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Constitutively expressed DHAR and MDHAR influence fruit, but not foliar ascorbate levels in tomato

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Abstract

Vitamin C (*L*-ascorbate, AsA) is an essential nutrient required in key metabolic functions in humans and must be obtained from the diet, mainly from fruits and vegetables. Given its importance in human health and plant physiology we sought to examine the role of the ascorbate recycling enzymes monodehydroascorbate reductase (MDHAR) and dehydroascorbate reductase (DHAR) in tomato (*Solanum lycopersicum*), an economically important fruit crop. Cytosolic-targeted tomato genes *Mdhar* and *Dhar* were cloned and over-expressed under a constitutive promoter in tomato var. Micro-Tom. Lines with increased protein levels and enzymatic activity were identified and examined. Mature green and red ripe fruit from DHAR over-expressing lines had a 1.6 fold increase in AsA content in plants grown under relatively low light conditions (150 $\mu\text{mol m}^{-2} \text{s}^{-1}$). Conversely, MDHAR over-expressers had significantly reduced AsA levels in mature green fruits by 0.7 fold. Neither over-expressing line had altered levels of AsA in foliar tissues. These results underscore a complex regulation of the AsA pool size in tomato.

1. Introduction

Ascorbate (AsA, vitamin C) is synthesized in a number of taxa in the plant kingdom including unicellular algae [1], macroalgae [2], and multicellular plants [3]. Within the plant cell, reactive oxygen species (ROS) are formed from internal biological reactions and triggered by external factors such as biotic and abiotic stresses. Regardless of their source, ROS can cause oxidative damages to proteins, nucleic acids, and lipid membranes, compromising cellular and organismal viability. The major antioxidant in plants poised to deal with ROS is AsA [4], being present at millimolar levels. In animals, vitamin C serves as a cofactor for the hydroxylation of proline and pro-collagen involved in the formation of normal structures of subcutaneous tissue, cartilage, bone and teeth and is implicated in proper immune system functioning, wound healing, allergic defenses, neurodegenerative and cardiovascular diseases, and even cancer [5–10]. In plants, AsA is also essential for the hydroxylation of proline as in the case of extensin and arabinogalactan proteins, and is also required for gibberellins and ethylene synthesis in reactions catalyzed by specific AsA-dependent dioxygenases [11, 12]. However, several lineages of invertebrates, insects, fish,

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some birds, flying mammals, and primates lack the capacity to synthesize AsA [13] which must be obtained from dietary sources. Thus, AsA is an important vitamin, especially in populations where antioxidant nutrients may be limiting. Understanding the synthesis and maintenance of vitamin C in plant sources would facilitate the development of nutrient rich foods by biotechnological or traditional breeding methods.

There are several published reports, as well as reviews, on the over-expression of AsA biosynthetic genes [14–25]. Of particular interest are the studies showing an increase in AsA or redox ratios via the over-expression of the enzymes that recycle oxidized forms of ascorbate, monodehydroascorbate reductase (NADH:monodehydroascorbate oxidoreductase; EC 1.6.5.4; MDHAR) and dehydroascorbate reductase (glutathione:dehydroascorbate oxidoreductase; EC 1.8.5.1; DHAR) (Table 1). Published literature on this topic has shown that AsA increases of 2–4 fold are possible in photosynthetic tissue and 1.5–6 fold can be attained in storage tissues such as potato tubers [26] and maize kernels [23], respectively. The majority of studies to date have focused on photosynthetically active tissues and mostly in model plants such as *Arabidopsis thaliana* [24, 25] and tobacco (*Nicotiana tabacum*) [14–16, 21]. To our knowledge, reports of over-expressing DHAR in a fleshy fruit like tomato are absent from the literature, as are over-expression of MDHAR in any storage organ. The effects of over-expressing these recycling genes in a nutritionally and economically important fruit crop, such as tomato, provide both fundamental and applied insights into strategies to modify AsA levels.

