Mapping and Function Characterization of the Tomato Spotted wilt Virus Resistance Gene SlCHS3 in *Solanum Lycopersicum*

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Abstract

Tomato spotted wilt virus (TSWV) poses a serious threat to tomato (*Solanum lycopersicum*) production. In this study, a tomato inbred line YNAU335 was developed without the *Sw-5* locus, which showed resistance or immunity to TSWV (absence of infection). Genetic analysis demonstrated that the immunity to TSWV was controlled by a dominant nuclear gene. The candidate genes were mapped into a 20 kb region in the terminal of the long arm of chromosome 9 using the bulk segregant analysis (BSA) and linkage analysis. In this candidate region, a chalcone synthase (CHS)-encoding gene (*SlCHS3*) was found to be a strong candidate gene for the TSWV resistance. The result showed that *SlCHS3* silencing reduced the synthesis of flavonoids, and overexpression of *SlCHS3* increased the content of flavonoids. The increased flavonoids could improve the ability of TSWV resistance in the tomato. These findings indicate that *SlCHS3* is indeed involved in the regulation of the flavonoids synthesis and play a significant role in the TSWV resistance of YNAU335, it could provide new insights and lay the foundation for analyzing the TSWV resistance mechanisms.

1. Introduction

Tomato spotted wilt virus (TSWV) is an important virus in the *Tospovirus* genus of the *Bunyaviridae* family (Lopez et al. 2011; Peiro et al. 2014). The virus is vectored by several species of thrips, most importantly the western flower thrip (Boonham et al. 2002). It has an extensive host range of more than 800 plant species in 82 families (Soler et al. 2003; Chung et al. 2018). The virus has spread to most countries in the world and caused serious losses to the production of crops such as tomatoes (Hoffmann et al. 2001; Boonham et al. 2002; Lopez et al. 2011).

Tomato resources that are resistant to TSWV are mostly wild species such as Peruvian (*Solanum peruvianum* Mill.) and Chilean (*S. chilense* Mill.) tomatoes (Stevens et al. 1994; Gordillo and Stevens, 2008). The resistance of tomato to TSWV is controlled by the *Sw-5* locus (Stevens et al. 1991; Boiteux and Giordano, 1993). Multiple molecular markers such as RAPD, RFLP, and SCAR have been developed for *Sw-5* (Stevens et al. 1995; Chague et al. 1996; Dianese et al. 2010). A series of technologies including marker linkage analysis (Stevens et al. 1995), YAC library construction (Brommonschenkel and Tanksley, 1997), and homology cloning (Folkertsma et al. 1999) revealed two CC-(NB-ARC)-LRR genes, *Sw5-a* and *Sw5-b*, but only the latter increased resistance to TSWV (Spassova et al. 2001). TSWV resistance-breaking isolates have emerged in different countries after using resistant cultivars carrying the *Sw-5* including the T992 isolate in Italy (Ciuffo et al. 2005) and Pujol1TL3 isolate in Spain (Debreczeni et al. 2015). Mutations in the TSWV genome, for example, a substitution of C to Y at position 118 or of T to N at position 120 in the TSWV movement protein have been found to inhibit the resistance induced by *Sw-5* (Hoffmann et al. 2001; Lopez et al. 2011).

Flavonoids are important secondary metabolites in plants that play significant roles in the regulation of plant growth and development (Peer et al. 2004; Wu et al. 2018). Flavonoids in plants are induced by stresses such as ultraviolet light, free radicals, and pathogens, thereby increasing resistance to these

stresses (Yamasaki et al. 1997; Ryan et al. 2002; Silva et al. 2015; Wu et al. 2018). Chalcone synthase (CHS) is a key enzyme in the plant flavonoid synthesis pathway (Nagamatsu et al. 2007; Wang et al. 2010). Based on the whole tomato genome sequence, eight *CHS* genes were identified with protein sequence lengths varying from 160 to 438 distributed on chromosomes 1, 5, 6, 9, and 12 (Ruan et al. 2013). The *CHS* genes are induced by a variety of pathogens including viruses and fungi, thus further increasing the synthesis of flavonoids and potentially enhancing resistance to these pathogens (Gutha et al. 2010; Samac et al. 2011). Moreover, the expression levels of *CHS* genes are induced by abiotic stresses such as exposure to UV-light, mechanical damage, and salt (Christie and Jenkins, 1996; Dehghan et al. 2014).

