Eur. J. Hortic. Sci. *84*(3), 161–170 | ISSN 1611-4426 print, 1611-4434 online | https://doi.org/10.17660/eJHS.2019/84.3.6 | © ISHS 2019

Original article

Possible involvement of flavonoids in response of mulberry (*Morus alba* L.) to infection with *Ralstonia solanacearum* (Smith 1896) Yabuuchi *et al.*, 1996

Fanwei Dai, Guoqing Luo, Zhenjiang Wang, Zheshi Kuang, Zhiyi Li, Jing Huang and Cuiming Tang
Sericultural & Agri-Food Research Institute, Guangdong Academy of Agricultural Sciences, Dongguan Village, Tianhe District, Guangzhou, Guangdong, China

Summary

Bacterial wilt in mulberry (*Morus atropurpurea*), caused by the phytopathogenic bacteria *Ralstonia solanacearum*, is one of the most important production constraints in many tropical and subtropical areas. In our previous work, we have investigated gene expression changes in mulberry at the early stages of *R. solanacearum* infection. In this work we have investigated transcriptional changes in mulberry that occur during all infection process caused by *R. solanacearum* in mulberry. Analysis of gene expression changes in two mulberry cultivars, following infection with *R. solanacearum*, revealed that expression of a series of genes associated with flavonoid biosynthesis was significantly involved into infection at all treated time points. Specifically, the expression of genes encoding the key-enzymes involved in flavonoid biosynthesis – such as chalcone synthase, chalcone isomerase, and flavonoid 3'-hydroxylase – were altered, and most of these genes were regulated more significantly in resistant cultivar KQ10 than in susceptible cultivar YSD10 after infection with *R. solanacearum*. We obtained full-length sequences of these three genes and additional transcriptional and phytochemical evidence to verify this result using qPCR and aluminum chloride colorimetry. This work indicates that flavonoids may play important roles in bacterial wilt resistance for mulberry plants and provides valuable gene targets for breeding disease-resistant mulberry cultivars.

Keywords
bacterial wilt, chalcone, disease resistance, flavonoid, gene expression, transcriptome

Significance of this study

What is already known on this subject?

- In our previous work, we investigated gene expression changes in mulberry at the early stages of *R. solanacearum* infection, and the results suggested that cell-wall modifications may play an important role in resistance levels in mulberry.

What are the new findings?

- Now we have investigated transcriptional changes in mulberry that occur during the entire *R. solanacearum* infection processes. The results suggested that flavonoids may also play important roles in bacterial wilt resistance for mulberry plants, and additional transcriptional and phytomedical data further supported this hypothesis using qPCR and aluminum chloride colorimetry. Meanwhile, we obtained the full-length sequences of these three key enzymes genes.

What is the expected impact on horticulture?

- Our results will be helpful for elucidating the molecular mechanisms of the resistance response to *R. solanacearum* in mulberry.

Introduction

Wilting caused by the phytopathogenic bacteria *Ralstonia solanacearum* (Smith 1896) Yabuuchi *et al.*, 1996 is a devastating soil-borne disease resulting in severe reductions in crop yields (Michel et al., 2013). Mulberry (*Morus alba* L.) is an economically important plant used under greenhouse condition in many developing countries in Asia (China, India, Thailand, and Vietnam). Bacterial wilt in mulberry caused by *R. solanacearum* is an important agricultural disease that results in severe yield and quality losses. South China, located in the tropical and subtropical region, is a high-incidence area of mulberry bacterial wilt, and the diseased areas accounted for 95% of all the mulberry areas in the 70s of last century. However, there is no efficient chemical pesticide against *R. solanacearum*. The resistant variety – KQ10 bred in the 90s of last century was widely planted in bacterial wilt areas with 80% resistance ability to *R. solanacearum*, but there is a shortcoming of low yield and low quality (Luo et al., 2010). Therefore, mulberry bacterial wilt is still a serious problem that negatively affects sericulture production .

Transcriptome effects of *R. solanacearum* infection have been studied in seedlings of *Solanum commersonii* (Narancio et al., 2013), leaves of Arabidopsis (*Arabidopsis thaliana*) and ginger (*Zingiber officinale*) (Hu et al., 2008; Prasath et al., 2014), roots of wild potato (*Solanum commersonii*) and *Arachis duranensis* (Chen et al., 2014; Zuluaga et al., 2015), and stems of tomato (*Lycopersicon esculentum* Mill.) (Ishihara et al., 2012). The number of expressed sequence tags analyzed ranges from 2,000 in *A. duranensis* (Chen et al., 2014) to tens of thousands in *A. thaliana* and tomato (Hu et al., 2008; Ishihara et al., 2012). Significant transcriptome differences induced by *R. solanacearum* infection have been shown to be dependent on inoculation time, plant species, organs, and tissues analyzed; however, a strong overlap in altered gene function was also observed.

Flavonoids are important and widely distributed plant secondary metabolites that play a key-role in interactions between plants and environmental conditions (Kim et al., 2012). They can be divided into different subgroups: flavonols, flavones, flavanones, flavanols, isoflavonoids, and anthocyanidins (Iker et al., 2009). Flavonoids possess a wide range of biological activities including energy transfer, auxin transport inhibition, allelopathy, control of respiration, and photosynthesis (Cushnie and Lamb, 2005). Flavonoids also play a significant role in plant resistance (Treutter, 2005).

The biosynthesis of flavonoids is a complex metabolic process controlled by a series of enzymes including chalcone synthase (CHS), chalcone isomerase (CHI), flavonoid 3'-hydroxylase (F3'H), flavanone 3-hydroxylase (F3H), and flavonoid-3',5'-hydroxylase (F3'5'H) (Koes et al., 1994). Flavonoids are very abundant in mulberry and considered to be beneficial for human nutrition and health (Graf et al., 2005; Jia et al., 2010). Clinical studies have reported that flavonoids can be used to help treating many diseases including cancer, hepatitis, and coronary heart disease (Gani et al., 2012).

In Arabidopsis leaves, gene expression is only slightly altered in the early stages of *R. solanacearum* infection; however, significant changes at the transcriptional level were observed upon the onset of wilt symptoms at 5 and 8 days-post-infection (dpi) (Hu et al., 2008). In our previous work, investigations of gene expression levels in mulberry roots in response to *R. solanacearum* infection revealed that modifications in the cell wall may play an important role in resistance only at early stages of infection (Dai et al., 2016). In this work instead we have investigated gene expression changes in mulberry root that occur during the whole *R. solanacearum* infection process. To reach this purpose we have analyzed transcriptional and phytochemical changes following *R. solanacearum* infection of two mulberry cultivars, one resistant and the other susceptible to wilting, and discovered that flavonoid biosynthesis was significantly affected to infection throughout the whole infection process. Additionally, we have obtained full-length sequences of three genes encoding key-enzymes in flavonoid biosynthetic pathways.

Materials and methods

Starting material

Two cultivars of mulberry, the first one resistant (KQ10) and the second one susceptible (YSD10) to *R. solanacearum*, were tested. Young plants of mulberry, propagated by stem-cuttings, were grown in a greenhouse (25 ± 5°C) at Sericultural and Agri-Food Research Institute, Guangdong Academy of Agricultural Sciences, Guangzhou, China. The methods of root inoculations were modified according to the procedures described in Arabidopsis and tomato (Deslandes et al., 1998; Ishihara et al., 2012). Inoculations were performed according to the following protocol: approximately 2 cm was cut from the bottom of each pot, and the exposed roots of the plants were immersed for 10 min in the *R. solanacearum* (accession no. JX112351) suspension containing 1.0×10^8 CFU (Colony Forming Unit) mL^{-1}. The plants were then transferred to a growth chamber held at 25°C for 12 h day^{-1}. Samples of the uninfected and infected roots at 0, 1, 3 and 8 dpi, excluding the cut areas, were frozen at -80°C until RNA extraction. Monitoring of bacterial density was carried out according to methods described by Dai et al. (2016).