2. Results and Discussion

2.1 Generation of MDHAR and DHAR over-expressing lines

In order to better understand the contribution of the recycling enzymes DHAR and MDHAR to overall levels of AsA in tomato, we generated transgenic lines that over-expressed tomato genes encoding these two enzymes. Cytosolic *Dhar* and *Mdhar* coding sequences were isolated from Ailsa-Craig tomato and cloned under the regulation of the constitutive FMV34S promoter. The resulting constructs were then transformed into tomato (*Solanum lycopersicum*, cv. Micro-Tom) plants. Homozygous lines were selected in the T₂ generation by screening on kanamycin-containing media and confirmed by PCR.

2.2 Identification of over-expressing transgenic lines

To investigate the levels of DHAR and MDHAR protein expression, these two enzymes were examined by Western Blot using enzyme-specific polyclonal antibodies. Preliminary data showed consistent trends in protein levels between fruit and leaf tissues and leaves were used to screen all of the transgenic lines. Total protein extracts of leaf tissue from homozygous lines (T₂) showed elevated levels of DHAR and MDHAR in transgenic lines relative to WT (Fig. 1). Earlier experiments using chitin-column purified recombinant DHAR and MDHAR enzymes expressed in bacteria confirmed that the cDNAs selected to transform tomato plants were indeed enzymatically active against their respective targets *in vitro* (data not shown). However, since activities from heterologous expression systems might not mirror activities *in planta*, nor do increased protein levels necessarily translate to increased activity, we analyzed extracts from leaf tissues for enzymatic activity.

Enzymatic activities from leaf extracts of DHAR and MDHAR over-expressing tomato lines showed increases compared to WT counterparts (Fig. 2). In DHAR lines, approximately 3 fold increases in enzyme activity were observed ($p < 2.5 \times 10^{-9}$), and in MDHAR lines, significant increases from 3–8 fold ($p < 0.0004$) were also noted. The results in leaves were similar to data reported for DHAR [14, 15, 27] and MDHAR [16] in transgenic tobacco and *Arabidopsis*.

2.3 Analysis of ascorbate levels in transgenic lines

Total vitamin C content was subsequently measured in both foliar and fruit tissues of lines identified as having increased enzymatic activity for the two AsA recycling enzymes, DHAR and MDHAR. We had previously observed that DHAR and MDHAR over-expressing plants grown under greenhouse conditions had higher levels of total AsA compared to plants grown in growth chambers. However, differences in AsA content among transgenic lines compared to WT plants were not statistically significant in leaf tissue or red ripe fruit for greenhouse grown plants (data not shown). To investigate if this was the case under different lighting conditions, plants were grown under relatively low light ($150 \mu\text{mol m}^{-2} \text{s}^{-1}$) and samples were taken during the first hours of the light period in order to minimize the contribution of *de novo* synthesized AsA. Samples taken from foliar tissue of Micro-Tom plants at the 5-leaf stage showed no significant differences between transgenic and WT lines ($p \geq 0.05$) in spite of the increased DHAR and MDHAR enzyme activities observed in these transgenic lines (Table 2). In addition, nested one-way ANOVA indicated that differences between WT, MDHAR and DHAR over-expressers as a group were not significant ($p = 0.79$). The experimental results compiled in Table 1 showed that in a handful of studies, over-expressing cytoplasmic-targeted DHAR [27, 28] or MDHAR [29], did not result in significant changes in AsA. Yet, the majority of previously reported studies indicate that over-expression of DHAR tended to increase AsA levels at least 20% relative to WT controls.

