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Research article

Flavonoid accumulation in spontaneous cotton mutant results in red coloration and enhanced disease resistance

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ABSTRACT

Cotton, the leading natural fiber, is cultivated worldwide, but its production is seriously threatened by pathogens. Accordingly, the selection of resistant cultivars has become a key priority of cotton breeding programs. In this study, a spontaneous mutant with red coloration (S156) and a control cultivar (S78) were used as experimental materials for a comparative analysis. Metabolomic analysis revealed the enrichment of flavonoids in S156 leaves compared with S78 leaves, and transcriptomic analysis revealed the upregulated expression of flavonoid biosynthesis genes in S156 leaves relative to S78 leaves. In addition, the red mutant showed a significantly increase in resistance to *Verticillium dahliae*, a fungal pathogen that poses a major threat to cotton production. The pathogen invasion process was suppressed in the red cotton cultivar. This study reveals the mechanism underlying the red coloration of S156 cotton and indicates the great potential of red cotton in pathogen- and insect-resistant breeding of cotton.

1. Introduction

Cotton (*Gossypium* spp.) is a crop plant in the family Malvaceae that has great agricultural and economic importance and is grown in more than 50 countries worldwide. *G. hirsutum* accounts for about 97% of all cultivated cotton, with the remainder comprised of *G. arboretum*, *G. barbadense*, and *G. herbaceum* (Ashraf et al., 2018). Cotton fiber is a major source of natural fiber in the textile industry. Cotton seeds contain both oil and nutritional protein fit for human and animal consumption. In addition, cotton straw is rich in cellulose and hemicellulose, which offers a promising source for biofuel production (Sunilkumar et al., 2006). However, global cotton production is restricted by various diseases that severely affect the cotton industry and cause huge economic losses annually (Gao et al., 2013a; Xu et al., 2018). One of the most devastating diseases of cotton is Verticillium wilt which is a soil-borne vascular disease caused by the fungal pathogen *Verticillium dahliae*. When *V. dahliae* infects cotton through roots, it spreads upwards along the vascular tissues and secretes toxins that kill the surrounding cells and block vascular transport. Diseased cotton plants exhibit chlorosis, wilting, and even death (Fradin et al., 2011). Techniques for chemically controlling Verticillium wilt have had limited effect because of pathogen infection strategies and current cotton farming practices; meanwhile, there have been increases in *V. dahliae* pathogenicity and diseased fields. Planting disease-resistant varieties is the most economical and effective measure to prevent the disease, and the study of cotton pathogen defense mechanisms may help to accelerate the process of cotton disease resistance breeding (Ashraf et al., 2018; Gao et al., 2013a; Xu et al., 2011).

Flavonoids are phenolic metabolites produced by plants. The core structure of flavonoids is a 15-carbon phenylpropanoid (C_6–C_3–C_6) in which the two aromatic rings are linked by a three-carbon bridge cyclized with oxygen. The saturation and oxidation status of the C_6–C_3–C_6 skeleton differentiates several classes of flavonoids, including chalcones, flavanones, flavones, flavonols, isoflavones, anthocyanins, and aurones (Vukics and Guttman, 2010). Chemical modification (e.g., glycosylation, hydroxylation, acetylation, etc.) and polymerization of the C_6–C_3–C_6 skeleton causes the vast diversity of flavonoids, hence ensuring flavonoids participate in many aspects of plants growth, reproduction, and defense. Moreover, some flavonoids exhibit substantial human health benefits via their antioxidant capabilities (Corradini et al., 2011).

Previous studies have revealed the involvement of flavonoids in pathogen defense (Mierziak et al., 2014). The specific defense mechanism depends on the chemical structures of flavonoids, that is, the defense mechanism vary, with most defense being nonspecific. Flavonols, isoflavones, and flavanones have been reported to have

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antibacterial activity and can also directly inhibit spore germination and mycelial elongation of pathogens. Flavonoids can inactivate cell envelope transport proteins and disrupt microbial membranes and the respiratory chain, and their antibacterial activity can be weakened or enhanced via methylation and hydroxylation of flavonoids (Naoumkina et al., 2010; Candiracci et al., 2012). For example, genistein, a yellow lupine isoflavone, functions as a phytoalexin that strongly inhibits infections and diseases caused by the pathogenic fungi *Aspergillus flavus*, *Fusarium oxysporum*, and *Sclerotinia sclerotiorum* (Morkunas et al., 2005). The flavan-3-ols, known as condensed tannins, are an effective chemical defense against infection by the biotrophic rust fungus *Melampsora larici-populina*, as the flavan-3-ols directly inhibit spore germination and hyphal growth (Ullah et al., 2017). Sakuranetin is a rice flavanone with a substantial inhibitory effect on the major rice pathogens *Pyricularia oryzae* and *Rhizoctonia solani* (Katsumata et al., 2018). Other studies have also indicated that flavonoids inactivate pathogenicity enzymes, such as enzymes that digest plant cell walls, thus restricting pathogen infection (Treutter, 2005). The flavonoids stored in all plant organs maintain homeostasis. Pathogen invasion induces flavonoid biosynthesis pathways and transportation of flavonoids. The abundance of flavonoids accumulated around infected cells induces hypersensitive responses and programmed cell death, thereby limiting the spread of pathogens (Beckman, 2000; Ullah et al., 2017). In addition, flavonoids were revealed to alter auxin transport and activity to tighten plant structures, thus promoting callus and tylose formation, which blocks the vascular system thereby preventing pathogen invasion and colonization (Beckman, 2000). Breeding crops with increased flavonoid production is a promising approach to creating more resistant cultivars with better nutritional value. One typical example of this is the purple tomato, which accumulates more anthocyanins, thus delaying fruit overripening and increasing *Botrytis cinerea* resistance relative to red tomato (Klee, 2013; Zhang et al., 2013). Notably, the functions of flavonoids are largely dependent on their chemical structure and show specificity among species. Despite the common antimicrobial effects of flavonoids, some flavonoid derivatives actually promote the growth and even pathogenicity of microorganisms. For example, legumes can accumulate flavonoids in their root systems and secrete flavonoids into the soil to promote spore germination and mycelia growth of arbuscular mycorrhizal fungi (Abdel-Lateif et al., 2012; Mollavali et al., 2018). The biotrophic pathogen *Ustilago maydis* promotes anthocyanin production in maize to repress lignin synthesis, which would otherwise restrict the spread of the pathogen (Tanaka et al., 2014).

Cotton is rich in secondary metabolites, includes flavonoids. Flavonoid biosynthesis genes have been cloned from cotton, yielding a total of 52 flavonoids representing seven classes (Nix et al., 2017). Despite the conservation of flavonoid biosynthesis and functions among plant species, studies of cotton flavonoids have been focused on natural colored cotton fiber. A spontaneous mutant of upland cotton (S156) and a control cultivar (S78) were used for the present comparative analysis of metabolism and disease resistance. Our results reveal S156 cotton shows increased production of flavonoids and expression of flavonoid biosynthesis genes compared to S78 cotton. In addition, S156 cotton was more resistant to the fungal pathogens *V. dahliae* and *B. cinerea*. The extracts containing flavonoids from S156 cotton also had stronger antibacterial activity against *V. dahliae* than did those from S78 cotton.

