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Identification and Functional Analysis of *1-Deoxy-D-xylulose-5-phosphate Synthase* Gene in Tomatoes (*Solanum lycopersicum*)

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Abstract: 1-deoxy-D-xylulose-5-phosphate synthase (DXS) is a rate-limiting enzyme in terpene synthesis that can affect the accumulation of secondary metabolites in plants. In this study, three *DXS* gene family members were identified in the tomato genome-wide database. Using bioinformatics methods, we analyzed the gene structure, evolutionary affinities, and cis-acting elements of the *SlDXS* gene family members. Promoters of *SlDXS* genes contain plant hormone-responsive elements such as the CGTCA-motif, TGACG-motif, ABRE, TCA-element, TGA-element, ERE, CAT-box, and AACA-motif, which suggested that the *SlDXS* gene family may play an important role in hormone response. The RT-qPCR analysis showed that the tomato *DXS2* gene was able to respond upon exposure to methyl jasmonate (MeJA). The construction of a virus-induced gene silencing (VIGS) vector for the *SlDXS* gene showed that the *SlDXS2* gene was also able to respond to MeJA in silenced plants, but the induction level was lower relative to that of wild-type plants. The *SlDXS1* gene is associated with the synthesis of photosynthetic pigments. This study provides a reference for the further elucidation of the *DXS* gene's biological function in the terpenoid synthesis pathway in tomatoes.

Keywords: tomato; *DXS*; expression analysis; gene silencing

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1. Introduction

Terpenoids are the most common and diverse class of secondary metabolites in plant volatiles; they can be produced by almost all plant organs, including roots, stems, leaves, flowers, fruits, and seeds [1], and they play important roles in the growth and development of plants themselves. Terpenoids have isoprene as their structural unit and mainly include monoterpenes, sesquiterpenes, diterpenes, and triterpenes [2,3]; monoterpenes and sesquiterpene compounds are the main volatile substance components produced by plants. Terpenoids can act as signaling molecules to mediate plant defense responses to phytophagous insect feeding and play an important role in the resistance to pathogenic microbial attacks, among other roles [4,5].

There are two pathways that are important for the synthesis of plant terpenoids: the mevalonate (MVA) pathway located in the cytoplasm and the methylerythritol-4-phosphate (MEP) pathway located in the plastid [6]. 1-deoxy-D-xylulose-5-phosphate synthase (DXS) is the first key enzyme of the MEP pathway that converts pyruvate and 3-phosphoglycerol aldehyde to produce the first key intermediate, 1-deoxy-xylulose-5-phosphate (DXP). This is considered to be the rate-limiting step in the synthesis of isopentenyl pyrophosphate

(IPP) and dimethylpropenyl pyrophosphate (DMAPP) via the MEP pathway [7,8]. The overexpression of the *DXS* gene in *Arabidopsis* leads to a significant increase in the terpene content [9,10]. The Ginkgo *DXS* gene is expressed in all trophic organs, and it is induced and regulated by methyl jasmonate, whereas the synthesis of ginkgolides is positively correlated with *DXS* expression [11]. These observations confirm that the *DXS* gene is an important regulatory site in the terpene metabolic pathway and can effectively influence the accumulation of secondary metabolites in plants.

Tomatoes (*Solanum lycopersicum* L.), belonging to the tomato genus of the Solanaceae family, are an important vegetable crop around the world, widely cultivated in the north and south of China. Tomatoes are rich in soluble sugar, organic acids, vitamins, and other nutrients, and consumers love their rich flavor. Tomatoes can be infested by phytophagous insects during growth, resulting in a decrease in the yield and quality of tomatoes [12]. Plants stimulate their own defense mechanisms when attacked by phytophagous insects. For example, plants can produce physical barriers and secondary metabolites, and they can induce the expression of relevant genes for direct defense [13,14]. Flavonoids are important secondary metabolites and are phenolic compounds. They are capable of hindering the feeding of pests and of affecting their growth, development, and reproduction [15,16]. Additionally, plants release volatile organic compounds as an indirect defense after an infestation to attract natural enemies to feed on or parasitize the pest [17]. Using the plants' own defense mechanisms to control pests is an environmentally friendly approach. It has been found that all tissue parts of a tomato are rich in terpenoids. Terpenoids are not only able to participate in the plant's defense response, acting directly or indirectly against phytophagous insects, but they can also act against disease infiltration [18,19]. The expression of the tomato *DXS* gene and the regulation of the synthesis of volatile terpenoids may influence a tomato's defense response to adversity.

The *DXS* gene encodes the first key enzyme in the terpenoid MEP pathway in tomatoes. In this study, we identified the *DXS* family members and bioinformatically analyzed three *SlDXS* gene-encoded proteins. We also explored the effects of different abiotic stresses on the expression of the tomato *DXS* gene. A virus-induced gene silencing (VIGS) vector was constructed for the silencing of a *SlDXS* gene, and the content of photosynthetic pigments in the silenced plants was analyzed. Real-time fluorescence quantification PCR (RT-qPCR) was used to analyze its induction of exogenous MeJA on silenced plants. This study provides a basis for an in-depth investigation of terpenoid metabolic pathways and molecular regulatory mechanisms in tomatoes in addition to a theoretical basis for further research on tomatoes' resistance to external stress.

2. Materials and Methods

2.1. Identification of the DXS Gene Family in Tomatoes

The tomato genome-wide data files and genome annotation files (ITAG4.0) were downloaded from the tomato genome website (https://solgenomics.net/organism/Solanum_lycopersicum/genome, accessed on 14 August 2022), and the Arabidopsis genome files were downloaded from the official Arabidopsis website (https://www.arabidopsis.org/, accessed on 14 August 2022). The protein sequences of the three identified *AtDXS* family members in Arabidopsis were compared with those of tomatoes using BLASTP to screen candidate genes. The hidden Markov model (HMM) of the *DXS* gene-specific Lyase aromatic structural domain (PF13292) [20] was downloaded from the Pfam database (http://pfam.sanger.ac.uk/, accessed on 14 August 2022). *SlDXS* family members were screened from the tomato genome database using the HMMER3.0 [21] software with the screening criterion of an E-value of $\leq 1 \times 10^{-5}$. The redundant sequences between the HMM search and BLASTP were removed, and candidate members were submitted to the online website SMART (http://smart.embl-heidelberg.de/, accessed on 14 August 2022) and the NCBI Conserved Structural Domain Database CDD (https://www.ncbi.nlm.nih.gov/Structure/cdd, accessed on 14 August 2022) [22] to verify the integrity of the

conserved structural domains of the candidate gene proteins. Finally, we obtained the *SlDXS* gene family members.

