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Overexpression of *RrGGP2* and *RrDHAR* Increases Ascorbic Acid Content in Tomato

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Abstract: Ascorbic acid (AsA) is the most abundant antioxidant in plants and is an important nutritional index for agricultural products. Some plants, such as *Rosa roxburghii* Tratt., contain exceptionally high levels of AsA, but are relatively unpalatable. In view of its role in human health, as well as plant growth and development, we examined the effects of two important AsA regulatory genes from *R. roxburghii* in tomato, with the aim of producing a crop of higher nutritional quality. *RrGGP2* and *RrDHAR* were cloned from *R. roxburghii* fruit. The overexpression vectors were made using 35S promoters and mediated by *Agrobacterium tumefaciens* to obtain the overexpression lines. A PCR and qRT-PCR verified that the two genes had been inserted and overexpressed in the tomato leaves and fruits. The results showed that the overexpression of *RrGGP2* increased tomato leaf and fruit AsA content by 108.5% and 294.3%, respectively, while the overexpression of *RrDHAR* increased tomato leaf and fruit AsA content by 183.9% and 179.9%. The overexpression of *RrGGP2* and *RrDHAR* further changed the expression of genes related to AsA metabolism, and the upregulation of one such gene, *SlGGP,* may have contributed greatly to the increase in AsA. Results here indicate that *RrGGP2* contributes more towards fruit AsA accumulation in tomato than *RrDHAR*.

Keywords: *Rosa roxburghii* Tratt; tomato; ascorbic acid; *RrGGP2*; *RrDHAR*

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1. Introduction

Ascorbic acid (AsA, Vitamin C) is an essential antioxidant in plants that scavenges reactive oxygen species (ROS) generated during exposure to biotic or abiotic stresses and those produced during normal growth and development [1–3]. Studies have also found that AsA regulates genes which control plant development [4,5], as well as flowering time regulation, premature senescence, and programmed cell death [6]. Unlike plants, humans cannot synthesize AsA independently due to the absence of a crucial enzyme called L-gulonolactone oxidase, and insufficient dietary AsA intake can cause a deficiency and related diseases in humans [7]. The predominant source of AsA is plant products such as fresh fruits or vegetables [8]; therefore, genetic engineering techniques have been extensively studied to enhance the AsA content of horticultural plant products [9].

Several pathways for AsA biosynthesis have been identified, including the D-mannose/L-galactose pathway [10], the D-galacturonate pathway [11], the myoinositol pathway [12], and the L-gulose pathway [13]. Among them, the L-galactose pathway has been identified as the principal pathway for AsA biosynthesis in many higher plants, such as apple [14], kiwifruit [15], *R. roxburghii* [16], blueberry [17], and tomato [18], and is the only pathway present in *Arabidopsis* [19]. In this pathway, D-glucose-6-phosphate is used as a substrate, which sequentially passes through glucose-6-phosphate isomerase (GPI), phosphomannose

isomerase (PMI) [20], phosphomannose mutase (PMM) [21], GDP-mannose pyrophosphorylase (GMP) [22], GDP-mannose-3′,5′-phenotypic isomerase (GME) [13], GDP-L-galactose phosphorylase (GGP) [19,23], L-galactose-1-phosphatase (GPP) [24,25], L-galactose dehydrogenase (GDH) [26], and L-galactose-1,4-lactone dehydrogenase (GLDH) [27]. As a vital antioxidant and electron donor, AsA is metabolized to monodehydroascorbic acid (MDHA) and dehydroascorbic acid (DHA) by the reactions of ascorbate oxidase (AO) and ascorbate peroxidase (APX) [28], and hydrogen peroxide (H_2O_2) is consumed to form malonyl H_2O and O_2. MDHA and DHA are catalyzed by monodehydroascorbate reductase (MDHAR) and dehydroascorbate reductase (DHAR) [29,30], respectively.

All genes in this pathway have been cloned, with several identified as key genes for AsA accumulation in various plants. Interestingly, studies on the same plant have shown inconsistent findings regarding the key genes for AsA accumulation. For instance, in kiwifruit, GGP was originally believed to be the key gene [15,31], but later, GPP was found to play this role [32]. In apple, initial evidence suggested that GDH, GPP, and GME expression were closely linked to AsA accumulation [14], but later studies showed that GGP was the key gene [33]. In tomato, GME was initially believed to be the key rate-limiting gene for AsA regulation [34,35], but recent research indicates that GGP is the actual key gene [33,36]. These variations in conclusions could be due to varietal differences or caused by experimental methods [33], and this controversy also exists in *R. roxburghii*. One research study has suggested that *RrDHAR* plays a pivotal role in achieving the high levels of AsA accumulation seen in *R. roxburghii* fruit [37], while another study has indicated that *RrGGP2* plays a more significant regulatory role in *R. roxburghii* AsA biosynthesis [38,39]. In this study, we aim to explore the roles of *RrGGP2* and *RrDHAR* in AsA biosynthesis and determine which makes the greatest contribution to the accumulation of AsA in *R. roxburghii*.

R. roxburghii belongs to the Rosaceae family, which is cultivated as a functional fruit rich in a variety of nutrients and health-promoting compounds [40–42]. It is primarily found in Southwest China and referred to as the 'King of Vitamin C' because of its high vitamin C content [43,44]. Therefore, understanding the mechanism behind this exceptional AsA accumulation and identifying the key genes responsible for its regulation would be significant. However, due to the absence of effective genetic transformation and regeneration systems, *RrGGP2* and *RrDHAR*'s functional validation is best conducted in model organisms, such as *Arabidopsis* and tobacco [37,39]. Such an approach, however, has its limitations, as they are not food crop plants and hence do not produce fruits for human consumption. As a consequence, this study aims to shed light on the functions of *RrGGP2* and *RrDHAR* by performing genetic transformation experiments in tomato, a food crop plant that bears fruit, to provide more impactful and informative evidence regarding the mechanism of high AsA accumulation in *R. roxburghii*.

2. Materials and Methods

2.1. Plant Materials and Growth Conditions

The donor material used was a three-year-old asexual line of *R. roxburghii* 'Guinong 5', while tomato plants ('Ailsa Craig') were selected as the receiver plants. Wild and subsequently obtained transgenic plants were grown in a greenhouse under specific conditions, including a temperature range of 25–28 °C, a humidity level of 60–80%, a light intensity between 30,000–50,000 lux, and a light/dark alternation time of approximately 13/11 h. The tomato plants were irrigated with Hoagland's nutrient solution every five days and watered every 2–3 days, according to standard production practices. The tomatoes were managed until maturity, which typically occurred after around 50 days of self-pollination. The 4th–6th leaves and mature fruits were then harvested and snap-frozen using liquid nitrogen, before being stored in an ultra-low temperature refrigerator at −80 °C for subsequent experiments.