AsA content was also examined in red ripe and mature green whole fruits from DHAR and MDHAR over-expressing lines (Fig. 3). At the mature green (MG) stage, tomatoes are firm enough to be picked and transported in bulk, yet are developed enough to be ethylene responsive, allowing for post-harvest ripening. Red ripe (RR) fruit is the stage most commonly associated with market produce, thus understanding AsA recycling in both stages is important from a commercial perspective. When red ripe fruit were tested, 1.5–1.6 fold increases were evident in DHAR lines compared to controls ($p < 0.018$). Similar differences were also observed in mature green fruit ($p < 0.002$). The AsA content of red ripe MDHAR fruits was not significantly different from WT, however, mature green fruits of this genotype were significantly lower in total AsA by approximately 30–50% ($p < 0.014$). The differences between lines over-expressing DHAR and MDHAR may indicate differential regulation of these two enzymes. In agreement with this concept, Jimenez and colleagues [30] showed an inverse relationship in DHAR and MDHAR activities in tomato fruit as it transitioned from mature green to breaker stages and then again during the over-ripe stage. Systematic expression profiling in tomato fruits revealed that transcript levels from two *Mdhar* isoforms were negatively correlated to rising AsA levels during fruit ripening [31]. Conversely, in an ascorbate-QTL mapping study [32], Stevens and colleagues showed that MDHAR activity had a modest positive effect on tomato AsA levels under field-grown conditions, but under chilling stress MDHAR activity strongly correlated with AsA levels, and explained 84% of the variation of AsA levels in tomato fruit. Furthermore, an earlier study showed MDHAR activity negatively correlated with AsA content, but *Mdhar* transcripts were over-expressed when tomato fruit was wounded [33]. Thus, although these correlations do not provide direct evidence for differential regulation of MDHAR and DHAR, they suggest that MDHAR may become an important determinant of AsA levels under stress-inducing conditions.

At the tissue level, AsA content is determined by a number of factors including biosynthesis, turnover, long-distance transport, tissue oxidative burden, and developmental programs. We do not know which of these factors had the most influence in the strikingly different effects of DHAR and MDHAR over-expression between leaves and fruits we describe here. A report in which the actual contribution of AsA biosynthesis and recycling were evaluated in excised maize embryos by blocking biosynthesis, the authors found elevated DHAR activity [34]. These data suggest that all components of the AsA system are highly interconnected,

and that DHAR activity may have an important role under conditions of reduced AsA biosynthesis.

Species-specific differences in biosynthetic and endogenous recycling capacities might also explain the differences between our results in tomato and other model plant systems where increases in AsA levels of up to 6 fold and 2 fold are reported for DHAR and MDHAR over-expressers, respectively (Table 1). Our results serve to illustrate that conclusions drawn from model plant systems do not necessarily translate to other crops. Attempts to increase vitamin C in tomato for applications in field-settings are not likely to be successful using constitutively over-expressed cytosolic *Dhar* and *Mdhar* cis-genes. In addition, results obtained in closely related tobacco and potato plants may not necessarily be mirrored when implemented in tomato. A very important redox and signaling molecule such as AsA is likely highly regulated. Strategies to increase AsA levels beyond current levels may require perturbations in entire regulatory networks using transcription factors or multi-gene approaches.

3. Conclusion

Contrary to previous reports in *Arabidopsis*, tobacco, and potato, over-expression of DHAR and MDHAR enzymes did not result in increases of AsA levels in photosynthetic tissues. However, under low lighting conditions, increases in AsA levels of DHAR over-expressing lines relative to WT were observed in red ripe and mature green fruit, while MDHAR lines had reduced AsA levels in mature green fruit only. This is the first report of over-expressing *Dhar* and *Mdhar* in a fleshy fruit like tomato. The roles of these two genes may be developmentally, and independently regulated as fruit matures and do not appear to be a significantly limiting factor in the maintenance of AsA in tomato leaves under normal growth conditions. However, they may be important in maintaining AsA levels in fruit when endogenous levels are low due to intrinsic or environmental factors.

Further research to determine how MDHAR over-expressing lines perform under cold conditions may be of interest, since MDHAR activity is closely correlated with AsA under chilling stress [32]. As a potential post-harvest application, MDHAR over-expressing tomatoes may serve to maintain AsA levels under low temperature storage conditions, a possibility that warrants examination in the near future.

4. Methods

4.1 Isolation of *Dhar* and *Mdhar* cDNA and Plant Transformation

Full-length *Dhar* (GenBank ID: AY971873.1) and *Mdhar* (GenBank ID: L41345.1) cDNAs were generated from reversed transcribed mRNA isolated from Ailsa-Craig tomato fruit. Resulting cDNAs were sequenced and *in silico* analysis confirmed the predicted open reading frames (ORFs) and respective amino acid sequences. ORFs from each gene were cloned into a plant binary vector, pCAMBIA 2301, having the polylinker replaced by the Figwort Mosaic Virus strong constitutive viral promoter (FMV34S) [35, 36] and a pea ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO) small subunit terminator (rbcS-E9) [37]. Bioinformatic analyses using the TargetP [38] and SignalP [38, 39] algorithms indicated cytoplasmic targeting for both DHAR and MDHAR.