Illumina sequencing, data processing, annotation, and analysis

Methods described by Dai et al. (2016) were used for RNA sequencing, data processing, annotation, and sequence analysis. RNA sequencing of total RNA extracts from roots at 0, 1, 3, and 8 dpi were used to compile each sequencing library composed of 50-bp single-end reads sequenced at the

Table 3. Primers used for qPCR and full length cloning.

	Gene ID	Gene annotation	Primer sequence (5' to 3')
qPCR	CL3598	Chalcone synthase	F: TGACGGGCACTTGAGGGAAATAG R: TATGGAGTTCCAGTCGCTGATCC
	Unigene23165	Chalcone synthase	F: CACCAACAGCGAGCACCTGATTC R: TTCATAAAGCTGCAAAGACTAGGGT
	Unigene15633	Chalcone synthase C	F: GTGACGGATTGAAGTGGGGAGT R: GCATTTTTTCATAATCTAAGCAACAA
	Unigene30900	Chalcone synthase	F: AGCCAGCCAGTGATCCGACGAG R: TTCTCTTTCAATTCTGTCTTGTCCTC
	Unigene28095	Flavonoid 3' hydroxylase-like	F: AGATTCTATTCCGTGGCTCGATTTC R: ACCAGCTATTAAGTCCTGGGTAA
	Unigene34977	Flavonoid 3' hydroxylase	F: GGTGGCGGTTGATGCGGAAGAT R: CGAAAACTCTGTACCCTAGCACTGC
	CL518	Flavonoid 3' hydroxylase	F: CGGTGAACGCCTTAGGACGTACAAT R: ATCGACTTAAATTCATCCGCCCTCG
	Unigene27928	Chalcone isomerase	F: GGCTCCGACAAGACTCTGTTTCTT R: CCTCCGCCGACTTCCCCTTC
	KT793030	Actin4 (*Morus alba* L.)	F: TGTTGCTCCACCAGAGAGAAAGTAC R: GGACAATTGATGGACCAGACTCG
Full length cloning	MaCHS4	Chalcone synthase	F: ATGGCGACACCCTCCTCCGTC R: TTAATTATTGATGGGAAGGCTGTGG
	MaCHS5	Chalcone synthase C	F: ATGGCACCGAATAACGTGTCCG R: CTAGCAGAACGTGTCCCGAGGCA
	MaF3'H1	Flavonoid 3' hydroxylase-like	F: ATGAAAGGCACAACATGGGTTTCCT R: TCACAGGGCATAGAGATGGGGTG

Beijing Genomics Institute, Shenzhen, China using the Illumina HiSeq 2000 platform (San Diego, CA). Approximately 12 million clean reads were acquired from each sample, and all clean reads were mapped onto the reference mulberry transcriptome using SOAP2 software (Li et al., 2009; Dai et al., 2015). Expression of each gene was estimated using the frequency of clean reads normalized to the number of reads per kilobase of transcript per million sequenced reads (RPKM) (Hoen et al., 2008), and fold-changes of transcription were calculated by $RPKM_{treatment}/RPKM_{control}$. Genes with ≥2-fold changes in expression ($\log_2$ ($RPKM_{treatment}/RPKM_{control}$) ≥ 1) were considered to indicate differential expression. Raw data have been deposited in the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo). For annotation, all sequences were mapped onto the reference sequence, which was composed of unigenes from the mulberry transcriptome (NCBI accession Nos. SRP026705 and GBZO00000000) (Li et al., 2009). Raw data and the differential gene expression analysis data have been submitted to the GEO database under accession number GSE60030.

qPCR and statistical analysis

qPCR and statistical analysis were carried out according to methods described by Dai et al. (2016). Primers used for qPCR are shown in Table 3. Mulberry *Actin4* (GenBank accession number: KT793030) gene was used as the reference gene. Three biological replicate reactions per sample, consisting of nucleic acids extracted from three individual plants, were used. Student's *t* test was used to detect differences between cultivars at the usual probability level p = 0.05.

Cloning of the genes encoding key enzymes

Total RNA was extracted from mulberry root using Trizol (Invitrogen, USA). The full-length cDNAs of *MaCHS4*, *MaCHS5*, and *MaF3'H1* were obtained by RACE using SMART RACE cDNA Amplification kit (Clontech, USA). The cDNA fragments were inserted into pGEM-T Easy Vector (Promega, USA) for sequencing. The primers used for full length cloning of genes encoding key enzymes are listed Table 3. Full length samples of *MaCHS4*, *MaCHS5*, and *MaF3'H1* were deposited into GenBank under accession numbers KX377353, KX377354, and KX377355.

Total flavonoid content in mulberry root

In order to evaluate the total flavonoid content in mulberry root, fresh roots were washed and freeze-dried. The freeze-dried samples were ground into powder using an electric grinder and stored at -20°C until extraction. Flavonoids were extracted from root dried powder (MRDP) using 80% ethanol in water, according to Zhou et al. (2012). Briefly, 1 g of MRDP was mixed with 40 mL of aqueous ethanol and macerated with sonication for 40 min at 50°C. After centrifugation, the solvent was removed in vacuo over a water bath kept below 50°C. The dried extracts were stored at -20°C.

Total flavonoid content of each extract was determined using a slightly modified aluminum chloride colorimetry method described by Do et al. (2013) with extracts which were brought to a concentration of 25 mg mL^{-1} MRDP in ethanol. A calibration curve was prepared by diluting the flavonoid glycoside rutin in ethanol at concentrations between 0 and 50 µg mL^{-1}. Diluted extract or rutin solution (2.0 mL) was mixed with 0.1 mL of 10% (w/v) aluminum chloride solution and 0.1 mL of 0.1 mM potassium acetate solution. The mixture was kept at room temperature for 30 minutes. Then the maximum absorbance of the mixture was measured at 415 nm using a UV spectrophotometer. Total flavonoid content was expressed as milligram rutin equivalent per gram mulberry root dried powder (mg g^{-1} MRDP). Student's *t* test was used to detect differences between cultivars at the usual probability level p = 0.05.

Results

Identification of genes differentially expressed in response to *R. solanacearum* infection

To assess the physical responses of mulberry to infection with *R. solanacearum*, two mulberry cultivars – one disease-susceptible (YSD10) and the other resistant (KQ10) – were inoculated with *R. solanacearum*. Eight days after in-

