Fig. 2c) resulted in multiple categories clustered to similar biological processes as the categories from Fig. 2b. We found clusters of GO categories that include the metabolism of small molecules, phenylpropanoid biosynthesis and metabolism, ABC-type transporter activity, lipid metabolism, regulation of enzymatic activity, diterpenoid biosynthetic processes, and phytohormone signaling-related processes.

The phenylpropanoid pathway in plants is the starting point for other metabolic pathways that produce critical secondary metabolites, including flavonoids, coumarins, and lignans, which have roles in plant resistance to pathogens [15]. Gene-by-gene analysis of Arabidopsis orthologs (Supplementary Dataset 1) allowed the identification of genes that encode key enzymes involved in the phenylpropanoid pathway, including PHENYLALANINE AMMONIA LYASE 1 and 2 (PAL1,2), which catalyze the initial step of the phenylpropanoid pathway [17], and CHORISMATE MUTASE 2 (CM2) which catalyzes the first committed step in the synthesis of Phenylalanine (Fraser and Chapple, 2011). We also found an ortholog of CHALCONE SYNTHASE (CHS) that encodes the enzyme that catalyzes the initial step in the synthesis of flavonoids [18]. Root exudation of secondary metabolism compounds through ABC-type transporters has also been shown to play a role in plant-microbe interactions and resistance to fungal pathogens [19, 20, 21]. Concomitantly, we found multiple cotton genes encoding ABC-type transporters orthologous to multiple Arabidopsis ABC-type transporters. Some of these transporters have been reported to play roles in fungal pathogen resistance, like PLEIOTROPIC DRUG RESISTANCE 12 (PDR12), by facilitating root exudation of secondary metabolites [21]. The differential transcriptional response of Pima-S6 compared with susceptible genotypes Pima S-7 and Pima 3–79 indicates that secondary metabolism might play a key role in the FOV4 resistance mechanism.

Non-targeted metabolomics analysis reveals Pima-S6 cotton has a robust root metabolome that is key to FOV4-resistance

To further explore the differences in secondary metabolism between the resistant and the susceptible genotypes, we performed mass spectrometry-based non-targeted metabolomics analysis in the roots of Pima-S6, Pima S-7, and Pima 3–79 seedlings (Fig. 3). We detected 3115 compounds in the roots of the three studied genotypes (Fig. 3a). Cluster profiling of the normalized relative abundance of the identified compounds indicates that Pima-S6 and Pima S-7 metabolomes are more closely related to each other

than to Pima 3–79 (Fig. 3a). We performed pairwise comparisons to determine statistically significant differences in relative compound concentration of these 3115 metabolites between FOV4-resistant Pima-S6 and the susceptible genotypes, namely, Pima-S6 vs Pima S-7 and Pima-S6 vs Pima 3–79 (Fig. 3b, c). We found 2251 metabolites with higher levels and 18 with lower levels in Pima-S6 relative to Pima 3–79, indicating that Pima-S6 has a more diverse root metabolome than Pima 3–79 (Fig. 3b). The Pima-S6 vs Pima S-7 comparison showed that 204 metabolites have higher levels and 190 lower levels in Pima-S6 compared to Pima S-7 (Fig. 3c), which agrees with the clustering profiling analysis (Fig. 3a).

To get functional insights into the metabolomic differences between Pima-S6 and FOV4-susceptible genotypes (Pima-S6 vs P Pima S-7 and Pima-S6 vs Pima 3–79), we performed a functional analysis using MS Peak-to-Pathway analysis [22, 23], which harnesses metabolite changes at pathway level to calculate enrichment and combines it with metabolite set enrichment analysis to determine an overall pathway enrichment P-value (Fig. 3d; Supplementary Dataset 1). In the case of the pairwise comparison between Pima-S6 and Pima 3–79, it was determined that the “cutin, suberin, and wax biosynthesis” pathway was significantly more enriched in Pima-S6 than in Pima 3–79 (Fig. 3d). Along with lignin, cutin, suberin, and wax are secondary cell wall components that, amidst other functions, provide protection against pathogen attack and interact metabolically with the phenylpropanoid synthesis pathway [24, 25]. In the case of the pairwise comparison between Pima-S6 and Pima S-7, six pathways were found significantly more enriched in Pima-S6 than in Pima S-7, including “flavonoid biosynthesis”, “lysine biosynthesis”, “ubiquinone and another terpenoid-quinone biosynthesis”, “flavone and flavonol biosynthesis” and “phenylpropanoid biosynthesis” (Fig. 3d). All these categories, except for lysine biosynthesis, are closely related to secondary metabolism, specifically to the phenylpropanoid pathway.

We rationalized that the set of metabolites key to FOV4-resistance in Pima-S6 must have enhanced levels compared to susceptible genotypes. To test this hypothesis, we performed Venn diagram analysis to determine more abundant metabolites in Pima-S6 compared to FO4-susceptible Pima 3–79 and Pima S-7 genotypes (Fig. 3e). We identified 175 compounds that are more abundant in Pima-S6 than in the two susceptible genotypes. Their identity was determined by matching their spectra to known databases using the Compound Discoverer software (Thermo Scientific). We found 57 hits to known molecules (~ 32%) among the Pima-S6-enhanced metabolites. Inspection of the list revealed multiple molecules related to secondary metabolism, specifically phenylpropanoid metabolism, as suggested by the presence of a phenyl group in their structure (Supplementary Dataset 1), which agrees with the pathways presented in Fig. 3d. Interestingly, we found enhanced levels of the coumarin esculetin in the roots of Pima-S6 (Fig. 3f). Root coumarin exudation has been recently shown to inhibit *Fusarium oxysporum* f. sp. *raphani* growth *in vitro* and promote the establishment of plant-beneficial microorganism communities in the rhizosphere [26].

