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Research Article

Increased antioxidant capacity in tomato by ectopic expression of the strawberry D-*galacturonate reductase* gene

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Increasing L-ascorbic acid (AsA, vitamin C) content in fruits is a common goal in current breeding programs due to its beneficial effect on human health. Attempts to increase AsA content by genetic engineering have resulted in variable success likely due to AsA's complex regulation. Here, we report the effect of ectopically expressing in tomato the D-galacturonate reductase (FaGalUR) gene from strawberry, involved in AsA biosynthesis, either under the control of the constitutive *35S* or the tomato fruit-specific *polygalucturonase* (*PG*) promoters. Although transgenic lines showed a moderate increase on AsA content, complex changes in metabolites were found in transgenic fruits. Metabolomic analyses of ripe fruits identified a decrease in citrate, glutamate, asparagine, glucose, and fructose, accompanied by an increase of sucrose, galactinol, and chlorogenic acid. Significant metabolic changes also occurred in leaves of *35S-FaGalUR* lines, which showed higher non-photochemical fluorescence quenching (NPQ), indicative of a higher constitutive photoprotective capacity. Overall, overexpression of *FaGalUR* increased total antioxidant capacity in fruits and the results suggest a tight control of AsA content, probably linked to a complex regulation of cellular redox state and metabolic adjustment.

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1 Introduction

Humans must acquire vitamin C (ascorbic acid, AsA) from regular intake of fruit and vegetables because the biosynthetic pathway of AsA is non-functional due to a mutation in the L-gulono-1,4-lactone oxidase gene [1]. Although deficiency of AsA is not common in developed countries, diets enriched or supplemented with AsA have been associated with a decreased incidence of several human diseases and disorders [2].

In plants, AsA influences multiple aspects of plant biology [3–5]. As the main soluble antioxidant, AsA exerts a crucial role in the detoxification of reactive oxygen species generated during photosynthesis, cellular respiration, and abiotic stress responses [6]. During photo-protection, AsA is involved in scavenging hydrogen peroxide via ascorbate peroxidase, regeneration of α-tocopherol, electron donation to photosystem II (PSII), and as a cofactor for violaxanthin de-epoxidase in the xanthophyll cycle for dissipation of light energy as heat. AsA plays additional roles in cell division and expansion during plant

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Abbreviations: **ABTS,** 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonate); **AsA,** L-ascorbic acid; **DHA,** dehydroascorbate; **FaGalUR,** D-galacturonate reductase; **FW,** fresh weight; **GALDH,** L-galactono-1,4-lactone dehydrogenase; **NPQ,** non-photochemical fluorescence quenching; **PG,** polygalacturonase; **PSII,** photosystem II; **SSC,** soluble solids content; **TA,** titratable acidity; **TCA,** tricarboxylic acids; **TEAC,** trolox equivalent antioxidant capacity

development [7, 8], senescence [9], defence against pathogens [10, 11], and hormonal regulation [12]. From an applied point of view, higher AsA content has been associated with improved postharvest quality in apple and pear fruits [13, 14].

For all these reasons, biotechnological approaches have been undertaken to increase AsA content in different crops [5, 15]. Attempts to engineer increased AsA have produced considerable advances in our understanding of metabolic pathways but have also underlined the necessity of cross-disciplinary approaches in order to gain insight into the complex regulatory mechanisms that control its content [16].

Different biosynthetic pathways have been described in plants, with AsA being produced via de novo synthesis or through carbon skeleton re-cycling networks [3, 5, 17]. It is generally accepted that the prevalent de novo pathway, known as the Smirnoff–Wheeler pathway, uses GDP-mannose and proceeds through the key intermediate L-galactose [18]. Additionally, it has been postulated that AsA can also proceed through GDP-mannose via L-gulose [19]. In a recovery pathway first described in strawberry, AsA is synthesized through the reduction of D-galacturonic acid (likely resulting from pectin degradation) to L-galactonic acid by the D-galacturonate reductase enzyme FaGalUR [20]. L-Galactonic acid is then converted to L-galactono-1,4-lactone and further oxidized to AsA by the mitochondrial L-galactono-1,4-lactone dehydrogenase (GALDH), which is common to the main Smirnoff–Wheeler pathway. The prevalence of each pathway is likely dependent on the species, tissue, and developmental stage.