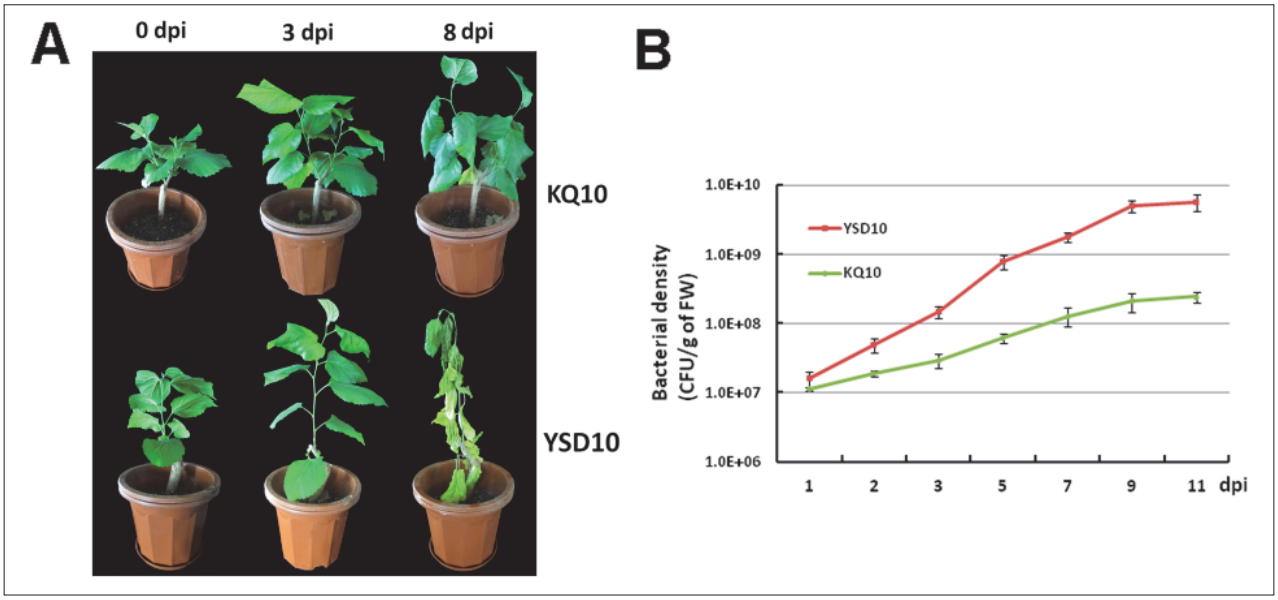


Figure 1. Physical responses of mulberry after inoculation with *Ralstonia solanacearum* and bacterial density over time. (A) Physical responses at 0, 3, and 8 days-post-inoculation (dpi); (B) Average bacterial density in roots of six randomly selected plants at 1, 2, 3, 5, 7, 9, and 11 dpi. Bars represent standard error.

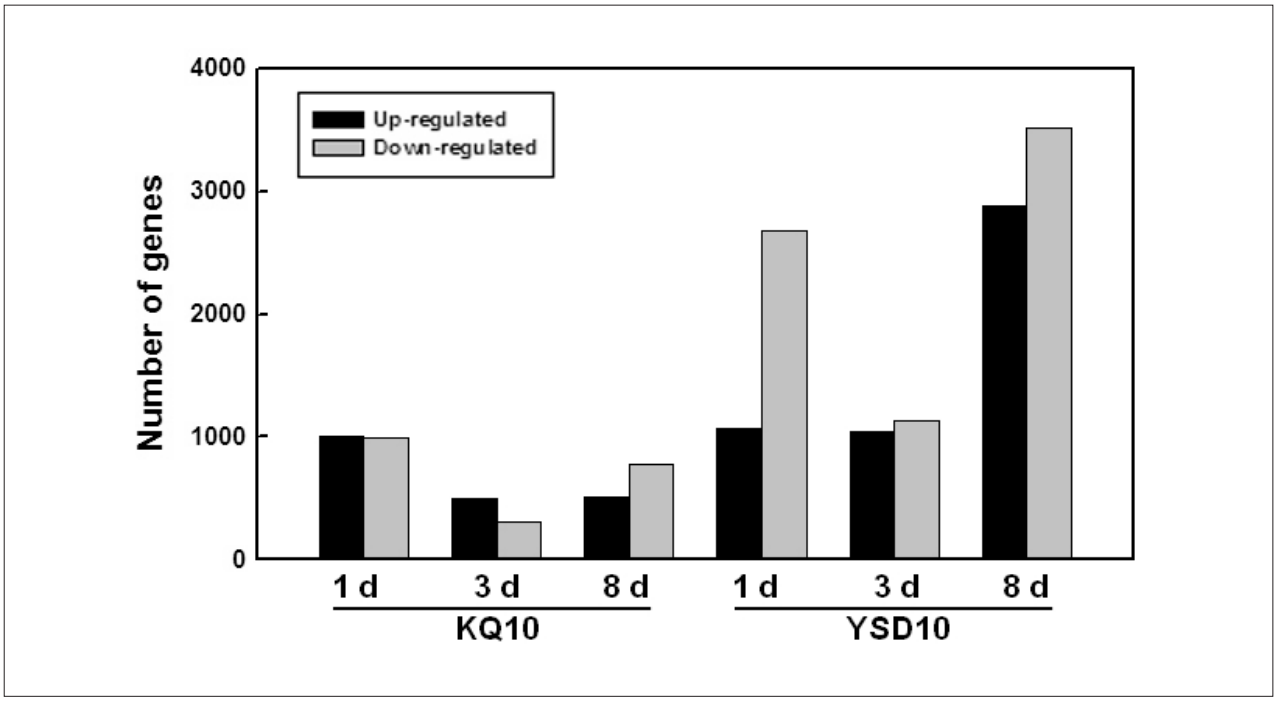


Figure 2. Number of differentially expressed genes in mulberry susceptible cultivar (YSD10) and resistant cultivar (KQ10) 1, 3, and 8 days-post-infection with *R. solanacearum*.

Table 1. Top KEGG pathways enriched with differentially expressed genes in susceptible mulberry cultivar YSD10 after inoculation with *R. solanacearum*.

Treatment	Sequence number	KEGG ID	KEGG Pathway (YSD10)	No. of genes		p-Value
				A[a]	B[b]	
RS 1d	1	ko00944	Flavone and flavonol biosynthesis	59	141	2.17E-26
	2	ko01110	Biosynthesis of secondary metabolites	363	2663	4.82E-20
	3	ko00940	Phenylpropanoid biosynthesis	91	396	2.86E-18
	4	ko00945	Stilbenoid, diarylheptanoid and gingerol biosynthesis	66	287	1.23E-13
	5	ko00360	Phenylalanine metabolism	41	152	2.78E-11
	6	ko00941	Flavonoid biosynthesis	62	298	6.31E-11
	7	ko02010	ABC transporters	56	257	8.23E-11
	8	ko04626	Plant-pathogen interaction	191	1464	3.80E-09
	9	ko00903	Limonene and pinene degradation	48	226	4.38E-09
	10	ko04075	Plant hormone signal transduction	147	1124	2.28E-07
	11	ko00943	Isoflavonoid biosynthesis	28	119	8.15E-07
RS 3d	1	ko01110	Biosynthesis of secondary metabolites	216	2663	1.61E-16
	2	ko00944	Flavone and flavonol biosynthesis	28	141	7.48E-11
	3	ko00196	Photosynthesis - antenna proteins	11	20	2.60E-10
	4	ko00940	Phenylpropanoid biosynthesis	49	396	6.05E-10
	5	ko00945	Stilbenoid, diarylheptanoid and gingerol biosynthesis	40	287	7.19E-10
	6	ko00591	Linoleic acid metabolism	14	48	2.46E-08
	7	ko04141	Protein processing in endoplasmic reticulum	60	620	9.38E-08
	8	ko00941	Flavonoid biosynthesis	36	298	2.07E-07
RS 8d	1	ko01110	Biosynthesis of secondary metabolites	812	2663	1.81E-38
	2	ko00941	Flavonoid biosynthesis	127	298	3.36E-18
	3	ko00944	Flavone and flavonol biosynthesis	75	141	9.94E-18
	4	ko00940	Phenylpropanoid biosynthesis	154	396	2.75E-17
	5	ko00500	Starch and sucrose metabolism	215	620	8.47E-17

[a] Number of genes with altered expression in response to *R. solanacearum* infection.
[b] Number of mulberry genes located in various biosynthetic pathways.

