

RESEARCH ARTICLE

Manipulation of the Rice L-Galactose Pathway: Evaluation of the Effects of Transgene Overexpression on Ascorbate Accumulation and Abiotic Stress Tolerance

Gui-Yun Zhang^{1,2}, Ru-Ru Liu¹, Chang-Quan Zhang¹, Ke-Xuan Tang³, Ming-Fa Sun², Guo-Hong Yan², Qiao-Quan Liu^{1*}

1 Key Laboratory of Crop Genetics and Physiology of Jiangsu Province, Co-Innovation Center for Modern Production Technology of Grain Crops, Yangzhou University, Yangzhou 225009, Jiangsu, China, **2** Agricultural Science Institute of Coastal Region of Jiangsu, Yancheng 224002, Jiangsu, China, **3** School of Agriculture and Biology, Shanghai Jiao Tong University, Shanghai 200240, China

* qqliu@yzu.edu.cn



OPEN ACCESS

Citation: Zhang G-Y, Liu R-R, Zhang C-Q, Tang K-X, Sun M-F, Yan Guo-Hong, et al. (2015) Manipulation of the Rice L-Galactose Pathway: Evaluation of the Effects of Transgene Overexpression on Ascorbate Accumulation and Abiotic Stress Tolerance. PLoS ONE 10(5): e0125870. doi:10.1371/journal.pone.0125870

Academic Editor: Jin-Song Zhang, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, CHINA

Received: January 11, 2015

Accepted: March 26, 2015

Published: May 4, 2015

Copyright: © 2015 Zhang et al. This is an open access article distributed under the terms of the [Creative Commons Attribution License](https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Funding: This study was supported by the National Key Basic Research Projects (2012CB944803), the National Major Special Project for the Development of Transgenic Organisms (2014ZX08001006-005), the National Science Foundation of China (31471459) to QQL, and the Independent Innovation Fund of Jiangsu Province (CX (13) 5084), China to GYZ. The funders had no role in study design, data collection

Abstract

Ascorbic acid (AsA) is the most abundant water-soluble antioxidant in plants, and it plays a crucial role in plant growth, development and abiotic stress tolerance. In the present study, six key *Arabidopsis* or rapeseed genes involved in AsA biosynthesis were constitutively overexpressed in an elite *Japonica* rice cultivar. These genes encoded the GDP-mannose pyrophosphorylase (GMP), GDP-mannose-3',5'-epimerase (GME), GDP-L-galactose phosphorylase (GGP), L-galactose-1-phosphate phosphatase (GPP), L-galactose dehydrogenase (GDH), and L-galactono-1,4-lactone dehydrogenase (GalLDH). The effects of transgene expression on rice leaf AsA accumulation were carefully evaluated. In homozygous transgenic seedlings, *AtGGP* transgenic lines had the highest AsA contents (2.55-fold greater than the empty vector transgenic control), followed by the *AtGME* and *AtGDH* transgenic lines. Moreover, with the exception of the *AtGPP* lines, the increased AsA content also provoked an increase in the redox state (AsA/DHA ratio). To evaluate salt tolerance, *AtGGP* and *AtGME* transgenic seedlings were exposed to salt stress for one week. The relative plant height, root length and fresh weight growth rates were significantly higher for the transgenic lines compared with the control plants. Altogether, our results suggest that GGP may be a key rate-limiting step in rice AsA biosynthesis, and the plants with elevated AsA contents demonstrated enhanced tolerance for salt stress.

Introduction

L-ascorbic acid (AsA), also known by its popular name vitamin C, is the most abundant water-soluble antioxidant in plants. It protects plants from oxidative damage by scavenging the free radicals and reactive oxygen species generated by photosynthesis, oxidative metabolism, and

and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

environmental stresses [1]. AsA has been shown to play a critical role in plant growth and development, as well as in tolerance of abiotic stresses such as drought [2], salinity [3,4], ozone [5], and high light intensity [6]. Additionally, AsA is a cofactor for many enzymes that are involved in multiple processes, including flavonoid and phytohormone biosynthesis as well as the xanthophyll cycle [7, 8].

To date, four alternative AsA biosynthesis pathways have been proposed in plants: the L-galactose [9], galacturonate [10,11], glucose [12] and myo-inositol pathways [13]. Among these pathways, the L-galactose pathway might have an important role in cereal AsA biosynthesis (Fig 1) [14]. For example, when rice seedlings were supplied with L-galactose and L-galactono-1,4-lactone, shoot AsA contents increased up to 5- and 4-fold, respectively; in contrast, for D-galacturonic acid, myo-inositol, D-glucuronolactone and L-gulono-1,4-lactone, AsA levels were similar to those of the untreated control plants [15]. At least ten enzymes in the L-galactose pathway are involved in converting D-glucose to AsA through the formation of GDP-D-mannose, GDP-L-galactose, L-galactose-1-P, L-galactose, and L-galactono-1,4-lactone intermediates (Fig 1).

Most of the genes involved in this pathway, e.g., GDP-mannose pyrophosphorylase (GMP) [16,17], GDP-mannose-3',5'-epimerase (GME) [3], GDP-L-galactose phosphorylase (GGP)

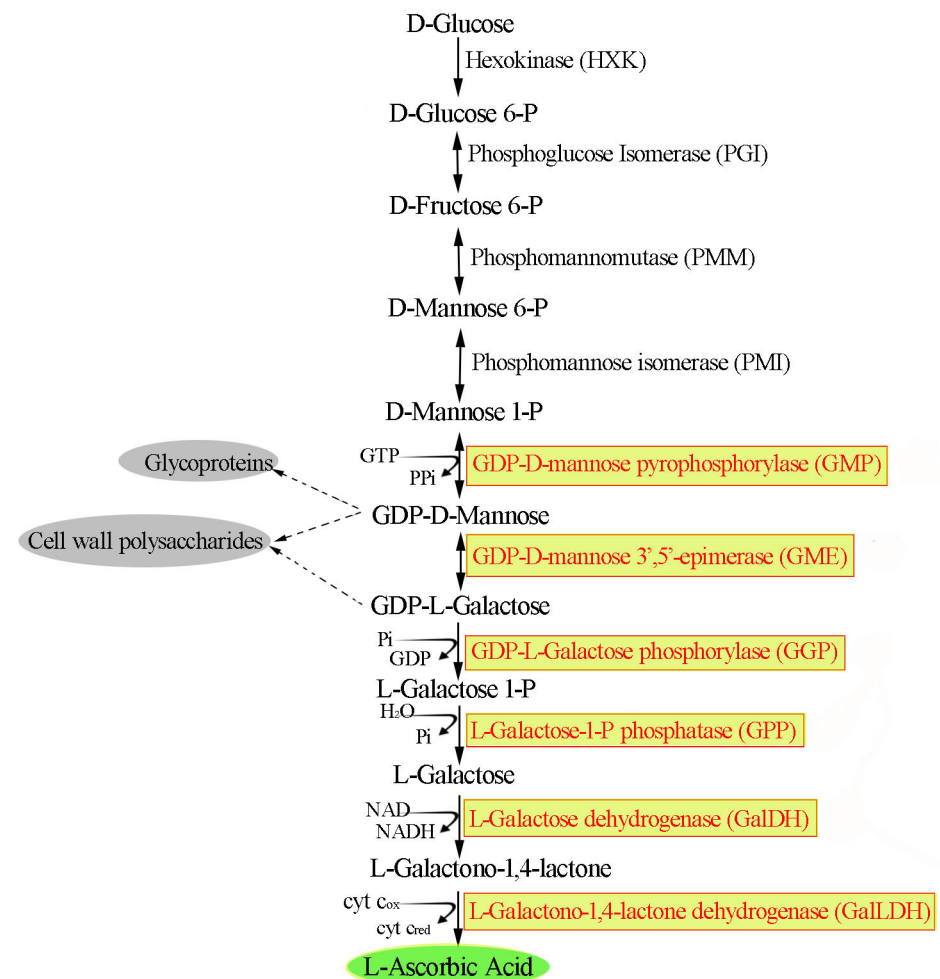


Fig 1. The L-galactose pathway in higher plants [14].