Plasmids were electroporated into *Agrobacterium tumefaciens* GV3101 strain and used to transform Micro-Tom tomato plants at the Ralph M. Parsons Plant Transformation Facility at the University of California, Davis. Transgenic tomatoes were allowed to self-pollinate and resulting T₁ progeny were screened on 0.5X MS media supplemented with 1% sucrose

(w/v), 1% agar (w/v), containing 50 $\mu\text{g mL}^{-1}$ kanamycin. Surviving seedlings were transplanted and allowed to set seed for subsequent generations and homozygote selection.

4.2. MDHAR and DHAR Polyclonal Antibodies

Mdhar and *Dhar* cDNAs were sub-cloned into the IMPACT protein expression vector (New England Biolabs) and used to transform *E. coli* ER2566 cells (New England Biolabs). Recombinant protein expression was induced and purified as recommended using chitin-based affinity columns. Anti-MDHAR and anti-DHAR polyclonal rabbit antiserum was raised against column purified full-length recombinant protein (Antibodies Inc., Davis, CA). Rabbit sera were prescreened to avoid non-specific background reactivity with the target proteins. Competition immunoblots were performed to confirm antibody specificity against their respective targets.

4.3 Growth Conditions and Sampling

Growth chamber conditions (PAR=150 $\mu\text{mol m}^{-2} \text{s}^{-1}$, 16 h day length, 25°C constant, and 65% humidity) were used in experiments for ascorbate quantification and sampling was performed immediately after lights were turned on (ASU, Jonesboro, AR).

4.4 Western Blot and Enzyme Assays

Total tomato protein was extracted using Extraction Buffer A [1.3 M NaCl, 0.2 M CaCl_2 , 13 mM CyDTA, 50 mM Tris-HCl pH 8.0, 0.1% (v/v) Igepal CA-630, 0.75% (w/v) PVPP, 0.25% (w/v) PVP, 5 mM TCEP, 1X Protease Inhibitor Cocktail (Sigma)]. Ten μg of total protein per lane was separated by electrophoresis in a precast 4–20% SDS-PAGE (Bio-Rad). Protein was immobilized by semi-dry transfer to Immuno-Blot PVDF (Bio-Rad) membranes and blocked in 1% (w/v) ECL Advance Blocking Reagent (GE Healthcare) in TBS-T Buffer [50 mM Tris pH 7.5, 150 mM NaCl, 0.025% (v/v) Tween-20]. Three 5 min washes with TBS-T were performed after each antibody incubation. MDHAR and DHAR antisera were used at a 1:3,000 and 1:5,000 dilutions respectively in 0.5% (w/v) blocking reagent TBS-T for 1 h. Secondary antibody incubation of 1:20,000 goat anti-rabbit conjugated with horseradish peroxidase for 1 h was performed. Chemi-luminescent detection with ECL-Lightening (Perkin Elmer) was carried out as per manufacturer's recommendations.

Active protein from leaves was extracted using Extraction Buffer B [50 mM MES pH 7.5, 40 mM KCl, 2 mM CaCl_2 , 13 mM CyDTA, 1 mM AsA, 0.1% (v/v) Igepal CA-630, 0.75% (w/v) PVPP, 0.25% (w/v) PVP, 1X Protease Inhibitor Cocktail (Sigma)]. DHAR, MDHAR activity were quantified as previously described [40] with the exception that volumes were modified for use in disposable 1.0 mL acrylic cuvettes and 20 μL of protein extract was used. Activity was calculated using extinction coefficients as described [40], and total protein was measured using the method of Bradford [41]; activity was normalized for total protein input.