Tomato is a highly consumed fruit and therefore an important crop in an agronomical and economical context. However, commercial tomato fruits have a relatively low AsA content, about 15–20 mg/100 g fresh weight (FW), in comparison with other fruits [5, 21]. Besides, tomato has long been a model organism for studying fruit development and ripening, and many studies have unraveled metabolic networks during these developmental processes (i.e. [22]). Therefore, tomato represents an appropriate target for increasing AsA content. The role of FaGalUR for AsA biosynthesis through the D-galacturonate pathway has been studied in strawberry fruit [20, 23] and overexpression in Arabidopsis and potato tubers increased AsA by two- to three-fold [20, 24] but there are not conclusive studies in tomato fruits. While transcript analysis supported the L-galactose pathway in this species [25], recent studies using Micro-Tom tomatoes reported the prevalence of the L-galacturonate pathway in ripe fruits [26]. In this report, we overexpressed *FaGalUR* in tomato using two different promoters and analyze the effect on AsA content and associated metabolic changes.

2 Materials and methods

2.1 Plant material and growth conditions

Ten plants of each tomato (*Solanum lycopersicum* L. cv. Moneymaker) transgenic line were grown in soil within a polyethylene greenhouse at Estación Experimental La Mayora, Algarrobo Costa, Málaga, Spain. During the fructification stage, mean daily maximum/minimum temperatures within the greenhouse were 31 ± 3.5/21.5 ± 2.5°C. Leaf tissue for Western blot, AsA and metabolite determinations was sampled from 4-wk-old plants by sampling the two youngest leaves larger than 3 cm from each of the 10 plants of each line and pooling them into three biological replicates. Green fruits were harvested in the immature green ripening stage. Metabolites, AsA content, and antioxidant capacity of fruits were determined in the light red stage, when 100% of the fruit is red and about 2 days before the red ripe stage. Ripening stages are named according to Gonzalez-Bosch et al. [27]. Three fruits harvested from each of the 10 plants of each line were divided into three pools that were used as replicates. Soluble solids content (SSC) and titratable acidity (TA) were determined in fully mature red fruits. Second batches of plants were grown under controlled conditions (see below) in a growth chamber for leaf photosynthetic capacity evaluation.

2.2 Plant transformation

For constitutive overexpression, the *FaGalUR* open reading frame (ORF) was cloned under the control of the constitutive cauliflower mosaic virus (CaMV) 35S promoter in the pBINPLUS plasmid [28] containing the *nptII* gene for kanamycin resistance. For fruit specific expression, the *FaGalUR* ORF was used to replace the GUS-intron insert of plasmid pPGGUSI, which contains a 4.8 Kb promoter fragment of the polygalacturonase (PG) gene and its terminator [29]. Each of three different binary vectors, namely (i) the binary empty vector pBINPLUS as control, (ii) the 35S-*FaGalUR*, and the (iii) PG-*FaGalUR* constructs were mobilized from *Escherichia coli* into *Agrobacterium tumefaciens* strain LBA4404 by triparental mating [30] and used to transform tomato plants. Transformation of tomato was performed essentially as described by Ellul et al. [31] with the exception that regenerated shoots were transferred for elongation to a medium with zeatin instead of IAA, and no hormone was added to the rooting medium.

To confirm the identity of transgenic plants, genomic DNA was isolated following standard procedures [32] and the inserted *FaGalUR* gene was confirmed by PCR using specific primers. Independent transgenic tomato lines expressing *FaGalUR*, as tested by Western blot (Supporting information, Fig. S1), were self-pollinated and resulting T1 progeny was screened on 0.5× MS media supplement-

ed with 1% sucrose (w/v), 7% agar (w/v), and 50 mg/mL kanamycin. In the following generation, homozygote lines were selected based on kanamycin resistance.