In this study, we developed the inbred line YNAU335, which was not infected after inoculation with the TSWV isolate YNAU2015. We found that the immunity was controlled by a dominant nuclear gene encoding a *CHS*, *SlCHS3*, which was successfully located at the terminal of the long arm of chromosome 9. Its presence resulted in altered expression patterns in different resistant inbred lines and affected the resistance of tomato to TSWV.

2. Materials And Methods

2.1 Tomato materials and TSWV isolate

The tomato materials used in this experiment included YNAU335, No. 5, 96712I and L. peruvianum LA2823, LA3858, PI128657. YNAU335, No. 5, and 96712I were developed from the local tomato varieties collected from Yuanmou County, Yunnan Province of China. L. peruvianum LA2823, LA3858, and PI128657 were introduced from the Vegetable and Flower Research Institute of the Chinese Academy of Agricultural Sciences. TSWV isolate YNAU2015 was identified and preserved in our laboratory.

2.2 Sw-5 locus determination

Genomic DNA was extracted from the leaves of tomato plants using a DNA secure Plant Kit (BioTeke) and used as a template for PCR amplification. The co-dominant SCAR marker ‘Sw-5-2’ (Dianese et al., 2010) linked with Sw-5 was employed to determine the phenotyping of the tomato materials. The primer pSw-5-2 for Sw-5 is shown in Table S1.

2.3 TSWV inoculation

The TSWV mechanical inoculation method used was based on that of Sundaraj et al. (Sundaraj et al., 2014) with minor changes. TSWV was stored on tobacco (*Nicotiana tabacum* L.) and spread by mechanical inoculation, as follows. Diseased tobacco leaves were ground in 0.1 M phosphate buffer at a ratio of 1:10 (wt/vol). The buffer (pH = 7.0) contained 0.2% sodium sulfite, 0.01 M mercaptoethanol, 0.01 g/mL emery, and 0.01 g/mL Celite 545. The whole grinding process was performed in an ice bath, and the grinding solution was used as the viral homogenate. Tomato leaves were gently wiped with emery, and then the homogenate was applied to the leaves. For the non-inoculated control group, phosphate buffer alone was applied. After inoculation, all seedlings were placed in an incubator for 3 weeks. For the

inoculation with TSWV using thrips, healthy tomato seedlings were transferred to the Vegetable Cognitive Center of Yunnan Agricultural University, where a population of TSWV-harboring thrips has existed for many years.

2.4 Bulk segregant analysis (BSA) genome resequencing and genetic map construction

The parental YNAU335 and No. 5 inbred lines and segregated populations of the F2 generation were established for genome resequencing to detect SNPs (single nucleotide polymorphisms) and InDels between the parents and F_2 generation, SNP index and the delta SNP were used to identify the candidate chromosomal regions related to TSWV resistance gene (Win et al. 2017). The polymorphic InDel markers were developed in this region to identify the genotypes of F_2 individuals in expanded F_2 populations and construct the genetic map (Chi et al. 2010). A total of 460 recessive include the expanded F_2 individuals were used for linkage analysis. The primers are shown in Table S1.