2.2. Total DNA and RNA Extraction, cDNA Synthesis

The Genome DNA Extraction Kit (BioTeke, Wuxi, China) was used to extract total genomic DNA from the leaves of both 'Guinong 5' and 'Ailsa Craig'. Moreover, total RNA was extracted from the fruits of 'Guinong 5' and the leaves and fruits of 'Ailsa Craig' using the RNAprep Pure Plant Plus Kit (TIANGEN, Beijing, China). To synthesize the first strand of cDNA, the PrimeScript™ RT reagent Kit (Takara, Dalian, China) was used.

2.3. Cloning of Target Gene and Constructing of Overexpression Vector

The transformation vector was constructed using pcambia1301-ky (Figure 1), and the target gene was integrated into the vector by homologous recombination. To achieve this, homologous cloning primers were designed based on the sequences of the target gene [45] and vector. The forward and reverse cloned primers both included the *Kpn*I restriction enzyme site at the 5′ end of each. Subsequently, the full-length *RrGGP2* and *RrDHAR* were amplified via PCR from 'Guinong 5′ cDNA using homologous recombination clone primers (Table 1), while the vector was digested using the *Kpn*I enzyme. Finally, *RrGGP2* and *RrDHAR* were introduced into the *Kpn*I polyclonal site located between the 35S promoter and the nos terminator of the vector through the action of recombinase (ClonExpress II One Step Cloning Kit, Vazyme). The resultant recombinant vector containing the *RrGGP2* or *RrDHAR* gene sequence was then transferred into the *Agrobacterium tumefaceiens* strain LBA4404 and saved for transformation into tomato plants.

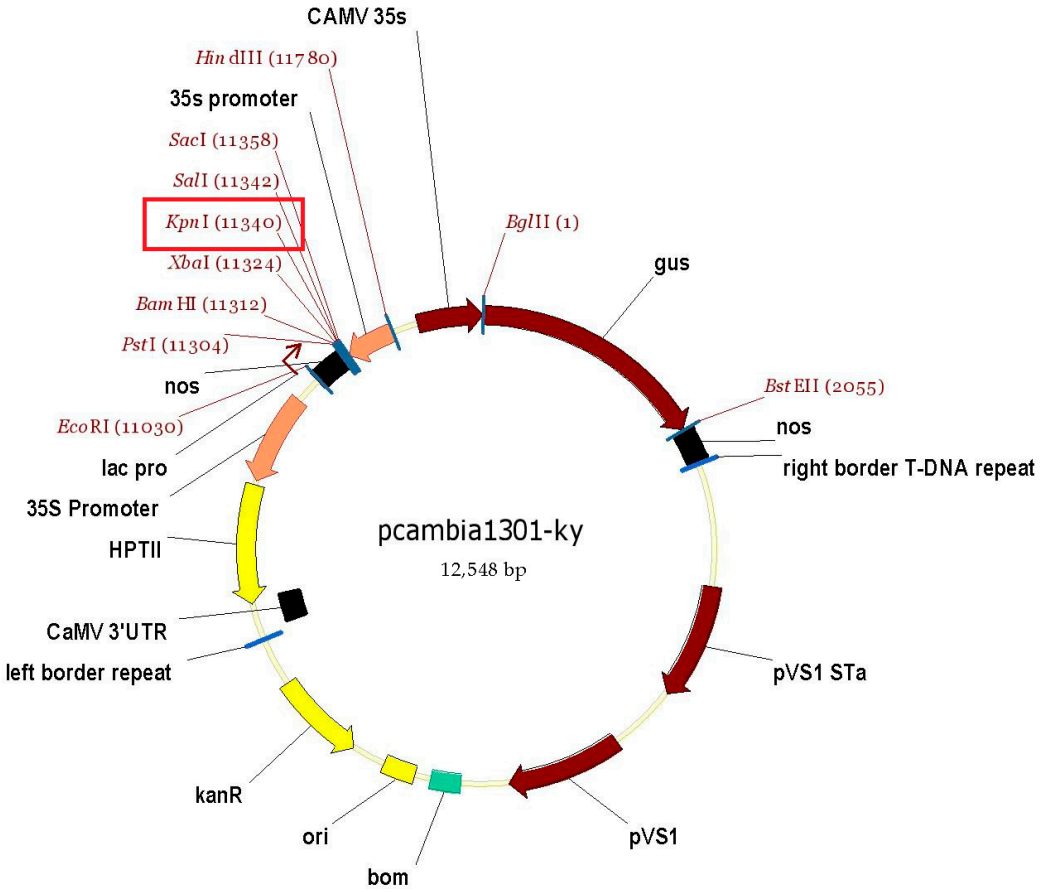

Figure 1. A map of the pcambia1301-ky overexpression vector, which is regulated by the 35S promoter and the nos terminator. The vector includes a reporter gene (gus) and is designed with both kanamycin (KanR) and hygromycin (HPTII) resistance genes for screening transformed plants. The restriction enzyme site is at the red rectangle.

Table 1. Primer names and sequences used in this experiment.