doi:10.1371/journal.pone.0125870.g001

[18,19], L-galactose-1-phosphate phosphatase (GPP) [20], L-galactose dehydrogenase (GDH) [21], and L-galactono-1,4-lactone dehydrogenase (GalLDH) [22,23], have been cloned, and several researchers have attempted to regulate AsA content by overexpressing them in transgenic plants. However, these studies each only focused on one gene, and few works have examined the effects of transgenic regulation of all of the above genes in one experiment, especially in rice, one of the most important crops consumed worldwide.

Feeding with AsA precursors during rice growth led to an increase in leaf AsA content, shedding light onto the development of cultivars with enhanced tolerance for key environmental stresses [24–26]. In contrast, the ND6172 rice mutant had approximately 20–30% lower AsA levels and exhibited negative effects on agronomic traits at ozone levels exceeding the current ambient concentrations [27]. Moreover, reducing *GalLDH* expression in rice was reported to increase lipid peroxidation, but also decrease foliar AsA levels [22]. These results revealed that the AsA content of rice leaves is a sensitive characteristic that might be consistent with stress tolerance. Furthermore, the AsA redox state, i.e., the ratio between ascorbate and dehydroascorbate (AsA/DHA), also plays important role in plant senescence, defense, and stress response [28–30]. The AsA/DHA ratio may depend on the plant species; in general, it is approximately 1.0 in 4-week-old rice seedlings [31, 32].

In the present study, the expression levels of genes involved in the rice L-galactose pathway were compared by quantitative RT-PCR. Six L-galactose pathway genes from *Arabidopsis* or rapeseed, including *GMP*, *GME*, *GGP*, *GPP*, *GDH* and *GalLDH* (Fig 1), were then overexpressed in rice and their effects on rice seedling AsA accumulation were carefully compared. This results of this analysis showed that *GGP* overexpression had the best AsA accumulation, followed by *GME* and *GDH*, and the abiotic stress tolerances of *GGP* and *GME* transgenic rice plants were subsequently evaluated.

Materials and Methods

Plant materials

The *Japonica* rice variety Nipponbare was used for quantitative expression analysis of the endogenous L-galactose pathway genes. Another elite *Japonica* cultivar, Wuyujing 3 (WY3), was used for generation of transgenic lines. All rice plants were grown in the field at the experimental farm of Yangzhou University (32°39'N, 119°42'E) under normal culture conditions. Developing endosperm, stems, sheaths and old leaves were sampled and collected at 20 days, and young leaves and roots were harvested from 4-week-old seedlings. The collected samples were immediately frozen in liquid nitrogen and stored at –80°C.

Transgenic vector construction

Arabidopsis AtGGP, *AtGMP*, *AtGME*, *AtGPP*, *AtGDH* and rapeseed *BoGalLDH* cDNAs (GenBank accession numbers AY093138, AF076484, NM_122767, NM_001035549, AJ417563, and Z97060, respectively) were previously cloned by RT-PCR [20]. To construct the transgenic vectors, the cDNAs were amplified to add suitable restriction enzymatic sites using the primers listed in S1 Table. The amplified cDNAs were then inserted into the pMD18-T vector (TAKARA, Dalian, China) and confirmed by sequence analysis. The full-length cDNAs, regulated by the maize *Ubi* or *CaMV 35s* promoters and NOS terminator, were cloned into the binary pCAMBIA1300 vector (www.cambia.com.au). With the exception of *AtGGP*, which was driven by the *CaMV 35s* promoter, the target genes were regulated by the maize *Ubi* promoter. The resulting binary vectors (Fig 2) containing the target genes and the corresponding empty pCAMBIA1300 control vector were transformed into *Agrobacterium tumefaciens* strain EHA105 and subsequently used for rice transformation.