Discussion

Breeding programs have successfully developed FOV4-resistant cotton germplasm genome [6, 11, 12]. However, little is known about the molecular mechanisms underlying FOV4 resistance in this crop. To

address this question, we harnessed our recent assembly of the Pima-S6 genome [8] to conduct RNA-seq profiling in the roots of *G. barbadense* resistant (Pima-S6) and susceptible cultivars (Pima S-7 and Pima 3–79) and determined the genes that are differentially expressed in Pima-S6 in response to FOV4 infection. RNA-seq data indicates that the transcriptional response of Pima-S6 differs from that of susceptible genotypes in magnitude (a smaller number of differentially expressed genes) and functional enrichment (Fig. 1). GO functional analysis of the genes activated in Pima-S6 in response to FOV4 infection revealed a differential activation of metabolic pathways that do not occur in susceptible genotypes Pima 3–79 and Pima S-7 (Fig. 2a,b).

Enhancement of secondary metabolism in response to FOV4 treatment, particularly of pathways related to the production of phenylpropanoids, is a key difference that sets the transcriptional response of Pima-S6 apart from that of Pima S-7 and Pima 3–79. Among the differentially enriched GO categories activated in the Pima-S6 transcriptional response to FOV4 treatment, we identified categories related to phenylpropanoid metabolism, including “ammonia-lyase activity” and “chorismate metabolic process”. The finding of these categories is interesting because an enhanced phenylpropanoid metabolism has been previously related to disease resistance in tobacco, rice, and barley [27, 28, 29, 30]. Identification of ortholog Arabidopsis genes to genes differentially expressed in FOV4-infected cotton plants revealed that genes orthologous to AtPAL1 are overexpressed in Pima-S6 with respect to the susceptible cotton cultivars (Supplementary Dataset 1; Fig. 4a). PAL1 has been thoroughly related to plant defense responses [31, 32, 17]. Our metabolomic analysis further corroborated transcriptomic findings as we found significant enrichment of the phenylpropanoid pathway in the roots of Pima-S6 compared to Pima S-7. We also identified enhanced transcript levels of *AtCHS1* orthologs in Pima-S6 (Supplementary Dataset 1; Fig. 4a), suggesting a possible role of flavonoids in the FOV4 resistance mechanism in this cotton cultivar. Supporting this notion, we also found significant enrichment of flavonoids in the root metabolome of Pima-S6 compared to Pima S-7 (Fig. 3d). Coding for the enzyme that catalyzes the first step in the synthesis of flavonoids, the expression of chalcone synthase genes is activated by plants in response to multiple stress conditions, including fungal infection [18]. A previous study demonstrated that *AtCHS1* protects against the pathogenic fungi *Verticillium dahliae* in Arabidopsis and that knockdown of orthologs of this gene in cotton (*G. hirsutum*) results in enhanced susceptibility to *V. dahliae* infection [33].

Phenylpropanoid metabolism is closely linked to lignin deposition in the root; the synthesis of the main lignin subunits, monolignols, starts with the phenyl ammonia lyase reaction and continues with subsequent biochemical reactions that give rise to monolignols which are then polymerized during secondary cell wall formation [15]. It was recently reported that higher levels of lignin are associated with a higher resistance of Pima-S6 to FOV4 [34]. The significant enrichment of the phenylpropanoid pathway found in our transcriptomic (Fig. 2b,c) and metabolomic (Fig. 3d) analyses supports increased lignification in Pima-S6 as one of the mechanisms underlying FOV4-resistance in this cultivar. Enhanced expression of Pima-S6 orthologs of *AtHCT* (Supplementary Dataset 1; Fig. 4a), whose expression is positively correlated with lignin deposition [35], provides candidate genes to explain the enhanced lignification in Pima-S6. Interestingly, we also found significant enrichment of cutin, suberin, and wax

biosynthesis in Pima-S6 compared to Pima 3–79 (Fig. 3d). Phenylpropanoid metabolism has recently been shown to be essential for tissue-specific suberin deposition in the root endodermis [25]. Both suberin and lignin contribute to forming root cell barriers that, among other functions, protect the plant from pathogen invasion. Concomitantly, lignin deposition has been recently shown to be part of a disease-resistance mechanism against *Verticillium dahlia* and *Fusarium oxysporum* in Upland cotton [36].