2.2. Physicochemical Analysis of Proteins of the DXS Gene Family in Tomatoes and Prediction of Their Subcellular Localization

The protein physicochemical properties of SlDXS were analyzed using the online website expasy (https://web.expasy.org, accessed on 18 August 2022). The NovoPro website (https://www.novopro.cn, accessed on 18 August 2022) was utilized for protein signal peptide prediction. A subcellular localization prediction analysis was performed using Plant-mPLoc (http://www.csbio.sjtu.edu.cn/bioinf/plant-multi/, accessed on 18 August 2022).

2.3. Prediction of Secondary and Tertiary Structures of Tomato DXS Gene Family Members

The DXS protein's secondary structure was predicted using Prabi (https://npsa-prabi.ibcp.fr, accessed on 5 October 2022) (Supplementary Table S5). The tertiary structure was structurally modeled using Swiss-model (https://swissmodel.expasy.org, accessed on 5 October 2022).

2.4. Phylogenetic Analysis of the Tomato DXS Gene

MEGA7 software was used to conduct a multiple sequence comparison of the DXS gene families of 13 species, including *Solanum lycopersicum*, *Arabidopsis thaliana*, *Zea mays*, *Oryza sativa*, *Populus trichocarpa*, *Capsella rubella*, *Ricinus communis*, *Medicago truncatula*, *Nicotiana tabacum*, *Ginkgo biloba*, *Hevea brasiliensis*, *Salvia miltiorrhiza*, and *Pinus densiflora*, and a phylogenetic tree was constructed using the neighbor-joining (NJ) method. The parameters were set as the Poisson correction, pairwise deletion, and bootstrap test (1000 repetitions), and the phylogenetic tree was beautified using the iTOL webpage (https://itol.embl.de/itol.cgi, accessed on 3 October 2023) to embellish the evolutionary tree.

2.5. Gene Structure and Conserved Motif Analysis of DXS Gene Family in Tomatoes

The gene structure information of the tomato *DXS* gene family members was analyzed using the Tbtools software v1.098775 [23], and the *SlDXS* gene structure was analyzed using the NCBI's online website (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi, accessed on 3 April 2023). The conserved motifs of proteins encoded by *SlDXS* gene family members were analyzed using the MEME website (https://meme-suite.org, accessed on 3 April 2023), and the gene structure and conserved motifs were mapped using Tbtools.

2.6. Analysis of Promoter Cis-Acting Elements of the DXS Gene Family in Tomatoes

The 2000 bp sequences upstream of the transcription start site of the SlDXS gene were extracted using TBtools, and the cis-acting elements in the promoter regions were predicted using PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/, accessed on 26 October 2023), and the prediction results were visualized through TBtools.

2.7. Abiotic Stress Treatment of Plant Materials

A 'Micro Tom' tomato was used as the material for stress treatment. The tomatoes were subjected to abiotic stress until they reached the six-leaf stage under normal conditions (light/dark for 16 h/8 h, 25 °C/20 °C, photosynthetic photon flux density of 200 $\mu mol \cdot m^{-2} \cdot s^{-1}$). The tomato leaves were sprayed with concentrations of 100 µmol/L of methyl jasmonate, gibberellin, and abscisic acid and 100 mmol/L of NaCl. The fifth and sixth true leaves at 0 h, 1 h, 3 h, 6 h, 12 h, 24 h, and 48 h were taken for quantitative analysis. After liquid nitrogen quick-freezing, the leaves were stored in the refrigerator at −80 °C, and three biological replicates were performed.

2.8. Expression Analysis of the DXS Gene in Tomatoes

The total RNA of tomato was extracted using the Vazyme FastPure® Plant Total RNA Isolation Kit (https://www.vazyme.com/product/164.html, accessed on 18 February 2023), and the first-strand cDNA was synthesized with the FastKing One-Step Reverse Transcription Kit from TIANGEN (https://www.tiangen.com/content/details_40_21180.html, accessed on 18 February 2023). RT-qPCR was performed with GenStar's 2×RealStar Fast SYBR qPCR Mix. Detection was performed using a LightCycler96 Real-Time PCR instrument with three biological replicates and three technical replicates set up for each sample. Primers were designed using the Primer 5.0 software (Supplementary Table S1), and the specificity of the primers was tested using the NCBI tool (https://www.ncbi.nlm.nih.gov/tools/primer-blast/, accessed on 10 February 2023). The expression of the *SlDXS* gene was verified using *Actin* as an internal reference gene, and the relative expression level of the gene was calculated using the $2^{-\Delta\Delta ct}$ method [24].

2.9. Cloning the SlDXS Gene and Constructing the VIGS Silencing Vector

SnapGene was used to design primers specific to the *SlDXSs* gene (Supplementary Tables S2 and S4), and tomato cDNA was used as a template to amplify the target gene. The PCR program was as follows: pre-denaturation at 95 °C for 3 min, denaturation at 95 °C for 15 s, annealing at 59 °C for 15 s, and extension at 72 °C for 30 s, for a total of 34 cycles.

The *Kpn*I and *Eco*RI restriction endonucleases were selected to double digest the pTRV2 vector plasmid, and the *SlDXS* fragment was inserted into the pTRV2 silencing vector. The pTRV2-*SlDXS* recombinant vector was verified through digestion. pTRV1 and the pTRV2-*SlDXS* recombinant vector were transformed into Agrobacterium GV3101. The single colonies containing pTRV1 and recombinant vector pTRV2-*SlDXS* were picked and inoculated into 400 μL of LB liquid medium (Kan 100 mg/L, Rif 50 mg/L), and then incubated for 6 h at 28 °C at 200 rpm. An amount of 200 μL of the above bacterial solution was added into 10 mL of LB liquid medium (Kan 100 mg/L, Rif 50 mg/L), and then incubated for 12 h at 28 °C at 200 rpm. The bacterial cells were collected by centrifugation at 8000 rpm for 10 min, the bacteria were resuspended in a VIGS infiltration solution (10 mmol/L of $MgCl_2$; 10 mmol/L of MES; 200 μmol/L of AS), and the OD_{600} was adjusted to about 0.8. The cells were allowed to stand at room temperature for 4 h, and the resuspension of pTRV1 with pTRV2-*SlDXS* was mixed at a volume ratio of 1:1 and injected into tomato cotyledons. The inoculum was aspirated with a 1 mL syringe and gently injected into the dorsal surface of the plants' cotyledons, which were protected from light for 24 h. After that, the plants were transferred to a light incubator for further incubation.