2.5 Generation of transgenic lines

To generate virus-induced gene silencing (VIGS) transgenic plants, the gene segments of *Solyc09g091500*, *Solyc09g091510*, and *Solyc09g091520* were cloned and inserted independently into the pTRV2 plasmid. The primers p500-V, pSlCHS3-V, and p520-V for the three candidate genes are shown in Table S1. The positive Agrobacterium tumefaciens strain GV3101 containing pTRV2, pTRV2-PDS, and pTRV2-target genes segments were each co-injected with positive GV3101 containing pTRV1 into cotyledons of the YNAU335 inbred line (Sheng et al. 2015).

To generate overexpressing transgenic No. 5 plants, full-length *SlCHS3* cDNA was amplified using the specific primer pSlCHS3-O (Table S1). The PCR product was fused to the binary plant transformation vector pBI121. Then, the pBI121 plasmid and pBI121-35S-SlCHS3 fusion plasmid were introduced into GV3101. Positive GV3101 was transformed into No. 5 cotyledons (Sheng et al. 2015). The primers used in this study are shown in Table S1.

2.6 DAS-ELISA and reverse transcription and real time quantitative PCR (RT-qPCR)

The inoculated tomato plants were used to detect TSWV accumulation using DAS-ELISA and RT-qPCR employing the primer pN-Q for the TSWV nucleoprotein (N) gene. A 100 μL tomato leaf extract was used for detection using the TSWV DAS-ELISA kit from Agdia (Elkhart, IN, USA) according to the manufacturer's instructions. Healthy tomato leaves were used as the control. The chromogenic reaction took place for 30 min in darkness, following which the optical density (OD) readings were taken at 415 nm in iMark Microplate Reader (Bio-RAD, Hercules, CA, USA). If the sample OD415/control OD415 $\geq$ 2, the sample was judged as positive, while if the sample OD415/control OD415 < 2, the sample was judged as negative (Canady et al. 2001). Each analysis was performed in biological and technical triplicate.

Gene expression profiles of the housekeeping ribosomal protein L2 (*RPL2*) (Løvdal and Lillo, 2009) and *Solyc09g091500*, *SlCHS3*, and *Solyc09g091520* genes of transgenic tomatoes were estimated by RT-qPCR using the Eppendorf Mastercycler ep Realplex real-time PCR system. Total RNA was extracted using the Quick RNA Isolation Kit (HuaYueYang Biotech Co., Ltd., Beijing, China) and then treated with RNase-free DNase I (TAKARA, Japan) to remove genomic DNA. Total RNA (2 μg) was reverse transcribed into first-strand cDNA using the M-MLV Reverse Transcriptase Kit (TAKARA, Japan), according to the manufacturer's protocol, and oligo(dT) primer and random primers were used in the reverse transcription reactions. The cDNA samples were diluted 5-fold and used as a template for RT-qPCR. Forty PCR cycles were performed according to the following temperature scheme: 95°C for 15 s, 60°C for 15 s, and 72°C for 20 s. The cycle threshold (Ct) values were read from the quantification curves. Each analysis was performed in biological and technical triplicate using the 2-ΔΔCt method (Zhao et al. 2013). The primers pN-Q, pRPL2-Q, p500-Q, pSlCHS3-Q, and p520-Q for the *N*, *RPL2*, *Solyc09g091500*, *SlCHS3*, and *Solyc09g091520*, respectively, are shown in Table S1.

2.7 Flavonoids content measurement

The total flavonoid content was measured using an aluminum chloride colorimetric method (Djeridane et al. 2006). In brief, 3.0 g of tomato leaf was weighed, and flavonoids were extracted using an ultrasonic method. Rutin was used as the standard substance to generate a standard curve (Rasha 2017). Flavonoid extracts were stained using aluminum chloride, and the absorptions were measured at 415 nm. The total flavonoid content was calculated using the following formula:

$$C\ (mg \cdot g{-}1) = C1 \times V/m,$$

where C represents the total flavonoid content; C1 represents the concentration of the sample extract; V represents the volume of the extract; and m represents the weight of the fresh sample.