Experiment Purpose	Gene Abbreviation	Gene ID	Primer Sequences (5′-3′)
Homologous recombination clone	*RrGGP2*	evm.model.Contig360.207	F:GCGGGTCGACGGTACCATGCTGAAGATC-AAGAGGGTGCGGGTCGACGTACCATGGGT R:TAGACATATGGGTACCTTACTGCAGAA-CGAGGCATTCTAGATATATGGGGTATTC
	RrDHAR	evm.model.Contig319.146	F:GCGGGTCGACGGTACCATGGCTCTTGAGGTT-GCTGCGCGGTCGACGTACCATGGCTGC R:TAGACATATGGGTACCCTATTTGGGGTTGACTTT CGG-TAGACATATGGGTACCCTATTTGGGGTTGACTTTCGG
PCR identification	*RrGGP2*	evm.model.Contig360.207	F:ATGCTGAAGATCAAGAGGGTTCCCAC R:TTACTGCAGAACGAGGCATTCCTGT
	RrDHAR	evm.model.Contig319.146	F:ATGGCTCTTGAGGTTGCTGC R:CTATTTGGGGTTGACTTTCGGCT
qRT-PCR	*RrGGP2*	evm.model.Contig360.207	F:AAGCTCCTGGCTGAGGTCTCT R:CCATCATCGCCACCACAAGCAAT
	RrDHAR	evm.model.Contig319.146	F:ACAAGCCCCAATGGTTTACAGA R:CCTCAGAATCCCAGCAAGCAC
	SlGPI	Solyc04g076090	F:TGCTCTTCAAAAGCGTGTCC R:CGGCAATAAGTGCTCTGTCA
	SlPMI	Solyc02g086090	F:TACATTGTGGTGGAACGAGGA R:ACCCCATTTGGCAAGAACAG
	SlPMM	Solyc05g048760	F:TTTACCCTCCATTACATTGCTGA R:CTTCTTGACTACAGTTTCTCCCA
	SlGMP	Solyc03g096730	F:AAACCTGAAATCGTGATGTGAGA R:TGAAGAAGAGGAGAACTGGAAAC
	SlGME	Solyc01g097340	F:AATCCGACTTCCGTGAGCC R:CTGAGTTGCGACCACGGAC
	SlGGP	Solyc06g073320	F:GAAATCTGGTCTGTTCCTCTGTGA R:TTCACACACCAACTCCACATTACA
	SlGPP	Solyc04g014800	F:AGCCGCTACAAACCCTCATCT R:TGTCCGCTTTCCATCTCCTAT
	SlGDH	Solyc01g106450	F:CTTCTTACTGAGGCTGGTGGTC R:AACCTCTTTAACAGACTTCATCCC
	SlGLDH	Solyc10g079470	F:ATTGAGGTTCCCAAGGACATAG R:ATGTTATTAGATAGGATGCGGTTT
	SlAO	Solyc04g054690	F:AGGATGGCTCAGAGTGTT R:ATCAGGTAAGGCGTATGG
	SlAPX	Solyc06g005150	F:TGGAGCCCATTAGGGAGCA R:GCCAGGGTGAAAGGGAACAT
	SlDHAR	Solyc05g054760	F:CCTACCTTCGTCTCATTTCCG R:TGAACAAACATTCTGCCCATT
	SlMDHAR	Solyc09g009390	F:GGTGATGTTGCCACTTTTCCTTT R:CGACAGACTTCCCTTGCTCACT
	Actin	Solyc11g005330	F:GTCCTCTTCCAGCCATCCA R:ACCACTGAGCACAATGTTACCG

Note: *RrGGP2* and *RrDHAR* are the numbers in the *R. roxburghii* genome data file; Actin was used as the internal control; F = forward, R = reverse.

2.4. Plant Transformation

The transgenic tomato was created through genetic transformation mediated by *Agrobacterium tumefaciens*. Initially, tomato cotyledons were cultured under light for 7 days, following which they were infected with *Agrobacterium tumefaciens* for 10 min and cocultured for 2 days. A screening culture was then carried out to encourage the growth of adventitious buds using the MS medium supplemented with 2.0 mg/L zeatin (ZT), 1 mg/L indole-3-acetic acid (IAA), 300 mg/L timentin, and 10 mg/L hygromycin. Finally, the adventitious buds were transferred to a 1/2 MS medium containing 150 mg/L timentin and 10 mg/L hygromycin to induce root growth and recover the whole plant.

2.5. Transgenic Plants Identification and qRT-PCR Analysis

To identify transgenic tomato plants resulting from plant transformation, we used a PCR with genomic DNA from leaves and a qRT-PCR with cDNA from both leaves and

fruits. Additionally, a qRT-PCR was utilized to analyze the gene expression related to AsA biosynthesis and metabolism in the leaves and fruits of both wild-type and transgenic plants. The genes analyzed included GPI, PMI, PMM, GMP, GME, GGP, GPP, GDH, and GLDH in the L-galactose pathway, AO and APX in the catabolism pathway, as well as DHAR and MDHAR in the recycling pathway. Previous studies [46,47] were referenced for primer design. The *Acitin* gene of *Solanum lycopersicum* (Solyc11g005330) was utilized as an internal reference gene to normalize expression data. All the primers were synthesized through Sangon Bioengineering Co., Ltd., (Shanghai, China) (Table 1). The qRT-PCR was performed on an ABI ViiA 7 DX system (Applied Biosystems, Waltham, MA, USA) using TB Green® Premix Ex Taq™ II kit (Takara). The qRT-PCR reaction system was 20 μL: SYBR Premix Ex Taq (2×) 10 μL, 10 μmol/L forward and reverse primers 1 μL each, c-DNA 1 μL, ddH_2O 7 μL. Reaction procedure was 95 °C 30 s; 95 °C 5 s, 55~60 °C 20 s, 40 cycles; 72 °C 20 s. Melting curve procedure was 95 °C 15 s; 55~95 °C 1 min in 0.3 °C increments; 95 °C 15 s. All reactions were performed in three replicates and the relative expression was calculated using the $2^{-\Delta\Delta CT}$ method.

2.6. Determination of AsA and DHA Content

The concentration of AsA and DHA were determined according to a previous method with minor modifications [48]. An amount of 0.5 g of leaf or fruit was ground into a homogenate in 5% metaphosphoric acid, centrifuged at 7500 rpm for 20 min at 4 °C, and the supernatant was collected. A total of 100 μL of supernatant was pipetted with 2.9 mL of 100 mmol/L potassium phosphate buffer (pH 6.8), and the change of absorbance value at 265 nm was recorded when 1 U of AAO was added. For the determination of DHA, 100 μL of the extract was added to 1.9 mL of 100 mmol/L potassium phosphate buffer (pH 6.8), and the absorbance at 265 nm was recorded after the addition of 2 mmol/L DTT. At the same time, standard curves were made with known concentrations of AsA and DHA solutions using the same method to calculate the AsA and DHA contents of the samples. Each tomato line was measured in triplicate.

2.7. Statistical Analysis

All data were determined in three independent biological replicates for each experiment. Data were counted and graphs were made using Excel 2019. Significant differences were tested by Duncan's method and correlation analysis was performed using Pearson's method in SPSS 26.0 software. The significance levels remained $p < 0.05$ and $p < 0.01$, respectively.

3. Results

3.1. Positive Identification of Transgenic Tomato Lines

A total of four tomato lines each overexpressing *RrGGP2* and *RrDHAR* were obtained by genetic transformation. The genome DNA of the transgenic tomato was used as the template, wild-type tomato DNA was utilized as a negative control, and *R. roxburghii* DNA was used as a positive control, using the specific primers (Table 1) to determine the presence of *RrGGP2* and *RrDHAR* in the transgenic tomato lines. After agarose gel electrophoresis, the electrophoretic bands of the four lines overexpressing *RrGGP2* (Figure 2A) and the four lines overexpressing *RrDHAR* (Figure 2B) were of the same intensity as the positive control. However, in the case of the wild-type tomato, the target fragments were not amplified (Figure 2A,B), indicating that the *RrGGP2* and *RrDHAR* genes of *R. roxburghii* have been successfully integrated into the tomato genome. The results of the qRT-PCR further confirmed that the expression levels of *RrGGP2* and *RrDHAR* in the leaves and fruits were significantly higher than the control (Figure 2C,D), thus proving that the two genes were successfully overexpressed in the tomato lines.

