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RESEARCH PAPER

Metabolic Engineering of Vitamin C Production in *Arabidopsis*

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Abstract Vitamin C (L-ascorbic acid, AsA) is a compound which provides major nutritional value, both for plants and humans. In this study, three distinct metabolic engineering strategies, including overexpression of biosynthesis enzyme, suppression of catabolism enzyme and switching the sub-cellular localization of compartment enzyme, were employed to enhance the production of AsA in *Arabidopsis thaliana.* The results showed that (1) overexpression of L-Galactose-1-P Phosphatase (GalPPase) enhanced AsA content to 3.96 μmol/g FW, which was 1.6-fold more than that in their wild-type (WT) counterparts; (2) RNAi suppression of ascorbate oxidase (AO) resulted in a significant increase of AsA accumulation (0.86 μmol/g FW) in apoplast; (3) both mitochondrion-target and none-target overexpression of L-Galactose dehydrogenase (GalDH) did not significantly promote AsA production compared with WT (1.96 μmol/g), however a dramatic enhancement was observed following infiltration with L-galactono-1, 4-lactone (L-GalL), both in transgenic and WT plants. The best line produced AsA with the content of 3.90 μmol/g FW, which was about 2-fold of that in the untreated control (1.99 μmol/g FW). This study provides new strategies including *GalPPase* overexpression, *AO* suppression as well as L-GalL feeding for modern breeding aimed at stimulating the AsA content in plants.

Keywords: AO, *Arabidopsis thaliana*, GalDH, GalPPase, metabolic engineering, vitamin C

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1. Introduction

L-Ascorbic acid (AsA) is a multifunctional metabolite in plants. It is an antioxidant and redox buffer, as well as an enzyme cofactor, so it has multiple roles in growth control, redox signaling, metabolism and also plant responses to abiotic stresses and pathogens [1,2]. Plant-derived AsA also provides the major source of vitamin C in the human diet, so the factors that control AsA content in plant are of great interest [3,4]. An understanding of how AsA is synthesized should provide a basis for engineering or otherwise manipulating its accumulation [5].

Early evidence from radiolabelling studies indicated that plant AsA was synthesized from GDP-D-mannose *via* L-galactose (D-Man/L-Gal pathway) (Fig. 1) [6]. The enzymes involved have been identified and some of them have been successfully used as critical metabolic control points for the production of target AsA in several plants. For example, expression of a yeast-derived GDP-mannose pyrophosphorylase (GMPase) in tomato increased AsA levels of up to 70% in leaves, 50% in green fruit, and 35% in red fruit [7]. Overexpression of L-galactono-1,4-lactone dehydrogenase (GLDH) led to approximately two-fold enhancement

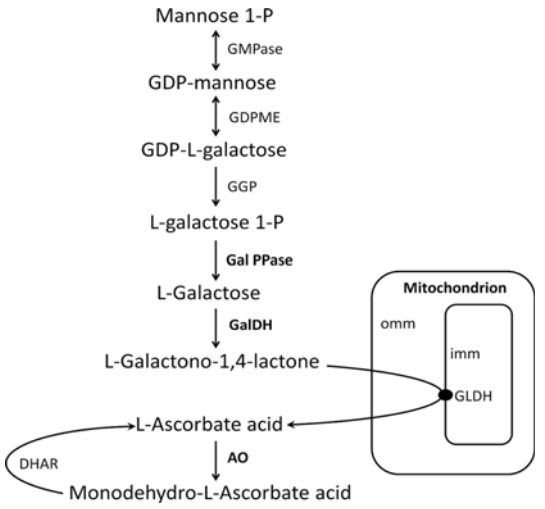

Fig. 1. General scheme of biosynthesis of AsA in plants. GMPase: GDP-mannose pyrophosphorylase; GDPME: GDP-mannose-3,5-epimerase; GGP: GDP-L-galactose phosphorylase; GalPPase: L-Galactose-1-P Phosphatase; GalDH: L-galactose dehydrogenase; GLDH: mitochondrial L-galactono-1,4-lactone dehydrogenase; AO: ascorbate oxidase; DHAR: dehydroascorbate reductase. Other abbreviations: imm, inner mitochondrial membrane; omm, outer mitochondrial membrane.

of AsA content in tobacco suspension cells [8], and our laboratory reported AsA level in *Arabidopsis* was enhanced 2- ~ 4.25-fold by stimulation of the ascorbate recycling pathway *via* overexpressing dehydroascorbate reductase (DHAR) [9]. AsA content of single GDP-galactose guanylyltransferase (GGT) transformation *Arabidopsis* lines were 2.9-fold higher as compared to the control, and co-transformation GGT-GPP (L-galactose-1-phosphate phosphatase) and GGT-GLDH (L-galactono-1,4-lactone dehydrogenase) led up to 4.1-fold AsA enhancement [10]. Nevertheless, the potential of engineering some other important genes for enhancing AsA production should still be exploited.