3. Results

3.1 Phenotyping identification of the tomato materials

The 'Sw-5-2' primer pair was used to fingerprint the TSWV susceptible and resistant tomato lines in our experiment. The result showed that the Sw-5-derived amplicon of 574 bp was observed in L. peruvianum LA3858, LA2823, and PI128657 (Figure S1A, lanes 1-3). However, YNAU335, No. 5, and 96712I displayed only a smaller amplicon of 464 bp (Figure S1A, lanes 4-6). A sequence comparison of 'Sw-5-2' PCR amplicons from the six tomato varieties is shown in Figure S2. Mechanical inoculation with TSWV (YNAU2015 isolate) was performed in LA3858 and YNAU335 to evaluate resistance to TSWV using double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) and RT-qPCR. Both LA3858 and YNAU335 showed no TSWV symptoms after being inoculated with phosphate buffer (Figure S1B). After being inoculated with TSWV, LA3858 showed typical TSWV symptoms with a Ct value of 23.2 following RT-qPCR and was judged as positive by ELISA with an OD415 ratio of 3.1. YNAU335 displayed no TSWV symptoms and a Ct value which was judged as negative with an OD415 ratio of ~1 (Figure

S1C). The results suggested that the YNAU335 inbred line without the *Sw-5* locus showed immunity to the TSWV.

3.2 Resistance identification and genetic control

TSWV was inoculated using a mechanical method in the laboratory and by thrips in the field. The resistance levels of YNAU335 and No. 5 inbred lines, as well as their F_1 generation, were determined. Both YNAU335 and the F_1 generation had no necrotic lesions in the inoculation zones. Contrary to the Ct values of the TSWV N gene in the infected No. 5 (24.4 using the mechanical method and 23.7 following thrip-based infection), the Ct values of the N gene were not detected in the TSWV-inoculated plants of the YNAU335 line and the F_1 generation of YANAU355 and No. 5 lines. The OD415 ratios of the TSWV-inoculated YNAU335 and F_1 plants were ~1, while the OD415 ratios of TSWV-inoculated No. 5 were ~3 (Fig. 1).The F_2 and BC_1 population was constructed by the resistant and susceptible parental inbred lines of YNAU335 and No. 5. The segregation ratios of the F_2 and BC_1 generations between the YANAU355 and No. 5 lines were 1:3 and 1:1, respectively (Table 1).

Table 1
Genetic ratios of YNAU335 and No. 5 inbred lines resistant to TSWV

Samples	Numbers of immune plants	Numbers of susceptible plants	Ratios (immune:susceptible)	χ^2
P1: YNAU335	67	0	——	——
P2: No. 5	0	84	——	——
F2: P1♀×P2♂	512	165	3:1	0.58
RF2: P1♀×P2♂	431	139	3:1	1.02
BCP2: (P1×P2)♀×P2♂	121	116	1:1	2.03
RBCP2: (P1×P2)♀×P2♂	140	131	1:1	0.01

3.3 Fine mapping of TSWV resistance genes

We established parental inbred lines of YNAU335 and No. 5 as well as susceptible and resistant selections from the F_2 generation. BSA genome resequencing was used to map the candidate chromosomal region, which was located at the terminal of the long arm of chromosome 9. Nine InDel markers in this region were used to construct a genetic map using the screening results obtained using polymorphic InDel markers in the 304 recessive individuals from the F_2 segregating population, and the recombinant numbers identified with the markers. Eventually the candidate region was reduced to a physical interval about 20 kb which was between the InDel 2 and InDel 3 markers (Fig. 2). Three candidate genes were identified between InDel2 and InDel3 markers according to the tomato reference genome: *Solyc09g091500*, *Solyc09g091510* (*SlCHS3*), and *Solyc09g091520* (Table 2).