2.10. Treatment of Silencing Plant Materials

A 'Micro Tom' tomato was used as the material for the silencing treatment, and the infested plants were cultured in a light incubator (photoperiod: 16 h/8 h light/dark cycle; temperature: 25 °C/20 °C day/night; PPFD: 200 $\mu mol \cdot m^{-2} \cdot s^{-1}$) for three weeks. RT-PCR was performed on the silenced plants to validate the transcription of TRV2 in order to obtain the positive plants. The expression level of the *SlDXS2* gene was measured using *Actin* as an internal reference gene, and the relative expression level of the gene was calculated using the $2^{-\Delta\Delta ct}$ method. The content of photosynthetic pigments in tomato leaves was determined by the ethanol extraction colorimetric method [25]. The leaves of the positive plants were treated with MeJA, and the samples were collected 6 h later. Flavonoids were determined using a kit from Comin Biotechnology (Suzhou, China). Three biological replicates were set up.

3. Results

3.1. Identification and Physicochemical Analysis of the DXS Gene Family in Tomatoes

Three members of the *DXS* gene family in tomatoes were identified using bioinformatics methods, and according to the existing nomenclature of *Arabidopsis* and phylogenetic developmental analyses, they were sequentially named *SlDXS1*, *SlDXS2*, and *SlDXS3*. Pro-

tein characterization revealed (Table 1) that the amino acid lengths of the proteins encoded by the family members were 719, 714, and 709aa, and the molecular weights ranged from 77,605.63 to 77,172.8 Da. The theoretical pI values of the *SlDXS* family members were all less than 7, and all of them were acidic proteins. SlDXS proteins do not have a signal peptide for any of the amino acids and are non-secretory proteins, and both SlDXS1 and SlDXS2 are hydrophilic, with a hydrophobicity of less than 0. The instability index was greater than 40, which made them unstable acidic proteins. An analysis of the subcellular localization of the proteins showed that SlDXS proteins are all predicted to be localized in the chloroplasts [26].

Table 1. Sequence characteristics of the *SlDXS* proteins.

Gene Name	Gene ID	AA [1] (aa)	Mw [2] (kDa)	pI [3]	II [4]	Gravy [5]	Subcellular Localization
SlDXS1	01g067890	719	77.60	6.32	40.36	−0.063	chloroplast
SlDXS2	11g010850	714	77.08	6.61	40.85	−0.094	chloroplast
SlDXS3	08g066950	709	77.17	5.85	35.56	0.041	chloroplast

[1] AA, amino acid; [2] Mw, molecular weight; [3] pI, theoretical isoelectric point; [4] II, instability index; [5] GRAVY, grand average of hydrophobicity.

3.2. Secondary and Tertiary Structure Analyses of the DXS Gene Family in Tomatoes

A predictive analysis of the secondary structures of tomato DXS proteins revealed (Table 2) that all three proteins consisted of α-helices, extended strands, β-turns, and random coiling in their secondary structures. The number of amino acid residues accounted for was dominated by α-helices and random coils, followed by extended chains, and β-turns. Further analysis of the tertiary structure (Figure 1) showed that the templates for SlDXS1, SlDXS2, and SlDXS3 proteins were all 7bzx.1.A, and the sequence identities were 88.75%, 73.93%, and 58.24%, respectively.

Table 2. Secondary structures of proteins in the tomato *DXS* gene family.

Gene Name	α-Helix/%	Extended Strand/%	β-Turn/%	Random Coil/%
SlDXS1	38.94	15.30	7.51	38.25
SlDXS2	38.10	16.25	7.70	37.96
SlDXS3	41.47	14.67	7.19	36.67

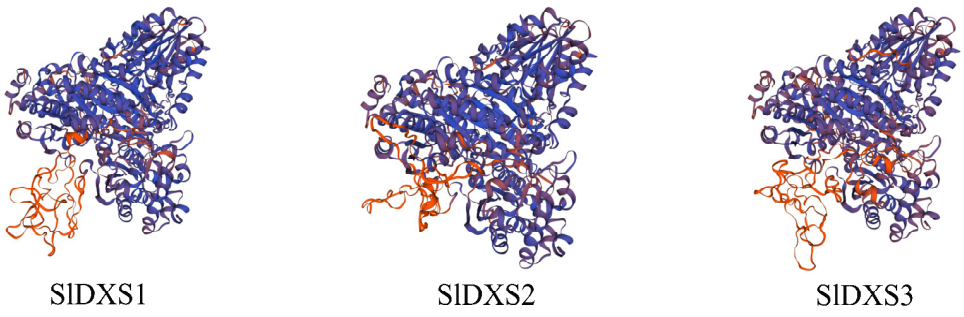

Figure 1. Tertiary structure of the tomato *DXS* gene family.

3.3. Phylogenetic Analysis of the DXS Gene Family in Tomatoes

The *SlDXS* proteins were combined with the sequences of proteins encoded by the *DXS* genes of 12 species, including *Arabidopsis thaliana*, *Oryza sativa*, *Zea mays*, *Nicotiana tabacum*, *Salvia miltiorrhiza*, and *Ricinus communis*, to perform phylogenetic analyses and construct a phylogenetic tree using the neighbor-joining (NJ) method (Figure 2). The results of the phylogenetic analysis showed that the *DXS* gene family members in all plants co-clustered into three branches, and the three tomato *DXS* genes were located in different

groups. The DXS I branch had the largest number of members, including *AtDXS1* and *AtDXS2* in *Arabidopsis*, and *SlDXS1* was more closely related to *NtDXS1* and *SmDXS1*. *SlDXS1* and *NtDXS2* clustered into one branch, suggesting a high degree of affinity. There were fewer members in the DXS III branch, including species such as *Arabidopsis thaliana*, *Oryza sativa*, *Populus trichocarpa*, *Zea mays*, *Ricinus communis*, and *Capsella bursa-pastoris*, whereas the tomato *SlDXS3* was closer to *AtDXS3*. Therefore, it was hypothesized that the *SlDXS1*, *SlDXS2*, and *SlDXS3* genes may have different functions.