L-Galactose-1-P Phosphatase (GalPPase) catalyzes the synthesis of L-galactose (L-Gal), whose role as a key intermediate of AsA biosynthesis pathway has been demonstrated by strong biochemical and genetic evidence [11,12]. Thus this step is suggested probably more important in the formation of AsA. Conklin *et al.* [13] indicated GalPPase played a critical role in plant AsA biosynthesis by investigation of a low ascorbate mutant (*vtc4-1*) of *Arabidopsis*. Ioannidi *et al.* [14] showed that *GalPPase* expression was up-regulated during fruit development, suggesting an important control point in AsA biosynthesis. However, until now there are no reports with enhanced AsA content as a result of overexpression of this gene.

AsA pool size is influenced not only by its biosynthesis but also by recycling, it might be expected that the enhancement of the AsA recycling pathway or the down-regulation of AsA oxidation would also affect the AsA accumulation. Ascorbate oxidase (AO) is an apoplastical oxidase that catalyzes the aerobic oxidation of ascorbate to monodehydroascorbate (DHA) [15]. Sense and antisense reduction of AO in transgenic tobacco significantly increased AsA content in apoplast and altered the proportion of ascorbate/DHA [16]. RNAi is a powerful reverse genetic approach to analyze gene functions, and has been employed successfully in metabolic engineering for improvement of several plant species, such as increasing their nutritional value, overall quality and conferring resistance against pathogens and diseases, *etc* [17]. We suggested it might be important to consider employing this technology to reduce AO expression level and ultimately increase AsA content.

L-Galactose dehydrogenase (GalDH) catalyzes L-Gal to form L-galactono-1,4-lactone (L-GalL). The function of GalDH in AsA biosynthesis was investigated by antisense suppression in *Arabidopsis* and overexpression in tobacco [18]. *Arabidopsis*, transformed with an antisense *GalDH* construct, produced lower AsA content compared with wild-type (WT) counterpart. However, *GalDH* overexpression in tobacco did not lead to an increase in AsA concentration. Since GLDH, the enzyme following L-GalDH in the D-Man/L-Gal pathway, located on the inner mitochondrial membrane [19], we proposed targeting *GalDH* to specific cellular compartment (mitochondrion) and thereby providing high concentration of L-GalL would be potentially useful for GLDH producing a marked effect.

In the present study, we applied three distinct strategies, including overexpression of *GalPPase*, RNAi suppression of AO (*AO*i), as well as switching the sub-cellular localization of *GalDH*, to engineering AsA biosynthesis in *Arabidopsis*, the most widely used model plant, and explore their application potential in plant biotechnologies. As a result of *GalPPase* overexpression and *AO* suppression, AsA content was significantly enhanced. Although a cellular compartment target construct to switch the sub-cellular localization of *GalDH* did not stimulate AsA content significantly, we proved that L-GalL accumulation was important to AsA pool. These results provide important insights into the potential application of *GalPPase* and *AO* in plant biotechnologies and indicate the possibility to enhance AsA yield by L-GalL enrichment.

2. Materials and Methods

2.1. Construction of expression cassette and transformation

Construction of *GalPPase* overexpression vector: Full-length cDNA of *GalPPase* (GenBank accession number NM001035549) was isolated from total RNA of *Arabidopsis*

(Columbia ecotype) seedling by one-step RNA PCR. The 6×*Myc* tag was cloned into *Bam*HI and *Spe*I sites of pBluescript SK+ (pBS; Stratagene, Shanghai, China). The *GalPPase* gene was amplified from the pMD-*GalPPase* construct using PCR with an introduced appropriate restriction site at the 5′ends (PCR primer pairs are given in Table S1) and was cloned into *Hin*dIII and *Pst*I sites of pBS-6×*Myc*. After confirmation by sequencing, the fragment encoding *GalPPase-myc* was excised with *Hin*dIII and *Spe*I and subsequently inserted into the PHB binary vector (supplied by Professor H. Yang, SIPPE, CAS) containing a hygromicin resistant gene *hph* and anti-herbicide gene *bar* inside the T-DNA for the selection of transformants (Fig. S1A).