2. Materials and methods

2.1. Materials and growth conditions

Seeds of the control cotton cultivar *G. hirsutum* L. S78 and red cotton mutant *G. hirsutum* L. S156 were germinated in a high humidity environment. The seedlings were grown in a greenhouse (28 °C for 16 h during the daytime, 25 °C for 8 h at night), and the adult plants were planted in an outdoor field. The leaves were subsequently harvested for metabolomic analyses.

Spores of *V. dahliae* and *B. cinerea* that had been stored at −80 °C were placed onto PDA culture media for spore germination and hyphal growth. After 5 days, 0.5-cm-diameter hyphae were transplanted to Czapek's culture medium for spore production at 25 °C and 100 rpm in the dark for 5 days (Gao et al., 2013a).

2.2. Metabolomics analysis

The cotton leaves were freeze-dried and grinded into a powder. For metabolite extraction, 100 mg of leaf powder was added into 1.0 mL of 70% aqueous methanol and extracted at 4 °C in darkness overnight. The liquid supernatants of the extracts were absorbed with a CNWBOND Carbon-GCB SPE Cartridge (ANPEL, Shanghai, China) and filtrated with a 0.22-μm millipore filter SCAA-104 (ANPEL, Shanghai, China). A LC-ESI-MS/MS system was employed to analyze the metabolites, and the effluent was alternatively connected to an ESI-triple quadrupole-linear ion trap (Q TRAP)-MS. The primary and secondary mass-spectrometry data were subjected to qualitative analysis based on a public database and a self-built MetWare database (Chen et al., 2013; Zhu et al., 2013). The metabolites with fold changes ≥ 2 or ≤ 0.5 were consider significant in this study.

2.3. Comparative transcriptome and heat map analysis

To comprehensively study the expression of flavonoid-related genes in S78 and S156, fresh leaves were grinded up with liquid nitrogen to extract the total RNA. The RNA quality was then evaluated and sequenced using Illumina HiSeqTM 2000 (Illumina, California, USA). The data filtering and assembly were performed following our previous method description (Long et al., 2019). The flavonoid synthesis genes in cotton were identified using the homologous genes from *Arabidopsis* as query, and the *G. hirsutum* genome (http://mascotton.njau.edu.cn/info/1054/1118.htm) was used as a search database. The RPKM (reads per kilobase of transcript per million mapped reads) values of flavonoid-related genes were analyzed using the Genesis 1.8.1 program to generate the heat maps (Sturn et al., 2002).

2.4. Pathogen inoculation

Pathogen inoculation was performed using protocols from previous studies (Gao et al., 2013a, 2016). Pathogen spore concentrations were adjusted to 10^6 spores per mL with sterile water before use. For the whole-plant inoculation with *V. dahliae*, 4-weeks-old cotton seedlings were soaked in a spore suspension for 1 min and placed back into the soil for pathogen invasion. The disease grading methods used were based on the national standards of China (GB/T 22101.5-2009). The recovery culture and the fungal biomass analysis were conducted as described in previous publications (Fradin et al., 2011). The pathogen inoculation of detached leaves was performed as described by Gao et al. (2016), and lesion sizes were measured with ImageJ software. For the visualization of fungal invasion, transgenic *V. dahliae* with GFP expression was used for detached leaf inoculation (Zhao et al., 2014). The hyphae labeled with green fluorescence were observed under microscopy.

2.5. In vitro bacteriostatic test

The antifungal activities of flavonoids in cotton leaves against *V. dahliae* were assayed as described by Ullah et al. (2017). The extracts of 0.1-g samples of fresh leaves were mixed with 1 mL of *V. dahliae* spore suspension (10^7 mL^{-1}), spread carefully onto PDA culture medium, and incubated in a dark cabinet with high humidity at 25 °C. The spore germination rate and hyphae growth were observed and calculated at the indicated time.

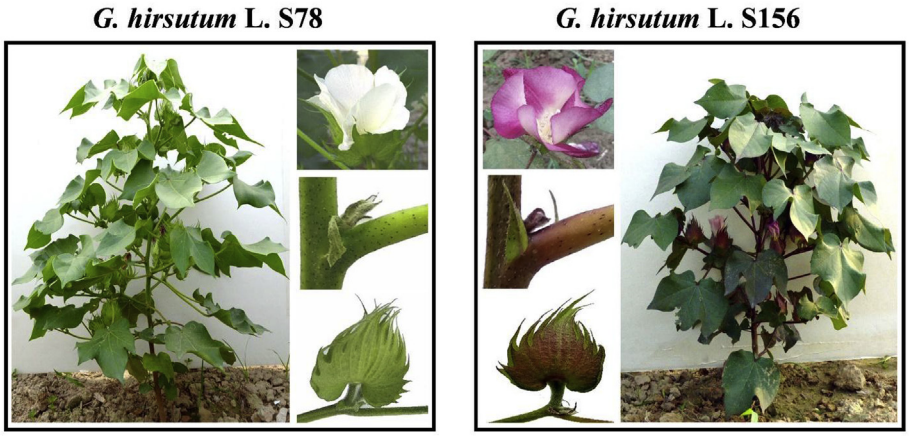

Fig. 1. Phenotype of the control cultivar S78 and the red mutant S156. The phenotypic differences between the adult plant, blooming flower, branch, and buds of normal cotton (S78) and the red mutant (S156). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

2.6. Histochemical staining

DMACA staining of the anthocyanins and proanthocyanins was performed as described by a previous study (Long et al., 2018).

2.7. Statistical analysis

Three biological replicates were performed for all experiments to calculate the standard deviations presented as mean ± standard deviation values.

3. Results

3.1. Morphological differences between S78 and S156

A spontaneous mutant of upland cotton cultivar S156 exhibits an obvious color change of the whole plant under field conditions. As shown in Fig. 1, compared to the control cultivar S78, red coloration appeared on various organs of the S156 cultivar throughout its lifespan. Stem, branch, leaf, and bud tissues were green for S78 plants but red for S156 plants. The flower of cotton plants undergoes a dramatic change in petal color following its blooming, such that the petals of the typical upland cotton cultivar change from cream at 0 days post-anthesis to red by 2 days post-anthesis (Tan et al., 2013). While S78 petals exhibited the color change of a typical upland cotton, S156 petals were red from blooming to abscission. Besides the color change, no significant differences in plant morphology were observed between S78 and S156 plants.