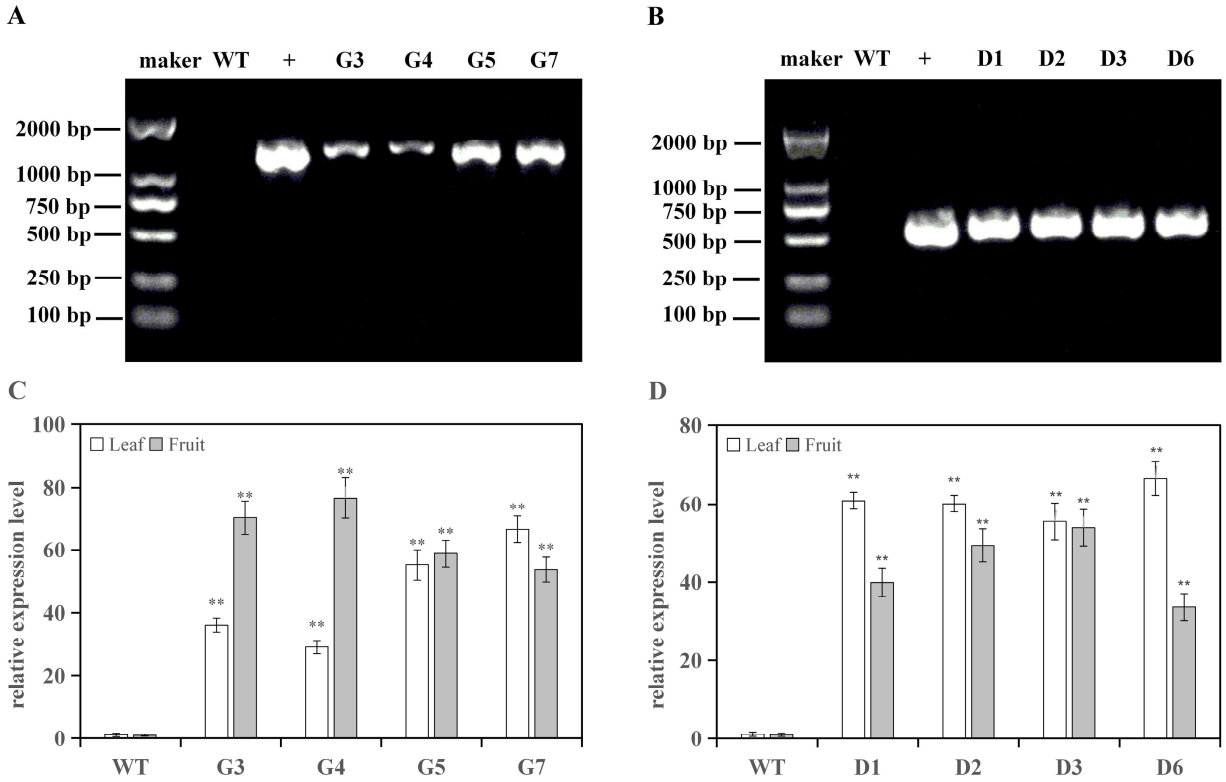

Figure 2. Positive identification and overexpression verification of transgenic tomato lines. (**A**) confirmation of *RrGGP2* by PCR analysis; (**B**) confirmation of *RrDHAR* by PCR analysis; (**C**) relative expression of *RrGGP2* in transgenic tomato plants; (**D**) relative expression of *RrDHAR* in transgenic tomato plants. *RrGGP2* transgenic lines are represented by G3, G4, G5, and G7, and *RrDHAR* transgenic lines are represented by D1, D2, D3, and D6. M = marker; WT = wild-type plant; + = positive control (PCR amplification products of the GGP2 and DHAR gene of *R. roxburghii*); bp—number of bases. The relative quantification of *RrGGP2* and *RrDHAR* expression was calculated using the comparative Ct ($2^{-\Delta\Delta CT}$) method; three replicate experiments were performed; error bars represent standard error, means ± SE. The asterisks above the bar chart represent significant differences from WT (** $p < 0.01$).

3.2. Analysis of Ascorbate Levels in Transgenic Lines

3.2.1. Analysis of Ascorbate Levels in Transgenic Tomato Fruits

The determination results indicate that the overexpression of *RrGGP2* and *RrDHAR* genes substantially augmented the levels of AsA and DHA in tomato fruits (Figure 3A,B). The content of AsA increased significantly from 209.7% to 384.3% in all the four tomato lines that overexpressed *RrGGP2*, and there was an increase in DHA that ranged from 69.2% to 309.0% (Figure 3A). Similarly, in the four tomato lines overexpressing *RrDHAR*, AsA content was increased by 175.4% to 213.6%, and DHA content was increased from 116.8% to 261.2% (Figure 3B). On average, the overexpression of *RrGGP2* in the tomato lines resulted in an increase of 294.3% for AsA and 190.8% for DHA, compared to an increase of 179.9% for AsA and 182.6% for DHA in the tomato lines overexpressing *RrDHAR* (Figure 3A,B). Hence, it is clear that the overexpression of *RrGGP2* is more effective in enhancing the AsA content of tomato fruits than the overexpression of *RrDHAR*.

3.2.2. Analysis of Ascorbate Levels in Transgenic Tomato Leaves

The overexpression of *RrGGP2* and *RrDHAR* genes also significantly increased AsA and DHA contents of transgenic tomato leaves (Figure 3C,D). Notably, the AsA content in the leaves of the four tomato lines overexpressing *RrGGP2* was significantly increased, ranging between 99.1% and 116.3%, while DHA content increased from 2.6% to 188.9%;

however, only two lines (G4 and G7) of transgenic lines showed a significant increase (Figure 3C). In contrast, the AsA content in the leaves of the four overexpressing *RrDHAR* tomato lines was significantly increased between 136.6% and 243.5%, and DHA content increased from 55.8% to 220.5% (Figure 3D). On average, the AsA and DHA levels in the leaves of tomato lines overexpressing *RrGGP2* increased by 108.5% and 75.7%, respectively. Similarly, in the tomato plants overexpressing *RrDHAR*, both the AsA and DHA levels in the leaves increased by 183.9% and 127.3%, respectively (Figure 3C,D). Therefore, it is evident that the overexpression of *RrDHAR* leads to a more pronounced increase in the AsA and DHA content in tomato leaves than *RrGGP2*.