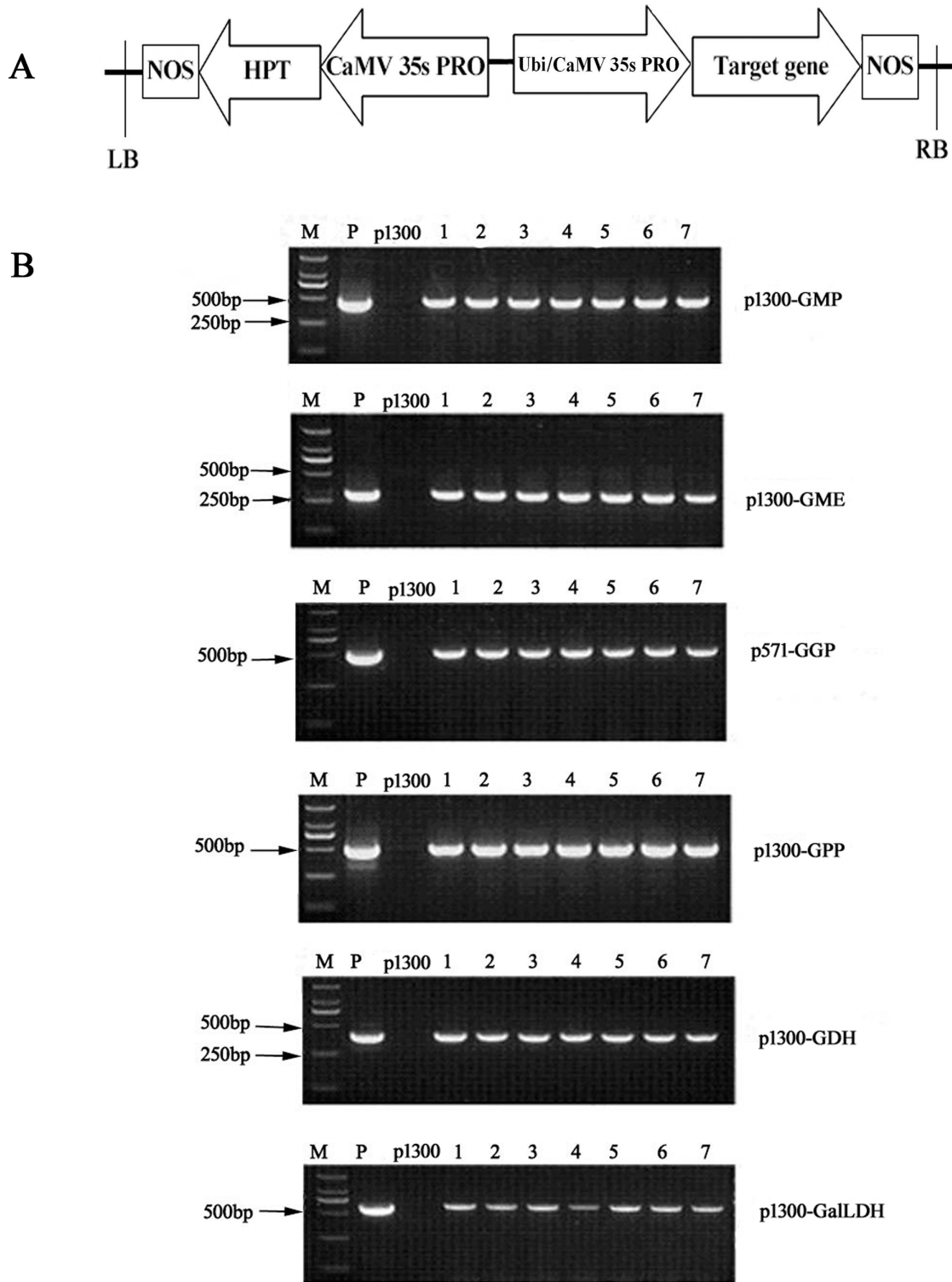


Fig 2. Rice transformation constructs (A) and PCR amplification of transformed genes (B). A. Schematic representation of the transformation vectors used in this study. *CaMV 35S pro*, cauliflower mosaic virus 35S promoter; *Ubi pro*, maize ubiquitin promoter; *NOS*, nopaline synthase gene terminator; *HPT*, hygromycin resistance gene; LB, left border; RB, right border. B. PCR analysis of transgenic and control lines using specific primers for amplification of each transgene. Lanes 1–7, transgenic plants (T_0 generation); P, positive control; M, DL2000 DNA marker (TAKARA, Dalian, China).

doi:10.1371/journal.pone.0125870.g002

Rice transformation and confirmation

Calli derived from either immature or mature WY3 rice cultivar embryos were used as explants for *Agrobacterium*-mediated transformation. Tissue culture, transformation, selection and re-generation of transgenic plants were performed as described by Liu et al. [33]. Genomic DNA was extracted from leaves using the CTAB method [34], and PCR analysis was performed using transgene-specific primer sets (S1 Table) to detect the transgenes.

Quantitative RT-PCR

Total RNA was isolated as previously described [35]. A 1 µg sample of each RNA preparation was used for reverse transcription using the SuperScript First-Strand Synthesis System (Invitrogen, Carlsbad, CA) and oligo(dT)₁₈ according to the manufacturer's instructions. Aliquots of cDNA were used in 25 µl real-time PCR assay reactions using the SYBR Premix ExTaq kit (TaKaRa, Tokyo, Japan). The results were normalized against rice *actin* mRNA. The sequences of the real-time PCR assay primers are listed in S1 Table.

AsA and DHA measurements

AsA was extracted according to the method described by Zhou et al. [20]. Briefly, 50 mg of leaves from 4-week-old seedlings were harvested at 6 pm, ground in liquid nitrogen, mixed with 1 ml 0.1% oxalic acid in dim light, and placed on ice for 1 h. The samples were centrifuged at 10 000 g for 10 min and the supernatants were then pooled and filtered for measurement. For AsA assays measuring the reduced + oxidized forms, 25 µL (25 mmol L⁻¹) DTT and 25 µL (320 mmol L⁻¹) K₂HPO₄ were added to 500 µL supernatant, incubated for 15 minutes at room temperature, centrifuged, and then filtered for measurements. The amounts of DHA were determined as the difference between the two assays. Standard L-AsA solutions (1 mg ml⁻¹) (Sigma, St. Louis, MO, USA) were freshly prepared prior to use. L-AsA HPLC analysis was performed using a C-18 reversed phase column (5 µm, 150×4.6 mm). Samples (10 µL) were injected using a Waters 2695 autosampler. The solvent system was 0.1% oxalic acid at a 1.0 mL min⁻¹ flow rate, and the samples were analyzed with a UV detector at 245 nm.

Salt stress treatment

Germinated seeds from transgenic and control lines were placed in the 96-well PCR plate (without bottom) and pre-grown in modified Hoagland's solution (S2 Table). The culture was under a light intensity of about 700 µmol photons m⁻² s⁻¹, 75% humidity and a 16/8-h light/dark photoperiod at 25°C for three weeks. Seedlings were then cultured in solutions containing 100 mM NaCl for one week. The solutions were renewed everyday, and three replications were used in salt stress evaluation experiments. The plant heights, root lengths and fresh weights were measured before and after salt stress, and the relative plant height, root length and fresh weight growth rates were calculated.

Relative membrane ion leakage analysis

The oxidative stress tolerance of the plants was assessed by ion leakage analysis as described by Wang et al. [36]. Ten leaf discs of 6 mm diameter were placed in a cuvette containing 20 mL distilled water, vacuumized for 30 min, and then surged for 10 h at room temperature to measure the initial electronic conductance (S1) using an osmometer (DDS-11A, SuoShen, Shanghai, China). The cuvette was heated in boiling water for 30 min and cooled to room temperature to determine the final electronic conductance (S2). The relative membrane ion leakage (%) was defined as 100× (S1/S2).

Statistical analysis

The data were analyzed using a statistical software package (SPSS v16.0). One-way analysis of variance (ANOVA) was used to compare the differences between the transgenic and control lines, followed by Tukey's post hoc test. A p-value below 0.05 was considered to be statistically significant.

Results

Enriched expression of the L-galactose pathway in young rice leaves

The rice genome contains only one copy each of the *OsPMM*, *OsGGP* and *OsGPP* genes, while there are two copies of the *OsGME* genes (*OsGME1* and *OsGME2*) and *OsGalLDH* (*OsGalLDH1* and *OsGalLDH2*) and three copies of *OsGMP* genes (*OsGMP1*, *OsGMP2* and *OsGMP3*) [37]. To increase our understanding of rice AsA biosynthesis, we first performed expression profiling of the genes involved in the final six L-galactose pathway steps in various tissues. As there is high identity (more than 90%) among the coding sequences of three *OsGMP* genes, and also very high identity (98%) between *OsGalLDH1* and *OsGalLDH2*, the expression analysis was only carried out by using the primer according to the *OsGMP2* or *OsGalLDH1*, which might represent the whole of three *OsGMP* genes or two *OsGalLDH* genes, respectively. As shown in Fig 3, this analysis demonstrated expression of all tested genes in all tissues; however, their transcription levels were quite low in root, stem and developing endosperm samples compared with their levels in leaves. Moreover, their expression in young leaves from