4.5 Ascorbate Assay

Foliar and fruit ascorbate content were determined by the ascorbate oxidase assay adapted to a 96-well plate format and setup essentially as described [22]. Leaf samples were collected during the first hours of the light period and frozen immediately in liquid nitrogen. Whole fruits were collected at the proper ripening stage during the first hours of the light period and immediately used for AsA measurements. Tissues were ground in fresh 6% (w/v) *meta*-phosphoric acid and centrifuged at 15,000 g for 5 min. Reduced AsA was determined by measuring the decline in A_{265} after addition of 0.5 U of ascorbate oxidase (Sigma, St. Louis, MO) to 300 μL of the reaction mix including tissue extract and 100 mM potassium phosphate pH 6.9. Oxidized AsA was determined in a 300 μL reaction mixture including 40

μM dithiothreitol and incubating at room temperature in the dark for 30 min. Total AsA was the sum of reduced and oxidized AsA. Calculations were based on a standard curve with pure AsA run in parallel.

4.6 Statistics

Statistics were performed in Excel using the 2-tailed Student's T-Test function, assuming unequal variances. Nested one-way ANOVA was also performed for leaf ascorbate content. An asterisk in figures denotes statistically significant values. Results were reported to be statistically significant if P-values were <0.05 .

Abbreviations

AsA	ascorbate
BSA	bovine serum albumin
CyDTA	1,2-cyclohexylenedinitrotetraacetic acid
DHA	dehydroascorbate
DHAR	DHA reductase
Igepal CA-630	(octylphenoxy)polyethoxyethanol
MDHA	monodehydroascorbate
MDHAR	MDHA reductase
MES	2-(N-morpholino)ethanesulfonic acid
MS	Murashige and Skogg (1962)
PVP	polyvinylpyrrolidone
PVPP	polyvinylpolypyrrolidone
TBS	tris-buffered saline
TCEP	tris(2-carboxyethyl)phosphine
WT	wild type

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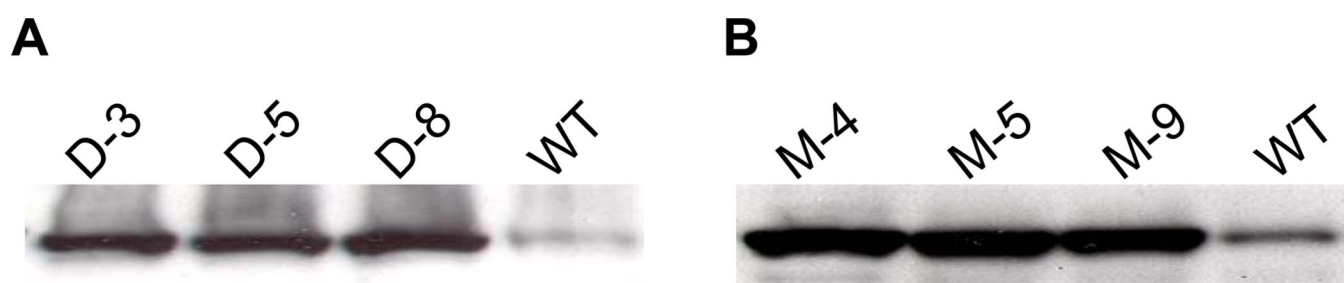


Fig. 1. Western blot analysis of AsA recycling enzymes in leaf total protein extracts from WT and three independent (A) DHAR and (B) MDHAR transgenic lines. Ten μg of total protein was loaded per lane, resolved on a 4–20% gradient SDS-PAGE, transferred to PVDF and probed with respective antibodies.