2.3 Protein extraction and immunoblot analysis

Tomato leaves and fruits were ground using liquid nitrogen and proteins extracted using 2× and 6× Laemmli buffer, respectively. Protein were separated on 12% polyacrylamide gels and blotted onto nitrocellulose membranes, as described [23]. Equal volumes of protein sample (15 μL) were loaded on the gels and equal loading checked using Coomassie blue (Supporting information, Fig. S2). Membranes were blocked using 5% milk and incubated with anti-FaGalUR polyclonal antibodies [20]. Signal was developed using anti-rabbit antibodies conjugated to horseradish peroxidase and the ECL detection kit (Amersham Biosciences, http://www.gelifesciences.com/), and exposed to an X-ray film. Protein extract from ripe strawberry fruit was used as control in Western blots (Supporting information, Fig. S2).

2.4 Agronomic and fruit quality evaluation

Basic agronomic and fruit quality traits were evaluated in plants grown in standard greenhouse conditions, equivalent to those used for commercial tomato production. Plant yield (in g) and fruit number per plant were evaluated for each line (10 plants for each line). SSC (in Brix) was evaluated in five ripe fruits per line with a digital refractometer (Atago PR32, Japan). Tomato juice was obtained from frozen and ground fruit tissue after centrifugation for 5 min at 3000*g*. TA was measured in three replicates of each line with an automatic titrator fitted with pH meter and autoburete (Titroline Easy, Schott Instruments, Germany), diluting 1 g of tomato juice in 100 mL of distilled water. Titrations were carried out to an end pH of 8.1 with 0.01 N NaOH and the results were referred to meq NaOH per 100 mL of juice.

2.5 Ascorbic acid determination

AsA content in leaf and fruit tissue was determined using high-performance liquid chromatography as previously described [33]. The level of total ascorbate (reduced and oxidized or AsA + dehydroascorbate (DHA), respectively) was determined as reported by Davey et al. [34].

2.6 Total antioxidant capacity

Polar compounds were extracted from 0.3 g of frozen fruit tissue homogenized in 1.5 mL of 80% v/v aqueous methanol. Samples were then incubated for 2 h at room temperature on the dark, centrifuged at 13 000 rpm for 10 min and the supernatant transferred to a new tube. The Trolox equivalent antioxidant capacity (TEAC) assays based on the ability of antioxidant molecules to quench the long-lived ABTS+ radical cation [2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonate); Sigma–Aldrich] were performed as described [35, 36]. Ten μL of sample was added to 1 mL ABTS+ solution and the Abs at 734 nm recorded. Results were compared with the ability of Trolox (a water-soluble vitamin E analog) to quench the ABTS+ radical. Results were expressed as TEAC in mmol of Trolox per kg of FW. Three replicates of each line (with two technical replicates each) were analyzed and used for statistical analysis.

2.7 Metabolite analysis

Metabolite extraction, derivatization, standard addition, and sample injection for gas chromatography–mass spectrometry (GC–MS) were performed according to Osorio et al. [37]. Both chromatograms and mass spectra were evaluated using TAGFINDER [38]. Three biological replicates of each line were analyzed and used for statistical analysis.