3.2. Red mutant S156 accumulates abundant flavonoids compared to S78

Plant often have red coloration when they accumulate abundant flavonoids under unfavorable environmental conditions. To explore whether the red mutant S156 accumulates more flavonoids, a LC-ESI-MS/MS system was used to compare the metabolic profiles of S78 and S156 plants. A total of 193 flavonoid metabolites were identified from tested samples, and 74 flavonoids with fold changes ≥ 2 or ≤ 0.5 were identified in the red mutant S156 compared with S78 plants (Table 1). Of these flavonoids, 19 of them (25.7%) were less abundant in S156 and 55 (74.3%) were more abundant. Based on their structural differences, the 74 differentially expressed metabolites (DEMs) were divided into seven groups, including 3 isoflavones, 10 anthocyanins, 26 flavones, 20 flavonols, 7 flavone *C*-glycosides, and 6 flavanones (Fig. 2A). Flavones were the most abundant group, constituting 35% of the total DEMs. The epicatechin gallate (a catechin derivative) and the tricin 4′-*O*-syringic acid (flavonolignan) showed lower concentrations in S156 relative to S78 plants and were categorized into the group 'other flavonoids' (Fig. 2A and Table 1). The top 10 increased and decreased DEMs are listed in Fig. 2B. Chrysoeriol *O*-acetyl hexoside (flavone) was the most increased flavonoid, showing a 15,778 × higher level in S156 relative to S78 plants. Isorhamnetin 3-*O*-neohesperidoside (a flavonol) was the most reduced flavonoid, with a concentration in S156 plants that accounted for only 0.01% of that in S78 plants. Notably, there are 10 anthocyanins with different concentrations between S78 and S156 plants, and 9 of them were increased in S156 plants compared to S78 plants. Among these 9 anthocyanins, cyanidin 3-*O*-malonylhexoside, mirtillin, cyanidin *O*-acetyl hexoside, tulipanin, and peonidin *O*-hexoside respectively increased to 15 ×, 10.7 ×, 8.8 ×, 6.4 ×, and 5.4 × higher levels in S156 plants relative to S78 plants. The red coloration of the S156 cultivar may be attributed to the increased production of these anthocyanins.

The 74 DEMs were annotated using the KEGG database, revealing that these DEMs were mainly enriched among four biosynthesis pathways, including isoflavonoid biosynthesis, flavonoid biosynthesis, flavone and flavonol biosynthesis, and anthocyanin biosynthesis (Fig. 3A). The flavonoid biosynthesis pathway was most enriched, with 58% of annotated DEMs being classified in this pathway (Fig. 3B).

DMACA staining was performed to visualize anthocyanins and proanthocyanins, which are the downstream products of the flavonoid pathway. Anthocyanins and proanthocyanins turn red and blue, respectively, in plant tissue after treatment with DMACA acid solution. As shown in Fig. 4A, the DMACA staining of S156 fresh leaves resulted in dark red staining of the whole leaf and blue staining in some parts, while S78 leaves were stained only yellow with light pink. Anthocyanin production by fresh leaves from S78 and S156 plants was quantized using HPLC. The anthocyanin production of S156 leaves was thus observed to be 6.3 × higher than that of S78 leaves (Fig. 4B).

3.3. Flavonoid biosynthesis genes were upregulated in S156 compared to S78

To reveal the molecular mechanisms underlying flavonoid abundance in S156 plants, the expression of related genes in flavonoid biosynthesis pathways were studied in S78 and S156 leaves through transcriptome analysis (Fig. 5). Most of the flavonoid biosynthesis steps showed upregulated expression of one or more genes in S156 leaves compared to S78 leaves, except for 4CL and *F3′5′H*, which exhibited similar expression levels across the two cultivars. PAL is the first enzyme of the phenylpropane secondary metabolic pathway. The expression of two *PAL* genes in S156 leaves was respectively 3 × and 2 × of that in S78 leaves. CHS is the rate-limiting enzyme that regulates an early step of flavonoid biosynthesis and thus controls phenolic metabolic pathway flow into flavonoid metabolism. CHS and the directly downstream enzyme CHI produce naringenin, a major substrate for different branches of flavonoid metabolic pathways (Ferrer et al., 2008; Petrussa et al., 2013). The expression of two *CHS* genes and two *CHI*

Table 1
The differencially expressed flavonoids.