Table 2
Annotation of the disease-resistance candidate genes

Gene ID	Chromosome	Function description
Solyc09g091500	ch09	U6 snRNA-associated Sm-like protein LSm5 IPR006649 Like-Sm ribonucleoprotein, eukaryotic and archaea-type, core.
Solyc09g091510	ch09	Chalcone synthase IPR011141 Polyketide synthase, type III.
Solyc09g091520	ch09	60S acidic ribosomal protein P0IPR001790 Ribosomal protein L10.

3.4 Functional verification of TSWV resistance candidate genes

The VIGS system was used to preliminarily verify the functions of the three candidate genes: *Solyc09g091500*, *Solyc09g091510*, and *Solyc09g091520*. The YNAU335 inbred line was susceptible to TSWV after silencing *Solyc09g091510*, resulting in an extremely significant reduction in the Ct values of the N gene and increase in the OD415 ratios (Fig. 3). However, the resistance of YNAU335 to TSWV was not changed after silencing *Solyc09g091500* or *Solyc09g091520* (Figure. S3). Therefore, *Solyc09g091510* appeared to be the disease-resistance gene in YNAU335, which encoded CHS and was named *SlCHS3*. A *SlCHS3*-overexpression transgenic system in transgenic No. 5 lines showed increased resistance to TSWV with an increase in the Ct values of N gene and reduction in the OD415 ratios (Fig. 4).

3.5 Impact of TSWV on flavonoids contents in different tomato material

The flavonoid contents in *SlCHS3*-overexpressing No. 5 inbred lines were higher than those of the wild type, and the flavonoid contents in silenced YNAU335 inbred lines were lower than that of wild type. TSWV decreased the flavonoid contents in wild type and *SlCHS3*-overexpressing No. 5 inbred lines, and increased the flavonoid content in wild type and *SlCHS3*-silenced YNAU335 inbred lines (Fig. 5).

4. Discussion

TSWV-resistant tomato resources are concentrated in wild species, and interspecific hybridization challenges the genetic-based improvement of these crop resources (Stevens et al. 1994; Gordillo and Stevens 2008). To date, eight TSWV resistance genes have been discovered: *Sw-1a*, *Sw-1b*, *Sw-2*, *Sw-3*, *Sw-4*, *Sw-5*, *Sw-6*, and *Sw-7* (Finlay 1953; Stevens et al. 1991; Boiteux and Giordano 1993; Rosello et al. 1998; Dockter et al. 2009). Of these, *Sw-5* is the only verified gene that has been applied to the production of tomatoes (Spassova et al. 2001). However, TSWV isolates that break *Sw-5* resistance have been observed (Lopez et al. 2011). In this study, we produced a tomato inbred line YNAU335 without the *Sw-5* locus that is immune to TSWV. YNAU335 is an indeterminate growth inbred line with an average fruit weight of 335 g. It hybridizes with other tomato inbred lines normally, and its immunity to TSWV is

controlled by a dominant gene. We successfully mapped this resistance gene in the YNAU335 inbred line and verified the gene functions.

The TSWV resistance of YNAU335 is regulated by the quality gene *SlCHS3*, which was identified using BSA genome sequencing, genetic mapping, and functional identification. *SlCHS3* encodes CHS, which is the first key enzyme to regulate flavonoid synthesis (Nagamatsu et al. 2007; Wang et al. 2010). The *CHS* gene can be induced by a variety of pathogens (Gutha et al. 2010; Samac et al. 2011). Flavonoids regulated by *CHS* also play important roles in pathogen resistance (Nagamatsu et al. 2007; Wang et al. 2010). In summary, *SlCHS3* is the TSWV-resistance quality gene in the YNAU335 inbred line.