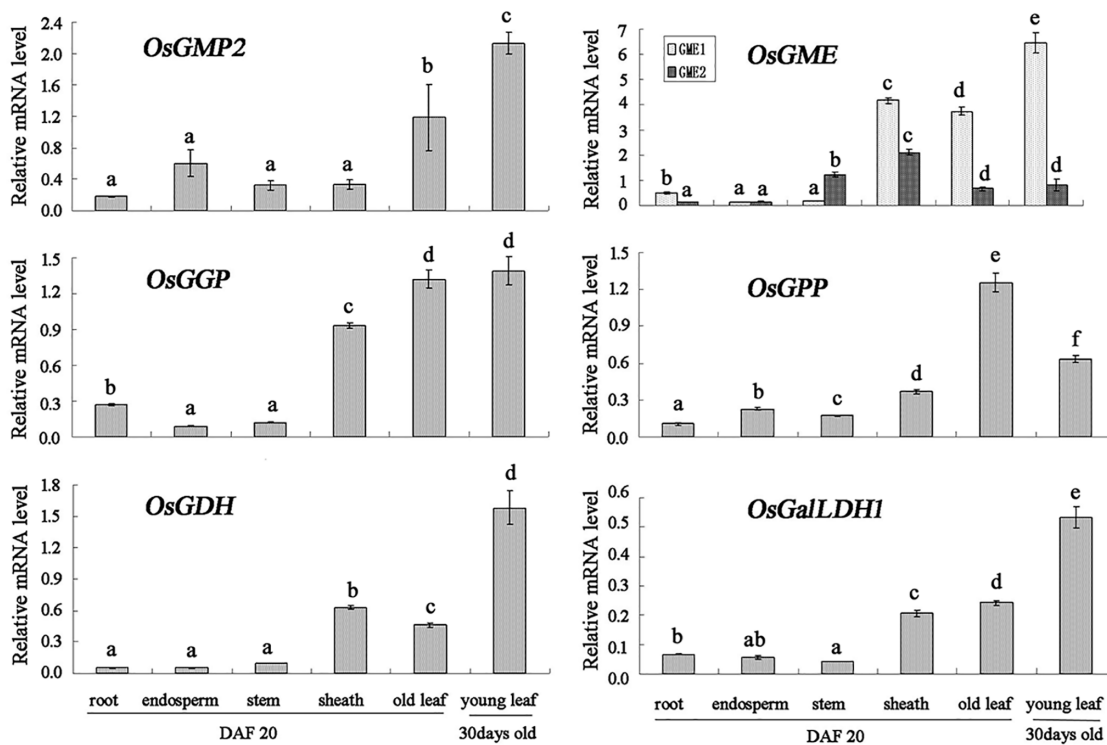


Fig 3. Expression of ascorbate biosynthetic genes in rice roots, endosperm, stems, sheaths, old and young leaves. All tissues except young leaves were taken from DAF20 (20 days after flowering) plants. The young leaves were taken from 30 day-old plants. Data obtained by real-time RT-PCR were normalized using *actin* mRNA expression. Data shown are the average of three measurements with standard errors. Different letters indicate statistically significant differences at $P < 0.05$ (ANOVA, Tukey post-hoc). The GeneBank accession numbers for *OsGMP2*, *OsGME1*, *OsGME2*, *OsGGP*, *OsGPP*, *OsGDH*, and *OsGalLDH1* are AK122126, AK069385, AK102348, AK071188, AK071149, AK067039, and AK241565, respectively.

doi:10.1371/journal.pone.0125870.g003

4-week-old seedlings were much greater than in old leaves from plants 20 days after flowering, implying that the expression of L-galactose pathway was enriched in young rice leaves.

Generation and confirmation of homozygous transgenic rice

Expression cassettes (Fig 2A) containing the target gene or empty vector were transferred into WY3 Japonica rice by *Agrobacterium*-mediated transformation. More than 20 independent transformants were regenerated for each construct. All T₀ transgenic rice plants were confirmed by PCR using primers specific to each target gene (Fig 2B). The results from field studies showed that there was no significant difference of the phenotype such as plant height, tiller number, flower time and seed production between the transgenic plants and their wild type under normal field condition. For each construct, at least three independent lines with homozygous and high-levels of transgene expression were chosen for further analysis. The transgenic plants derived from the empty vector p1300 (empty control) were also selected for further testing.

Transgene expression in rice leaves

Transgene expression was detected by real-time RT-PCR, and the results of this analysis showed that the foreign transgenes could be expressed at high levels in rice leaves (Fig 4A–4C). The expression of endogenous genes in transgenic rice plants was also analyzed and compared with the empty control (Fig 4D–4F). In *AtGDH* transgenic rice leaves, endogenous *OsGDH*

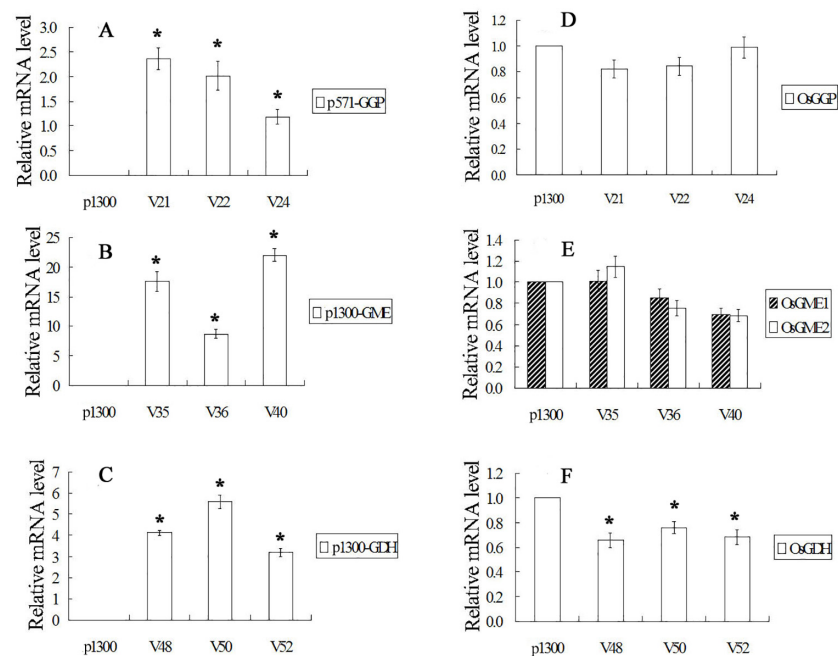


Figure 4

Fig 4. Relative gene expression in *AtGPP*, *AtGME* and *AtGDH* transgenic lines. A, *AtGPP*; B, *AtGME*; C, *AtGDH*; D, *OsGPP*; E, *OsGME1* and *OsGME2*; F, *OsGDH*. A-C, data obtained by real-time RT-PCR were normalized using *actin* mRNA expression. E-F, data obtained by real-time RT-PCR were normalized using *actin* expression and are expressed as a percentage of the control. Asterisk indicates values that are significantly different from those of empty control plants (Tukey's test, P < 0.05).

doi:10.1371/journal.pone.0125870.g004

expression was slightly lower than empty control (Fig 4F); however, the expression levels of endogenous genes related to AsA biosynthesis in the other transgenic plants were quite similar to those of the empty control (Fig 4D and 4E). The results of this analysis suggested that the transgenes had limited or no effects on L-galactose pathway gene expression.