Compounds	Class	S78	S156	Fold Change	Log_2 FC	Type
Epicatechin gallate (ECG)	Catechin derivatives	14300	6200	0.43	−1.21	down
Peonidin O-hexoside	Anthocyanins	2780000	15000000	5.40	2.43	up
Cyanidin 3-O-malonylhexoside	Anthocyanins	249000	3750000	15.06	3.91	up
Rosinidin O-hexoside	Anthocyanins	359000	128000	0.36	−1.49	down
Cyanidin O-syringic acid	Anthocyanins	865000	2300000	2.66	1.41	up
Cyanidin O-acetylhexoside	Anthocyanins	7900	69400	8.78	3.14	up
Delphinidin	Anthocyanins	843000	1740000	2.06	1.05	up
Pelargonidin	Anthocyanins	44200	105000	2.38	1.25	up
Delphinidin 3-O-glucoside (Mirtillin)	Anthocyanins	2950000	31800000	10.78	3.43	up
Delphinidin 3-O-rutinoside (Tulipanin)	Anthocyanins	92700	593000	6.40	2.68	up
Cyanidin	Anthocyanins	5550000	11900000	2.14	1.10	up
Selgin 5-O-hexoside	Flavone	239000	536000	2.24	1.17	up
Chrysoeriol 5-O-hexoside	Flavone	3130000	8600000	2.75	1.46	up
Selgin O-malonylhexoside	Flavone	271000	8370000	30.89	4.95	up
Chrysin O-malonylhexoside	Flavone	29600	70500	2.38	1.25	up
Tricin 7-O-acetylglucoside	Flavone	7620	9	0.00	−9.73	down
Chrysin O-hexoside	Flavone	121000	347000	2.87	1.52	up
Velutin	Flavone	190000	55800	0.29	−1.77	down
Tricin	Flavone	73100	155000	2.12	1.08	up
Syringetin 5-O-hexoside	Flavone	170000	57000	0.34	−1.58	down
Chrysin 5-O-glucoside (Toringin)	Flavone	102000	323000	3.17	1.66	up
Chrysoeriol O-glucuronic acid-O-hexoside	Flavone	78400	207000	2.64	1.40	up
Luteolin 3′,7-di-O-glucoside	Flavone	83300	261000	3.13	1.65	up
Chrysoeriol 7-O-hexoside	Flavone	4290000	11100000	2.59	1.37	up
Chrysoeriol O-malonylhexoside	Flavone	26000	241000	9.27	3.21	up
Luteolin O-hexosyl-O-hexosyl-O-hexoside	Flavone	4290	1840	0.43	−1.22	down
Acacetin O-acetyl hexoside	Flavone	18600	9070	0.49	−1.04	down
Luteolin O-hexosyl-O-gluconic acid	Flavone	3720	8400	2.26	1.18	up
Chrysoeriol O-acetylhexoside	Flavone	9	142000	15777.78	13.95	up
Tricin O-glycerol	Flavone	1560	5200	3.33	1.74	up
Tricin O-saccharic acid	Flavone	1540000	696000	0.45	−1.15	down
Tricin 7-O-hexoside	Flavone	19200	59800	3.11	1.64	up
Tricin O-eudesmic acid	Flavone	34800	9	0.00	−11.92	down
Luteolin	Flavone	109000	896000	8.22	3.04	up
Chrysoeriol	Flavone	85400	284000	3.33	1.73	up
Apigenin	Flavone	94800	196000	2.07	1.05	up
Tricetin	Flavone	614000	2910000	4.74	2.24	up
Quercetin-3,4′-O-di-beta-glucopyranoside	Flavonol	148000	589000	3.98	1.99	up
Syringetin 3-O-hexoside	Flavonol	60200	9	0.00	−12.71	down
Quercetin 5-O-malonylhexosyl-hexoside	Flavonol	89300	5000000	55.99	5.81	up
Quercetin 7-O-malonylhexosyl-hexoside	Flavonol	335000	9010000	26.90	4.75	up
Isorhamnetin O-acetyl-hexoside	Flavonol	48300	1750000	36.23	5.18	up
Quercetin O-acetylhexoside	Flavonol	28900	1400000	48.44	5.60	up
Di-O-methylquercetin	Flavonol	3720000	1650000	0.44	−1.17	down
Kaempferol	Flavonol	827000	2360000	2.85	1.51	up
Quercetin	Flavonol	382000	1070000	2.80	1.49	up
Kaempferol 7-O-rhamnoside	Flavonol	129000	732000	5.67	2.50	up
Myricetin	Flavonol	4110000	12600000	3.07	1.62	up
Kumatakenin	Flavonol	157000	63400	0.40	−1.31	down
Isorhamnetin 3-O-neohesperidoside	Flavonol	62000	9	0.00	−12.75	down
Isorhamnetin	Flavonol	140000	514000	3.67	1.88	up
Kaempferol 3-O-rhamnoside (Kaempferin)	Flavonol	245000	709000	2.89	1.53	up
Fustin	Flavonol	64400	135000	2.10	1.07	up
Quercetin 7-O-β-D-Glucuronide	Flavonol	959000	2090000	2.18	1.12	up
Kaempferol-3-O-robinoside-7-O-rhamnoside	Flavonol	184000	25300	0.14	−2.86	down
Myricetin 3-O-galactoside	Flavonol	2720000	7350000	2.70	1.43	up
Morin	Flavonol	372000	1040000	2.80	1.48	up
Tricin 4′-O-syringic acid	Flavonolignan	7410	16700	2.25	1.17	up
Acacetin C-hexoside	Flavone C-glycosides	73600	9570	0.13	−2.94	down
Hesperetin C-hexosyl-O-hexosyl-O-hexoside	Flavone C-glycosides	35100	83200	2.37	1.25	up
Eriodictiol 6-C-hexoside 8-C-hexoside-O-hexoside	Flavone C-glycosides	15600	36100	2.31	1.21	up
6-C-hexosyl-luteolin O-hexoside	Flavone C-glycosides	90200	303000	3.36	1.75	up
Eriodictiol C-hexosyl-O-hexoside	Flavone C-glycosides	348000	1200000	3.45	1.79	up
C-hexosyl-luteolin O-p-coumaroylhexoside	Flavone C-glycosides	313000	129000	0.41	−1.28	down
Eriodictyol C-hexoside	Flavone C-glycosides	2050000	6190000	3.02	1.59	up
Eriodictyol O-malonylhexoside	Flavanone	156000	12100000	77.56	6.28	up
Hesperetin O-malonylhexoside	Flavanone	72000	27800	0.39	−1.37	down
Naringenin chalcone	Flavanone	3890000	9530000	2.45	1.29	up
Isoliquiritigenin	Flavanone	4940	11100	2.25	1.17	up
Afzelechin (3,5,7,4′-Tetrahydroxyflavan)	Flavanone	333000	146000	0.44	−1.19	down
Butein	Flavanone	27100	75500	2.79	1.48	up
2′-Hydroxygenistein	Isoflavone	122000	409000	3.35	1.75	up
Rotenone	Isoflavone	38600	8280	0.21	−2.22	down
Formononetin 7-O-glucoside (Ononin)	Isoflavone	63700	138000	2.17	1.12	up

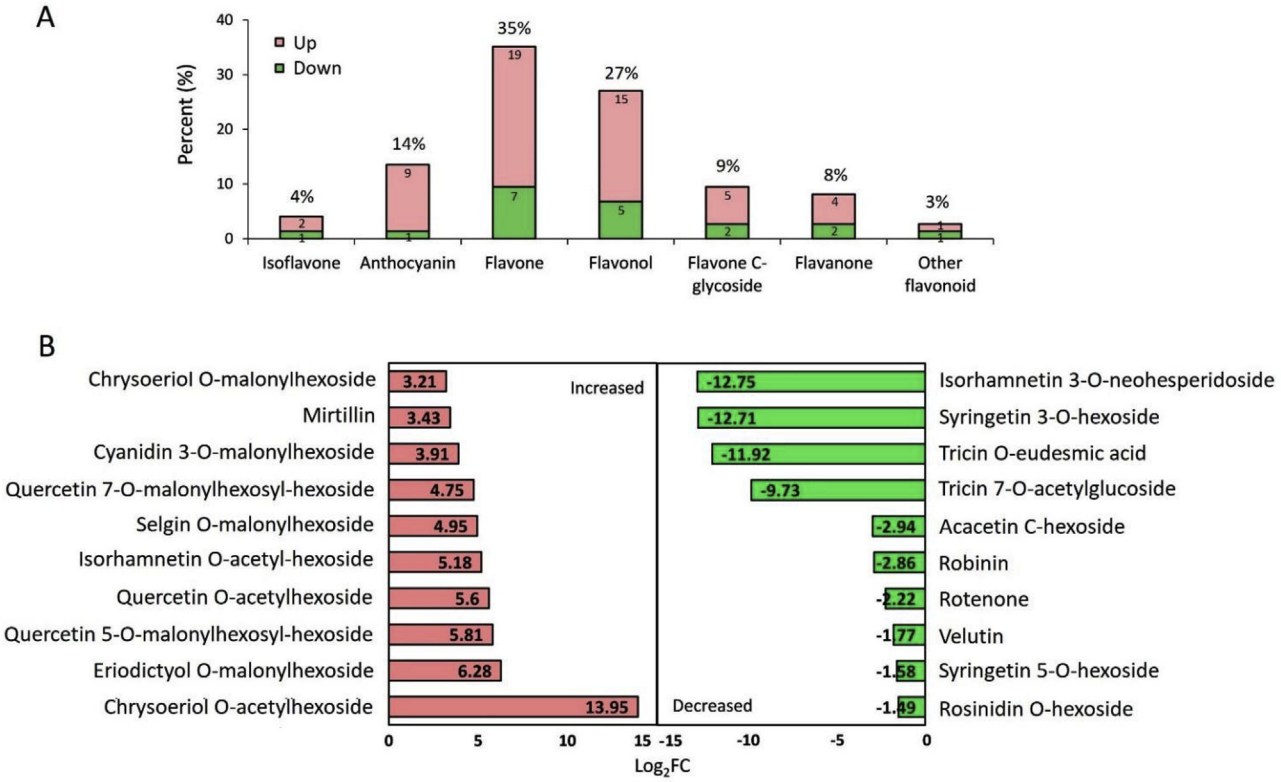

Fig. 2. The red mutant accumulates abundant flavonoids. (A) The classification of 74 differentially expressed flavonoids. The numbers of metabolites are denoted in columns, and the percentage of metabolites are noted above the columns. (B) The fold change of the top 10 increased (left, red columns) and decreased (right, green columns) flavonoids. The fold change values were $\log_2$ transformed. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

genes were approximately 2-fold higher in S156 leaves compared to S78 leaves. The enhanced expression of *PAL*, *C4H*, *CHS*, and *CHI* indicate that S156 leaves have more active flavonoid production than do S78 leaves.