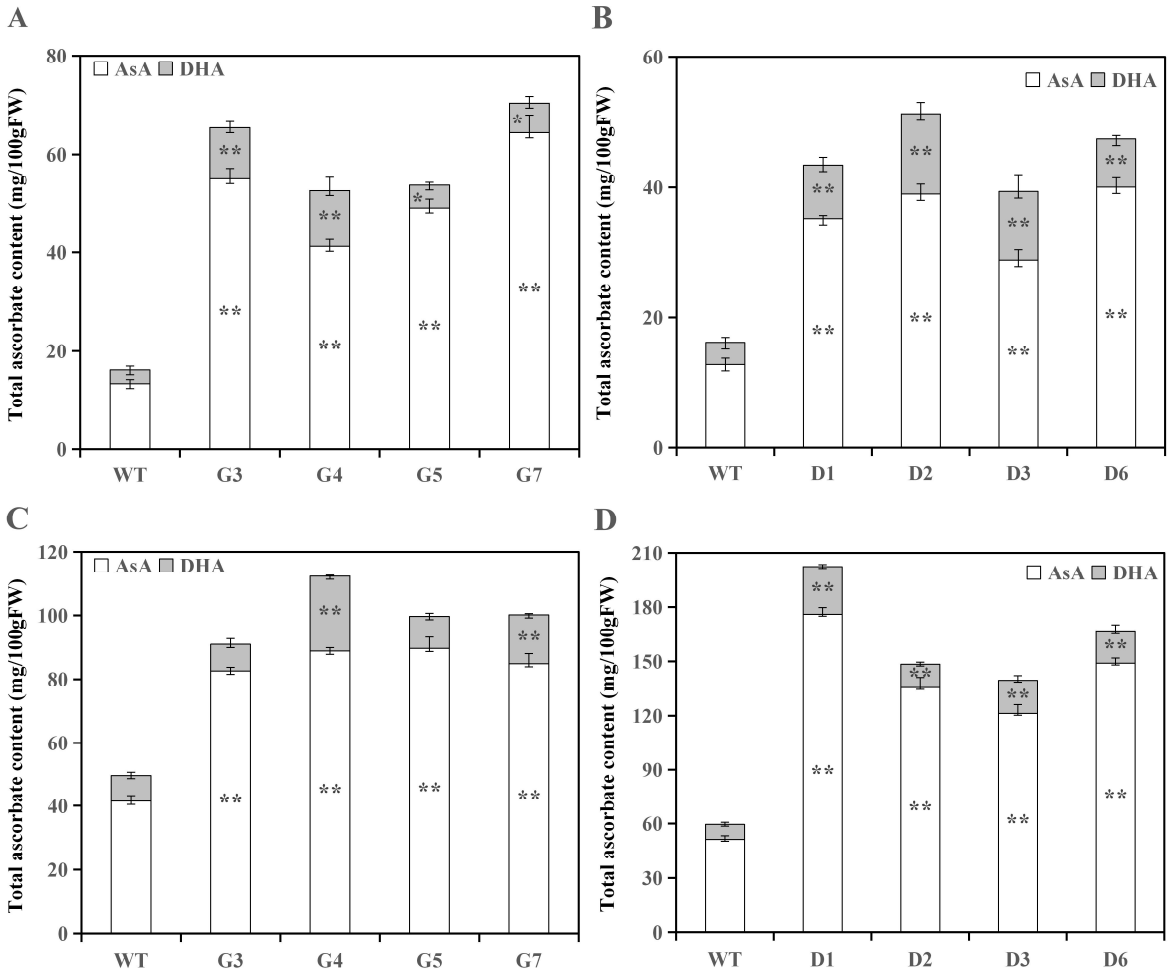

Figure 3. Determination of AsA and DHA contents in tomato lines overexpressing *RrGGP2* and *RrDHAR*. (**A**) AsA and DHA contents in transgenic *RrGGP2* fruits; (**B**) AsA and DHA contents in transgenic *RrDHAR* fruits; (**C**) AsA and DHA contents in transgenic *RrGGP2* leaves; (**D**) AsA and DHA contents in transgenic *RrDHAR* leaves. Three replicate experiments were performed; error bars represent standard error, means ± SE; FW—fresh weight. The asterisks represent significant differences from WT (* $p < 0.05$; ** $p < 0.01$).

3.3. Analysis of Genes Expression in Transgenic Lines

3.3.1. Analysis of Genes Expression in Transgenic Tomato Fruits

The overexpression of *RrGGP2* and *RrDHAR* affected the expression of a range of genes related to the biosynthesis, catabolism, and recycling of AsA in transgenic tomato line fruits. In four tomato lines overexpressing *RrGGP2*, the expression of *SlPMI*, *SlGME*, *SlGGP*, *SlAO*, *SlDHAR*, and *SlMDHAR* were upregulated, and *SlGPI*, *SlPMM*, *SlGMP*, *SlGPP*, *SlGLDH*, and *SlAPX* were downregulated; *SlGDH* was significantly upregulated in three lines, while in line G7 it was significantly downregulated (Figure 4A). Furthermore, in

the four tomato lines overexpressing *RrDHAR*, the expression of *SlGMP*, *SlGME*, *SlGLDH*, *SlAO*, *SlAPX*, *SlDHAR*, and *SlMDHAR* was upregulated, while *SlGPI*, *SlPMI*, *SlGGP*, and *SlGPP* were downregulated. The expression of *SlPMM* and *SlGDH* was not significantly different from the control (Figure 4B).