Table 2. Top KEGG pathways enriched with differentially expressed genes in resistant mulberry cultivar KQ10 after inoculation with *R. solanacearum*.

Treatment	Sequence number	KEGG ID	KEGG Pathway (KQ10)	No. of genes		p-Value
				A[a]	B[b]	
RS 1d	1	ko01110	Biosynthesis of secondary metabolites	210	2663	7.53E-16
	2	ko00941	Flavonoid biosynthesis	50	298	1.44E-15
	3	ko00944	Flavone and flavonol biosynthesis	31	141	2.29E-13
	4	ko00360	Phenylalanine metabolism	28	152	2.76E-10
	5	ko00940	Phenylpropanoid biosynthesis	49	396	2.76E-10
RS 3d	1	ko04712	Circadian rhythm - plant	18	195	2.18E-08
	2	ko00941	Flavonoid biosynthesis	20	298	6.95E-07
	3	ko00940	Phenylpropanoid biosynthesis	22	396	4.47E-06
	4	ko01110	Biosynthesis of secondary metabolites	80	2663	5.05E-06
	5	ko00500	Starch and sucrose metabolism	28	620	1.21E-05
	6	ko00040	Pentose and glucuronate interconversions	19	340	1.84E-05
	7	ko00360	Phenylalanine metabolism	12	152	2.43E-05
	8	ko04626	Plant-pathogen interaction	48	1464	6.54E-05
	9	ko00906	Carotenoid biosynthesis	12	184	0.000154
	10	ko00591	Linoleic acid metabolism	6	48	0.000231
	11	ko00944	Flavone and flavonol biosynthesis	10	141	0.000276
RS 8d	1	ko01110	Biosynthesis of secondary metabolites	143	2663	8.03E-13
	2	ko00906	Carotenoid biosynthesis	26	184	4.35E-11
	3	ko02010	ABC transporters	29	257	8.13E-10
	4	ko00904	Diterpenoid biosynthesis	20	129	1.46E-09
	5	ko00940	Phenylpropanoid biosynthesis	35	396	1.05E-08
	…	…	…	…	…	…
	18	ko00941	Flavonoid biosynthesis	18	298	0.003646
	19	ko04626	Plant-pathogen interaction	60	1464	0.007086
	20	ko00905	Brassinosteroid biosynthesis	5	50	0.015987
	21	ko00943	Isoflavonoid biosynthesis	8	119	0.025557

[a] Number of genes with altered expression in response to *R. solanacearum* infection.
[b] Number of mulberry genes located in various biosynthetic pathways.

oculation, 'YSD10' showed considerable leaf wilt, but no obvious physical changes were observed in 'KQ10' (Figure 1A). Root bacterial density quickly increased after infection with *R. solanacearum* in both cultivars; however, increases in bacterial density were smaller in 'KQ10' than in 'YSD10' (Figure 1B).

To evaluate global gene expression alterations caused by infection with *R. solanacearum* in the two mulberry cultivars, we used RNA-Seq technology to analyze transcriptome changes following infection. Genes with a ≥ 2-fold change in expression were considered to have altered expression levels due to infection. Based on this criterion, approximately 2,006, 809, and 1,292 genes with altered expression in response to *R. solanacearum* infection were identified in samples collected 1, 3, and 8 dpi, respectively in 'KQ10', and approximately 3,752, 2,174, and 6,398 genes were identified at 1, 3, and 8 dpi, respectively in 'YSD10' (Figure 2).

Biochemical pathway effects in response to *R. solanacearum* infection

To further characterize the differentially expressed genes, we functionally classified them using the public Kyoto Encyclopedia of Genes and Genomes (KEGG) database (Kanehisa and Goto, 2000). This analysis suggested that *R. solanacearum* infection caused significant changes in a range of biochemical pathways including those involved in plant–pathogen interactions, biosynthesis of secondary metabolites, and starch and sucrose metabolism.

The top KEGG pathways enriched in genes with altered expression for both cultivars are shown in Tables 1 and 2. We found that many transcripts associated with polyphenolic biosynthesis pathways – including flavonoid, flavone, flavonol and isoflavonoid biosynthesis – changed significantly in both cultivars here tested at all treatment times (Pathways highlighted in yellow in Tables 1 and 2). These findings suggest that flavonoid biosynthesis is an important response factor to *R. solanacearum* infection in mulberry.

Characterization of genes that encode key flavonoid biosynthesis enzymes

We used RNA-Seq analysis to investigate the effects of *R. solanacearum* infection on the flavonoid biosynthesis–associated genes. Several genes that encode key-enzymes involved in flavonoid biosynthetic pathways were significantly up-regulated in one or both cultivars, including homologs to four CHS genes (*Unigene30900*, *Unigene23165*, *Unigene15633*, and *CL3598*), three F3'H genes (*Unigene28095*, *Unigene34977*, and *CL518*), and one CHI gene (*Unigene27928*) (Figure 3A, B, top).

The expression patterns of these eight genes, changed during infection with *R. solanacearum* identified by RNA-seq, were quantified by qPCR (Figure 3A, B, bottom). Among the