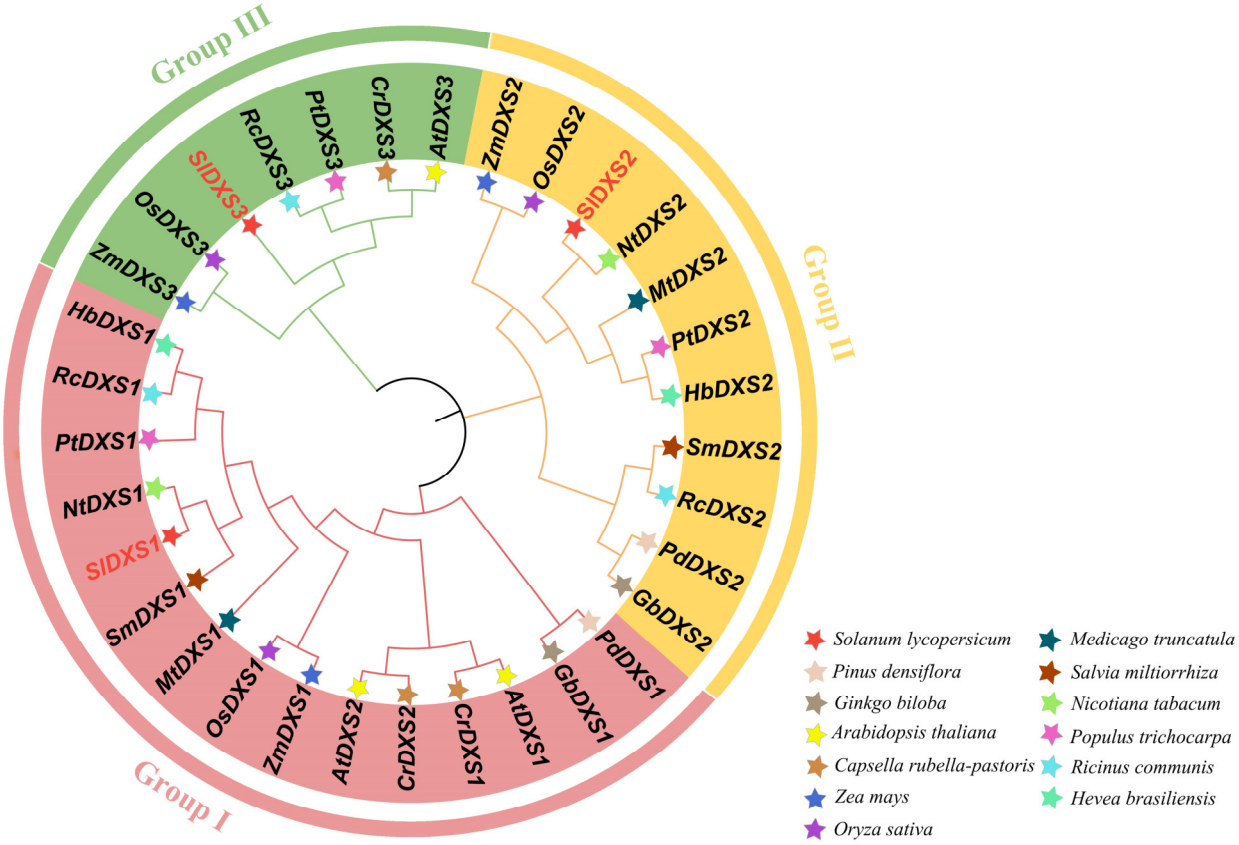

Figure 2. Phylogenetic tree analysis of tomato *SlDXS1*, *SlDXS2*, and *SlDXS3* proteins and *DXS* proteins from other species. The *SlDXS* proteins are marked with red stars.

3.4. Analysis of the Gene Structure and Conserved Motifs of the DXS Gene Family in Tomatoes

In order to further analyze the structural characteristics of the *SlDXS* gene family, three genes of the tomato *DXS* gene family were subjected to relevant intron and exon analyses (Figure 3A), and the results showed that the *SlDXS1*, *SlDXS2*, and *SlDXS3* genes all have 10 exons and 9 introns. The tomato DXS protein's conserved motifs were analyzed using the online MEME software, and eight motifs were used to analyze the gene sequences (Supplementary Table S3). There were eight motifs for all three genes, and the ranking order was fixed (Figure 3B). This indicates that there are highly conserved motifs in the DXS protein family and that the DXS gene family is highly conserved. A predictive analysis of the structural and functional domains of the tomato DXS proteins revealed that all three proteins had DXS structural domains (Figure 3C), indicating that the SlDXS1, SlDXS2, and SlDXS3 proteins all belong to the DXS superfamily.

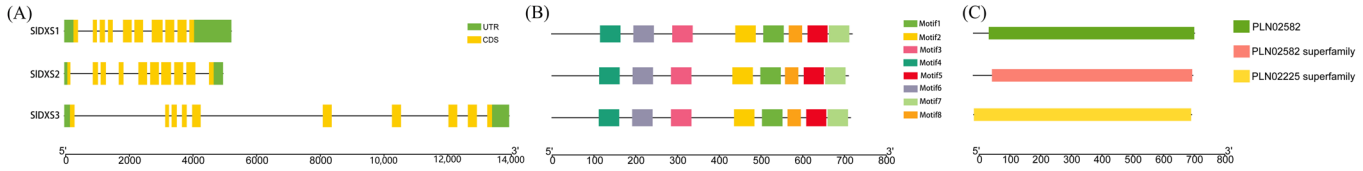

Figure 3. Analysis of proteins' conserved motifs and gene structure of *DXS* gene family in tomatoes. (**A**) Gene structure. (**B**) Protein conserved motifs. (**C**) Protein conservative domain.