Identified for the first time in the plant species formerly known as *Coumarona odorata*, coumarins is a family of secondary metabolites derived from the phenylpropanoid pathway [37]. Coumarins consist of a benzene ring linked to a pyrone ring and have been linked with Fe uptake and, recently, with regulating plant-microbe interactions [37, 38]. A recent report highlighted the role of the transcription factor *AtMYB72* in activating coumarin exudation to modulate plant-microbe interactions and protect *Arabidopsis* from *F. oxysporum* infection [26]. The activation of the expression of the *BETA-GLUCOSIDASE 42* (*AtBGLU42*) gene by *AtMYB72* promotes the de-glycosylation of scopolin into scopoletin, which facilitates its exudation into the rhizosphere. Scopoletin was reported to inhibit *F. oxysporum* growth *in vitro* [26]. Interestingly, we found enhanced levels of esculetin in the metabolome of Pima-S6 roots (Fig. 3f). Scopoletin (6-methoxy-7-hydroxicoumarin) and esculetin (6,7-dihydroxicoumarin) only differ in the presence of a methyl group. Moreover, we found higher transcript levels of two of *AtBGLU40* in Pima-S6 (Supplementary Dataset 1; Fig. 4a), which belongs to the same family of beta-glucosidases with coumarin-hydrolyzing activity as *AtBGLU42* [39] and whose enzymatic activity is enhanced upon *F. oxysporum* infection [40]. We also found that Pima-S6 presents an enhanced expression of *AtPDR11/12* orthologs in response to FOV4 treatment (Supplementary Dataset 1; Fig. 4a), which encode ABC-type transporters that facilitate the exudation of secondary metabolites to the rhizosphere [41, 21].

Following the *AtMYB72*-*GLU42* model [26], we looked for *GbMYB72* orthologs and found only one hit in our assembly of the Pima -S6 genome (Supplementary Dataset 1; Fig. 4a), but it was not significantly upregulated by FOV4 treatment in any of the tested genotypes. However, it has been reported that over-expression of this MYB72 does not enhance disease resistance [42], suggesting that this transcription factor is most likely subject to a post-translational regulation mechanism. Furthermore, it has been demonstrated that *AtMYB72* interacts with the *ETHYLENE INSENSITIVE LIKE 3* (*EIL3*), suggesting a role for this transcriptional activator in the regulation of *AtMYB72* activity [42]. Nonetheless, the nature of the relationship between these two transcriptional activators has yet to be elucidated. Interestingly, we found ethylene signaling as one of the phytohormone GO categories enriched in Pima-S6 (Fig. 3c). Moreover, we found two *GbEIL3* orthologs significantly upregulated in Pima-S6 in response to FOV4 treatment compared to the susceptible cotton genotypes tested (Supplementary Dataset 1; Fig. 4a).

In Fig. 4b, we provide an illustration summarizing the possible pathways and candidate genes contributing to FOV4-resistance in Pima-S6 cultivar. We provide metabolomic and transcriptomic evidence that indicates a central role for phenylpropanoid metabolism in FOV4-resistance. The enhanced expression of *GbCM2* and *GbPAL 1* genes in FOV4- infected Pima-S6 plants leads to an increased phenylpropanoid metabolism. In Pima-S6, lignification might be increased by the FOV-4-induced

phenylpropanoid metabolism and the increased expression of *GbHCT* genes, which contributes to forming a physical barrier for FOV4 infection. Transcriptional activation of *GbCM2* and *GbPAL1* can take place by transcription factors like *GbMYB72* and *GbEIL3*. These transcription factors might also interact to activate the expression of *GbPAL1* and *GbBGLU40* to facilitate coumarin de-glycosylation and further exudation, which is toxic for *F. oxysporum* [26]. Flavonoid synthesis, enhanced through the upregulation of phenylpropanoid metabolism and *GbHCT* and *GhCHS* genes, might also contribute to the FOV4 resistance. Lastly, enhanced expression of ABC-type transporters through *GbPDR* genes might ultimately contribute to the exudation of flavonoids and coumarins, which are toxic for FOV4. A previous QTL study linked *G. barbadense* chromosomes A2, A6, A8 and D2, D3, D6 with FOV4 resistance [3, 4, 13]. From the candidate genes listed in Fig. 4b, both *GbCM2* paralogs (*PimaS6.A06G072050*, *PimaS6.D06G069084*) are found in A6 and D6 chromosomes and *GbPDR12* (*PimaS6.D03G079859*) is found in chromosome D3. The contribution of these genes to the FOV4 resistance remains an interesting research prospect. Overall, our study indicates that genes linked to enhanced phenylpropanoid metabolism are attractive research targets for transferring FOV4 resistance to other cotton cultivars of economic relevance.

Conclusions

In conclusion, our results highlight an essential role for the phenylpropanoid synthesis pathway in FOV4 resistance in Pima-S6 cotton. The enhanced accumulation of secondary metabolites in the roots, specifically esculetin, might be part of the underlying mechanism of FOV4 cotton resistance as this coumarin has been shown to inhibit the growth of this pathogen. Using the transcriptomic data generated in this study, we identified potential gene candidates that can be manipulated through traditional and modern techniques to confer FOV4 resistance to other cotton cultivars. More importantly, some of these genes map to previously identified QTLs linked to FOV4 resistance in cotton. Therefore, they represent promising targets for cotton improvement.