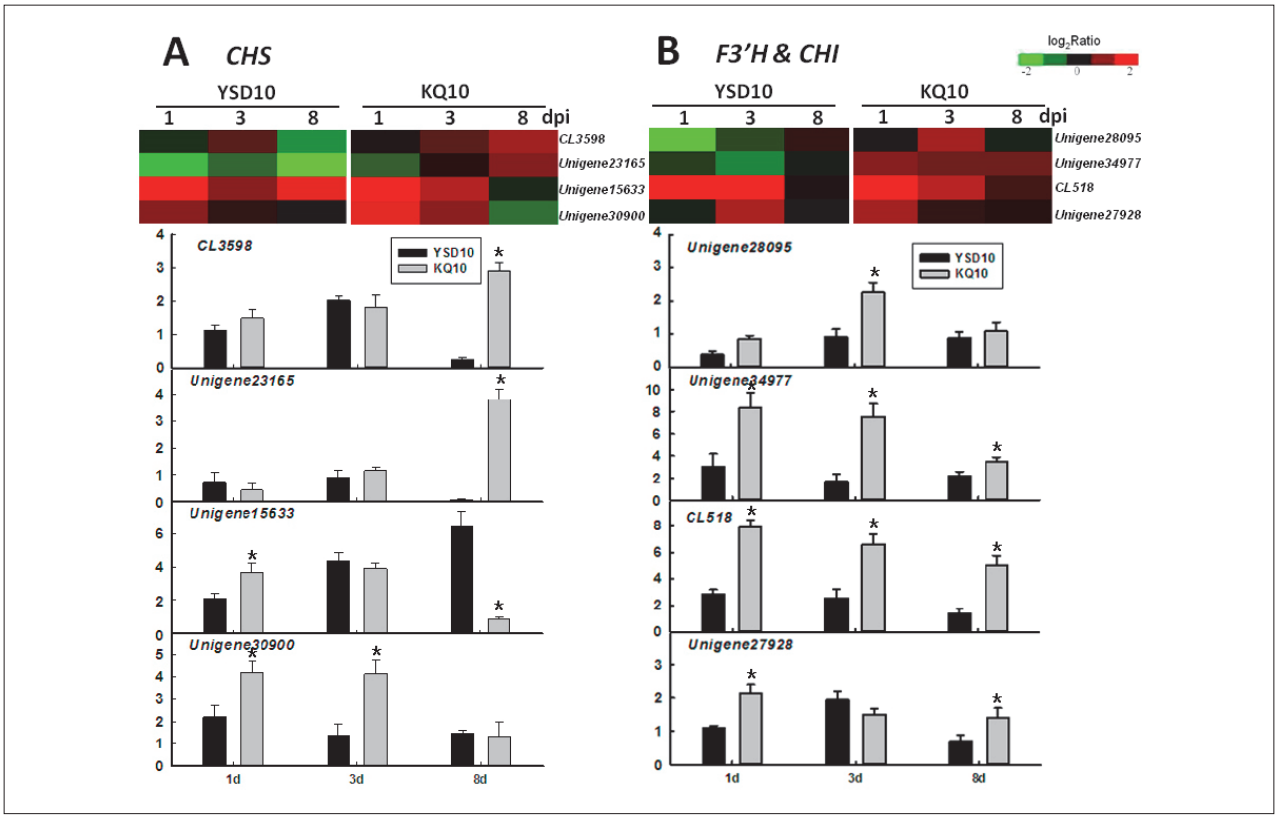


FIGURE 3. Detailed expression profiles of genes encoding key enzymes in flavonoid biosynthetic pathways. (A) Comparison of expression ratios obtained from RNA-Seq and qPCR for *CHS* genes. (B) Comparison of expression ratios obtained from RNA-Seq and qPCR for *F3'H* and *CHI* genes. Top: RNA-Seq heatmap. Bottom: qPCR analysis of four *CHS* genes, three *F3'H* genes, and one *CHS* gene, revealed by RNA-Seq to be preferentially expressed in response to *R. solanacearum* infection. Bars represent standard error for three independent experiments. CHS, chalcone synthase; *F3'H*, flavanone-3'-hydroxylase; CHI, chalcone isomerase. Student's *t* tests were used to detect differences between cultivars, and the asterisk indicates a significant difference with p value < 0.05.

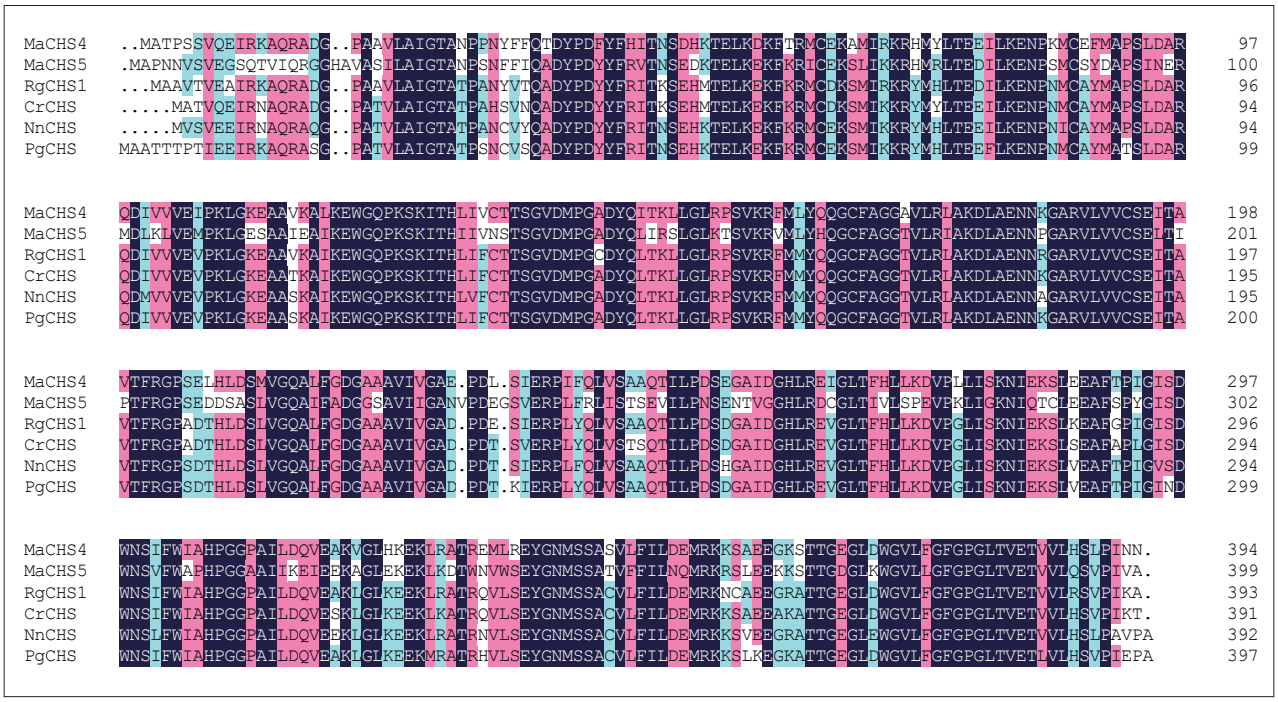


FIGURE 4. Amino acid sequence alignment of mulberry MaCHS4 (*Unigene15633*) and MaCHS5 (*CL3598*) with CHS proteins from other plants. The accession numbers of the amino acid sequences used are as follows: RgCHS1 (Rue, *Ruta graveolens*), Q9FSB9; CrCHS (Orange, *Citrus reticulata*), AKR05589; NnCHS (Lotus, *Nelumbo nucifera*), XP_010249317; PgCHS (Pomegranate, *Punica granatum*), AHZ97870.

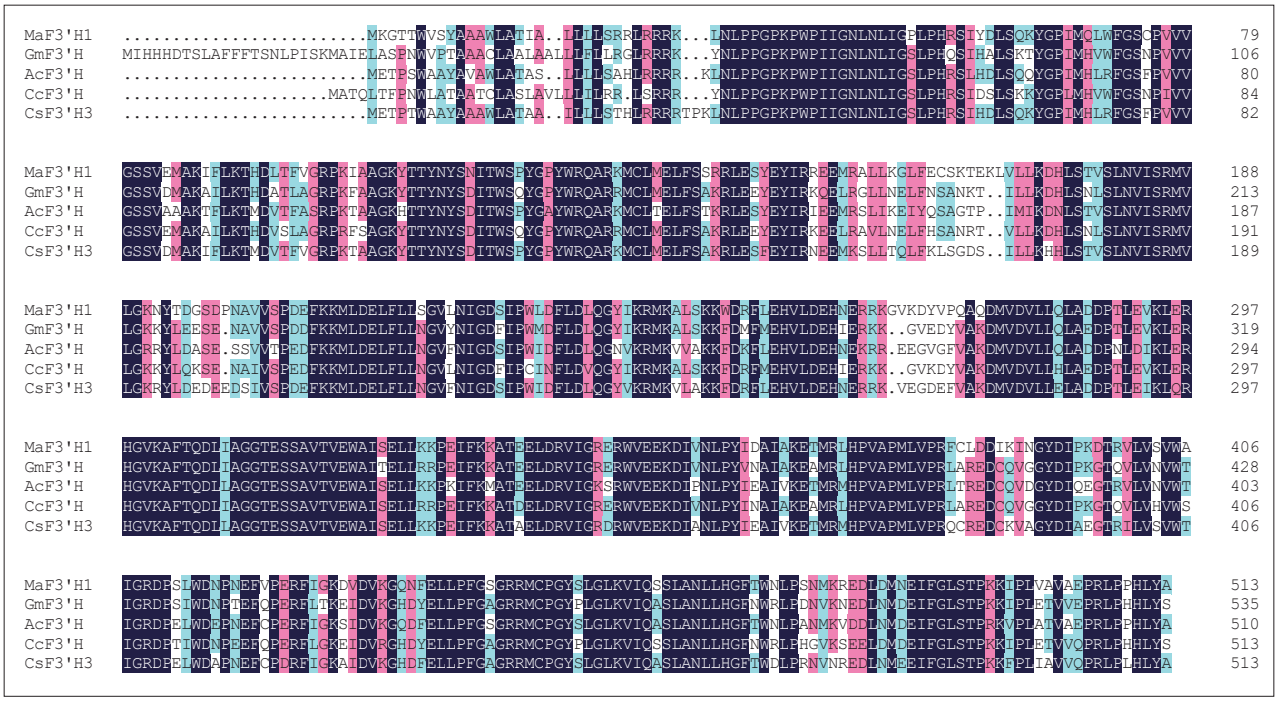
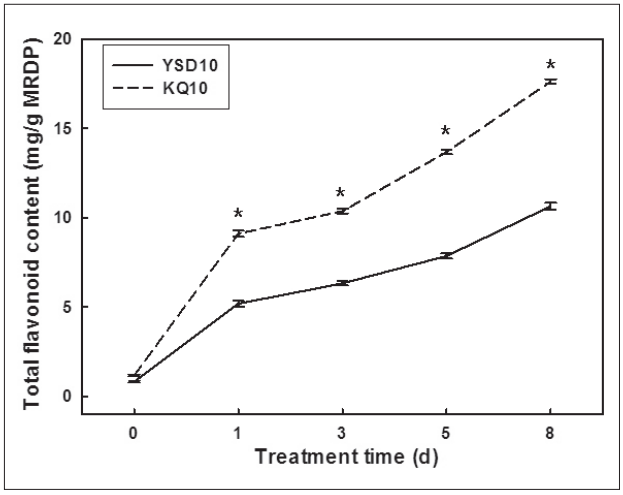