Effects of exogenous gene overexpression on AsA accumulation

To analyze and compare AsA levels among different transgenic rice plants, T₆ generation homozygous transgenic lines and the empty p1300 control were grown simultaneously in the same field under normal culture conditions. The young, fully expanded fresh leaves of 30 day-old plants were collected and used for HPLC measurement of AsA and DHA levels. We used 0.1% oxalic acid as the extraction buffer and found that it provided high AsA stabilization and minimal interaction with the chromatographic system (Fig 5). Compared with the p1300 empty control, AsA levels were significantly increased (up to 2.55-fold) for all six target transgenic lines (Fig 6). Among the transgenic lines, the p571-GGP lines showed the best AsA accumulation, followed by the p1300-GME and p1300-GDH transgenic lines.

Effects of exogenous gene overexpression on the ascorbate redox state

As shown in Fig 6, the DHA levels were not significantly altered in the leaves of transgenic rice overexpressing the target genes. As the AsA levels increased significantly in these transgenic leaves, the redox state (AsA/DHA ratio) also increased in the transgenic lines, with the exception of the p1300-GPP lines. The ratio of AsA to DHA was approximately 1.04 in the empty control (p1300) plants, while it was 3.42, 2.64 and 2.08 in the leaves of p1300-GGP transgenic lines V21, V22 and V24, respectively (Fig 6).

Increased AsA content leads to enhanced salt stress tolerance

To investigate the effects of increased AsA content on transgenic rice salt tolerance, p1300-GGP and p1300-GME transgenic rice plants which were the two best in AsA accumulation were selected for stress tolerance testing. The 3-week old rice seedlings were transferred to Hoagland's solution supplemented with 100 mM NaCl for one week. The plant heights, root lengths and fresh weights were measured before and after salt stress, and used to calculate the relative growth rates (Table 1). This analysis showed that the relative plant height and root length growth rates in most of the p1300-GME or p571-GGP transgenic lines tested were significantly greater ($p < 0.01$) than those of the p1300 control plants. The fresh weights of the p1300 control plants were reduced after salt treatment due to wilting under salt stress; in contrast, the extent of wilting was reduced for the p571-GGP and p1300-GME transgenic lines. Consequently, the relative fresh weight growth rates of the p571-GGP and p1300-GME transgenic lines were significantly higher ($p < 0.01$) than those of the p1300 control plants (Table 1). The ion leakage was measured to reflect the level of cellular damage after salt treatment. As shown in Fig 7, relative membrane ion leakage was not significantly different between wild-type and transgenic plants under normal conditions. However, after salt treatment, the relative membrane ion leakage of wild-type plants increased up to 67%, significantly higher ($p < 0.01$) than that of transgenic plants. The results indicated that overexpression of *AtGGP* or *AtGME* could significantly improve rice seedling salt tolerance.

Discussion

In the last decade, great progress has been made in metabolic engineering of the L-galactose pathway; however, it is difficult to systematically compare the contributions of L-galactose

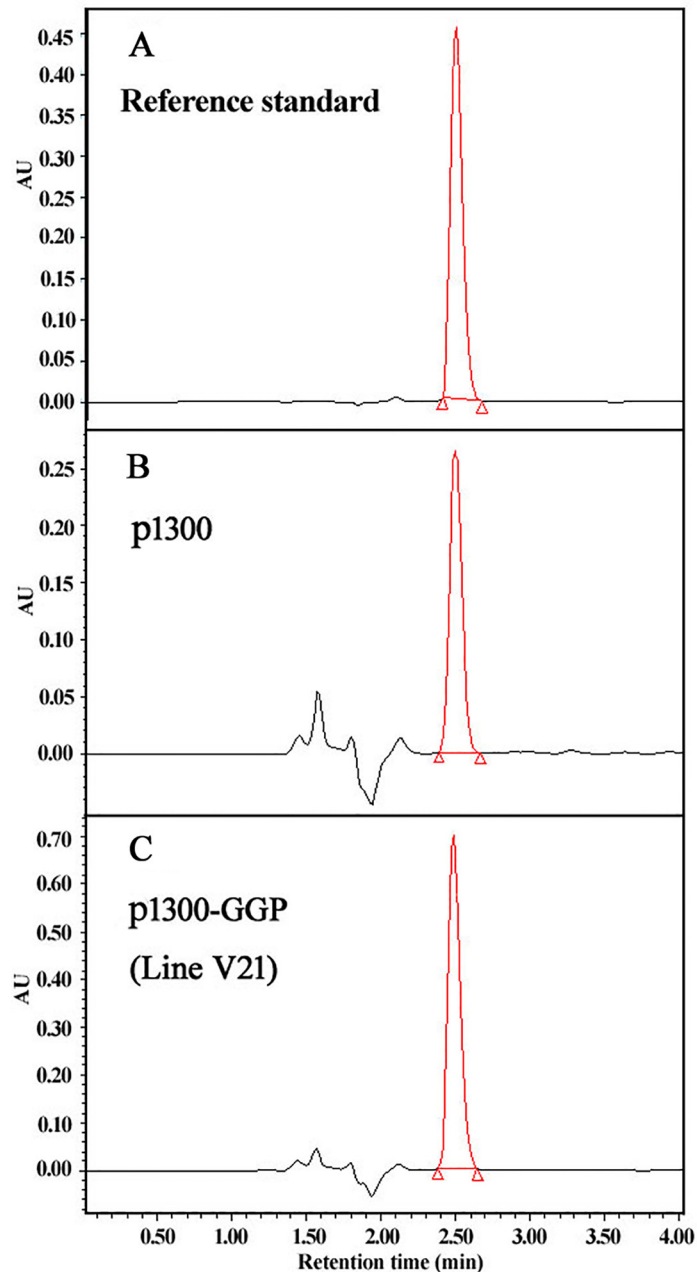


Fig 5. HPLC chromatograms of AsA standards (A), and extracts from the leaves of empty control (B) and p571-GGP-derived transgenic rice (C).

doi:10.1371/journal.pone.0125870.g005

pathway genes to AsA accumulation due to the differences in transgenes and plant species across studies.

In this study, six genes involved in the L-galactose pathway were overexpressed in rice. Our analysis showed that the p571-GGP transgenic lines had the highest AsA levels, with a 2.55-fold increase compared with the empty vector control; similar results were reported by Zhou et al. [20]. Transgenic tobacco plants overexpressing kiwifruit *GGP* showed an approximately 3-fold increase in AsA levels [38]. In 2014, Wang et al. [18] reported that overexpression of tomato

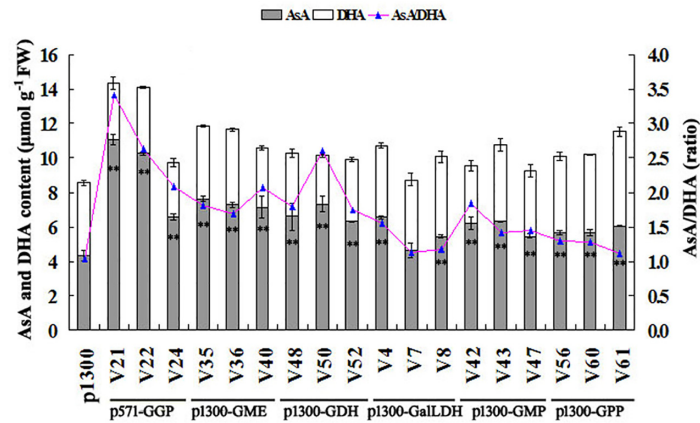


Fig 6. AsA and DHA contents and the ratio of AsA to DHA in each transgenic line. Data are the averages of thirty plants \pm standard deviation. Double asterisk indicates values of AsA content are significantly different from those of empty control plants (Tukey's test, $P < 0.01$).

doi:10.1371/journal.pone.0125870.g006

GGP in tobacco led to a significant increase in the AsA content and AsA/DHA ratio. Therefore, we can conclude that GGP may be a key rate-limiting step in plant AsA biosynthesis, especially in *Arabidopsis*, tobacco and rice.