Previous researches showed that the transgenic expression of flavanone 3-hydroxylase redirects flavonoid biosynthesis and alleviates anthracnose susceptibility in sorghum (Wang et al. 2020). The enriched flavonoid content may improve the defence response and increase the nutrition values of sorghum grain/bran (Hsu et al. 2009). During gene transcription and translation, 5′-UTRs play important regulatory roles (Wilkie et al. 2003) and participate in responses to environmental factors (Xiao et al. 2014). Mutated sequences in 5′-UTRs may lead to transcriptional and translational abnormalities, which in turn alter the biological features of microorganisms or plants (Yan et al. 2004; Oestreicher et al. 2009; Hyodo et al. 2017; Yang et al. 2018). In this study, the *SlCHS3* expression could influence the content of flavonoids, the reason of this may the exon, intron, and promoter sequences of *SlCHS3* had mutations between the TSWV-resistant and -susceptible inbred lines. Therefore, we hypothesized that the differential expression levels of *SlCHS3* will influence the resistance ability in the tomato.

5. Conclusions

We used bulk segregant analysis and linkage analysis to map the TSWV resistance gene into a 20 kb region in the chromosome 9, and there were three candidate genes in the region. Based on the results of gene silence and overexpression, a *SlCHS3* was taken as the strong candidate gene. *SlCHS3* silencing could reduce the synthesis of flavonoids, and overexpression of *SlCHS3* could increase the content of flavonoids. The increased flavonoids could improve the ability of TSWV resistance in the tomato, we speculated that the gene expression level could control the synthesis of flavonoids and influence the TSWV resistance ability in tomato. Our study could provide new insights and lay the foundation for analyzing the TSWV resistance mechanisms.

Declarations

Author contribution

Wrote first draft: JL, and KZ. Designed experimental work: JL, MD, SJ, HZ and KZ. Investigation: ZL, ZW and JL. Provided experimental materials: JL, ZY and YL. Analyzed data: JL, KZ and JX. Wrote original manuscript: JL, and KZ. Wrote and edit review: JL, and KZ. Visualization: JL. Supervised the whole work:

KZ. Project administration: KZ. All authors have read and agreed to the published version of the manuscript.

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Availability of data and materials

All of the whole genome resequencing data used in the BSA analysis is available from the NCBI Short Read Archive (SRA, BioProject ID: PRJNA751989), and the raw data are freely available at: http://www.ncbi.nlm.nih.gov/bioproject/751989. The other supporting data are included as Additional files.

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Competing interests The authors declare no competing interests.

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Figures

Figure 1

Identification of resistance in tomato materials infected with the TSWV isolate YNAU2015. Tomato materials were inoculated with phosphate buffer (A) and TSWV using a mechanical method (B) and thrips (C). The numbers in the first and second brackets indicate the Ct values of the N gene using RT-qPCR and the ratios of the sample OD415 to the control OD415 using ELISA, which are shown as the

means of the three biological replicates ± standard deviations (SDs), “-”: not detected.Fig. 2. Fine-mapping of the TSWV-resistance candidate genes.

Figure 2

Fine-mapping of the TSWV-resistance candidate genes.

Figure 3

Functional identification of the SlCHS3 gene using a transgenic silencing system. Identification of transgenic plants using a phenotypic analysis of phytoene desaturase (PDS)silencing and RT-qPCR. WT-1 and WT-2 represent empty vector-transformed plants, which were assigned a value of 1. The error bar on each column represents the SD of three biological replicates (Student’s t-test, *P < 0.05, **P < 0.01) (A). The resistance performances of transgenic plants after inoculation with TSWV using a mechanical method (VIGS-1, -3, and -4) (B) and thrips (VIGS-8, -9, and -17) (C). The numbers in the first and second brackets indicate the Ct values of the N gene using RT-qPCR and the ratios of the sample OD415 to the control OD415 using ELISA, which are shown as the means of three biological replicates ± standard deviations (SDs), “-”: not detected.