Materials and Methods

Fusarium wilt race 4 (FOV4) infection of Pima roots

Gossypium barbadense inbred lines Pima S-7 (PI 560140) and Pima 3–79 susceptible to Fusarium wilt [*Fusarium oxysporum* f.sp. *vasinfectum* (FOV)] race 4 (FOV4), and Pima-S6 resistant to FOV4 were used for the preparation of cDNA libraries/RNAseq analyses. An isolate of FOV4 (FOV CA-14) from a naturally infested field in Fresno County in the San Joaquin Valley, California, was used in this study [43]. We used no inoculation for control plants, and four replicates for FOV4-inoculated and non-inoculated control, respectively. Cultures from single spores were stored on filter paper at -20°C. To produce inoculum, the isolate was grown in 9-cm-diameter Petri dishes containing 20 ml potato dextrose agar (PDA) with 3 mM streptomycin per liter at room temperature for one week. Then plugs of 2-3-mm² of fresh-grown culture were cut and put into 500 ml flasks containing potato dextrose liquid medium with 3 mM streptomycin at 28 °C with rotation for 3–4 days. The conidial suspension was then filtered through eight layers of

cheesecloth to remove hyphae, quantified with a hemacytometer, and diluted with water to obtain 1×10^6 conidia per ml. The infection of the roots in the greenhouse was performed according to the root-cut dip method described by [3]. Cultivars or genotypes were germinated in seedling trays for one week, and seedlings were uprooted. Roots were rinsed with tap water and immediately dipped for 3 min into an aqueous suspension of FOV4 inoculum containing 1×10^6 conidia per ml. The non-inoculated control plants were processed similarly, except they were dipped into the water without FOV4. Seedlings were then transplanted into steam-sterilized U.C. Mix #2 soil in new pots with five plants in each pot and placed in a warm greenhouse maintained between 24°-28° C. At 11 days after inoculation (dai), the plant and soil ball were removed from the pots, and the soil was removed gently from the roots by washing under continuous water flow. The washed roots were placed immediately in 50ml screw-top plastic vials, liquid nitrogen was added, and the vials were placed in a -80° C freezer with the tops off to allow vaporization of the liquid N. Then the tops were screwed on, and the frozen roots were stored at -80°C until RNA extraction.

RNA-seq data analysis

Briefly, quality assessment of the reads from the Illumina platform was performed using FastQC (version 0.11.9; <https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>). Libraries were processed with Trimmomatic (version 0.39) [44] to remove adapter read sequences. Gene expression was quantified using the pseudo-alignment RNA-seq quantification program kallisto version 0.46.1 [45]. Pima cotton libraries were aligned to our assembly of the Pima S-6 *G. barbadense* genome [8]. Transcript-level abundances were integrated from kallisto to count-based statistical analysis in edgeR (version 3.130 [46] using the tximport package [47]. A gene was determined to be differentially expressed using a $-1 > \log_{2}FC > 1$ threshold and false discovery rate threshold (FDR < 0.05). To perform GO analysis, annotations were assigned as the GO annotations of the blastp reciprocal best hits versus Araport11 [48] and UniProt [49], Swiss-Prot proteins from nine plant species (*Glycine max*, *Oryza sativa subsp. japonica*, *Populus trichocarpa*, *Solanum lycopersicum*, *Sorghum bicolor*, *Vitis vinifera*, *Brachypodium distachyon*, *Physcomitrium patens*, and *Chlamydomonas reinhardtii*), the GO annotations from an Interproscan (v5.48.83) [50] analysis, and the GO annotations from the PANNZER2 [51] functional annotation webserver with a PPV value of at least 0.5. Analyses were collated into a non-redundant gtf file and used for GO enrichment analyses. GO enrichment analysis was performed using the clusterProfiler package [52].

Plant Culture for Metabolomic Analysis

Cotton seedlings were grown vertically in Petri dishes in a Conviron growth chamber set at 28°C, 150 $\mu\text{M}/\text{m}^2\text{s}$ light intensity, 16/8 h light/dark cycle, and 45% relative humidity. Culture media was 0.25 X MS with slight modifications (pH 6, 5 mM CaCl_2 and 1 mM MgCl_2) and 0.35% Phytigel (Sigma-Aldrich). Root samples for metabolomic analysis were harvested 8 days after germination. Five biological replicates were performed (n = 5).

Metabolomic Analysis

Non-targeted metabolomic analysis of root samples was performed using a reverse phase chromatography method and positive electrospray ionization mode mass spectrometry. Freeze-dried samples of cotton roots were powdered and extracted with a mixture of methanol-water (60:40 v/v) with 0.1% formic acid. The supernatant of this extraction was further concentrated in a vacuum centrifuge (Labconco, USA) and filtered before chromatographic analysis. Chromatographic separation was performed in a Vanquish Flex UHPLC system (Thermo Scientific, USA) using an Acclaim VANQUISH column (150 x 2.1 mm, with 2.2 μ m) and a solvent gradient method starting 5% phase B for 4 minutes, then increasing to 100% B from 4–17 minutes, and finally maintaining 100% B for 4 minutes prior to re-equilibration time of 4 minutes. Phases consisted of Water (A) and Methanol (B) supplemented with 0.1% formic acid. Detection was carried out in an Orbitrap Exploris 240 (Thermo Scientific, USA) with electrospray ionization source in positive mode scanning from 70–800 Da with a resolution of 120,000 and Data Dependent Acquisition (DDA) of MS/MS with cycle time of 3 seconds. Raw files were imported into Compound Discoverer 3.3 (Thermo Fisher Scientific) and processed with an untargeted metabolomics workflow. Chromatograms were aligned, compounds detected, and grouped with 5 ppm tolerance window. Identification of detected metabolites was done using MZCloud MS/MS database with a similitude factor of at least 50%. Exact masses were also searched against BioCyc and KEGG databases, and final lists were curated manually. Pathway enrichment analysis was done in the MetaboAnalyst online platform [23], using the exact m/z values of detected compounds for the MS Peak-to-Pathway enrichment [22], retention times and statistical values from Compound Discoverer analysis.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Data Availability Statement

The datasets supporting the conclusions of this article are available in National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) database repository under accession number GSE233711.