2.8 Chlorophyll a fluorescence measurements

In vivo chlorophyll *a* fluorescence signals were measured with a portable fluorometer PAM-2100 (Heinz Walz, Effeltrich, Germany). Plants were grown for 6 weeks in a growth-chamber under controlled conditions (i.e. long-day photoperiod, temperature ≈28°C and irradiance of 100 mol quanta m^{-2} s^{-1}) and transferred for three additional weeks to a shadow-house with 60% of natural light and daily average temperature of 25 ± 1.5°C, in order to achieve higher light intensities for light-induced quenching. Measurements were taken in 9 weeks-old plants. The third fully expanded leaf of nine plants per transgenic line was measured before dawn (darkness) and at midmorning (11:00 am), when photosynthetic photon flux density on the leaves was 355 ± 1.5 μmol m^{-2} s^{-1}. No appreciable differences in leaf morphology and thickness were observed among transgenic plants. The so-called saturation pulse method was used for determinations of the fluorescence parameters [39]. Maximal photochemical efficiency of PSII ($F_v/F_m = [F_m - F_o]/F_m$) was calculated after the determination of the initial minimal fluorescence (F_o) and the maximal fluorescence levels in the in the dark-adapted state (F_m). The quantum yield of non-regulated non-photochemical energy loss in PSII for the dark-adapted state (Φ_{NOpd}) was calculated as F_o/F_m, with healthy green leaves showing values of about 0.2, according to Klughammer and Schreiber [40].

Under light conditions, the steady-state fluorescence yield (F_s), and the maximal fluorescence under light conditions (F'_m) after a light-saturation pulse were determined. Non-photochemical quenching ($NPQ = [F_m - F'_m]/[F'_m]$), the relative quantum yield of PSII photochemistry ($\Phi_{PSII} = [F'_m - F_s]/F'_m$), the yield for regulated non-photo-

chemical dissipation ($\Phi_{NPQ} = F_s/F'_m - F_s/F_m$) and the yield of non-regulated non-photochemical losses in the steady-state light conditions ($\Phi_{NO} = F_s/F_m$) were determined according to Klughammer and Schreiber [40] and references therein. In this approach, non-photochemical losses (Φ_n) can be dissected into Φ_{NO}, which reflects non-light induced (basal or dark) quenching processes and Φ_{NPQ}, which reflects either all light-induced or only the rapidly reversible processes considered to be regulatory [41].

2.9 Statistical analysis

Data were analyzed using the analytical software Statistix 9.0 (Analytical Software, Florida, USA). Statistical differences among transgenic lines in all the variables measured (fruit quality, AsA, antioxidant capacity, metabolites, and chlorophyll fluorescence) were assessed by a one-way ANOVA. When significant differences ($p < 0.05$) were found, means were separated by the least significant differences test (LSD). Normality and homogeneity assumptions were tested prior to ANOVA by using the Kolmogorov–Smirnov and Cochran's *C* tests, respectively.

3 Results

3.1 Generation of tomato lines expressing a D-galacturonic acid reductase from strawberry

In order to ectopically express *FaGalUR* in tomato, we employed two different strategies, in the first one, *FaGalUR* expression was driven by the *CaMV 35S* promoter, which causes the expression of the gene in most parts of the plants, while in the second, *FaGalUR* was expressed under the control of the tomato fruit ripening-specific *polygalacturonase* (*PG*) promoter [29]. Tomato plants transformed with the empty binary vector were used as controls. Between 26 and 34 independent lines resistant to kanamycin were regenerated from each transformation. Presence of the transgene was confirmed first by PCR using DNA extracted from leaves, and second by Western blot analysis of ripe fruits using *FaGalUR*-specific polyclonal antibodies (Supporting information, Fig. S1). Two *35S-FaGalUR* (L2-4 and L2-11) and three *PG-FaGaLUR* lines (L3-1, L3-6, and L3-24) showing a single insertion and high content of FaGalUR protein in ripe fruits were selected and homozygous lines were generated for further analysis. Two homozygous control lines (L1-2 and L1-4) carrying one insertion of the empty vector were generated for the study.

We next examined the protein level of FaGalUR in leaf, green immature, and light red fruits (Fig. 1; Supporting information, Fig. S2). Both *35S-FaGalUR* and *PG-FaGalUR* lines displayed comparable levels of FaGalUR protein in light red fruits. As expected from the fruit specific expression of the *PG* promoter [29], only L2-4 and L2-11 expressed the protein in leaves (Fig. 1A) and immature green fruits (Fig. 1B).