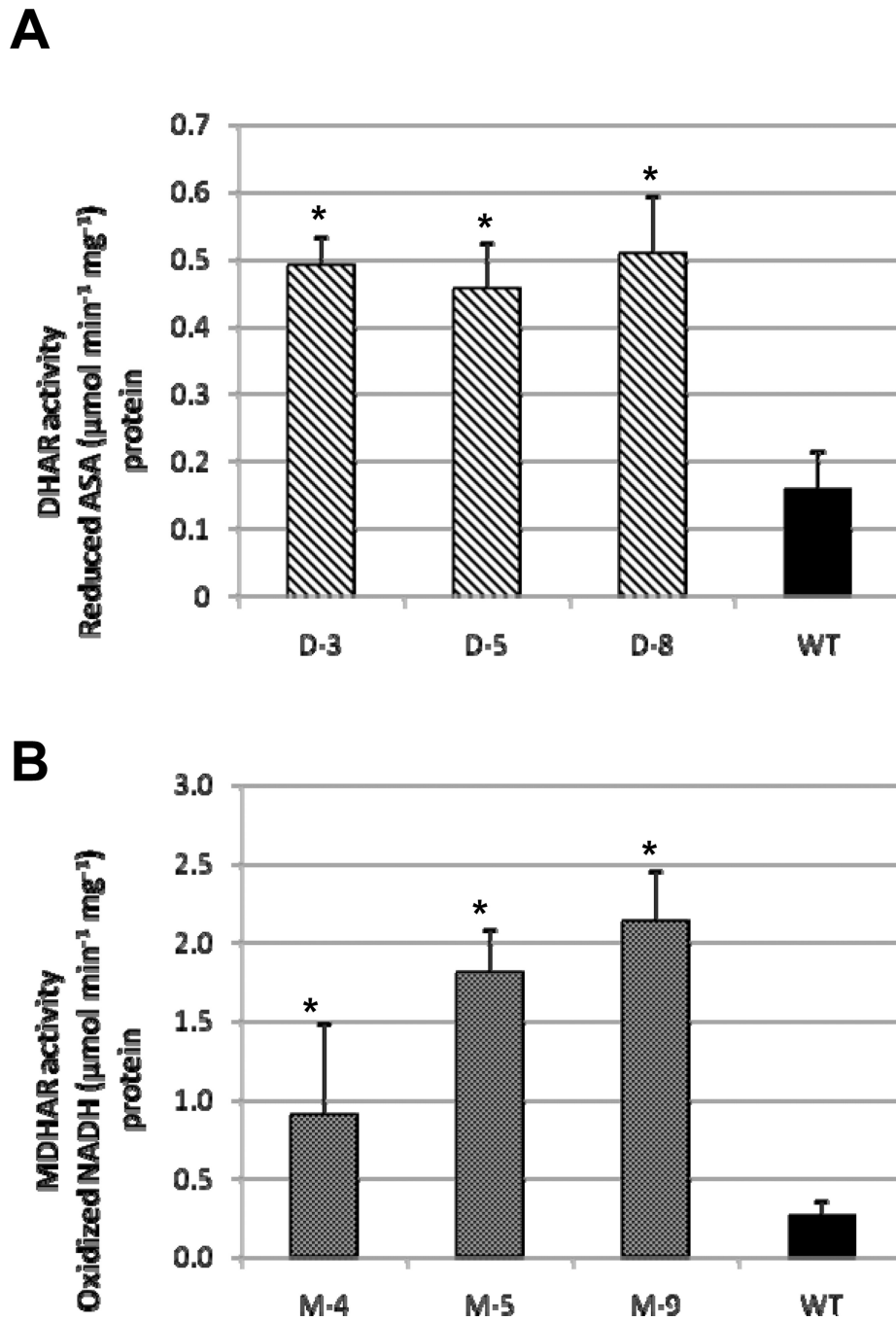


Fig. 2. *In vitro* enzymatic activity of (A) DHAR and (B) MDHAR in leaf protein extracts. Activity was normalized for total protein input as determined by Bradford quantification. Data represent the mean \pm standard deviation ($n=12$). Significance at $P<0.05$ is indicated by asterisks. Diagonal fill=DHAR, dotted fill=MDHAR, solid fill=WT.