3.5. Analysis of Cis-Acting Elements in Promoters of the DXS Gene Family in Tomatoes

The promoter sequences of the first 2000 bp of the tomato *SlDXS* genes were obtained using the TBtools software, and the promoter cis-acting elements of the *SlDXS* genes were analyzed using the PlantCARE online website. The results showed that the promoter regions of the *SlDXS* gene family contain a variety of cis-acting elements with different roles, indicating that the three *SlDXS* genes may be involved in different physiological and metabolic regulatory pathways. A total of 121 cis-acting elements were detected in the 2000 bp region of the upstream promoter of the tomato *SlDXS* gene family. They were classified into four categories: hormone-responsive elements, light-responsive elements, growth and development elements, and stress-related cis-acting elements (Figure 4). The results showed that there were 6 hormone-related regulatory elements, including 4 methyl jasmonate regulatory elements (CGTCA-motif and TGACG-motif), 10 ABA-responsive regulatory elements (ABRE), 2 salicylic acid-responsive regulatory elements (TCA-element), 1 growth hormone-responsive element (TGA-element), and 19 gibberellin-responsive regulatory elements (ERE). There were two elements related to growth and development, including meristematic tissue expression (CAT-box) and endosperm expression (AACA-motif). A total of 43 stress-related response regulatory elements were found, including MYC, ARE, and W-box. There were 11 different elements related to light response regulation, such as the 1-box, GA-motif, and ATCT-motif. Light-responsive elements were present in each gene family member, suggesting that expression of the *SlDXS* gene may be induced or repressed by light. The *SlDXS* gene family members contain hormone-responsive elements, and it is hypothesized that tomato *SlDXS* genes play an important role in hormone responses. The *SlDXS2* gene contains two methyl jasmonate regulatory elements (TGACG-motif), which presumably respond to MeJA expression.

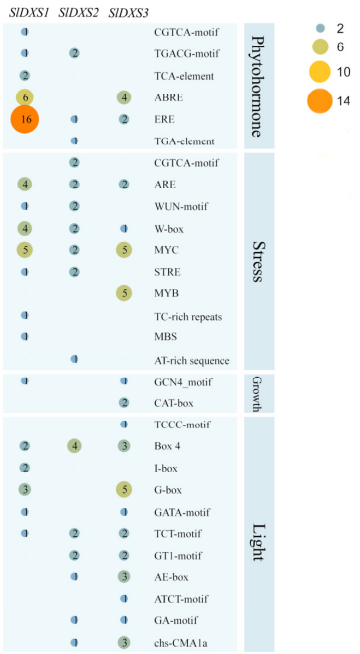

Figure 4. Distribution of cis-acting elements in the tomato *DXS* gene family promoter sequence.

3.6. Analysis of the Induced Expression Pattern of the Tomato DXS Gene Family

To examine the expression levels of the *SlDXS* gene family members, the tomato leaves were treated with MeJA, GA, ABA, and salt stress factors, respectively. The results showed that, after the tomato leaves were treated with MeJA, the expression levels of *SlDXS1* and *SlDXS3* showed decreasing trends, which were minimized at 24 h and 6 h, respectively. The expression level of *SlDXS2* increased at 3 h and 24 h, and it peaked at 3 h (Figure 5A). This indicates that MeJA was able to induce the expression of the *SlDXS2* gene and significantly increase its expression level. After the GA treatment was applied, the expression of *SlDXS2* increased from 1 h to 9 h, and it peaked at 1 h; its expression was 4.22 times higher than that of the control group. The expression of *SlDXS3* increased rapidly at 1 h and peaked at 1 h, and its expression was more than 2.23 times higher than that of the control group. The expression of *SlDXS1* showed a decreasing tendency (Figure 5B). This indicates that GA induced the expression of *SlDXS2* and *SlDXS3*, while SlDXS1 was not responsive to GA. After the tomato leaves were treated with ABA, the expression levels of both *SlDXS1* and *SlDXS2* showed decreasing trends, which were minimized at 12 h and 1 h. The expression of *SlDXS3* increased at 1 h and 9 h, and its expression levels were 1.55 and 1.51 times higher than that of the control, respectively (Figure 5C). This indicates that *SlDXS3* responded slower to ABA. Under different hormone treatments, *SlDXS1*, *SlDXS2*, and *SlDXS3* had different responses and presumably different functions. After the salt stress treatment was applied, the expression of *SlDXS1* increased transiently at 1 h and decreased from 3 h to 48 h. The expression of *SlDXS2* increased at 1 h, 6–12 h, and 48 h; peaked at 6 h; and was 7.64 times higher than that of the control group. The expression of *SlDXS3* increased at 3 h and 48 h and peaked at 48 h (Figure 5D). The expression levels of *SlDXS1* and *SlDXS2* increased rapidly at 1 h, indicating that they had more rapid responses to NaCl.

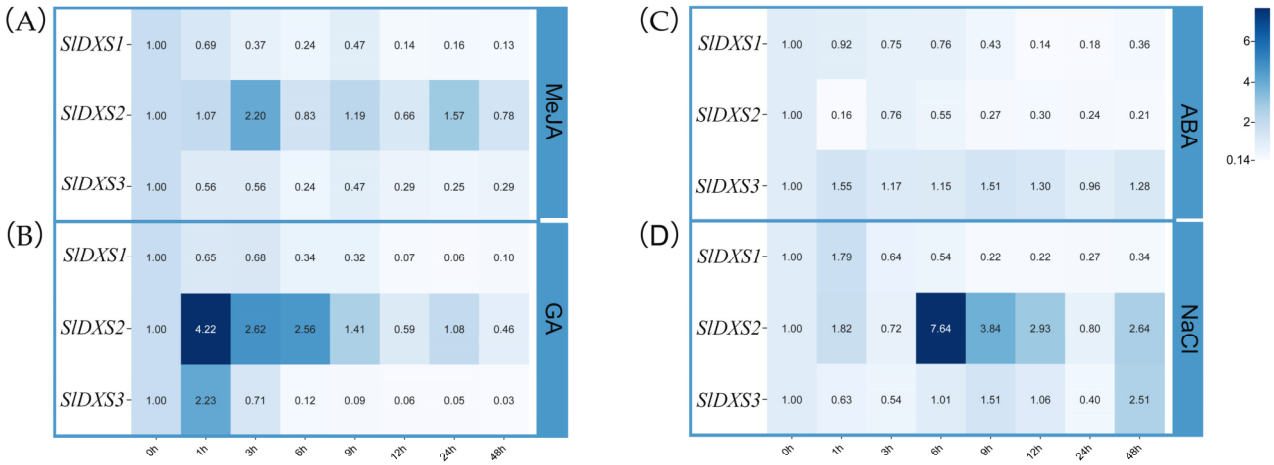

Figure 5. Expression patterns of tomato *DXS* gene under abiotic stress. Treatments with (**A**) 100 μM of MeJA; (**B**) 100 μM of GA; (**C**) 100 μM of ABA; and (**D**) 100 mM of NaCl. The *Actin* gene was used as the reference gene, and the expression data were used for the RT-qPCR. The data represent the mean of triplicates with three biological replicates (Supplementary Materials, Figure S1).