Construction of *AO*i vector: After using BLASTn to ensure the presence of homologous complementary DNA (cDNA) fragments above 30 bp in length, a 500 bp fragment was chosen to be the target of RNA silencing in this study. The DNA fragment was amplified by PCR, based on the sequence of the target cDNA of *AO* from *Arabidopsis* (*AtAO*, GenBank accession number BT003407). Appropriate restriction sites were added to the 5′ends of the primers to amplify the forward segment and the reverse segment, respectively (see the resulting primer sequences in Table S1). The DNA was digested using corresponding restriction enzymes and ligated into the pBS-GUS vector sequentially, with the sequence in the appropriate orientation under the transcriptional control of the *Cauliflower mosaic virus* (CaMV) 35S promoter and octopine synthase (OCS) terminator, which was expected to be expressed in *Arabidopsis* as an inverted repeat fragment. The entire expression element was then excised by restriction enzymes *Hin*dIII and *Bam*HI and incorporated into PHB (Fig. S1B).

Construction of *GalDH* mitochondrion-target expression vector: *GalDH* (accession number ATH417563) and mitochondrial target sequence *Cox*IV (accession number X01418) [20] were PCR amplified from *A. thaliana* and *Saccharomyces cerevisiae*, respectively (see primer sequences in Table S1). *Cox*IV was digested using corresponding restriction enzymes and ligated into the pCAMBIA1304 to form the mitochondrial target expression vector, designated as 1304-*Cox*IV-*GFP*. After sequencing confirmation, the obtained DNA sequence of *GalDH* was excised with *Bgl*II and *Bst*EII and subsequently inserted into 1304-*Cox*IV-*GFP* (Fig. S1C).

Construction of *GalDH* none-target overexpresion vector followed the methods as described above for that of *GalPPase* (Fig. S1D).

After sequencing confirmation, the above four plasmids (Fig. S1) were separately transferred into *Agrobacterium tumefaciens* GV3101 (rifampicin resistant) by triparental mating and the floral-dip method was used for transformation of *Arabidopsis* (Columbia ecotype) [21]. T0 seeds were screened on 15 cm Murashige–Skoog (MS) basal plates supplemented with 50 μg/mL hygromycin. Then, 30 ~ 50 independent lines expressing transgene were transferred to soil and screened on 1% PPT. PCR and Protein gel blot analysis were performed on 8 ~ 10 T3 independent transgenic lines to check for homozygosity (T4). Homozygous T4 seeds were used for final analyses. Chemical components were measured in plants which were 18 days old after 7 ~ 10 days of seed germination.

2.2. Molecular identification of transgenic plants

Integration of the introduced gene (*GalPPase*, *GalDH*) in transformed plants was separately examined by western blot analysis, carried out as described previously with minor modifications [22]. Aliquots of 50 μg total soluble proteins per sample, determined with the DC Protein Assay Kit (Bio-Rad, Hercules, CA, USA), was fractionated on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) mini-gel and blotted to a polyvinylidene difluoride membrane (Amersham, Stafford, UK). The blots were probed with the primary antibody-Myc (Santa Cruz Biotechnology), washed in PBST three times, reacted with goat anti-mouse IgG conjugated with horseradish peroxidase (1:5,000, Amersham), washed, and exposed to X-ray film using the enhanced chemiluminescence method according to the manufacturer's instructions (Amersham).

Molecular identification of *AO*i in transformed plants was achieved by PCR detection of the selectable marker *hph* gene and semi-quantitative RT-PCR analysis of *AO* expression level. Genomic DNA was isolated from both WT and transformed samples using the acetyl trimethyl ammonium bromide (CTAB) method [23]. The DNA was then used in PCR analysis for detecting the presence of *hph* gene (see primer sequences in Table S1). Total RNA was extracted using TRIzol Reagent (GIBCO BRL, Gaithersburg, MD) according to the manufacturer's instruction [24]. After establishing agreement between the OD values of the RNA, aliquot of 1 μg total RNA (1 μg/μL) was used as the template in one-step RT-PCR analysis using One-step RT-PCR Kit (TaKaRa) with *AO*-RT-F and *AO*-RT-R as primers (Table S1). Accordingly, the RT-PCR reaction for the housekeeping gene (*actin*) was performed using specific primers *actin*-RT-F and *actin*-RT-R (Table S1). The densities of the target bands were measured with a WEALTEC Dolphin-DOC ultraviolet analyzer (WEALTEC). Each sample was assayed in triplicate.