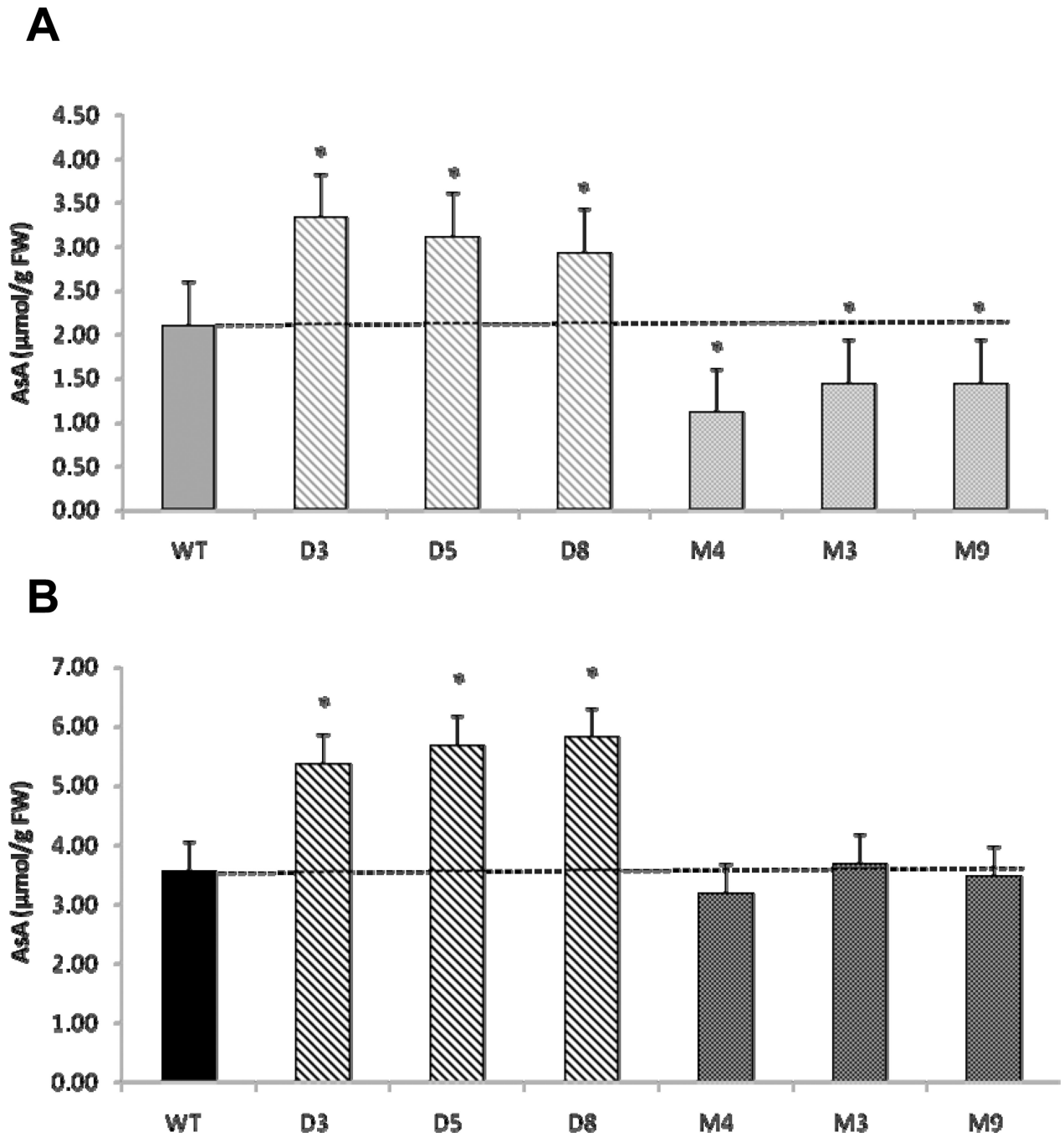


Fig. 3. Total ascorbate levels in (A) mature green and (B) red ripe whole fruit extracts. Data represent the mean \pm standard deviation ($n > 9$). Significance at $P < 0.05$ is indicated by asterisks. Diagonal fill=DHAR, dotted fill=MDHAR, solid fill=WT. Grey=mature green, black=red ripe.

Table 1

Summary of transgenic strategies to stably engineer elevated ascorbate levels in plants by overexpressing MDHAR or DHAR.