Conflict of Interest

The authors declare no competing interests.

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Authors' contributions

MU conceived the idea, organized and planned experiments, and reviewed and edited the manuscript. DL-A analyzed data, supervised bioinformatic work, and wrote the paper with JOO-R and LH-E. LH-E analyzed data, helped designed figures, and wrote the paper with JOO-R and DL-A. JOO-R performed RNA-seq data analysis, designed figures, and wrote the first draft of the manuscript. PR designed and performed experiments and reviewed and edited the manuscript. HRN-G performed metabolomics analysis. RCM generated GO annotations and search for Arabidopsis orthologs. All authors contributed to the article and approved the submitted version.

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Authors' information

Institute of Genomics for Crop Abiotic Stress Tolerance, Plant and Soil Science Department, Texas Tech University, Lubbock, TX 79409, USA.

Damar Lopez-Arredondo, Luis Herrera-Estrella, Jonathan Ojeda-Rivera, Rogelio Najera-Gonzalez, Ricardo Chavez Montes.

USDA-ARS, PA, CSRL, Plant Stress and Germplasm Development Research, Lubbock, TX 79415, USA.

Mauricio Ulloa

Department of Nematology, University of California, Riverside, CA 92521, USA.

Philip Roberts

Unidad de Genomica Avanzada/Langebio, Centro de Investigacion y de Estudios Avanzados, Irapuato, Guanajuato 36821, Mexico.