2.3. Enzyme assays

Protein extraction for AO assay followed the method as described by Sanmartin *et al.* [25]. Apoplast washing fluid (AWF) was extracted by vacuum infiltration, employing a

method outlined by Turcsányi *et al.* [26]. The activity of AO was assayed spectrophotometrically at 25°C following the oxidation of AsA at 265 nm as described by Moser and Kanellis [27]. The assay mixture contained 66 mM potassium phosphate (pH 5.3), 0.15 mM AsA and plant extract. One unit of AO activity was defined as the oxidation of 1 μmol AsA/min at 25°C, employing a coefficient for AsA of 14 mM/ cm at 265 nm in calculations.

Mitochondria were isolated by differential centrifugation as described by Moore and Proudlove [28]. GalDH was assayed as described by Gatzek *et al.* [18]. Plant tissue (0.2 g FW/mL) was homogenized in 50 mM Tris-HCl (pH 7.5) containing 20% glycerol, 1 mM EDTA, 5 mM dithiothreitol (DTT) and 1% polyvinylpyrrolidone and centrifuged in a microcentrifuge at maximum speed (12,000 g) for 5 min. The supernatants were assayed for GalDH activity by NADH formation at 340 nm in a reaction mixture containing 50 mM Tris-HCl (pH 7.5), 0.1 mM NAD and 5 mM L-Gal.

2.4. Growth evaluation

To evaluate the effects of *AO*i on growth performances, plants from 23 independent lines of *AO*i transgenic and 20 WT lines were respectively tested for their height and weight, on day-18 after seeds germination.

2.5. L-GalL infiltration

Leaves from *GalDH* mitochondrion-target expression, *GalDH* none-target overexpression and WT plants, were respectively infiltrated with L-GalL solution (30 mM) at 12 h intervals for a 60 h period, and then were harvested at hour-72 for AsA content determination [29].

2.6. AsA and DHA content determination

Leaves were ground in 2.5 M $HClO_4$ and after a 10 min centrifugation at 9,500 g, two volumes of 1.25 M Na_2CO_3 were added to the supernatant. One hundred microliters of the mixture after centrifugation was added to 895 μL of 100 mM K_2HPO_4-KH_2PO_4, pH 5.6. After 0.25 units of AO were added, AsA was determined by the change in absorbance at 265 nm. When DHA was reduced to AsA (100 mM K_2HPO_4/KH_2PO_4 at pH 6.5, 2 mM glutathione, and 0.1 μg of *At*DHAR protein, incubated at 25°C for 20 min) before measuring AsA, the total amount of reduced and oxidized ascorbic acid (*i.e.*, AsA and DHA) could be determined. The amount of DHA was determined as the difference between these two assays above [9]. AsA standard was purchased from Sigma-Aldrich (St. Louis, MO, USA, purity $\geqslant$ 99%).

2.7. Statistical analysis

Statistical analysis of the gene expression levels, enzyme activity and metabolites content among various transgenic plants and WT controls was performed with SPSS 13.0 software. Analysis of variance (ANOVA) was followed by Tukey's pairwise comparison tests, at a level of $p < 0.05$, to determine significant differences between means.

3. Results and Discussion

3.1. Enhance the activity of GalPPase to promote AsA biosynthesis

We transformed *Arabidopsis* with the pHB::*GalPPase* binary construct that contained both *GalPPase* and two selected marker genes (hygromicin resistant gene *hph* and anti-herbicide gene *bar*) on the same T-DNA fragment. Primary transformants (T0) were then selected on the basis of resistance to the antibiotic hygromycin (50 μg/mL) and 1% phosphinothricin (PPT) and established transgenic lines by single seed descent. The accumulation of the Myc-GalPPase fusion protein in transgenic plants was examined by western blot analysis using an antibody against Myc. A strong cross-reaction signal corresponding to a polypeptide with an expected molecular weight of 40 kDa for the Myc-GalPPase fusion protein was present in line 2, 5, 7, 8 and 9 among all the eight tested plant lines, and line 8 showed especially high level. As expected, no signal was observed using total protein extracted from WT (Fig. 2A). This result indicated that exogenous *GalPPase* was successfully expressed *via* transgene. These confirmed transgenic lines (2, 5, 7, 8 and 9) were selected for further phenotype analysis and AsA content determination.