The reactions catalyzed by GMP and GME are located in the upper steps of the end of AsA biosynthesis, and they are also important for cell wall formation and membrane protein modification. Overexpression of tomato GMP in tobacco resulted in a 2-4-fold increase in AsA and improved temperature stress tolerance [17]. Expression of GMP increased AsA levels in tomatoes up to 70% in leaves, 50% in green fruit and 35% in red fruit [39]. In this report, p1300-GMP transgenic lines exhibited a half-fold increase in AsA levels compared with the

Table 1. The relative growth rate of plant height, root length and fresh weight growth rates of transgenic lines transferred to 100 mM NaCl for one week.

Transgene	Line	Plant height (cm) before stress	Plant height (cm) after stress	The relative growth rate of plant height	Root length (cm) before stress	Root length (cm) after stress	The relative growth rate of root length	Fresh weight (mg/plant) before stress	Fresh weight (mg/plant) after stress	The relative growth rate of fresh weight
Empty	p1300	15.87 \pm 0.21	16.17 \pm 0.32	+0.02	9.57 \pm 0.31	10.73 \pm 0.25	+0.12	206.33 \pm 7.37	185.33 \pm 5.13	-0.10
AtGGP	V21	16.06 \pm 0.25	16.40 \pm 0.43	+0.02	10.28 \pm 0.21	11.98 \pm 0.15**	+0.17**	218.38 \pm 9.76	251.01 \pm 4.58**	+0.15**
	V22	16.02 \pm 0.14	16.78 \pm 0.65*	+0.05**	10.17 \pm 0.15	11.77 \pm 0.32**	+0.15*	214.34 \pm 6.31	286.49 \pm 4.16**	+0.34**
	V24	15.47 \pm 0.15	16.80 \pm 0.26**	+0.09**	10.13 \pm 0.15	11.37 \pm 0.21	+0.12	208.21 \pm 8.79	241.67 \pm 7.81**	+0.16**
AtGME	V35	15.41 \pm 0.21	16.94 \pm 0.21**	+0.10**	9.67 \pm 0.40	11.62 \pm 0.13**	+0.20**	209.46 \pm 7.10	235.08 \pm 6.03**	+0.12**
	V36	15.20 \pm 0.36	17.18 \pm 0.23**	+0.13**	9.54 \pm 0.17	11.53 \pm 0.26*	+0.21**	202.33 \pm 8.22	247.29 \pm 7.21**	+0.22**
	V40	15.16 \pm 0.37	17.43 \pm 0.26**	+0.15**	9.23 \pm 0.46	11.05 \pm 0.32	+0.20**	193.37 \pm 5.03	207.67 \pm 5.51*	+0.07**

The data is presented as mean \pm SD of at least twelve plants.

* and ** represent significant differences (Tukey's test) at $p \leq 0.05$ and $p \leq 0.01$, respectively.

doi:10.1371/journal.pone.0125870.t001

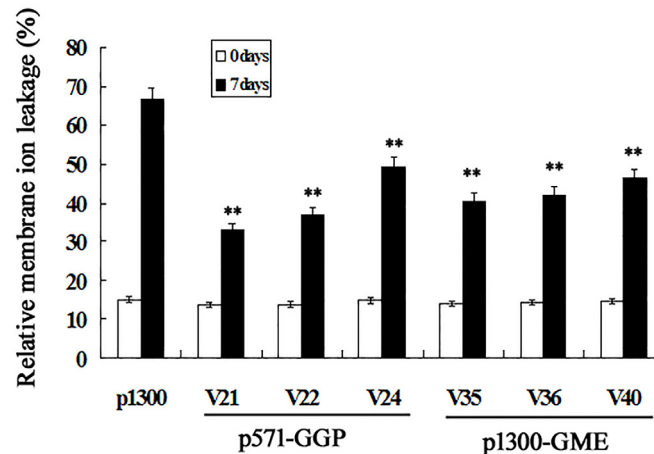


Fig 7. Relative membrane ion leakage in the leaves before and after salt stress treatment. The data presented are the mean values of twelve plants \pm standard deviation. Asterisk indicates values that are significantly different from those of empty control plants (Tukey's test, $P < 0.05$).

doi:10.1371/journal.pone.0125870.g007

controls; therefore, GMP does not appear to exert a major metabolic function during rice AsA biosynthesis. Overexpression of both *SIGME1* and *SIGME2* resulted in a 1.4-fold increase in the AsA contents of tomato leaves and fruits, and improved abiotic stress tolerance [3]. It was reported that the *OsGME* kinetic parameters seemed to be relatively low for rapid AsA biosynthesis [40]. Overexpression of *AtGME* in rice showed the next best enhancement of AsA levels. *GME* catalyzes the double epimerization of the D-mannose moiety at the 3' and 5' carbons to form L-galactose, and the reaction equilibrium is highly biased against AsA biosynthesis [10, 40]. Consequently, GDP-L-galactose formed by *GME* must be quickly converted to L-galactose-1-phosphate, otherwise abundant amount of GDP-L-galactose needs to accumulate in cells. Consequently, simultaneous overexpression of both *GME* and *GGP* may be suitable for strongly pulling the metabolic flux downwards and promoting rice AsA accumulation.

GDH and *GalLDH* are downstream genes in the L-galactose pathway. Overexpression of *GDH* in tobacco and *Arabidopsis* showed little effect on the increase in leaf AsA content [20, 21]. Our results revealed that *AtGDH* transgenic lines displayed the third best enhancement in AsA levels. Similar to our result, prior *OsGalLDH* overexpression in rice showed up to a 1.48-fold increase in AsA content [22]. However, Fukunaga et al. [15] reported that the activities of *OsGDH* and *OsGalLDH* were sufficient to convert an increased substrate to AsA. Thus, *GDH* and *GalLDH* might not be ideal candidates for co-transformation to increase AsA levels. Zhou et al. [20] reported that *GPP* overexpression in *Arabidopsis* resulted in an up to 1.5-fold increase in AsA levels compared with wild-type, and our analysis yielded similar results. Furthermore, for transgenic *Arabidopsis*, simultaneous overexpression of *GGP* and *GPP* increased leaf AsA contents up to 4.1-fold [20]. Consequently, it is suggested that the transgenic expression of both enzymes might shed light on the potential for increasing rice AsA content.

In general, plant responses to salinity by occurring in two phases, osmotic phase and ionic phase [41]. In this study, overexpression of *AtGGP* or *AtGME* in rice led to improved osmotic stress tolerance. The growth rate of root and leaf of these transgenic lines were significantly higher than that in the control. The result of ion leakage analysis revealed the lesser cellular damage in transgenic lines than that in empty control. Similar results were obtained for transgenic plants overexpressing genes involved in AsA biosynthesis

[2, 3, 18, 23, 42–45]. Therefore, AsA plays a key role in plants as an antioxidant in abiotic stress defense, and plants with higher AsA contents exhibited enhanced tolerance to these stresses.