3.7. Cloning the SlDXS Genes and Constructing the VIGS Silencing Vector in Tomatoes

To further investigate the biological function of *SlDXS* genes, VIGS technology was utilized to silence *SlDXS* genes in tomatoes. Fourteen days after the Agrobacterium infection of tomato cotyledons, photobleaching appeared on the leaves of tomato seedlings injected with the pTRV2-*SlDXS1* infection solution (Figure 6B). One month after inoculation, photobleaching appeared in most leaves of the plants infected with pTRV2-*SlDXS1* (Figure 6F). In contrast, the albino phenotype was not presented in wild-type plants and the plants injected with pTRV2-*SlDXS2* and pTRV2-*SlDXS3* infection solutions (Figure 6F–H).

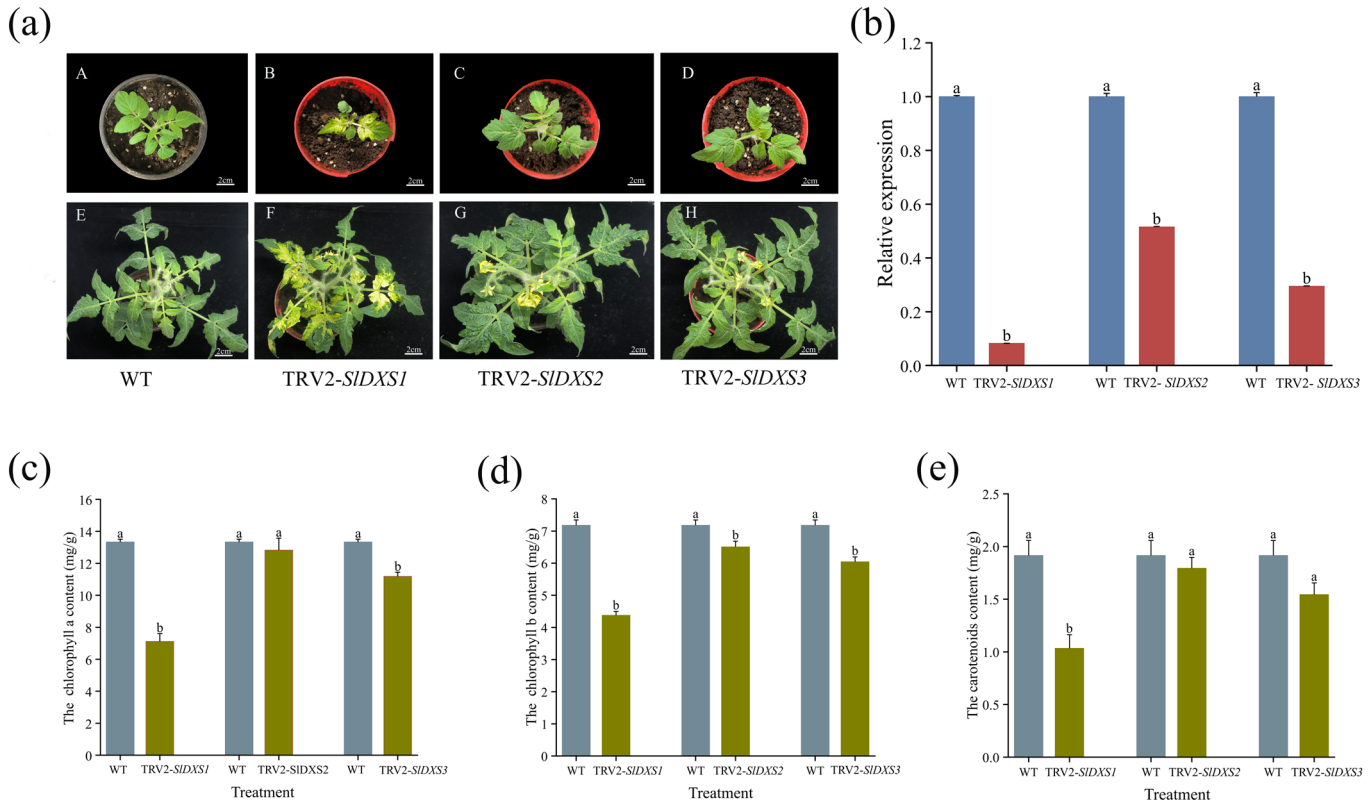

Figure 6. Analysis of silenced tomato plants. (**a**) Phenotype of plants silenced by tomato *SlDXS* genes. (**A**–**D**) are phenotypes from 7 d after infestation and (**E**–**H**) are phenotypes from one month after infestation. (**b**) Analysis of tomato TRV2-*SlDXS* gene expression. (**c**) Content of chlorophyll a. (**d**) Content of chlorophyll b. (**e**) Carotenoid content. The *Actin* gene was used as the reference gene for (**b**), and the expression data for the RT-qPCR. The data represent the mean of triplicates with three biological replicates. Error bars represent the standard errors (SEs). Different lowercase letters represent significant differences ($p < 0.05$).

The expression levels of the *SlDXS* genes in plants that underwent different treatments were detected through an RT-qPCR using Actin as an internal reference gene, and the results showed that pTRV2-*SlDXS* resulted in a significant decrease in the expression of the *SlDXS* genes compared to the control group (Figure 6b).

The content of photosynthetic pigments was determined in wild-type and silent plants. As shown in Figure 6c–e, the content of photosynthetic pigments was reduced in all silenced plants compared to the wild type. Silenced plants injected with pTRV2-*SlDXS1* showed significant reductions in chlorophyll a, chlorophyll b, and carotenoids by 46.63%, 38.99%, and 46.02%, respectively. Silenced plants injected with the pTRV2-*SlDXS3* infection solution showed a reduction of 16.14% and 15.84% in chlorophyll a and chlorophyll b, respectively, whereas there was no significant difference in carotenoid content. There was no significant difference in the photosynthetic pigment content of tomato plants silencing the *SlDXS2* gene. It was shown that the *SlDXS1* gene is associated with the synthesis of chlorophyll and carotenoids, and the silencing of the *SlDXS1* gene can cause a decrease in the content of photosynthetic pigments in tomatoes.

3.8. Analysis of TRV2-SlDXS2 Gene Response to MeJA

As shown in Figure 5A, MeJA increased the expression of the tomato *SlDXS2* gene. The following procedures were conducted in order to further investigate the response of *SlDXS2* to MeJA and its biological function.