Phenotype analysis indicated *GalPPase*-overexpression was not detrimental to the growth of transgenic plants compared with WT. *GalPPase* transgenic seedlings and WT showed similar physical appearances (Fig. 2B). No statistically significant differences ($P > 0.05$) were observed in plant height and number of rosette leaves after tallow and at the end of the culture, respectively (data not shown). Overexpression of *GalPPase* did not lead to chlorosis and necrosis or premature senescence. This result indicated GalPPase did not participate in the development process.

AsA production in *GalPPase* transgenic plants was showed in Fig. 2C. The transgenic lines produced higher levels of AsA compared to WT, and line 8, exhibiting the highest expression level of Myc-GalPPase fusion protein (Fig. 2B), was found to produce the most abundant AsA (3.96 μmol/g FW), which was over 1.6 times more than that in the WT (2.47 μmol/g FW). This result confirmed a strong correlation between GalPPase expression and its capacity to produce AsA. Since GalPPase catalyzes to form L-Gal, which was specialized for the synthesis of AsA [30], and L-Gal feeding increased total AsA contents by approximately five-fold in non-heading Chinese cabbage

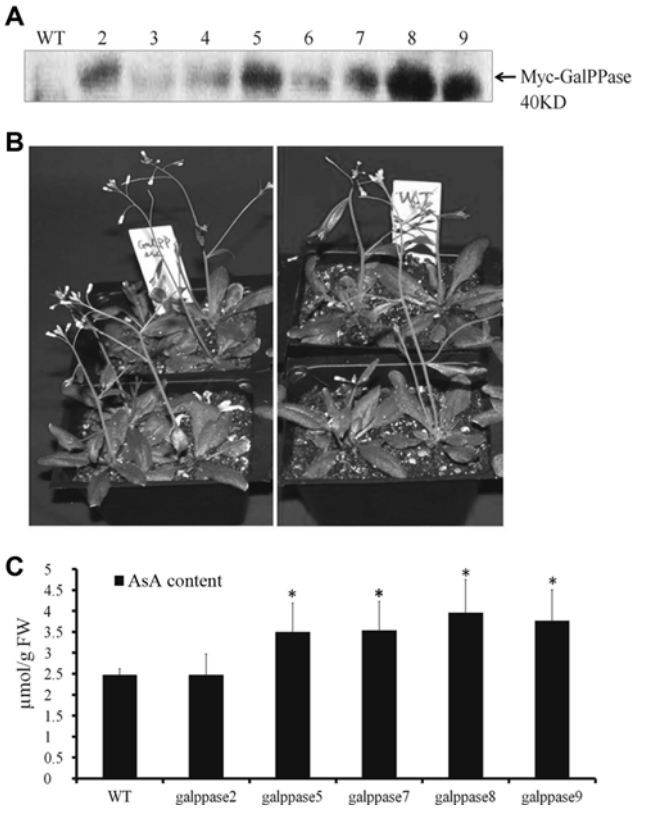

Fig. 2. Molecular identification, phenotype, and AsA content analysis of *GalPPase* transgenic *Arabidopsis*. (A) Western blotting of transgenic plants. (B) Transgenic plants and WT control were grown under normal condition. (C) AsA content in *GalPPase* transgenic and WT plants.