Figure 5. Amino acid sequence alignment of mulberry MaF3'H1 (*Unigene28095*) with F3'H proteins from other plants. The accession numbers of the amino acid sequences used are as follows: GmF3'H (Soybean, *Glycine max*), XP_003547756; AcF3'H (Kiwi, *Actinidia chinensis*), ADC34701; CcF3'H (Pigeonpea, *Cajanus cajan*), KYP50489; CsF3'H3 (*Camellia sinensis*), AKM12330.

four up-regulated CHS genes, *Unigene23165* was up-regulated at 8 dpi in the resistant cultivar KQ10 but not in the susceptible cultivar YSD10. The expression of *Unigene30900* and *CL3598* was up-regulated more significantly in 'KQ10' than in 'YSD10'. Among the three up-regulated F3'H genes, *Unigene34977* was up-regulated at all time points only in 'KQ10', and the expression of *Unigene28095* was up-regulated more significantly in 'KQ10' than in 'YSD10'. However, the rest of the genes (*Unigene15633*, *CL518*, and *Unigene27928*) were up-regulated to similar levels and showed similar expression patterns in both cultivars (Figure 3A, B).

Cloning genes encoding key flavonoid biosynthesis enzymes

To further characterize the genes encoding key enzymes in the flavonoid biosynthetic pathways, we cloned three full-length cDNAs for *Unigene15633*, *CL3598*, and *Unigene28095*.



The gene *Unigene15633* is 1,301 bp in length with a 101-bp 5'-UTR and encodes a 399-residue polypeptide. The gene *CL3598* is 1,185 bp in length and encodes a 394-residue polypeptide. Amino acid sequence analysis of these two clones indicated a high level of homology (more than 80%) to known CHS family proteins isolated from other plant species (Figure 4). Accordingly, *Unigene15633* and *CL3598* are designated as *MaCHS4* and *MaCHS5* respectively, because three other CHS genes (*MaCHS1*, *MaCHS2*, *MaCHS3*) have been described previously (Zhao et al., 2015).

The gene *Unigene28095* is 1,545 bp in length and encodes a 514-residue polypeptide. Amino acid sequence analysis of *Unigene28095* indicates that the protein is highly homologous (< 70%) to known F3'H family proteins (Figure 5), and is therefore designated as *MaF3'H1*.

Total flavonoid content of mulberry in response to *R. solanacearum* infection

We investigated total flavonoid content of both mulberry cultivars at 0, 1, 3, 5, and 8 dpi with *R. solanacearum*. Total flavonoid contents of mulberry root were similar in both cultivars before infection; however, at 1 dpi, total flavonoid levels were significantly higher in 'KQ10' than in 'YSD10'. Total flavonoid content continued to increase linearly in both cultivars. At 8 dpi, total flavonoid concentration in 'KQ10' reached nearly 20 mg g^{-1} and 10 mg g^{-1} in 'YSD10' (Figure 6).

Figure 6. Total flavonoid content in mulberry roots belonging to the cultivars YSD10 and KQ10. MRDP, mulberry root dried powder. Student's *t* tests were used to detect differences between cultivars, and the asterisk indicates a significant difference with p value < 0.05.

Discussion and conclusions

Biotechnology has facilitated investigation of the genetic basis of bacterial wilt disease resistance by targeting relevant genes for transcriptome analysis (Narancio et al., 2013; Prasath et al., 2014; Zuluaga et al., 2015). RNA-Seq analysis generates unprecedentedly large data sets that can be acquired for transcriptomics studies of non-model plants because a predefined measuring platform, for techniques such as microarrays, is not required (Lohse et al., 2012).

Gene expression changes in response to *R. solanacearum* infection of leaf, stem, and root tissue, measured using both RNA-seq and microarray analysis, have been reported for several plant species (Narancio et al., 2013; Prasath et al., 2014; Zuluaga et al., 2015). To understand the functional significance of genes affected by *R. solanacearum* infection, gene ontology function classification is commonly used (Gene Ontology Consortium, 2008). For example, genes responsive to *R. solanacearum* that fit within gene ontology molecular function groups were related to "catalytic activity", "transferase activity", and "hydrolase activity", and were most highly represented in tomato stems, ginger leaves, and wild potato (*S. commersonii*) (Hu et al., 2008; Prasath et al., 2014; Zuluaga et al., 2015). KEGG pathway analysis is another functional classification system that has been widely used as a database for compiling data understanding of biological pathways and functions of cellular processes (Kanehisa and Goto, 2000). In our study, we functionally classified genes with differential expression patterns in response to *R. solanacearum* infection using the KEGG database. Results showed a range of significantly altered biochemical pathways including those involved with plant–pathogen interactions, biosynthesis of secondary metabolites, and starch and sucrose metabolism (Tables 1 and 2).

On the other hand, transcriptional changes associated with the development of wilt disease have been studied in Arabidopsis leaves. The results showed that very few genes are differentially expressed in the early phases of the resistance response, whereas major changes take place upon the appearance of wilt symptoms at 5 and 8 dpi (Hu et al., 2008). In the present work, we further investigated gene expression changes in mulberry root during all OR infection process by *R. solanacearum*. We found that most transcripts associated with polyphenolic biosynthesis pathways changed significantly at all treatment time points – 1, 3, and 8 dpi – including those involved in flavonoid, flavone, flavonol, and isoflavonoid biosynthesis (Tables 1 and 2). The expression levels of several genes encoding key-flavonoid biosynthesis enzymes (CHS, F3'H, and CHI) were changed during infection with *R. solanacearum* in both mulberry cultivars (Figure 3). Total flavonoid content of both cultivars increased linearly after infection, although the increase was more significant in the resistant cultivar (KQ10) than the susceptible cultivar (YSD10) (Figure 6). These results suggest that flavonoid biosynthesis may play an important role in the resistance response to *R. solanacearum* infection in mulberry.