Taking previous evidence and our present study together, it can be concluded that it is easy to elevate AsA levels in plants via metabolic engineering of AsA biosynthesis; however, the effects differed greatly according to the target genes used. *GGP*, *GDH* and *GME* may be the best three targets, and combining two or three target genes is a better approach for generating transgenic lines with increased AsA accumulation. Consequently, further experimental efforts are necessary to verify whether simultaneous overexpression of *GGP* and *GME*, or *GGP* and *GPP* would lead to a greater increase in rice AsA levels.

Supporting Information

S1 Table. PCR Primers used in this study.

(DOC)

S2 Table. The formula of modified Hoagland solution (pH5.5).

(DOC)

Author Contributions

Conceived and designed the experiments: QQL KXT. Performed the experiments: GYZ RRL CQZ GHY. Analyzed the data: GYZ RRL. Contributed reagents/materials/analysis tools: CQZ MFS KXT. Wrote the paper: GYZ QQL.

References

1. Noctor G, Foyer CH. Ascorbate and glutathione: keeping active oxygen under control. *Annu Rev Plant Physiol Plant Mol Biol.* 1998; 49: 249–279. PMID: [15012235](#)
2. Hemavathi, Upadhyaya CP, Akula N, Kim HS, Jeon JH, Ho OM, et al. Biochemical analysis of enhanced tolerance in transgenic potato plants overexpressing D-galacturonic acid reductase gene in response to various abiotic stresses. *Mol Breed.* 2011; 28: 105–115.
3. Zhang C, Liu J, Zhang Y, Cai X, Gong P, Zhang J, et al. Overexpression of SIGMEs leads to ascorbate accumulation with enhanced oxidative stress, cold, and salt tolerance in tomato. *Plant Cell Rep.* 2011; 30: 389–398. doi: [10.1007/s00299-010-0939-0](#) PMID: [20981454](#)
4. Venkatesh J, Upadhyaya CP, Yu JW, Hemavathi A, Kim DH, Strasser RJ, et al. Chlorophyll a fluorescence transient analysis of transgenic potato overexpressing D-galacturonic acid reductase gene for salinity stress tolerance. *Hort Environ Biotechnol.* 2012; 53: 320–328.
5. Feng Z, Pang J, Nouchi I, Kobayashi K, Yamakawa T, Zhu J. Apoplastic ascorbate contributes to the differential ozone sensitivity in two varieties of winter wheat under fully open-air field conditions. *Environ Pollut.* 2010; 158: 3539–3545. doi: [10.1016/j.envpol.2010.08.019](#) PMID: [20855141](#)
6. Talla S, Riazunnisa K, Padmavathi L, Sunil B, Rajsheel P, Raghavendra AS. Ascorbic acid is a key participant during the interactions between chloroplasts and mitochondria to optimize photosynthesis and protect against photoinhibition. *J Biosci.* 2011; 36: 163–173. PMID: [21451257](#)
7. De Tullio MC, Arrigoni O. Hopes, disillusion and more hopes from vitamin C. *Cell Mol Life Sci.* 2004; 61: 209–219. PMID: [14745499](#)
8. Debolt S, Melino V, Ford CM. Ascorbate as a biosynthetic precursor in plants. *Ann Bot.* 2007; 99: 3–8. PMID: [17098753](#)
9. Wheeler GL, Jones MA, Smirnov N. The biosynthetic pathway of vitamin C in higher plants. *Nature.* 1998; 393: 365–369. PMID: [9620799](#)
10. Wolucka BA, Van Montagu M. GDP-mannose 3', 5'-epimerase forms GDP-L-gulose, a putative intermediate for the de novo biosynthesis of vitamin C in plants. *J Biol Chem.* 2003; 278: 47483–47490. PMID: [12954627](#)
11. Agius F, González-Lamothe R, Caballero JL, Muñoz-Blanco J, Botella MA, Valpuesta V. Engineering increased vitamin C levels in plants by overexpression of a D-galacturonic acid reductase. *Nat Biotechnol.* 2003; 21: 177–181. PMID: [12524550](#)