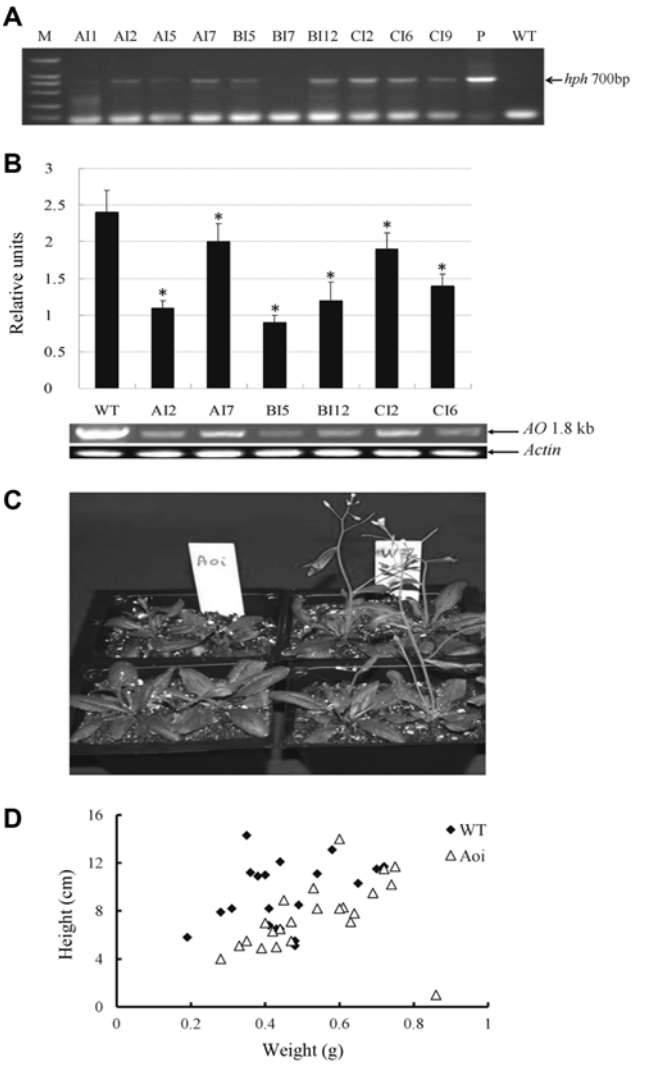

Fig. 3. Molecular identification and phenotype analysis of *AO*i transformed *Arabidopsis*. (A) Representative PCR analyses for *hph* gene in transgenic lines. (B) Representative RT-PCR analysis for the expression of *AO* in WT and six independent transgenic plants. (C) Transgenic plants expressing RNAi vector exhibited a dwarf phenotype compared with WT control. (D) statistical analysis of plant height and biomass.

[29], scientists presumed that enhancement of GalPPase activity would boost L-Gal biosynthesis, ultimately leading to a dramatic increase in the yield of AsA [13,14].

Here, for the first time, we investigated the contribution of *GalPPase* expression to AsA accumulation by means of transgene in *Arabidopsis*. However, the resulting increase of AsA level (only 1.6 times of WT) was not as excited as wished. We suggested higher copy numbers of GalPPase may be useful to further increase vitamin C production. On the other hand, since GDP-L-galactose phosphorylase (GGP), which catalyzes the formation of L-Galactose-1-P, has been demonstrated as one of the rate-limiting factors in AsA biosynthesis [31], we suggested that high concentrations of substrates (L-Galactose-1-P) may be essential for GalPPase producing a more marked effect.

3.2. Decrease the catabolism of AsA by *AO*i

*AO*i transgenic lines were identified by detecting the presence of the selectable marker hygromycin resistance gene *hph*. Result showed 9 lines (AI1, AI2, AI5, AI7, BI5, BI12, CI2, CI6 and CI9) were positive for the presence of *hph*. The expected product size of 700 bp for *hph* gene was observed in these lines and positive control (*AO*i plasmid), whereas not detected in WT (Fig. 3A), indicating *AO*i construct had integrated into the plant genome. Six lines (AI2, AI7, BI5, BI12, CI2 and CI6) were selected for RT-PCR analysis. Result showed that *AO* expression was significantly suppressed through RNAi manipulation ($P < 0.05$). In comparison with the level of WT control, *AO* transcript levels were down-regulated approx. 54, 63, and 42% in line AI2, BI5 and CI6, respectively (Fig. 3B).

The AI2, BI5 and CI6 lines were then used for further AO activity assay and AsA content determination. As shown in Table 1, leaf AO activity in transgenic lines (AI2: 0.02 µmol/mg/min; BI5: 0.01 µmol/mg/min; CI6:

Table 1. AO activity and ascorbate content in whole leaves and apoplasts of *AO*i transgenic and WT lines

	Leaf				Apoplast			
	WT	AI2	BI5	CI6	WT	AI2	BI5	CI6
AO activity[a]	0.04 ± 0.01	0.02 ± 0.00*	0.01 ± 0.00*	0.01 ± 0.00*	0.03 ± 0.00	ND	ND	ND
Total ascorbate[b]	3.87 ± 1.09	3.93 ± 1.21	3.7 ± 0.87	4.02 ± 1.55	ND	0.85 ± 0.23	1.02 ± 0.56	0.97 ± 0.11
AsA[b]	3.60 ± 0.74	3.44 ± 0.86	3.5 ± 0.92	3.58 ± 0.77	ND	0.77 ± 0.20	0.81 ± 0.31	0.86 ± 0.32
DHA[b]	0.3 ± 0.06	0.47 ± 0.16	0.16 ± 0.07	0.4 ± 0.18	ND	ND	0.2 ± 0.04	ND

Leaf samples were taken from 18-day-old-plants grown in controlled environment. Values are the mean of six independent samples per line. Total ascorbate: AsA plus DHA; a: Given as units μmol/mg/min; b: Given as units μmol/g FW; ND: Not detected. *$p < 0.05$.