According to the composition of metabolite production, the downstream metabolic pathway had flown into different branches. The synthesis of flavonoids, isoflavones, and flavonols were related to enzymes such as F3H, F3′H, F3′5′H, and FLS (Ferrer et al., 2008; Fan et al., 2016). The expression levels of two *F3H*s, three *F3′H*s, and two *FLS*s in S156 tissues were approximately 2 ×, 3 ×, and 2.5 × higher than those in S78 tissues, respectively. DFR, ANS, and UFGT catalyzed colorless dihydroflavonols into anthocyanins and proanthocyanins (Petrussa et al., 2013; Ferrer et al., 2008). The two *UFGT* genes identified in the transcriptomic study were both upregulated in S156 leaves; which were 5 × and 3 × higher than in the S78 leaves; and the expression of upregulated *DFR*s and *ANS*s was about 1.5 × higher in S156 relative to the S78 leaves. These results correspond to the contrasting coloration of S78 and S156 plants, indicating that the red cultivar accumulates more flavonoids and has higher expression of flavonoid biosynthesis genes.

3.4. S156 shows enhanced resistance to V. dahliae relative to S78

To reveal the involvement of flavonoids in cotton defense, both S156 and S78 seedlings were subjected to *V. dahliae* infection. The detached leaves were wounded and sprayed with a spore suspension, and the lesion expansion process was recorded daily. Significantly larger lesions were observed on S78 leaves compared to S156 leaves (Fig. 6A). By 1 day post-inoculation (DPI), *V. dahliae* hyphae invaded both S78 and S156 veins, possibly because the first defense barrier that prevents pathogen invasion were damaged by wounding. By 3 DPI, statistically significant differences in lesion size were observed between the two cotton varieties, and the difference increased as time elapsed. At 8 DPI, the lesions on S78 leaves were approximately 4 × larger than those on S156 leaves (Fig. 6B). To confirm this observation, the invasion process was visualized using transgenic *V. dahliae* with GFP expression. At 2 DPI, green fluorescence was observed in both S78 and S156 leaf veins, but colonized mycelia were more abundant in S78 tissues (Fig. 6C).

We also performed whole-plant inoculation of *V. dahliae* using the root-dipping method with root-wounded seedlings (Gao et al., 2013a). S156 plants appeared to be more resistant to *V. dahliae* than S78 plants, as demonstrated by their fewer leaves lost and lower seedling death rates (Fig. 6D). The rate of diseased S156 plants was only 70% that of S78 plants, and the disease index of S156 plants was 60% that of S78 plants (Fig. 6E). To further quantify pathogen colonization, stem sections were subjected to a pathogen recovery assay and biomass analysis (Fradin et al., 2011; Gao et al., 2016). After 5 days of recovery culture on PDA, typical *V. dahliae* colonies were observed growing from the inside of stem sections, which suggests that *V. dahliae* was the dominate pathogen causing cotton disease in our study (Fig. 6F). The fungal biomass analysis by qPCR also revealed that S156 plants contain lower pathogen levels than did S78 plants after infection (Fig. 6F). The above results indicated the enhanced resistance to *V. dahliae* of S156 plants over S78 plants.

3.5. Enhanced resistance of S156 to B. cinerea relative to S78

B. cinerea is one of the typical necrotrophic fungal pathogens that infects many crop species in nature and causes enormous economic losses. Previous studies have revealed the substantial role of flavonoids in resisting *B. cinerea* in other crops (Klee, 2013; Zhang et al., 2013). To investigate whether similar resistance effects also exist in cotton, S78 and S156 leaves were infected with *B. cinerea*. As shown in Fig. 7A,

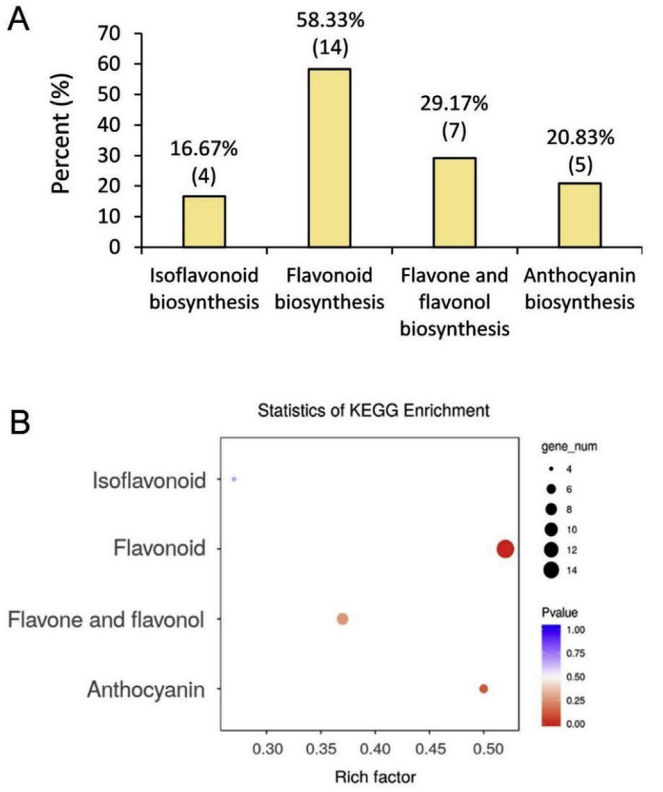

Fig. 3. The KEGG classification of DEMs. (A) The KEGG classification of the identified DEMs in S156 leaves compared to S78 leaves. The number and percentage of metabolites are noted above the columns. (B) Statistics summarizing KEGG pathway enrichment of the identified DEMs. The abscissae represent the enrichment factor of each biosynthesis pathway; the sizes of dots represent the number of DEMs, and the colors of dots represent *P*-values (with a color gradient ranging from blue to red, representing low and high significance, respectively). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

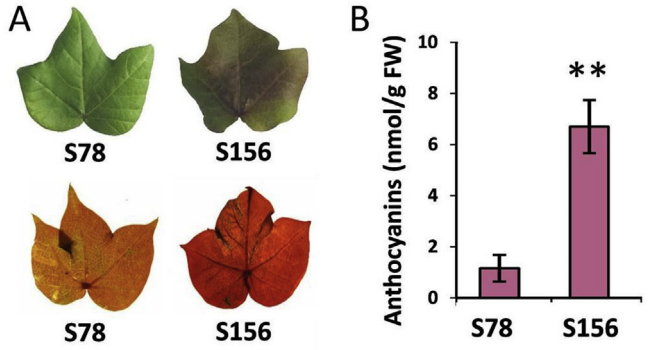

Fig. 4. The anthocyanin production in S78 and S156 leaves. (A) Representative fresh leaves of S78 and S156 cultivars before (upper row) and after (lower row) DMACA staining. (B) The measurement of the anthocyanin concentrations in representative S78 and S156 leaves ($n \geq 8$, $^{**}P < 0.01$, *t*-test).