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Figures

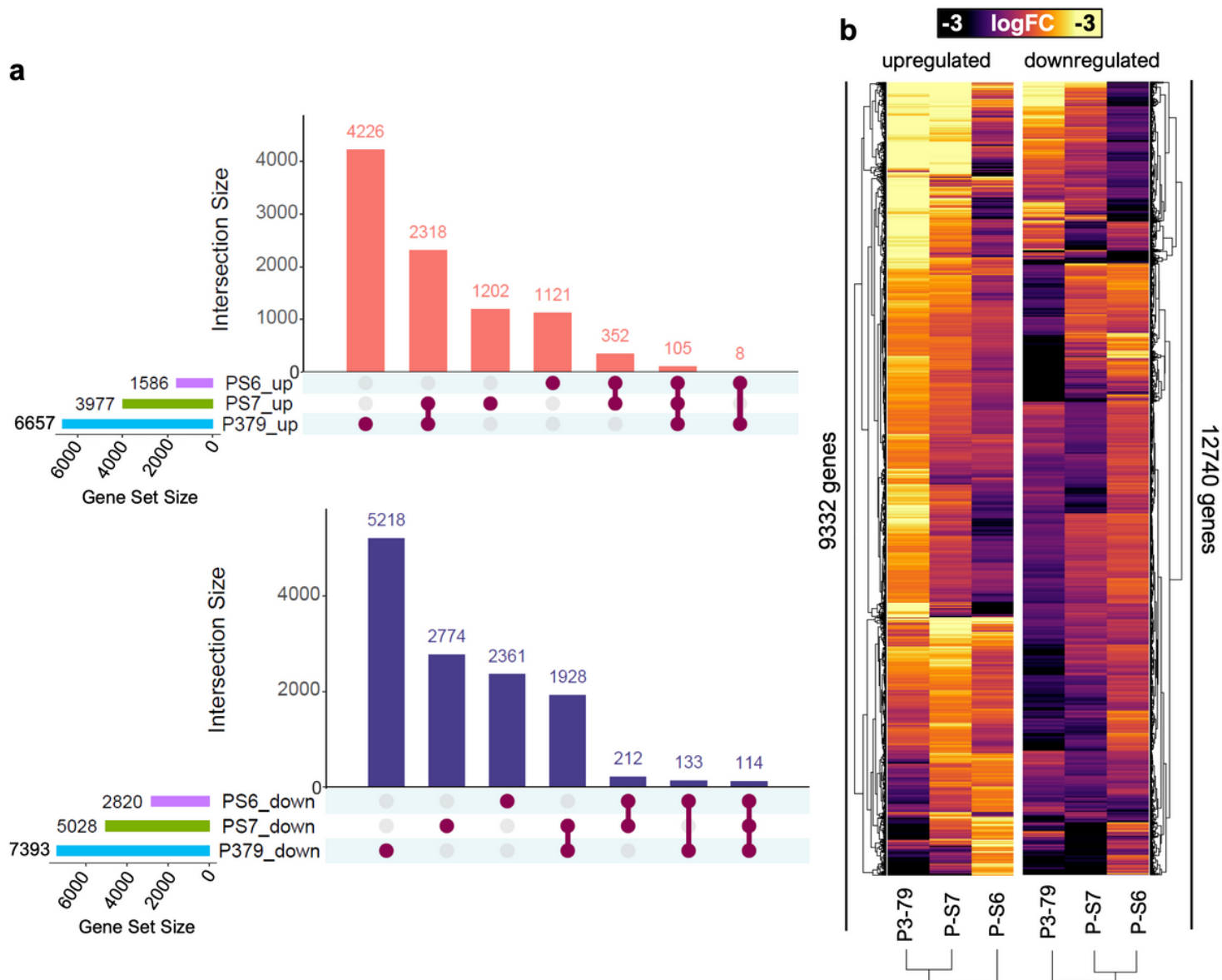


Figure 1

Pima-S6 presents a specific and compact transcriptomic response to FOV4 infection compared to Pima S-7 and Pima 3-79. (a) Gene set analysis of shared and unique differentially expressed genes in response to FOV4 treatment in Pima cotton cultivars. Connected dots represent common gene sets. Upper panel: upregulated genes; Lower panel: downregulated genes. (b) Heatmap of the logarithm of fold change (logFC) of genes that are differentially upregulated (left panel) or downregulated (right panel) with statistical significance in at least one of the three genotypes tested.

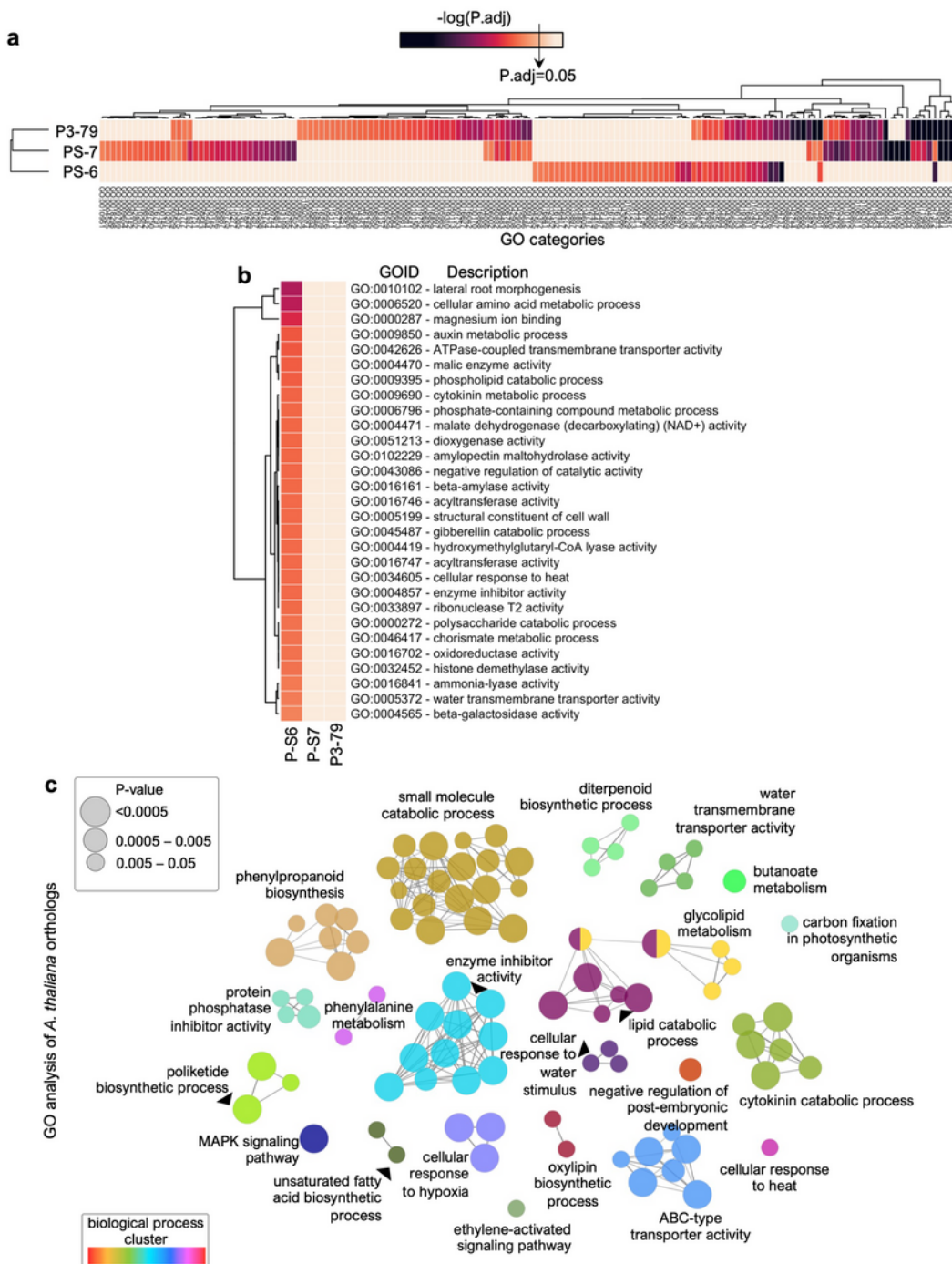


Figure 2

Differential analysis of Gene Ontology (GO) enriched categories in Pima cotton cultivars indicates secondary metabolism-related processes are activated in P-S6 in response to FOV4 infection. (a) Heatmap representing GO categories found enriched in at least one of the cultivars tested. The statistical significance key, $-\log(P_{adj})$, indicates the threshold for enrichment. (b) Enriched GO categories specific to the P-S6 resistant genotype in response to FOV4 treatment. (c) GO Enrichment analysis of the

Arabidopsis orthologs from (b). Each bubble represents a GO category and connections represent shared genes between categories. Categories are clustered according to the biological process. A full list of GO categories and descriptions from a-c is included in Supplementary Dataset 1.