0.01 μmol/mg/min) was significantly decreased compared with that in WT (0.04 μmol/mg/min, $P < 0.05$), and at the extreme, apoplast AO activity was undetectable in all the tested transgenic lines whereas its value in WT was 0.03 μmol/mg/min. However, the modulation of AO activity did not lead to the alteration of whole leaf ascorbate accumulation. In contrast to whole leaf ascorbate, modified AO expression resulted in dramatic changes in the apoplastic ascorbate pool. Line BI5 and line CI6 produced the highest level of total ascorbate (1.02 μmol/g FW) and AsA (0.86 μmol/g FW) respectively, while in WT line the content of ascorbate, AsA, and DHA were all undetectable. Similar results have been reported by Pignocchi's research group using transgenic tobacco expressing *AO* gene in sense and antisense orientation. The resultant large changes in AO activity in the transformed tobacco plants had little effect on whole leaf ascorbate content, but they had dramatic effects on apoplastic ascorbate levels [16]. The observation that such modulation in AO activity only changed apoplast ascorbate content but did not lead to changes in whole leaf ascorbate, may be explained by the different localization of the two components: *AO* is located in the apoplast, whereas most of the ascorbate is in the cytoplasm [32].

Phenotype analysis showed *AO*i transgenic lines exhibited a shorter phenotype than WT (Fig. 3C), and levels of plant height and weight of these transgenics were also generally lower than that of WT (Fig. 3D). These results indicated the reduction of AO activity by RNAi technology had a negative effect on plant growth, which was in accordance with the statement about AO's role in the regulation of plant growth that AO participated in a broad dialogue between signaling molecules and environmental cues, and therefore its expression stimulated cell growth and development [16,33,34].

Taken together, the engineering of AO is also potentially useful to the improvement of ascorbate accumulation. In order to enlarge total ascorbate pool more efficiently, we suggested it might be important to consider targeting gene products to specific cellular compartments such as cytoplasm in future study.

3.3. Switching the sub-cellular localization of *GalDH*

GalDH mitochondrion-target expression and none-target overexpression vectors were both constructed, and separately introduced into *Arabidopsis* to investigate their different effects on AsA accumulation. Molecular identification of *GalDH* none-target overexpression lines was achieved by examining Myc-GalDH expression using western blot. Result showed a well-defined band was detected at 45 kDa in all six tested lines, which was consistent with the predicted molecular weight values of the Myc-GalDH fusion protein. In contrast, no band was detected in WT line (Fig. 4A). This result indicated exogenous *GalDH* was successfully integrated in transformants. Three transformants

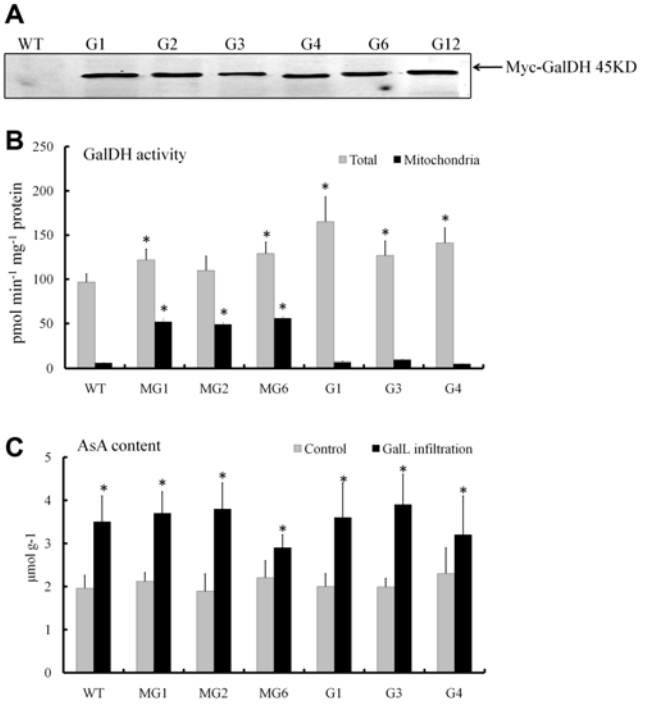

Fig. 4. Molecular identification, enzyme activities, and AsA content analysis of *GalDH* transgenic *Arabidopsis*. (A) Western blotting of *GalDH* none-target overexpression plants. (B) GalDH activity in foliar of transgenic plants and mitochondria sub-cellular compartments. (C) AsA content analysis in foliar of transgenic plants both before and after GalL infiltration.