S156 leaves showed only slight necrosis while severe necrosis appeared on S78 leaves. Pathogen colonies were quantified by measuring the size of S78 and S156 lesions caused by *B. cinerea*. S78 lesions were 2.5 × larger than S156 lesions (Fig. 7B), consistent with the abundance of flavonoids in cotton plants conferring greater resistance to S156 plants relative to S78 plants.

3.6. S156 shows antifungal activities against V. dahliae

To study the antifungal activities of flavonoids in cotton, in vitro bioassays with *V. dahliae* were conducted with extracts of S78 and S156 fresh leaves. *V. dahliae* spores were mixed with leaf extracts and cultured in a suitable environment for germination and growth. Spores had germinated by 7 h post-inoculation (HPI) and reached 100% by 10 HPI. During the germination period, the rate of germinated spores was relatively lower when spores were incubated with S156 extracts than with S78 extracts (Fig. 8A and C). After 24 h, hyphal growth was observed, and no significant differences were found between the two treatments (Fig. 8B). Thus, the S156 extracts slightly delayed spore germination, but did not affect the growth of *V. dahliae*.

4. Discussion

Cotton is a globally important economic crop. The average annual production of cotton fiber reaches 26 million tons, which meets most of the demand for natural fiber in the textile industry. In addition, cotton seeds are rich in proteins (24%), fatty acids (20%), carbohydrates, and vitamins and thus show great potential for meeting the global nutritional needs amidst growing food shortages (Sunilkumar et al., 2006). However, the productivity of cotton is seriously threatened by various kinds of disease. Selective breeding of resistant cultivars is the most effective way to prevent diminished cotton yields and quality (Gao et al., 2017; Ashraf et al., 2018; Shen et al., 2019).

Natural cotton mutants like the one examined in this study that exhibited red coloration of its vegetative organs are poised to be important genetic resources with wide application prospects but they have not yet been studied in depth. Three independent loci have been identified to be responsible for such a red cotton phenotype so far, including *Rd*, *Rs*, and *R1* (Zhao et al., 2009; Liu et al., 2015; Cai et al., 2014; Li et al., 2019; Gao et al., 2013b). *Rd* mutants exhibit red coloration with a dwarf phenotype. *Rs* mutants exhibit enhanced photosynthetic efficiency and have red flowers and reddish vegetative organs. *R1* is the most-studied loci controlling red coloration of cotton stems, leaves, flowers, and bolls. Traditional breeding has utilized *R1* mutants as a visible morphological marker for selecting target traits (Zhao et al., 2009; Liu et al., 2015; Cai et al., 2014). Marker-assisted selection mapped *R1* onto chromosome D07 of upland cotton, and further research identified *RLC1* (homologous to the anthocyanin biosynthesis gene *PAP1* in *Arabidopsis*) as the gene underlying red coloration of the *R1* mutant (Gao et al., 2013b). Silencing of *RLC1* in R1 seedlings reduces anthocyanin accumulation in leaves, causing green plants, while overexpression of *RLC1* in cotton and tobacco results in red transgenic plants resembling the *R1* mutant (Li et al., 2019; Gao et al., 2013b). The S156 cultivar is a newly produced mutant exhibiting red coloration of both its vegetative organs and reproductive organs. Through comparative metabolomic analysis, we identified that nine anthocyanins were significantly increased in S156 plants, suggesting its phenotype is associated with a similar mechanism of anthocyanin abundance resulting in red coloration as is observed in *R1* mutants.

Physiological and biochemical barriers play important roles in cotton defense. In order to prevent pathogen damage, cotton has accumulated a variety of antimicrobial compounds, including terpenoids, gossypols, flavonoids, and tannins. These substances can effectively inhibit infection by *Verticillium* spp. (González-Lamothe et al., 2009). In cotton, catechin and quercetin have been demonstrated to directly inhibit the sporulation, spore germination, and hyphal growth of fungal pathogens. Catechin production was obviously increased after cotton was subjected to a pathogen infection, and the catechin concentration in resistant cotton was higher than that in susceptible cotton (Gillmeister et al., 2019; Boots et al., 2008). The present metabolomic analysis revealed that 55 flavonoids, including quercetin and its derivatives, were increased in S156 tissues compared with S78 tissues. In addition, the red mutant exhibits significantly increased resistance to *V.*