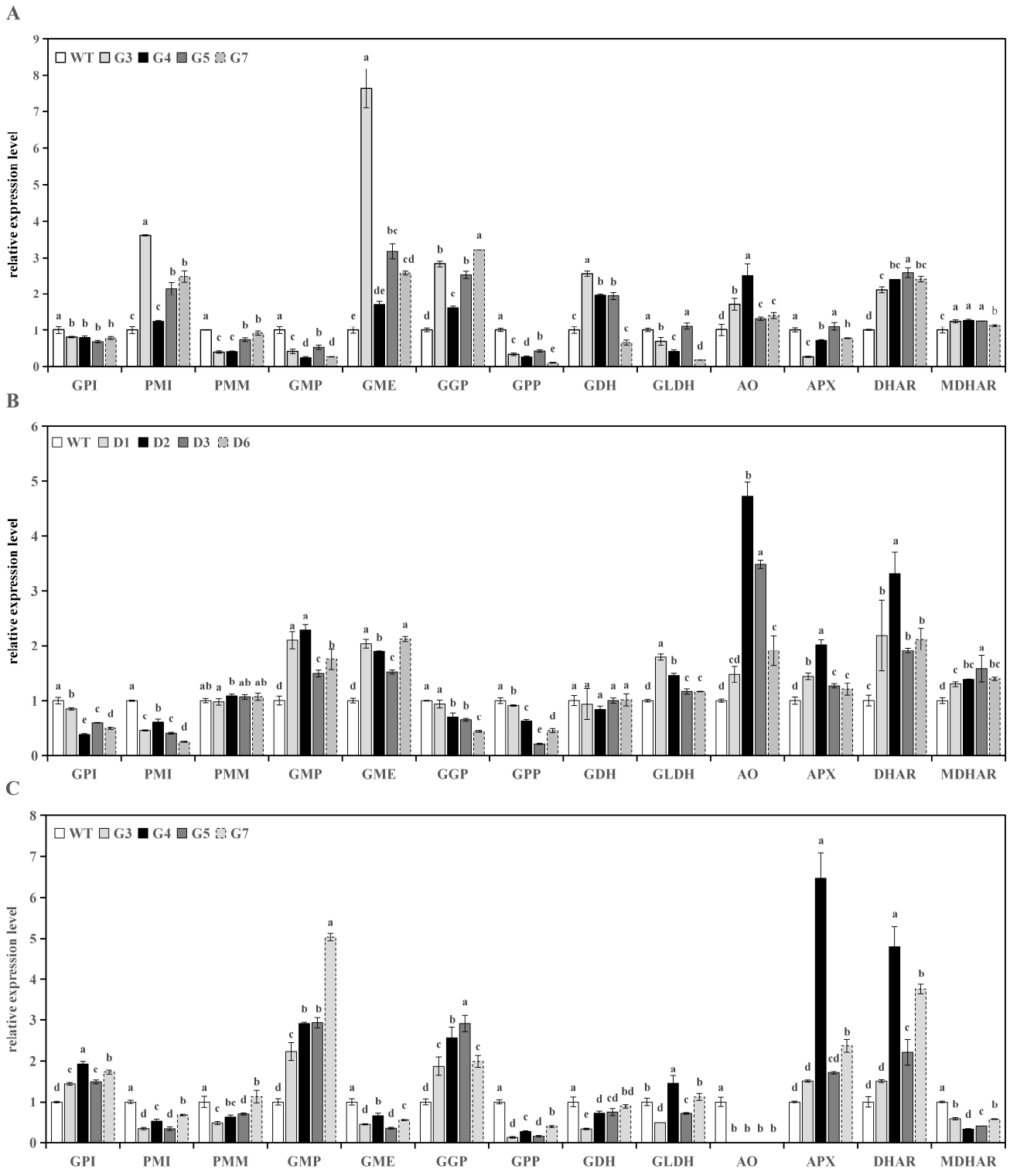

Figure 4. *Cont.*

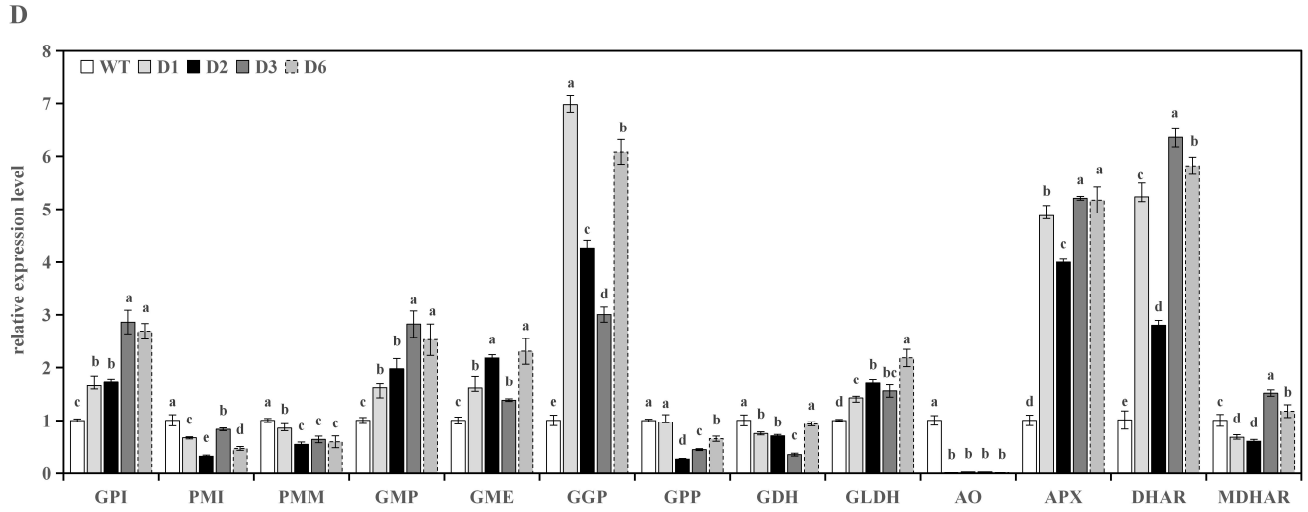

Figure 4. Relative expression of AsA biosynthesis, metabolism, and recycling-related genes in the WT and transgenic lines. (**A**) Expression of transgenic *RrGGP2* fruits; (**B**) expression of transgenic *RrDHAR* fruits; (**C**) expression of transgenic *RrGGP2* leaves; (**D**) expression of transgenic *RrDAHR* leaves. The relative expression levels of each gene were obtained using the comparative Ct ($2^{-\Delta\Delta Ct}$) method; three replicate experiments were performed; error bars represent standard error, means ± SE; the same letter above bars indicates a nonsignificant difference at the $p < 0.05$ probability level.

3.3.2. Analysis of Genes Expression in Transgenic Tomato Leaves

The overexpression of *RrGGP2* and *RrDHAR* also significantly affected the expression of genes related to AsA biosynthesis and metabolism in transgenic tomato line leaves. In the leaves of the four tomato lines overexpressing *RrGGP2*, the expressions of *SlGPI*, *SlGMP*, *SlGGP*, *SlAPX*, and *SlDHAR* were upregulated, and *SlPMI*, *SlPMM*, *SlGME*, *SlGPP*, *SlGDH*, *SlAO*, and *SlMDHAR* were downregulated (Figure 4C). *SlGLDH* was upregulated in G4 and G7 and downregulated in G3 and G5. Similarly, the expression of *SlGPI*, *SlGMP*, *SlGME*, *SlGGP*, *SlGLDH*, *SlAPX*, and *SlDHAR* was upregulated, and *SlPMI*, *SlPMM*, *SlGPP*, *SlGDH*, and *SlAO* were downregulated in the four tomato lines overexpressing *RrDHAR*. In addition, the expression of *SlMDHAR* was upregulated in G5 and G7, but downregulated in G3 and G4 (Figure 4D).

3.4. Correlation Analysis between Gene Expression and AsA Content

3.4.1. Correlation Analysis between Gene Expression and AsA Content in Transgenic Tomato Fruits

There was a significant correlation between the expression of *SlGGP* and the content of AsA in the fruits of tomato lines overexpressing *RrGGP2*; additionally, positive correlations were observed with *SlPMI*, *SlGME*, and *SlDHAR*. The expression of *SlAO* was significantly correlated with DHA (Table 2). In the *RrDHAR* overexpressing tomato lines, *SlGME* was significantly correlated with AsA content, while *SlGMP* and *SlDHAR* expression were also highly correlated with AsA levels. Furthermore, *SIAO* was observed to have a significant correlation with DHA (Table 2).