The tomato leaves were treated with MeJA, and the samples were collected after 6 h for a gene expression analysis and the determination of physiological indices. The tomato

SlDXS2 gene was analyzed using an RT-qPCR after MeJA treatment. The results showed that the expression of the *SlDXS2* gene was increased in both the wild-type and silenced plants, but MeJA promoted the expression of the *SlDXS2* gene at a lower level in the silenced plants compared with the wild-type plants (Figure 7A). This indicates that MeJA reduced the induction of *SlDXS2* in the silenced plants.

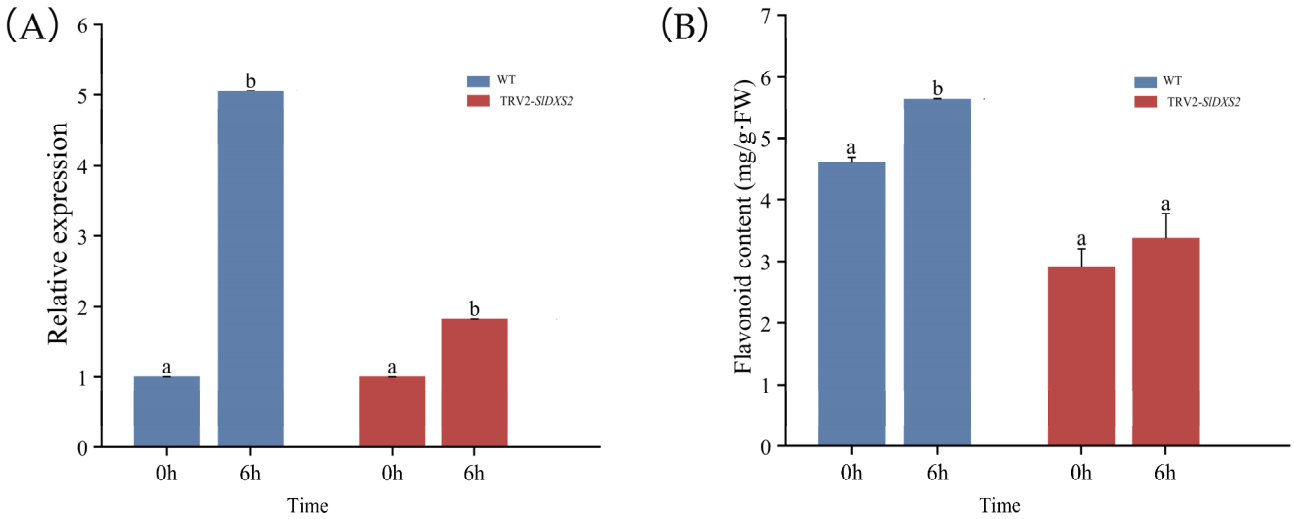

Figure 7. Analysis of TRV2-*SlDXS2* gene response to MeJA. (**A**) Analysis of gene expression in silenced plants in response to MeJA. (**B**) Flavonoid content of silenced plants. The data represent the mean of triplicates with three biological replicates. Error bars represent the standard errors (SEs). Different lowercase letters represent significant differences ($p < 0.05$).

Flavonoids are important secondary metabolites that help plants resist pests and diseases, whereas terpenoids have a role in helping plants avoid pests [27,28]. The flavonoid content was determined after the MeJA treatment was applied to silenced and wild-type tomato plants, and the results showed that the flavonoid contents of both the wild-type and silenced plants were elevated after the MeJA treatment; however, compared with the wild-type plants, the MeJA-induced synthesis of flavonoids was lower in the silenced plants (Figure 7B). This suggests that MeJA's promotion of secondary metabolism in tomatoes was reduced after *SlDXS2* silencing.

The physiological indicators and the results of the gene expression analyses indicated that MeJA was able to significantly induce the expression of the tomato *SlDXS2* gene and promote the synthesis of secondary metabolites; they also indicated that the *SlDXS2* gene was capable of responding to MeJA in silenced plants, but at a lower level of induction relative to that of wild-type plants.

4. Discussion

Terpenoids play important roles as chemical signaling substances during indirect defense responses, such as by helping plants avoid pests and natural enemies, and they are involved in plant-to-plant and plant–insect interactions [27]. The DXS enzyme is the first key enzyme in the MEP pathway, which is one of the terpene synthesis pathways, and the *DXS* gene is also the rate-limiting enzyme gene in the MEP synthesis pathway [29]. It was shown that the tissue expression pattern of the *DXS* gene in *C. blini* was positively correlated with the tissue accumulation pattern of the diterpene substance artemisinin, suggesting that the overexpression of the *DXS* gene may increase the synthesis of artemisinin in *C. blini* [30]. The overexpression of the *DXS2* gene in the hairy roots of *S. miltiorrhiza* was able to significantly promote the accumulation of tanshinones [31]. It was also demonstrated that the *DXS* gene is important for terpenoid synthesis.

DXS is currently confirmed to be a small gene family in several species, such as *A. thaliana* [32], *Z. mays* [33], and *A. annua* [34], usually containing between two and four

members. In this study based on the tomato genome, three tomato *DXS* genes were identified, which is the same number of *DXS* gene family members as in *A. thaliana*, *O. sativa*, and *Z. mays*, and *DXS* gene family members have similar gene structures and conserved motifs in many species. It was found that AtDXS3 is not involved in primary and secondary metabolism [35]. It was found that the SlDXS3 protein does not have the same conserved residues as SlDXS1 and SlDXS2. Therefore, the SlDXS3 protein may not be able to bind to TPP cofactors [26]. An analysis of the subcellular localization of the proteins showed that *SlDXS* proteins are all predicted to be localized in the chloroplasts. This is consistent with the fact that the three *DXS* genes of *Morus notabilis* are located in chloroplasts [36]. The MEP pathway occurs in plastids, so it is reasonable that the *SlDXS* genes are located in chloroplasts [36]. It was found that the *DXS* gene family contains three subfamilies, namely DXS I, DXS II, and DXS III, each with different functions [33]. Specifically, type I *DXS* genes are mostly housekeeping genes and may be involved in plants' primary metabolism, type II *DXS* genes mostly encode for proteins that participate in plants' secondary metabolism, and type III genes may be involved in the biosynthesis of related substances on which the survival of plants depends [37]. Through a phylogenetic analysis of tomatoes with *A. thaliana*, *Z. mays*, and *O. sativa* species, the *SlDXS* genes were divided into three different branches, and it was hypothesized that DXS enzymes have different functions. *SlDXS1* was in the same branch as the *AtDXS1* and *SmDXS1* species, which indicated that *SlDXS1* might play the role of housekeeping genes [38]. Additionally, *SlDXS2* was in the same branch as the *SmDXS2* and *MtDXS2* species [39], and *SlDXS3* is a type III gene that may be involved in the synthesis of MEP pathway derivatives, which is consistent with the function of *AaDXS4* in artemisinin.