Because of the cost of RNA-seq, we did not sequence biological duplicates, which may affect the quality of the sequence data in this study. However, having carried out RNA-seq analysis on roots of two different mulberry cultivars at multiple time points, our findings showed that gene expression changes after infection were somewhat similar in both varieties with different genes revealing trends that correlated with an increase in the severity of the infection over time (Tables 1 and 2). The RNA-seq analysis was further verified by results obtained from qPCR and phytochemical experiments (Figures 3 and 6). These subsequent experiments may partly compensate for the shortcomings of the limited RNA-seq analysis.

More studies reported in literature confirm our findings showing that flavonoids participate to plant resistance against abiotic environmental stresses, such as drought, salinity, chilling, UV damage, frost, light, and toxic metals, by different ways (Havaux and Kloppstech, 2001; Barceló and Poschenrieder, 2002; Tattini et al., 2004; Agati et al., 2011). Flavonoids are also involved in plant immunity during biotic stress responses such as insect pests (Abdel-Aal et al., 2001). A study of barley fungus disease showed that the flavonoid dihydroquercetin is involved in defense against *Fusarium* infections (Skadhauge et al., 1997). Flavonoids are also moderately effective in inhibiting bacteria that cause certain plant diseases (Taguri et al., 2006; Maddox et al., 2010). Rutin, for example, may function as a plant disease resistance activator by initiating the early stages of the salicylic acid signaling pathway (Yang et al., 2016). Quercetin was also identified as a primary defense barrier against *Pseudomonas syringae* in *A. thaliana*, where its concentration is mediated by H_2O_2 bursts (Jia et al., 2010). In this study, infection of *R. solanacearum* in mulberry was differentially expressed by different genes at all infection stages by a continuous enrichment of flavonoids biosynthesis in the pathways (Tables 1 and 2), showing that flavonoids were involved in plant disease resistance of mulberry. Several key-enzyme genes of flavonoids biosynthesis (CHS, F3'H, and CHI) were up-regulated during infection with *R. solanacearum* in two cultivars by qPCR method (Figure 3). Meanwhile, we detected total flavonoid contents of two cultivars were both increasing linearly after infection, and the increase was more pronounced in the resistant cultivar KQ10 (Figure 6). In the future research work, we will further explore the physiological and molecular mechanism of flavonoids involved in mulberry disease resistance, and gene function analysis of the key enzyme genes of flavonoids biosynthesis.

In this study, analysis of the transcriptional and phytochemical changes of two mulberry cultivars following infection with *R. solanacearum* during the entire infection process revealed that transcription of several enzymes of the flavonoid pathway and flavonoid levels were significantly affected by the entire infection process. We also obtained full-length sequences of three genes that encode key enzymes involved in flavonoid biosynthetic pathways changed during infection by *R. solanacearum*. More studies to explore the physiological and molecular mechanism of flavonoid-involved disease resistance in mulberry and functional analyses of the key genes encoding flavonoid biosynthesis enzymes are needed to facilitate breeding disease-resistant mulberry cultivars.

Acknowledgments

This work was supported by grants from the National Nature Science Foundation of China (No. 31500527), the Science and Technology Project of Guangzhou City, China (No. 201710010129), the Science and Technology Project of Guangdong Province, China (No. 2016A040402036).

References

Abdel-Aal, E.S.M., Hucl, P., Sosulski, F.W., Graf, R., Gillott, C., and Pietrzak, L. (2001). Screening spring wheat for midge resistance in relation to ferulic acid content. J. Agric. Food Chem. *49*, 3559–3566. https://doi.org/10.1021/jf010027h.

Agati, G., Biricolti, S., Guidi, L., Ferrini, F., Fini, A., and Tattini, M. (2011). The biosynthesis of flavonoids is enhanced similarly by UV radiation and root zone salinity in *L. vulgare* leaves. J. Plant Physiol. *168*, 204–212. https://doi.org/10.1016/j.jplph.2010.07.016.

Barceló, J., and Poschenrieder, C. (2002). Fast root growth responses, root exudates, and internal detoxification as clues to the mechanism of aluminium toxicity and resistance: A review. Environ. Exp. Bot. *48*, 75–92. https://doi.org/10.1016/S0098-8472(02)00013-8.

Chen, Y.N., Ren, X.P., Zhou, X.J., Huang, L., Huang, J.Q., Yan, L.Y., Lei, Y.C., Qi, Y., Wei, W.H., and Jiang, H.F. (2014). Alteration of gene expression profile in the roots of wild diploid *Arachis duranensis* inoculated with *Ralstonia solanacearum*. Plant Pathol. *63*, 803–811. https://doi.org/10.1111/ppa.12158.

Cushnie, T.P., and Lamb, A.J. (2005). Antimicrobial activity of flavonoids. Int. J. Antimicrob. Ag. *26*, 343–356. https://doi.org/10.1016/j.ijantimicag.2005.09.002.

Dai, F.W., Tang, C.M., Wang, Z.J., Luo, G.Q., He, Li, and Yao, L.H. (2015). De novo assembly, gene annotation and marker development of mulberry (*Morus atropurpurea*) transcriptome. Tree Genet. Genomes *11*, 26. https://doi.org/10.1007/s11295-015-0851-4.

Dai, F.W., Wang, Z.J., Luo, G.Q., and Tang, C.M. (2016). Transcriptional analysis of different mulberry cultivars in response to *Ralstonia solanacearum*. Can. J. For. Res. *46*, 152–162. https://doi.org/10.1139/cjfr-2015-0235.

Deslandes, L., Pileur, F., Liaubet, L., Camut, S., Can, C., Williams, K., Holub, E., Beynon, J., Arlat, M., and Marco, Y. (1998) Genetic characterization of *RRS1*, a recessive locus in *Arabidopsis thaliana* that confers resistance to the bacterial soilborne pathogen *Ralstonia solanacearum*. Mol. Plant Microbe Interact. *11*, 659–667. https://doi.org/10.1094/MPMI.1998.11.7.659.

Do, Q.D., Angkawijaya, A.E., Tran-Nguyen, P.L., Huynh, L.H., Soetaredjo, F.E., Ismadji, S., and Ju, Y.H. (2014). Effect of extraction solvent on total phenol content, total flavonoid content, and antioxidant activity of *Limnophila aromatica*. J. Food Drug Anal. *22*, 296–302. https://doi.org/10.1016/j.jfda.2013.11.001.

Gani, A., Wani, S.M., Masoodi, F.A., and Hameed, G. (2012). Whole-grain cereal bioactive compounds and their health benefits: A review. J. Food Process Technol. *3*, 3. https://doi.org/10.4172/2157-7110.1000146.

Gene Ontology Consortium. (2008). The Gene Ontology project in 2008. Nucleic Acids Res. *36*, D440–D444. https://doi.org/10.1093/nar/gkm883.

Graf, B.A., Milbury, P.E., and Blumberg, J.B. (2005). Flavonols, flavones, flavanones, and human health: epidemiological evidence. J. Med. Food *8*, 281–290. https://doi.org/10.1089/jmf.2005.8.281.

Havaux, M., and Kloppstech, K. (2001). The protective functions of carotenoid and flavonoid pigments against excess visible radiation at chilling temperature investigated in *Arabidopsis npq* and *tt* mutants. Planta *213*, 953–966. https://doi.org/10.1007/s004250100572.

't Hoen, P.A.C., Ariyurek, Y., Thygesen, H.H., Vreugdenhil, E., Vossen, R.H., Menezes, R.X., Boer, J.M., Ommen, G.J., and Dunnen, J.T. (2008). Deep sequencing-based expression analysis shows major advances in robustness, resolution and inter-lab portability over five microarray platforms. Nucleic Acids Res. *36*, e141. https://doi.org/10.1093/nar/gkn705.