Table 2. Correlation analysis between gene expression and AsA content in transgenic tomato fruits.

		Correlation Coefficient												
		GPI	**PMI**	**PMM**	**GMP**	**GME**	**GGP**	**GPP**	**GDH**	**GLDH**	**AO**	**APX**	**DHAR**	**MDHAR**
RrGGP2	AsA	−0.802	0.739	−0.328	−0.854	0.521	0.951 *	−0.938 *	0.171	−0.574	0.270	−0.416	0.848	0.603
	DHA	−0.270	0.228	0.030	−0.708	0.361	0.097	−0.55	0.617	−0.500	0.953 *	−0.699	0.445	0.697
RrDHAR	AsA	−0.789	−0.826	0.485	0.878	0.965 **	−0.664	−0.433	−0.397	0.555	0.481	0.634	0.83	0.677
	DHA	−0.806	−0.526	0.636	0.762	0.540	−0.447	−0.600	−0.637	0.444	0.910 *	0.819	0.874	0.819

The asterisks represent significance (* $p < 0.05$; ** $p < 0.01$).

3.4.2. Correlation Analysis between Gene Expression and AsA Content in Transgenic Tomato Leaves

In the leaves of tomato lines overexpressing *RrGGP2*, the expression of *SlGGP* was significantly correlated with AsA content, and *SlGPI*, *SlGMP*, *SlAPX*, and *SlDHAR* were also positively correlated with AsA. Notably, *SlAO* had very low expression but showed a highly significant negative correlation with AsA, which matches its function of AsA catabolism. Regarding the concentration of DHA, both *SlAPX* and *SlDHAR* showed a significant correlation (Table 3). In the transgenic *RrDHAR* tomato lines, the expression of *SlGGP* was significantly correlated with AsA content, and *SlGPI*, *SlGMP*, *SlGME*, *SlGLDH*, *SlAPX*, and *SlDHAR* were also positively correlated with AsA, whilst *SlAO* showed a significant negative correlation with AsA content. For DHA, *SlAPX* and *SlDHAR* were correlated with high coefficients of correlation of 0.761 and 0.765, respectively (Table 3).

Table 3. Correlation analysis between gene expression and AsA content in transgenic tomato leaves.

		Correlation Coefficient												
		GPI	**PMI**	**PMM**	**GMP**	**GME**	**GGP**	**GPP**	**GDH**	**GLDH**	**AO**	**APX**	**DHAR**	**MDHAR**
RrGGP2	AsA	0.844	−0.869	−0.435	0.684	−0.882 *	0.885 *	−0.943 **	−0.521	0.002	−0.990 **	0.463	0.626	−0.952 **
	DHA	0.856	−0.091	−0.039	0.452	−0.007	0.455	−0.239	0.097	0.856	−0.423	0.965 **	0.962 **	−0.623
RrDHAR	AsA	0.457	−0.658	−0.501	0.468	0.663	0.956 *	−0.214	−0.275	0.629	−0.906 *	0.872	0.718	−0.265
	DHA	0.379	−0.180	−0.106	0.326	0.203	0.816	0.147	−0.366	0.274	−0.697	0.761	0.765	−0.066

The asterisks represent significance (* $p < 0.05$; ** $p < 0.01$).

4. Discussion

4.1. Overexpressing RrGGP2 and RrDHAR in Tomato Indicates That RrGGP2 Is the Key Control Point of AsA Biosynthesis and Metabolism

Over the years, genes relating to AsA biosynthesis and metabolism in *R. roxburghii* have been identified and cloned, and their functions have been verified by heterologous overexpression. For instance, the overexpression of *RrGDH* and *RrGGP2* in tobacco increased the leaf AsA content by an average of 1.1-fold and 12-fold [16,39], while increasing the leaf AsA content by 3.02-fold and 2.11-fold was achieved by overexpressing *RrDHAR* and *RrGME* in *Arabidopsis* [37]. Indeed, *RrGGP2* and *RrDHAR* are particularly good candidates for increasing AsA biosynthesis in *R. roxburghii* [49]; however, further validation of these two genes was required in a model plant that has edible fruit, such as tomato, to assess their effectiveness in a plant with commercial applications. Our results reveal that overexpressing *RrGGP2* increases AsA content in tomato fruits to a greater extent than *RrDHAR*, which suggests that *RrGGP2* may have greater potential to enhance fruit AsA content in other fruit crops via molecular breeding.

GGP catalyzes the conversion of GDP-L-galactose into L-galactose-1-phosphate, making it the first specific enzyme in the L-galactose pathway. Numerous previous studies on a variety of crops have demonstrated that overexpressing GGP can significantly augment AsA content; these crops include *Arabidopsis* [15,50–52], tobacco [23,39,53–55], tomato [31,36,56,57], rice [2,58,59], kiwifruit [60], strawberry, and potato [31]. Although other genes in this pathway may also amplify AsA content, such as PMM in Acerola and GME in alfalfa [50,61], not all genes have the same effect on recipient plants. For instance, GMP and GME in peach [62] were found to not increase AsA content. After summarizing experimental data from previous studies and conducting a comparative analysis, we have determined that overexpressing native GGP has a greater impact on fruit AsA content compared to overexpressing other genes in the AsA pathway. For instance, overexpressing *SlGGP* in tomatoes resulted in a 3-fold increase in fruit AsA content [36], which was higher than the increases observed with *SlGMP* (1.22 to 1.60-fold) [63], *SlGME* (1.22 to 1.42-fold) [35], *SlDHAR* (1.4 to 1.5-fold), and *SlMDHAR* [64]. Furthermore, overexpressing *Arabidopsis* AsA biosynthesis-related GMP, GME, GGP, GPP, GDH, and GLDH in *Arabidopsis* leaves resulted in 1.3, 1.4, 2.9, 1.5, 1.2, and 1.8-fold increases in the leaves' AsA content, respectively [51]. Although not all the differences were significant, it offers initial evidence that indicates that the single transformation of GGP is more effective than other

genes in increasing AsA content, while other genes have additive effects compared to GGP alone. While other genes may not be as efficient as GGP, studies show that co-transforming genes such as GME, GPP, and GLDH can significantly increase the AsA content, ultimately leading to an increase in AsA accumulation in plants. Metabolic control analysis of known kinetic parameters in *Arabidopsis* can explain why GGP is the key gene in this conclusion, while manipulating other genes can have minimal impact on AsA content. This is due to feedback inhibition of the GGP catalytic step that provides high flow control coefficients [52,65]. Apart from physiological and biochemical evidence, bioinformatics analysis also identified tomato GGP as a key gene for AsA biosynthesis [33,36]. AsA accumulation in 11 wild and cultivated tomatoes and QTL analysis for ascorbic acid content in strawberry fruit reveals a complex genetic architecture and association with GDP-L-galactose phosphorylase [66,67]. As we have speculated, GGP was recently identified once again as a key gene in two kiwifruits with distinct AsA content based on transcriptomic data [60], further solidifying its role in AsA accumulation.