12. Jain AK, Nessler CL. Metabolic engineering of an alternative pathway for ascorbic acid biosynthesis in plants. *Mol Breed*. 2000; 6: 73–78.
13. Lorence A, Chevone BI, Mendes P, Nessler CL. myo-Inositol oxygenase offers a possible entry point into plant ascorbate biosynthesis. *Plant Physiol*. 2004; 134: 1200–1205. PMID: [14976233](#)
14. Imai T, Ban Y, Yamamoto T, Moriguchi T. Ectopic overexpression of peach GDP-D-mannose pyrophosphorylase and GDP-D-mannose-3',5'-epimerase in transgenic tobacco. *Plant Cell Tiss Organ Cult*. 2012; 111:1–13
15. Fukunaga K, Fujikawa Y, Esaka M. Light regulation of ascorbic acid biosynthesis in rice via light responsive cis-elements in genes encoding ascorbic acid biosynthetic enzymes. *Biosci Biotechnol Biochem*. 2010; 74: 888–891. PMID: [20378970](#)
16. Badejo AA, Tanaka N, Esaka M. Analysis of GDP-D-mannose pyrophosphorylase gene promoter from acerola (*Malpighia glabra*) and increase in ascorbate content of transgenic tobacco expressing the acerola gene. *Plant Cell Physiol*. 2008; 49: 126–132. PMID: [18037674](#)
17. Wang HS, Yu C, Zhu ZJ, Yu XC. Overexpression in tobacco of a tomato GMPase gene improves tolerance to both low and high temperature stress by enhancing antioxidation capacity. *Plant Cell Rep*. 2011; 30: 1029–1040. doi: [10.1007/s00299-011-1009-y](#) PMID: [21287174](#)
18. Wang L, Meng X, Yang D, Ma N, Wang G, Meng Q. Overexpression of tomato GDP-I-galactose phosphorylase gene in tobacco improves tolerance to chilling stress. *Plant Cell Rep*. 2014; 33: 1441–1451. doi: [10.1007/s00299-014-1627-2](#) PMID: [24832771](#)
19. Bulley S, Wright M, Rommens C, Yan H, Rassam M, Lin-Wang K, et al. Enhancing ascorbate in fruits and tubers through over-expression of the L-galactose pathway gene GDP-L-galactose phosphorylase. *Plant Biotechnol*. 2012; 10: 390–397. doi: [10.1111/j.1467-7652.2011.00668.x](#) PMID: [22129455](#)
20. Zhou Y, Tao QC, Wang ZN, Fan R, Li Y, Sun XF, et al. Engineering ascorbic acid biosynthetic pathway in *Arabidopsis* leaves by single and double gene transformation. *Biol Plant*. 2012; 56: 451–457.
21. Gatzek S, Wheeler GL, Smirnov N. Antisense suppression of I-galactose dehydrogenase in *Arabidopsis thaliana* provides evidence for its role in ascorbate synthesis and reveals light modulated I-galactose synthesis. *Plant J*. 2002; 30: 541–553. PMID: [12047629](#)
22. Liu Y, Yu L, Wang R. Level of ascorbic acid in transgenic rice for L-galactono-1, 4-lactone dehydrogenase overexpressing or suppressed is associated with plant growth and seed set. *Acta Physiol Plant*. 2011; 33: 1353–1363.
23. Liu W, An HM, Yang M. Overexpression of *Rosa roxburghii* I-galactono-1, 4-lactone dehydrogenase in tobacco plant enhances ascorbate accumulation and abiotic stress tolerance. *Acta Physiol Plant*. 2013; 35: 1617–1624.
24. Chao YY, Hong CY, Kao CH. The decline in ascorbic acid content is associated with cadmium toxicity of rice seedlings. *Plant Physiol Biochem*. 2010; 48: 374–381. doi: [10.1016/j.plaphy.2010.01.009](#) PMID: [20144872](#)
25. Yamane K, Rahman MS, Kawasaki M, Taniguchi M, Miyake H. Pretreatment with antioxidants decreases the effects of salt stress on chloroplast ultrastructure in rice leaf segments (*Oryza sativa* L.). *Plant Prod Sci*. 2004; 7: 292–300.
26. Guo Z, Tan H, Zhu Z, Lu S, Zhou B. Effect of intermediates on ascorbic acid and oxalate biosynthesis of rice and in relation to its stress resistance. *Plant Physiol Biochem*. 2005; 43: 955–962. PMID: [16310370](#)
27. Frei M, Wissuwa M, Pariasca-Tanaka J, Chen CP, Südekum KH, Kohno Y. Leaf ascorbic acid level—Is it really important for ozone tolerance in rice? *Plant Physiol Biochem*. 2012; 59: 63–70. doi: [10.1016/j.plaphy.2012.02.015](#) PMID: [22417733](#)
28. Barth C, Moeder W, Klessig DF, Conklin PL. The timing of senescence and response to pathogens is altered in the ascorbate-deficient *Arabidopsis* mutant vitamin c-1. *Plant Physiol*. 2004; 134: 1784–1792. PMID: [15064386](#)
29. López-Carbonell M, Munné-Bosch S, Alegre L. The ascorbate-deficient vtc-1 *Arabidopsis* mutant shows altered ABA accumulation in leaves and chloroplasts. *J Plant Growth Regul*. 2006; 25: 137–144.
30. Noctor G. Metabolic signalling in defence and stress: the central roles of soluble redox couples. *Plant Cell Environ*. 2006; 29: 409–425. PMID: [17080595](#)
31. Kwon SY, Choi SM, Ahn YO, Lee HS, Lee HB, Park YM, et al. Enhanced stress-tolerance of transgenic tobacco plants expressing a human dehydroascorbate reductase gene. *J Plant Physiol*. 2003; 160: 347–353. PMID: [12756914](#)
32. Wang Z, Xiao Y, Chen W, Tang K, Zhang L. Increased vitamin C content accompanied by an enhanced recycling pathway confers oxidative stress tolerance in *Arabidopsis*. *J Integr Plant Biol*. 2010; 52: 400–409 doi: [10.1111/j.1744-7909.2010.00921.x](#) PMID: [20377702](#)

33. Liu QQ, Zhang JL, Wang ZY, Hong MM, Gu MH. A highly efficient transformation system mediated by *Agrobacterium tumefaciens* in rice (*Oryza sativa* L.). *Acta Phytophysiol Sin.* 1998; 24: 259–271.
34. Murray MG, Thompson WF. Rapid isolation of high molecular weight plant DNA. *Nucleic Acids Res.* 1980; 8: 4321–4325. PMID: [7433111](#)
35. Zhang GY, Liu RR, Xu G, Zhang P, Li Y, Tang KX, et al. Increased α -tocotrienol content in seeds of transgenic rice overexpressing *Arabidopsis* γ -tocopherol methyltransferase. *Transgenic Res.* 2013; 22: 89–99. doi: [10.1007/s11248-012-9630-2](#) PMID: [22763462](#)
36. Wang LY, Li D, Deng YS, Lv W, Meng QW. Antisense-mediated depletion of tomato GDP-l-galactose phosphorylase increases susceptibility to chilling stress. *J Plant Physiol.* 2013; 170: 303–314 doi: [10.1016/j.jplph.2012.10.015](#) PMID: [23267461](#)
37. Höller S, Hajirezaei MR, von Wirén N, Frei M. Ascorbate metabolism in rice genotypes differing in zinc efficiency. *Planta.* 2014; 239: 367–379 doi: [10.1007/s00425-013-1978-x](#) PMID: [24173698](#)
38. Laing WA, Wright MA, Cooney J, Bulley SM. The missing step of the L-galactose pathway of ascorbate biosynthesis in plants, an L-galactose guanyltransferase, increases leaf ascorbate content. *Proc Natl Acad Sci USA.* 2007; 104: 9534–9539. PMID: [17485667](#)
39. Cronje C, George GM, Fernie AR, Bekker J, Kossmann J, Bauer R. Manipulation of L-ascorbic acid biosynthesis pathways in *Solanum lycopersicum*: elevated GDP-mannose pyrophosphorylase activity enhances L-ascorbate levels in red fruit. *Planta.* 2012; 235: 553–564. doi: [10.1007/s00425-011-1525-6](#) PMID: [21979413](#)
40. Watanabe K, Suzuki K, Kitamura S. Characterization of a GDP-d-mannose 3', 5'-epimerase from rice. *Phytochemistry.* 2006; 67: 338–346. PMID: [16413588](#)
41. Munns R, Tester M. Mechanisms of salinity tolerance. *Annu Rev Plant Biol.* 2008; 59: 651–681 doi: [10.1146/annurev.arplant.59.032607.092911](#) PMID: [18444910](#)
42. Eltayeb AE, Yamamoto S, Habora MEE, Yin L, Tsujimoto H, Tanaka K. Transgenic potato overexpressing *Arabidopsis* cytosolic *AtDHAR1* showed higher tolerance to herbicide, drought and salt stresses. *Breeding Sci.* 2011; 61: 3–10.
43. Lisko KA, Torres R, Harris RS, Belisle M, Vaughan MM, Jullian B, et al. (2013) Elevating vitamin C content via overexpression of myo-inositol oxygenase and L-gulonono-1, 4-lactone oxidase in *Arabidopsis* leads to enhanced biomass and tolerance to abiotic stresses. *In Vitro Cell Dev Biol-Plant.* 2013; 49: 643–655. PMID: [25767369](#)
44. Lim MY, Pulla RK, Park JM, Ham CH, Jeong BR. Over-expression of L-gulonono- γ -lactone oxidase (GLOase) gene leads to ascorbate accumulation with enhanced abiotic stress tolerance in tomato. *In Vitro Cell Dev Biol-Plant.* 2012; 48: 453–461.
45. Hemavathi, Upadhyaya CP, Young KE, Akula N, Kim HS, Oh OM, et al. Over-expression of strawberry D-galacturonic acid reductase in potato leads to accumulation of vitamin C with enhanced abiotic stress tolerance. *Plant Sci.* 2009; 177: 659–667.