Photosynthetic pigments are important substances for primary production in plants and their levels are related to plant growth and development [40]. When subjected to adversity stress, the chlorophyll content decreases [41]. The *CrDXS1* gene in *Citrus reticulata* is positively correlated with the accumulation of carotenoid content [42]. The overexpression of the *AtDXS1* gene from *Arabidopsis thaliana* increased the chlorophyll and carotenoid content [43]. The overexpression of the *GmDXS* gene of *Glycine max* significantly increased the photosynthetic pigment content [44]. Silencing the *SlDXS* genes in tomato leaves using VIGS technology showed that silencing the *SlDXS1* gene resulted in the photobleaching of plant leaves. Meanwhile, silencing *SlDXS2* and *SlDXS3* did not show a bleaching phenotype. This indicates that the *SlDXS1* gene is involved in chlorophyll synthesis.

The results of the prediction and analysis of the tomato *DXS* gene family promoter's cis-acting elements showed that *DXS* may respond to a variety of abiotic stresses and hormones. After the salt stress treatment, *SlDXS1*, *SlDXS2*, and *SlDXS3* peaked in expression at different times, suggesting that the expression of the tomato *DXS* gene can be increased by salt stress. The expression levels of both the *AaDXS2* and *AaDXS3* genes were significantly increased in *Artemisia annua* after salt stress treatment [34]. *Populus trichocarpa* seedlings showed an increased expression of the *PtDXS* gene after NaCl treatment [45]. The *PmDXS* gene showed an upward trend after salt stress in *Pinus massoniana* [46]. Under the treatment of different exogenous hormones, the expression of the tomato *SlDXS* genes varied and showed different patterns, which may be related to the different functions of the *SlDXS* gene family. After the tomato leaves were treated with ABA, the expression levels of both *SlDXS1* and *SlDXS2* showed decreasing trends, whereas *SlDXS3* was responsive to ABA. After the GA treatment, the expression levels of *SlDXS2* and *SlDXS3* increased rapidly, indicating that they had more rapid responses to GA. After the exogenous GA treatment of *Camellia sinensis*, the expression of the *CsDXS* gene reached its maximum at 4 h, which was 1.3 times of that of CK [47]. In the process of regulating a plant's metabolism, different stress conditions can have inducing, promoting, or inhibiting effects, thus affecting the formation and accumulation of secondary metabolites in plants.

It was found that exogenous MeJA could significantly regulate the accumulation of secondary metabolites, and exogenous MeJA induced the accumulation of volatile monoterpenes in grape pericarp [48]. WRKY transcription factors in *P. grandiflorus* can regulate the

synthesis of triterpenoid compounds in response to MeJA [49]. Exogenous MeJA treatment significantly increases lavender's monoterpene and sesquiterpene contents [50]. Applying exogenous MeJA treatment to Goosegrass rhizomes leads to an increase in the triterpenoid saponin content [51]. It was found that the expression of *MnDXS2A* and *MnDXS2B* is up-regulated by the exogenous MeJA treatment of mulberry seedling leaves, which was hypothesized to be possibly related to certain metabolites in the plant and plant defense system [36]. In this study, where MeJA was used to treat tomato leaves, *SlDXS1* and *SlDXS3* were not responsive to MeJA, whereas the expression of *SlDXS2* was up-regulated to the maximum at 3 h, indicating that *SlDXS2* can be significantly induced by exogenous MeJA. This may be related to the presence of a cis-acting element (TGACG-motif) in the tomato *SlDXS2* gene in response to methyl jasmonate, which may explain why the MeJA treatment significantly induced the expression of the key *SlDXS2* gene in tomatoes.

As a common signaling substance in plants, methyl jasmonate (MeJA) induces the production of volatiles to help plants avoid pests, thus reducing the damage caused to plants [52]. The silencing of the *SlDXS2* gene in tomato leaves using VIGS technology showed that *SlDXS2* was able to respond to an exogenous MeJA expression in silenced plants. However, the induction level was low. It was found that *SlDXS2* was trauma-responsive in RNAi plants, but the expression level was significantly reduced [53]. Flavonoids are important secondary metabolites synthesized by plants, that help them to develop insect resistance. After plants are infested by insect pests, flavonoids are synthesized and accumulated in a plant's body, and the higher their content, the higher the plant's ability to resist insects [28]. The flavonoid content of tomato leaves increased significantly after their treatment with MeJA. However, MeJA induced the synthesis of flavonoids at lower levels in the silenced plants compared to the wild-type plants. This indicates that MeJA promotes secondary metabolism in tomato plants less after *SlDXS2* silencing. This study lays the foundation for future studies.

5. Conclusions

In this study, three members of the tomato *DXS* gene family were identified, and their physicochemical properties, gene structures, phylogenetic relationships, and cis-acting elements were analyzed using bioinformatics. An RT-qPCR was used to analyze the expression patterns of the *DXS* genes under different stresses, and it was found that the expression of the *SlDXS2* gene was increased by an exogenous MeJA treatment. The silencing of the *SlDXS* gene verified that *SlDXS1* is associated with the accumulation of photosynthetic pigments through a virus-induced gene silencing (VIGS) method study. The silencing gene known as pTRV-*SlDXS2* was also verified to be capable of responding to an exogenous MeJA expression. This study provides a reference for the further elucidation of the biological function of *SlDXS* genes from the terpenoid synthesis pathway in tomato plants.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/horticulturae10030304/s1: Figure S1: Expression patterns of tomato DXS gene under abiotic stress; Table S1: Primer sequences used for RT-qPCR amplification of *SlDXS*; Table S2: Sequences of RT-PCR primers; Table S3: Consensus sequence of predicted *SlDXS* motifs in tomato; Table S4: *SlDXS* nucleotide sequences of tomato; Table S5: *SlDXS* protein sequences of tomato.

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