(G1, G3 and G4) were selected for further enzyme assay and AsA content determination. As expected, levels of total GalDH activity in these transgenic lines were significantly higher than WT ($P < 0.05$) (Fig. 4B).

CoxIV is a well-known transit peptide that can target the in-frame proteins into mitochondria without any misrouting [34] and has already been successfully used for targeting wheat U-ATP9 or sunflower or fH522 into tobacco mitochondria [36-38]. Here, we used this transit peptide for routing GalDH into mitochondria in *Arabidopsis*. As shown in Fig. 4B, GalDH activities in *Arabidopsis* mitochondria were dramatically enhanced in *Cox*IV-*GalDH* transgenic lines (MG1, MG2 and MG6), as compared with those in either WT or *GalDH* transgenic lines. In addition, we observed that *Cox*IV-*GalDH* transgenic lines also possessed higher total GalDH activities than WT, but lower than *GalDH* transgenic lines. This different capacity to enhance total GalDH activities between *GalDH* and *Cox*IV-*GalDH* transgenic lines could be explained by that the introduction of *Cox*IV as a pre-sequence of *GalDH* probably impacted *GalDH* expression. Taken together, these observations indicated the success of routing GalDH into *Arabidopsis* mitochondria.

However, the enhanced GalDH did not result in a significant increase in AsA accumulation, both in *GalDH* mitochondrion-target expression and none-target overexpression lines ($P > 0.05$) (Fig. 4C). In order to reveal the underlying reason, L-GalL, the catalytic product of GalDH, was directly fed to both transgenic (including mitochondrion-target and none-target transformants) and WT plants. Results showed AsA content was significantly enhanced following L-GalL infiltration ($P < 0.05$), the best line was found in G3, which produced AsA with the content of 3.90 μmol/g FW, values that was about 2-fold of that in corresponding untreated control (1.99 μmol/g FW). Moreover, on the part of capacities to produce AsA after L-GalL infiltration, there was no significant difference observed among WT, *GalDH* and *Cox*IV-*GalDH* transgenic plants. These results demonstrated supply of L-GalL indeed could stimulate AsA production, which paralleled our previous hypothesis that L-GalL pool size was one of the most important bottlenecks influencing AsA biosynthesis. The similar result has been found in non-heading Chinese cabbage, in which the AsA content increased by approximately three-fold [29]. However, why did positive modulation of GalDH not result in enhanced AsA accumulation? The reason was probably that the abundance of GalDH activity in *Arabidopsis* was relatively excessive in this specific catalytic reaction, thus GalDH activity enhancement could not make any positive effect. In plants, GalDH is a key enzyme in the biosynthesis of ascorbic acid (AsA), which is well known as a cofactor for many enzymes [39]. Therefore, we suggested that the mere overexpression of the *GalDH* gene was ineffective. High concentrations of L-Gal and other cofactors such as NADPH were necessary for GalDH producing a marked effect.

4. Conclusion

Metabolic engineering is an available approach to understand and use metabolic processes. D-Man/L-Gal pathway is the major AsA biosynthetic process in many plant species [40]. Characterization of synthesis pathways along with the expression profiling of AsA biosynthesis and recycling related genes could bring new insights into regulation mechanisms.

In the present study, for the first time, a systematically function profile of D-Man/L-Gal pathway-related genes were investigated. Three distinct metabolic engineering strategies, including *GalPPase* overexpression, *AO*i suppression and L-GalL feeding, successfully increased AsA accumulation in *Arabidopsis*, and we suggest a combination of these three strategies will be a more effective method to stimulate vitamin C production, which is now under intensive investigation in our laboratory. Taken together, the information gained here did not only elucidate the specific roles of *GalPPase*, *AO* and *GalDH* involved in AsA biosynthesis pathway, but also will certainly help to offer a solid foundation for modern breeding aimed at stimulating vitamin C content in plants such as crops, vegetables and fruits.

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