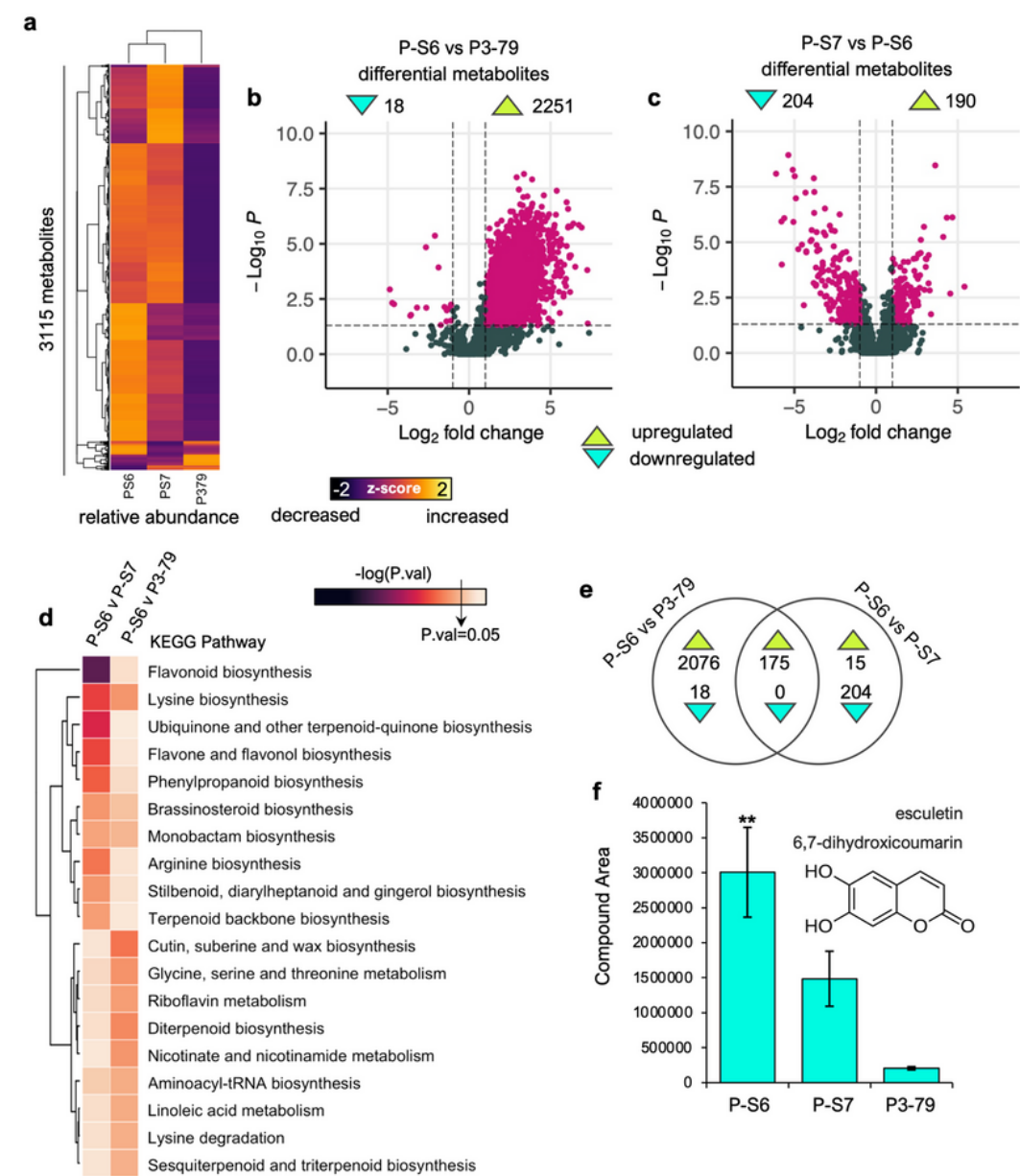


Figure 3

Mass spectrometry non-targeted metabolomic analysis of Pima-S6, Pima S-7 and Pima 3-79 corroborates differential enrichment of secondary metabolism-related compounds derived from the phenylpropanoid pathway in Pima-S6. (a) Heatmap of the normalized relative abundance (z-score) of the 3115 compounds identified using MS metabolomic profiling. (b,c) Volcano plots of the differential compounds found in the pairwise comparisons of P-S6 vs P3-79 (b) and PS-6 vs PS-7 (c). The threshold for a differential compound is ($-1 < \log FC > 1$; $P\text{-value} < 0.05$). (d) MS Peak-to-Pathway metabolite analysis. The key, $-\log(P\text{.val})$, indicates the threshold for statistically significant enrichment of a KEGG metabolic pathway. (e) Venn analysis of differentially accumulated metabolites in the pairwise comparisons of PS-6 vs. P3-79 and PS-6 vs. PS-7. (f) Relative abundance, in compound area units, of the coumarin esculetin in the three genotypes tested. Asterisks indicate that the level of this compound is significantly higher with statistical significance ($P\text{.val} < 0.05$).