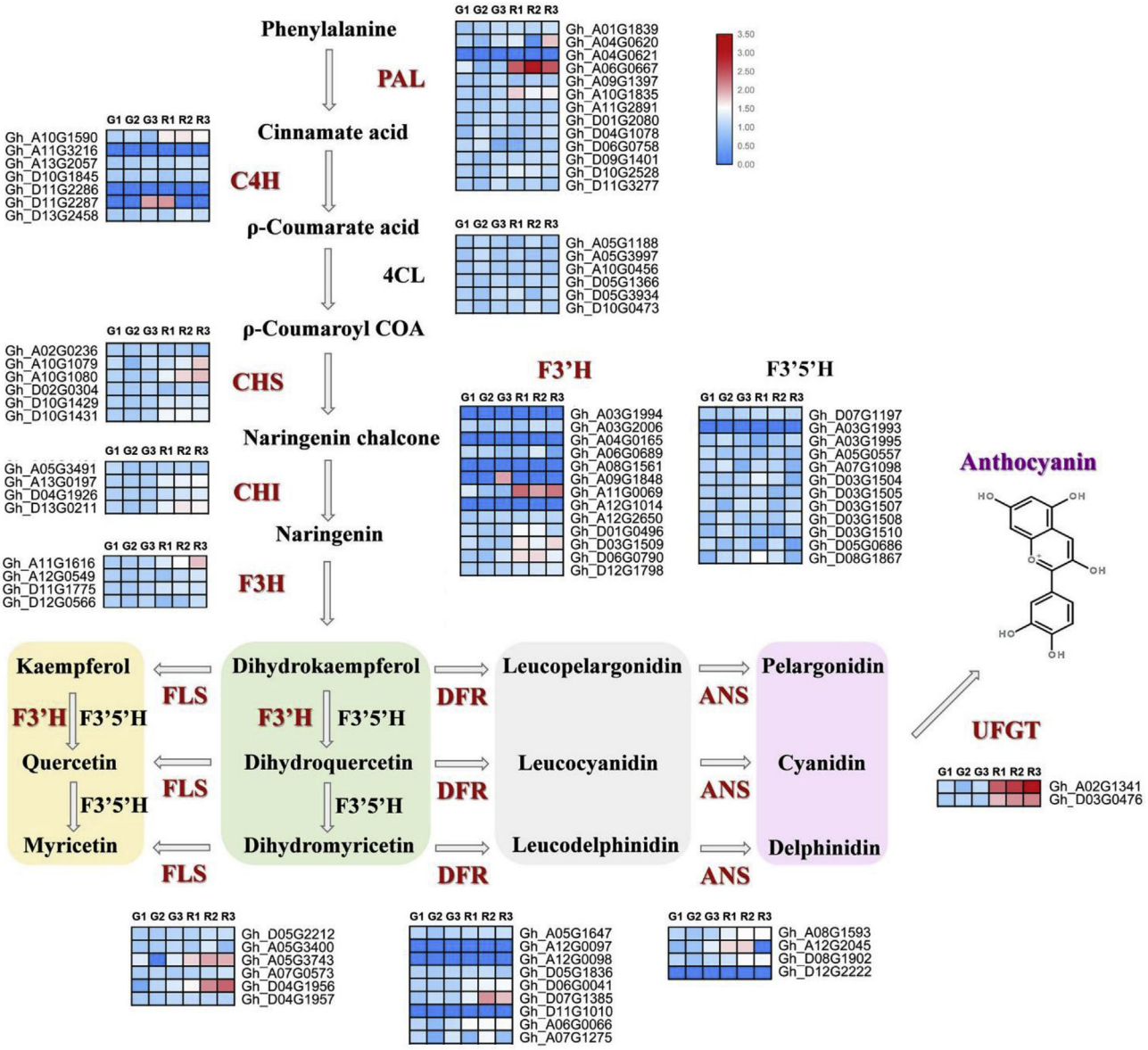

Fig. 5. The expression profile of flavonoid synthesis genes in S78 and S156 plants. The schematic model of the flavonoid biosynthetic pathway in cotton and heat map of the expression level of flavonoid biosynthesis genes. The G1, G2, G3 represent the three biological repeats of S78, and the R1, R2 and R3 represent the three biological repeats of S156. The relative expression values of flavonoid synthesis genes are shown by a color gradient from low (blue) to high (red). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

dahliae and *B. cinerea* infection, suggesting that the pathogen invasion processes are suppressed in cotton enriched with flavonoids. Interestingly, extracts of purple cotton did not affect fungal growth but did slightly delay spore germination on agar plates compared to extracts from green cotton. These results may suggest that the bacteriostatic role of flavonoids may function better in *vivo*. Transcriptome and proteome analyses have also revealed the involvement of flavonoid biosynthesis genes in cotton responses to *V. dahliae* infection (Gao et al., 2013a; Xu et al., 2011). The expression of *CHS*, *CHI*, *LDOX*, *DFR*, and *ANR* were increased by more than 10 × after *V. dahliae* invasion in resistant cotton cultivars relative to sensitive cultivars, revealing the close relation between cotton defense and flavonoid metabolism (Xu et al., 2011). Silencing of the biosynthesis genes could cause potential metabolism reprogramming and affect cotton resistance (Long et al., 2018). This research indicates that cotton may respond to pathogen infection through alterations of the metabolic flux of flavonoids via the regulation of its flavonoid biosynthesis genes.

The red cotton phenotype appears to confer resistance to a broad range of insect pests such as bollworms, spider mites, whiteflies, and boll weevils. Transgenic cotton overexpressing the *Lc* and *RLC* genes accumulated high levels of anthocyanin and was also shown to have resistance to cotton bollworms and spider mites (Fan et al., 2016; Li et al., 2019). Red cotton mutants have been widely cultivated as commercial insect-resistant varieties in some countries, yet their great potential in disease resistance breeding against pathogens has been undervalued in cotton compared to other crops. For example, the purple tomato, which is rich in anthocyanins, shows higher nutritional value. In addition, the enrichment of anthocyanins in purple tomato reduces their susceptible to *B. cinerea* and doubles their shelf life by delaying overripening. Similarly, a spontaneous ponkan mutant that accumulates high levels of polymethoxylated flavones shows higher postharvest resistance and a lower spoilage rate (Luo et al., 2015). Our results explore the enrichment of flavonoids in red cotton mutant S156 plants and demonstrate the cultivar's enhanced resistance to *V. dahliae* and *B. cinerea*. This provides valuable information for the study of secondary metabolites in cotton and has the potential to assist the