Why does GGP play such a crucial and distinctive role in the regulation of AsA biosynthesis and metabolism? With the rapid advancement of genome sequencing technology, we now have some answers from the perspective of gene evolution. GGP genes are present in all plants, and due to their unique whole-gene replication mode, GGP is significantly amplified in angiosperms. The majority of GGP proteins have similar catalytic functions, which can be attributed to their conserved motif arrangement and composition. This may explain why angiosperms have a higher AsA content and can adapt more readily to environmental changes [68]. Previous evolutionary analysis has shown that *R. roxburghii* and strawberries are closely related [39], and strawberries are known for their high AsA content, which may be due to their relatively conservative motif arrangement and composition. Furthermore, research has revealed that the expression of GGP in various plants is significantly regulated by light and undergoes drastic changes under stress [19,69]. Analysis of the GGP promoter sequence has shown that it contains numerous cis-acting elements associated with light response and stress response [70]. Additionally, the light and photosynthesis-dependent rate-limiting enzyme GGP is activated and plays a critical role in the regulation of the ascorbate pool size [71]. Recent studies on the mechanism of GGP translation regulation propose a model that allows for a feedback response to regulate AsA synthesis under adverse conditions. Under rapidly changing conditions, uORF directly regulates GGP translation without gene transcriptional modification, and this points to a more dependable way to regulate AsA concentration [72]. All the evidence mentioned above once again confirms that, from the perspective of genetic structure composition, GGP plays a key role in AsA biosynthesis.

4.2. Similarities and Differences for the Mechanism of AsA Accumulation in Tomato Fruit and Leaf

The accumulation of AsA depends on the interplay between biosynthesis, catabolism, and cycling [73]. In this study, we comprehensively analyzed these three pathways to investigate AsA accumulation in the fruits and leaves of transgenic tomatoes. Our findings revealed both similarities and differences.

GMP, GME, and GGP have been identified as good candidates for promoting AsA accumulation in tomato [63]. The overexpression of *SlGMP*, *SlGME*, or *SlGGP* has been found to increase AsA content in tomato [35,36,63]. In our experiment, at least two of the three key genes were simultaneously upregulated in the fruits and leaves of transgenic tomatoes, which is likely why the AsA content was higher in transgenic tomatoes than in the control. Furthermore, through comparison and summarization, we suggest that there may be an additive model centered on GGP. From the perspective of increasing AsA content, GMP-GME-GGP co-expression yields higher results than GGP-GMP/GGP-GME, which is higher than GMP-GME and also higher than GMP/GME/GGP [15,56,74,75]. This may explain why the AsA content in fruits of tomatoes overexpressing *RrGGP2* is higher than that of *RrDHAR* and why the AsA content in leaves of tomatoes overexpressing *RrDHAR* is higher than that of *RrGGP2*. In transgenic *RrGGP2* tomato fruits, the expression

of *SlGME* and *SlGGP* was upregulated simultaneously, while in transgenic *RrDHAR* tomato fruits, the expression of *SlGMP* and *SlGME* was upregulated and *SlGGP* expression was downregulated. The expression of *SlGMP*, *SlGME*, and *SlGGP* was simultaneously upregulated in transgenic *RrDHAR* tomato leaves, but only *SlGMP* and *SlGGP* were upregulated in transgenic *RrGGP2* tomato leaves, and *SlGME* was downregulated. Additionally, the expression of *SlGGP* in transgenic *RrDHAR* tomato leaves was much higher than that in *RrGGP2*, and the correlation results further confirmed that the upregulation of key genes in the biosynthetic pathway, especially *SlGGP*, may be the main factor contributing to the increase in AsA. Recent studies have shown that GMP, GME, and GGP proteins are located in the cytoplasm [65] and that these enzyme complexes likely interact, demonstrating channelization [76]. This finding confirms the complex regulation of AsA pool size in tomato.

In terms of catabolism and recycling pathways, *SlAPX* was found to be significantly upregulated in the leaves of both transgenic tomatoes. It was observed that the high expression of *SlAPX* could be detrimental to AsA accumulation, while almost no expression of *SlAO* was very beneficial to AsA accumulation [77,78]. Although the expression levels of the two genes were opposite, the final phenotypic results of tomatoes showed that AsA was still elevated. The correlation analysis results confirmed the positive effect of *SlAO* on AsA accumulation. However, it was not immediately clear why the significant downregulation of *SlAPX* expression did not result in AsA content remaining constant or decreasing. This may be related to the multiple functions of APX in plants; in addition to its oxidative function in AsA metabolism, APX also acts as an important antioxidant enzyme to scavenge ROS under various stress conditions. The overexpression of APX can improve tolerance to various stresses in tobacco [79], tomato [80], and *Arabidopsis* [81,82]. Therefore, the upregulation of *SlAPX* expression may not only affect the catabolism of AsA but also perform tasks related to stress resistance. Interestingly, the expression of *SlAO* or *SlAPX* in fruits was not significantly inhibited; this may be because fruits contain more abundant secondary metabolites and more hierarchical biological structures than leaves, thus having a potentially higher stress resistance effect. The expression of *SlDHAR* was significantly upregulated in the fruits and leaves of the two transgenic tomatoes; although the correlation coefficient was not statistically significant, it was still at a relatively high level with all biosynthetic and metabolic genes. We believe that the upregulation of *SlDHAR* expression in the recycling pathway is also an important factor for the accumulation of AsA in tomatoes. In addition to the function of DHA recovery, the AsA-GSH system involved in DHAR plays an important role in managing H_2O_2 and indirectly participates in ROS scavenging to protect plants from environmental stress and better accumulate AsA [83–88]. The expression of *SlMDHAR* has no obvious regularity in transgenic lines. Some studies have shown that MDHAR is not a key gene regulated by AsA [64].

5. Conclusions

In summary, this is the first functional validation of *RrGGP2* and *RrDHAR* in tomato, a valuable agricultural crop. The results indicate that the overexpression of *RrGGP2* and *RrDHAR* can increase AsA content in tomato leaves and fruits, with the effect of *RrGGP2* being more significant in fruits. Expression measurements of genes relating to AsA biosynthesis and catabolism in transgenic lines revealed both similarities and differences in the mechanisms of AsA accumulation in fruits and leaves, in which *SlGGP* may play a key role. Additionally, this study provides evidence that can lead to a better understanding of the crucial role that *RrGGP2* plays in *R. roxburghii* AsA biosynthesis.

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