Target	Plant engineered	Enzyme	Gene source	Promoter used	Fold Increase in total AsA	Other effects of modification / comments
<i>Leaf Tissue</i>						
	Tobacco[42]	Chl-DHAR	Human	NR	0.84	Increased GR activity 2X increase in AsA redox ratio Paraquat, cold and NaCl tolerance
	Tobacco[21]	Chl-DHAR	Human	NR	1.1	Increased SOD and APX activities in conjunction via triple gene construct Enhanced GSH levels Paraquat, and NaCl tolerance
	Tobacco[15]	DHAR	Arabidopsis	CaMV35S	1.9–2.1	Ozone, PEG, NaCl, and drought tolerance
	Arabidopsis[27]	DHAR	Rice	CaMV35S	1.1–1.2	NaCl tolerance
	Tobacco[29]	DHAR	Arabidopsis	CaMV35S	1.2–1.3	Aluminum tolerance in roots
	Arabidopsis[24]	DHAR	Arabidopsis	CaMV35S	3.3	Increased GSH level Paraquat, high light and temperature tolerance
	Arabidopsis[25]	Chl-MDHAR	Tomato	CaMV35S	1.2	Paraquat, low and high temperature stress tolerance
	Tobacco[29]	MDHAR	Arabidopsis	CaMV35S	1.1	No significant responses
	Tobacco[29]	MDHAR	Arabidopsis	CaMV35S	1.1	No significant responses
	Tobacco[16]	MDHAR	Arabidopsis	CaMV35S	2.0–2.2	NaCl and PEG stress tolerance
<i>Storage organ</i>						
	Maize[23]	DHAR	Rice	D-hordenin	6.2	Increased folate and β -carotene levels engineered in conjunction via triple gene construct
<i>Leaf & storage organ</i>						
	Potato[28]	Chl-DHAR, DHAR	Potato	CaMV35S	1.1–1.2	Increased AsA in leaves and tubers of cytosolic targeted Increased AsA only in leaves of chloroplast targeted
	Potato[26]	DHAR	Sesame	Patatin CaMV35S	1.1–1.3 1.5–1.6	Increased AsA in tuber only with patatin promoter Increased AsA in leaves and tuber with CaMV35S promoter
	Tomato*	DHAR	Tomato	FMV34S	1.5–1.6	No increase in foliar AsA Increased AsA in mature green and red-ripe fruit
	Tobacco and maize[14]	DHAR	Wheat	Shruken2 Ubiquitin CaMV35S	1.8–3.9	Increased GSH in maize kernel and leaves Increased GSH in tobacco leaves MDHAR activity reduced ~2X
	Tomato*	MDHAR	Tomato	FMV34S	0.6–1.6	No increase in foliar or red-ripe fruit AsA Decreased AsA in mature green fruit

Studies are grouped by targeted organ (leaf tissue, storage organ, or both); current study is indicated by asterisks.

Abbreviations: APX, ascorbate peroxidase; CaMV35S, 35S promoter of the Cauliflower mosaic virus; FMV34S, 34S promoter of the Figwort mosaic virus; Chl, chloroplastic enzyme; GR, glutathione reductase; GSH, glutathione, NR, not reported; PEG, polyethylene glycol; SOD, superoxide dismutase.

Table 2

Total ascorbate content in leaves of DHAR and MDHAR over-expressing tomato lines compared to WT controls. The content of oxidized AsA was less than 5% for all genotypes. Values represent the mean \pm standard deviation, and are reported as $\mu\text{mol}\cdot\text{g}^{-1}$ fresh weight. P-values comparing WT to transgenic lines are indicated below.

WT	D-3	D-5	D-8	M-4	M-5	M-9
3.22	2.22	2.80	3.35	2.43	2.58	2.61
3.30	2.14	2.92	3.38	3.24	2.44	2.41
2.79	4.22	4.01	3.42	3.99	4.43	3.10
3.09	3.93	3.95	3.57		5.50	3.43
3.10 ± 0.22	3.13 ± 1.10	3.42 ± 0.65	3.43 ± 0.10	3.22 ± 0.78	3.74 ± 1.48	2.89 ± 0.46
p-value	0.96	0.40	0.05	0.82	0.46	0.46