Hu, J., Barlet, X., Deslandes, L., Hirsch, J., Feng, D.X., Somssich, I., and Marco, Y. (2008). Transcriptional responses of *Arabidopsis thaliana* during wilt disease caused by the soil-borne phytopathogenic bacterium, *Ralstonia solanacearum*. PLoS One *3*, e2589. https://doi.org/10.1371/journal.pone.0002589.

Iker, H., Leonor, A., Frank, V.B., and Sergi, M.B. (2009). How relevant are flavonoids as antioxidants in plants? Trends Plant Sci. *14*, 125–132. https://doi.org/10.1016/j.tplants.2008.12.003.

Ishihara, T., Mitsuhara, I., Takahashi, H., and Nakaho, K. (2012). Transcriptome analysis of quantitative resistance-specific response upon *Ralstonia solanacearum* infection in tomato. PLoS One *7*, e46763. https://doi.org/10.1371/journal.pone.0046763.

Jia, Z.H., Zou, B., Wang, X.M., Qiu, J., Ma, H., Gou, Z., Song, S., and Dong, H. (2010). Quercetin-induced H_2O_2 mediates the pathogen resistance against *Pseudomonas syringae* pv. *tomato* DC3000 in *Arabidopsis thaliana*. Biochem. Bioph. Res. Co. *396*, 522–527. https://doi.org/10.1016/j.bbrc.2010.04.131.

Jia, Z.S., Tang, M.C., and Wu, J.M. (1999). The determination of flavonoid contents in mulberry and their scavenging effects on superoxide radicals. Food Chem. *64*, 555–559. https://doi.org/10.1016/S0308-8146(98)00102-2.

Kanehisa, M., and Goto, S. (2000). KEGG: Kyoto encyclopedia of genes and genomes. Nucleic Acids Res. *28*, 27–30. https://doi.org/10.1093/nar/28.1.27.

Kim, B.G., Lee, E.R., and Ahn, J.H. (2012). Analysis of flavonoid contents and expression of flavonoid biosynthetic genes in *Populus euramericana* Guinier in response to abiotic stress. J. Korean Soc. Appl. Biol. Chem. *55*, 141–145. https://doi.org/10.1007/s13765-012-0025-0.

Koes, R.E., Quattrocchio, F., and Mol, J.N.M. (1994). The flavonoid biosynthetic pathway in plants: Function and evolution. Bioessays *16*, 123–132. https://doi.org/10.1002/bies.950160209.

Li, R.Q., Yu, C., Li, Y.R., Lam, T.W., Yiu, S.M., Kristiansen, K., and Wang, J. (2009). SOAP2: an improved ultrafast tool for short read alignment. Bioinformatics *25*, 1966–1967. https://doi.org/10.1093/bioinformatics/btp336.

Lohse, M., Bolger, A.M., Nagel, A., Fernie, A.R., Lunn, J.E., Stitt, M., and Usadel, B. (2012). RobiNA: a user-friendly, integrated software solution for RNA-Seq-based transcriptomics. Nucleic Acids Res. *40* (Web Server issue), W622–W627. https://doi.org/10.1093/nar/gks540.

Luo, G.Q., Tang, C.M., Wang, Z.J., Wu, J.A., Wu, F.Q., and Xiao, G.S. (2010). Advances in Mulberry bacterial wilt disease caused by *Ralstonia solanacearum*. Acta Sericologica Sin. *31*, 28–41.

Maddox, C.E., Laur, L.M., and Tian, L. (2010). Antibacterial activity of phenolic compounds against the phytopathogen *Xylella fastidiosa*. Curr. Microbiol. *60*, 53–58. https://doi.org/10.1007/s00284-009-9501-0.

Michel, V.V., and Mew, T.W. (1998). Effect of a soil amendment on the survival of *Ralstonia solanacearum* in different soils. Phytopathology *88*, 300–305. https://doi.org/10.1094/PHYTO.1998.88.4.300.

Narancio, R., Zorrilla, P., Robello, C., Gonzalez, M., Vilaró, F., Pritsch, C., and Dalla Rizza, M. (2013). Insights on gene expression response of a characterized resistant genotype of *Solanum commersonii* Dun. against *Ralstonia solanacearum*. Eur. J. Plant Pathol. *136*, 823–835. https://doi.org/10.1007/s10658-013-0210-y.

Prasath, D., Karthika, R., Habeeba, N.T., Suraby, E.J., Rosana, O.B., Shaji, A., Eapen, S.J., Deshpande, U., and Anandaraj, M. (2014). Comparison of the transcriptomes of ginger (*Zingiber officinale* Rosc.) and mango ginger (*Curcuma amada* Roxb.) in response to the bacterial wilt infection. PLoS One *9*, e99731. https://doi.org/10.1371/journal.pone.0099731.

Skadhauge, B., Thomsen, K., and von Wettstein, D. (1997). The role of barley testa layer and its flavonoid content in resistance to *Fusarium*

infections. Hereditas *126*, 147–160. https://doi.org/10.1111/j.1601-5223.1997.00147.x.

Taguri, T., Tanaka, T., and Kouno, I. (2006). Antibacterial spectrum of plant polyphenols and extracts depending upon hydroxyphenyl structure. Biol. Pharm. Bull. *29*, 2226–2235. https://doi.org/10.1248/bpb.29.2226.

Tattini, M., Galardi, C., Pinelli, P., Massai, R., Remorini, D., and Agati, G. (2004). Differential accumulation of flavonoids and hydroxycinnamates in leaves of *Ligustrum vulgare* under excess light and drought stress. New Phytol. *163*, 547–561. https://doi.org/10.1111/j.1469-8137.2004.01126.x.

Treutter, D. (2005). Significance of flavonoids in plant resistance and enhancement of their biosynthesis. Plant Biol. *7*, 581–591. https://doi.org/10.1055/s-2005-873009.

Yang, W., Xu, X., Li, Y., Wang, Y.Z., Li, M., Wang, Yong, Ding, X.H., and Chu, Z.H. (2016). Rutin-mediated priming of plant resistance to three bacterial pathogens initiating the early SA signal pathway. Plos One *11*, e0146910. https://doi.org/10.1371/journal.pone.0146910.

Zhao, S., Park, C.H., Li, X., Kim, Y.B., Yang, J., Sung, G.B., Park, N.I., Kim, S., and Park, S.U. (2015). Accumulation of rutin and betulinic acid and expression of phenylpropanoid and triterpenoid biosynthetic genes in Mulberry (*Morus alba* L.). J. Agric. Food Chem. *63*, 8622–8630. https://doi.org/10.1021/acs.jafc.5b03221.

Zhou, H., Pan, B.H., Wang, L., and Liu, L. (2012). Optimization of ultrasonic-assisted extraction of total flavonoids from mulberry leaves using response surface methodology. Acta Sericologica Sin. *38*, 727–733.

Zuluaga, A.P., Solé, M., Lu, H., Góngora-Castillo, E., Vaillancourt, B., Coll, N., Buell, C.R., and Valls, M. (2015). Transcriptome responses to *Ralstonia solanacearum* infection in the roots of the wild potato *Solanum commersonii*. BMC Genomics *16*, 246. https://doi.org/10.1186/s12864-015-1460-1.

Received: Jun. 2, 2017
Accepted: May 18, 2018

Addresses of authors:
Fanwei Dai, Guoqing Luo, Zhenjiang Wang, Zheshi Kuang, Zhiyi Li, Jing Huang and Cuiming Tang*
Sericultural & Agri-Food Research Institute Guangdong Academy of Agricultural Sciences, 133 Yiheng Road, Dongguan Village, Tianhe District, Guangzhou 510610, Guangdong, China
* Corresponding author; E-mail: tangcuiming@21cn.com
Tel.: +86-20-87236511