Figure 4

Functional identification of the SlCHS3 gene using a transgenic overexpression system. SlCHS3 gene expression levels in transgenic lines were assessed by RT-qPCR. WT-1 and WT-2 represent empty vector-transformed plants, which were assigned a value of 1. The error bar on each column represents the SD of three biological replicates (Student’s t-test, **P < 0.01) (A). The resistance performances of transgenic lines after inoculation with TSWV using a mechanical method (L-1, -4, and -6) (B) and thrips (L-9, -10, and -12) (C). The numbers in the first and second brackets indicate the Ct values of the N gene using RT-qPCR and the ratios of the sample OD415 to the control OD415 using ELISA, which are shown as the means of three biological replicates ± SDs.

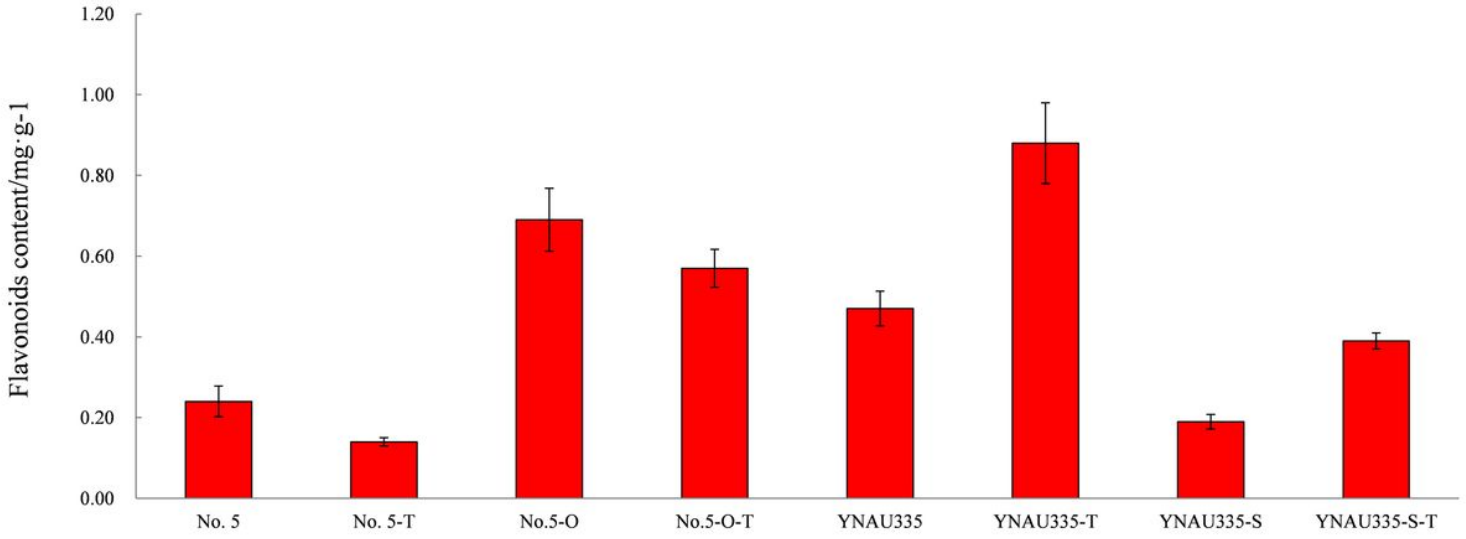

Figure 5

Flavonoids contents in different tomato materials. No. 5 and YNAU335 represent the No. 5 and YNAU335 inbred lines under normal growth conditions, respectively. No. 5-T and YNAU335-T represent the No. 5 and YNAU335 inbred lines inoculated with TSWV, respectively. No. 5-O represents the No. 5 inbred line overexpressing SlCHS3. No. 5-O-T represents the SlCHS3-overexpressing No. 5 inbred lines inoculated with TSWV. YNAU335-S represents the SlCHS3-silenced YNAU335 inbred line. YNAU335-S-T represents the SlCHS3-silenced YNAU335 inbred line inoculated with TSWV. The error bar on each column represents the SD of three biological replicates.

Supplementary Files

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