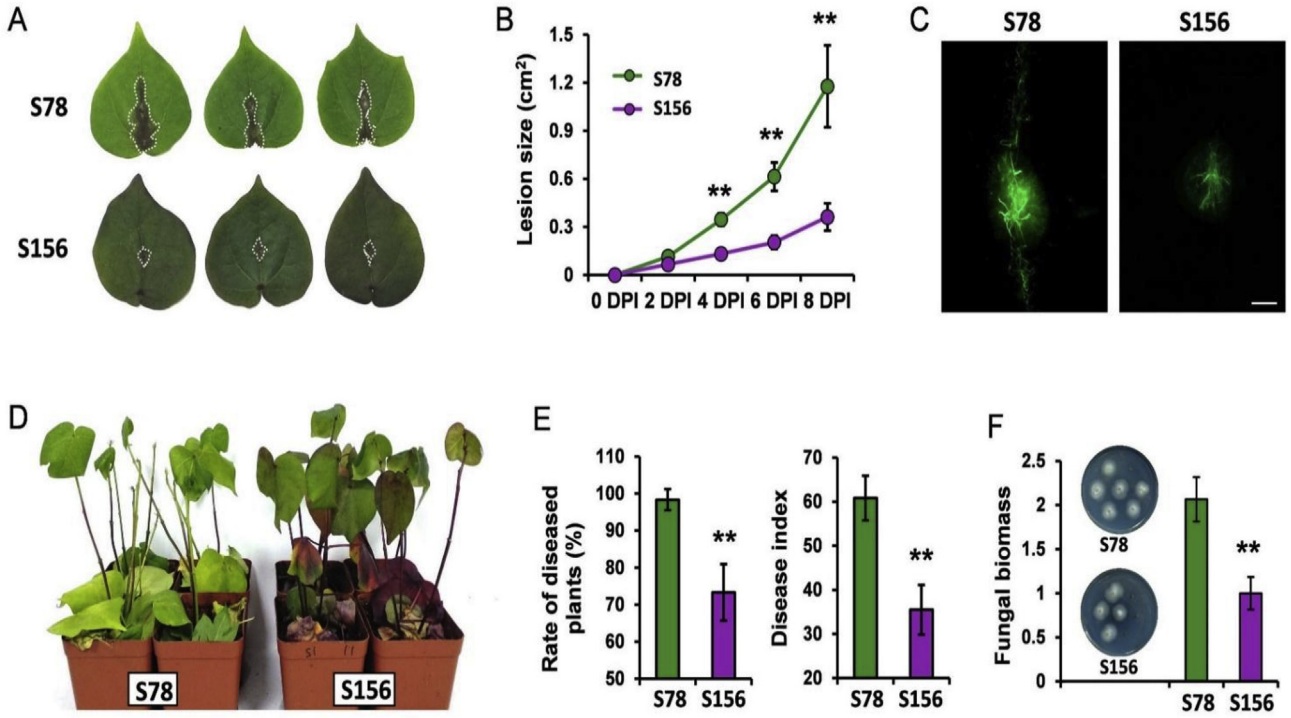

Fig. 6. The resistance assay of S78 and S156 to *V. dahliae*. (A) The lesion expansion of *V. dahliae* on cotton leaves at 7 DPI. (B) The calculation of the lesion size at the indicated times ($n \geq 12$, $^{**}P < 0.01$, *t*-test). (C) The pathogen colonization visualized by GFP-tagged *V. dahliae* in S78 and S156 leaves; bar = 1 mm. (D) Phenotypes of whole S78 and S156 plants infected by *V. dahliae* at 14 DPI. (E) The calculation of diseased plant percentage and disease index of S78 and S156 plants at 12 DPI ($n \geq 20$, $^{**}P < 0.01$, *t*-test). (F) The relative quantification of fungal biomass and the recovery culture of *V. dahliae* in S78 and S156 stem sections ($n \geq 20$, $^{**}P < 0.01$, *t*-test).

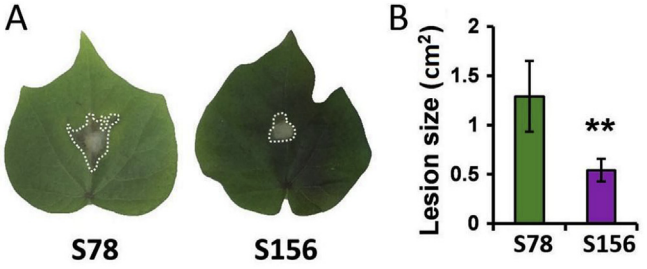

Fig. 7. S156 shows enhanced resistance to *B. cinerea* relative to S78. (A) Representative S78 and S156 leaves infected with *B. cinerea* at 3 DPI. (B) The calculation of lesion size on S78 and S156 leaves generated by *B. cinerea* infection at 3 DPI ($n \geq 8$, $^{**}P < 0.01$, *t*-test).

breeding of pathogen- and insect-resistant cotton varieties.

5. Conclusion

Natural cotton mutants that exhibited red coloration of its vegetative organs are poised to be important genetic resources with wide application prospects but they have not yet been studied in depth. In this study, we used a spontaneous mutant with red coloration (S156) and a control cultivar (S78) as experimental materials for comparative analysis. Our results revealed that the red mutant S156 accumulates more flavonoids compared to S78. In addition, the expression of related genes in flavonoid biosynthesis pathways was upregulated in S156 compared to S78. The red coloration of the S156 cultivar may be attributed to the increased production of anthocyanins. We also proved that the red mutant S156 shows enhanced disease resistance to pathogens relative to S78, and the S156 shows antifungal activities and restrict pathogen colonization. Our study provides valuable information for the study of secondary metabolites in cotton and has the potential to assist the breeding of pathogen- and insect-resistant cotton varieties.

Conflicts of interest

Authors declare no conflict of interest.

Author contributions

Lu Long and Ji Liu designed the study and wrote the manuscript. Ya Gao, Fuchun Xu, Jingruo Zhao and Bing Li performed the experiments. Wei Gao modified this manuscript. All authors reviewed and approved the manuscript.

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Abbreviations

GFP	green fluorescence protein
PDA	potato dextrose agar
DPA	day post anthesis
DPI	day post inoculation
HPI	hour post incubation
RLC	red leaf cotton
DEM	differentially expressed metabolite
KEGG	kyoto encyclopedia of genes and genomes
DMACA	4-(Dimethylamino)cinnamaldehyde
PAL	L-phenylalanin ammo-nialyase

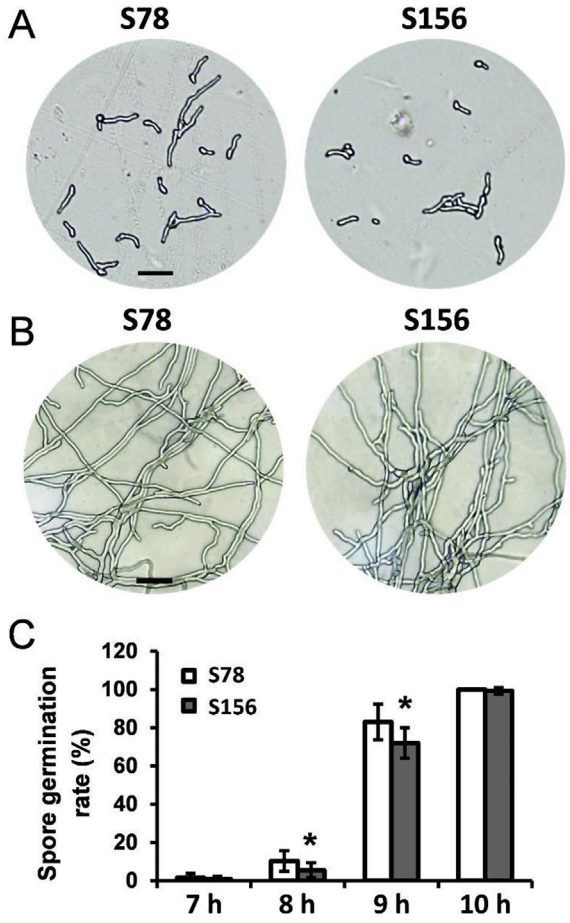

Fig. 8. The inhibition effects of S78 and S156 on *V. dahliae* germination and growth. (A) Representative image of spore germination at 8 HPI in S78 and S156 fresh leaf extracts; bar = 10 μm. (B) Representative images of mycelia growth at 24 HPI on PDA culture medium containing extracts of S78 and S156 leaves; bar = 50 μm. (C) The percentage of spore germination at the indicated times ($n \geq 5$, $^*P < 0.05$, *t*-test).

C4H	cinnamic acid-4-hydroxylase
4 C L	4-coumarate coenzyme A ligase
CHS	chalcone synthase
CHI	chalcone-flavanone isomerase
F3H	flavanone-3-hydroxylase
F3′H	lavonoid-3′-hydroxylase
F3′5′H	Flavonoid-3′5′-hydroxylase
FLS	flavonol synthase
DFR	dihydroflavonol-4-reductase
ANS	anthocyanidin synthase flavonoid-3-*O*-glucosyltransferase

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