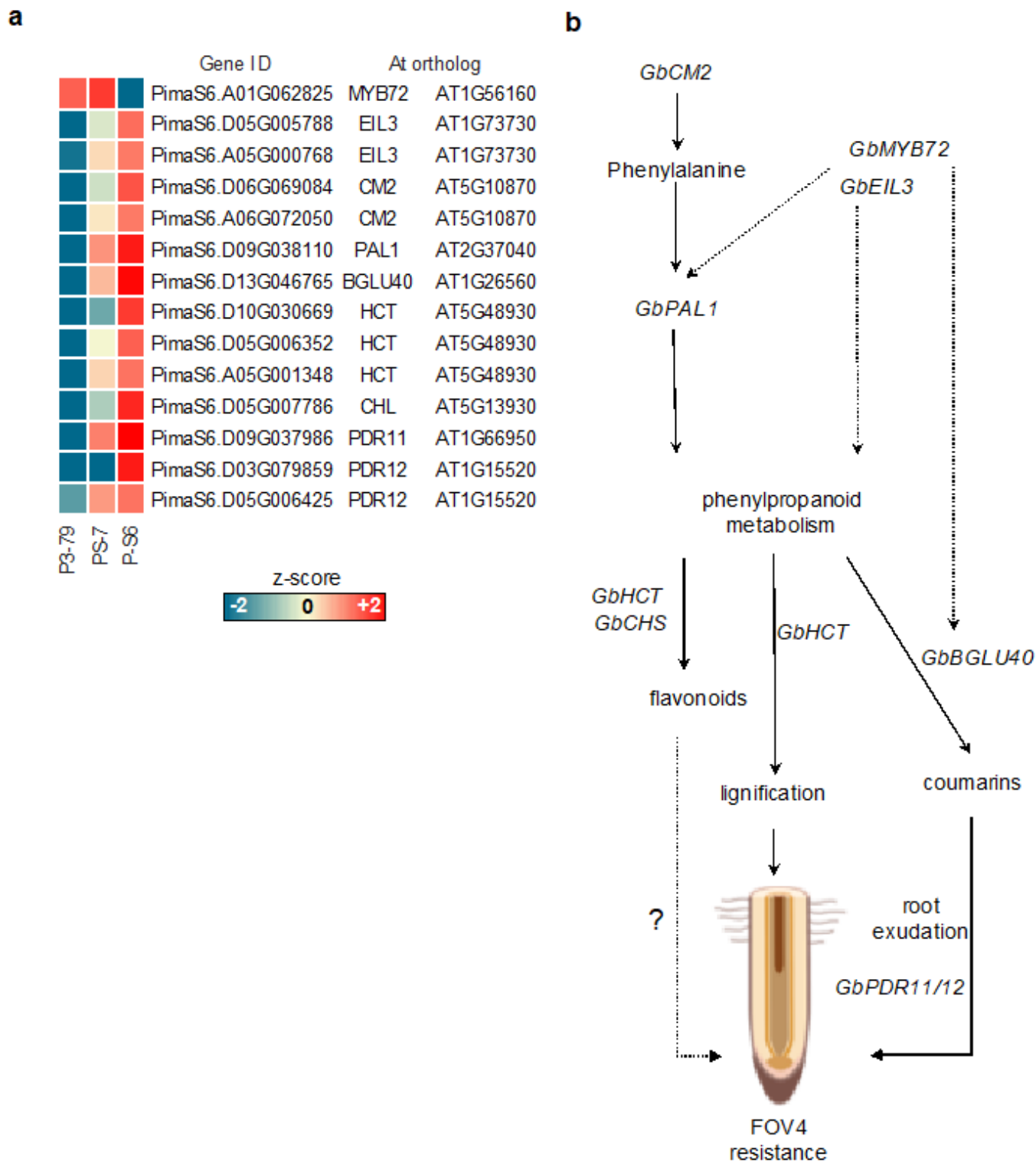


Figure 4

Phenylpropanoid metabolism and the possible genes and pathway that contribute to FOV4 resistance in Pima-S6. (a) Heatmap of relative expression (z-score) of candidate Pima-S6 genes that contribute to the enhanced phenylpropanoid metabolism observed in P-S6 and their Arabidopsis ortholog. (b) Putative gene regulatory pathway that controls the enhancement of phenylpropanoid metabolism and is upregulated in response to FOV4 in Pima-S6. Chorismate mutase (encoded by *GbCM2*) catalyzes

phenylalanine synthesis, which boosts the phenylpropanoid pathway via phenyl-ammonia lyase activity (encoded by *GbPAL 1*). Three possible pathways contribute to FOV4 resistance: coumarin exudation, lignification of the root, and flavonoid synthesis. We observed enhanced levels of the coumarin esculetin which suggests a basal synthesis of coumarins is enhanced in Pima-S6, the exudation of these types of compounds could be enhanced via *GbBGLU40* and root ABC-type transporter-encoding genes *GbPDR11/12*. The same root exudation mechanisms could be operating for flavonoid compounds whose synthesis could be heightened by enhanced hydroxycinnamoyltransferase activity (encoded by *GbHCT* genes) and chalcone synthase activity (encoded by *GbCHS* genes). Increased lignification of the root of P-S6, which we have observed in a previous study [34], could be caused by the enhanced activity of the encoded products of *GbCM2*, *GbPAL 1*, and *GbHCT*, which results in the secondary cell-wall thickening and provides a physical barrier of defense against the FOV4 threat.

Supplementary Files

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