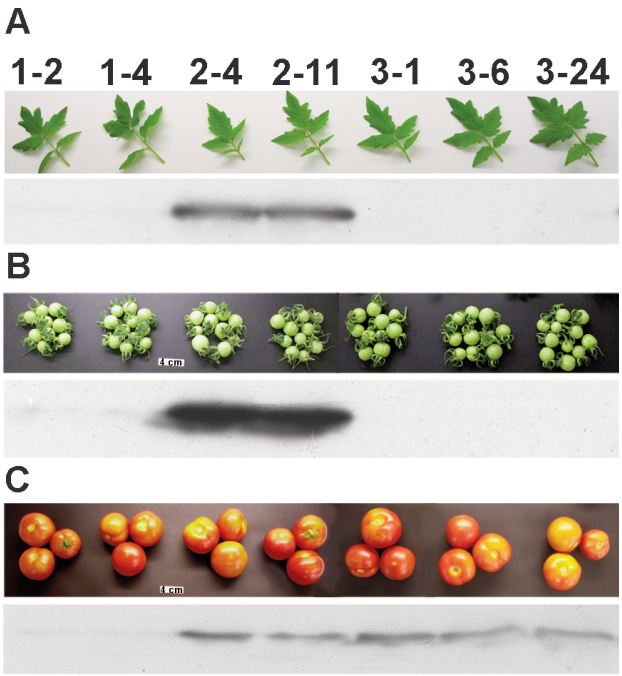

Figure 1. Western blot analysis of FaGalUR in total protein extracts of (**A**) leaf, (**B**) green, and (**C**) light red fruits from control (lines 1-2 and 1-4), two independent *35S-FaGalUR* (2-4 and 2-11) and three independent *PG-FaGalUR* (3-1, 3-6, and 3-24) transgenic lines. Fifteen µL of protein extract was loaded per lane, resolved on a 12% SDS–PAGE, transferred to nitrocellulose and probed with anti-FaGalUR polyclonal antibodies. Loading controls are shown in Supporting information, Fig. S2.

All of the transgenic lines were morphologically indistinguishable over different generations from control lines both in vegetative traits, such as leaf size or plant height, and fruit traits such as color or size (Supporting information, Fig. S3). However, the majority of transgenic plants displayed a slight increase in fruit yield, up to 1.4-fold for line 3-6, which was a consequence of an increase in the number of fruits rather than an increase in fruit weight (Fig. 2A). Fruit SSC and TA are important traits for tomato breeders and industry since they are key determinants of fruit quality and taste [42]. No significant changes were found in soluble solids (Fig. 2B), but a reduction in acidity was observed in L2-4, L3-1, and L3-6 transgenic fruits, showing a reduction of 25, 27, and 22%, respectively (Fig. 2C).

3.2 Expression of *FaGalUR* in tomato increased AsA content in light red fruit but not consistently in green tissues

AsA content was measured in leaf, green immature, and light red fruits of the selected transgenic lines. Because levels of AsA change during tomato fruit ripening [22], it

is essential to select the same ripening stage in the samples in order to effectively compare the changes among different lines. Therefore, fruits at the light red stage were selected for the analysis because it is easy and reliably established for tomato [27]. The content of AsA in leaf was only significantly increased 1.26-fold in L2-4 when compared with the average value of the two controls, which exhibited different AsA content (Fig. 3A). Total AsA content (AsA + DHA) was also measured in leaves and, albeit no significant differences were observed between the transgenic and control lines, both *35S-FaGalUR* lines displayed the highest values (Fig. 3A). The AsA/AsA + DHA ratio ranged from 0.65 to 0.85 among the lines with no association to any of the three genotypes (L1, L2, and L3). In green fruits, no significant differences in AsA content were found between control and transgenic lines, having an average value of 20 mg/100 g FW in all lines (Fig. 3B). However, light red fruits of transgenic lines (with the exception of L3-6) showed a slight but statistically significant increase in AsA, ranging from 1.22- to 1.36-fold (Fig. 3C). The content of DHA was very low in light red fruits and therefore AsA/AsA + DHA ranged from 1 to 0.96 among the lines (Fig. 3C). Regarding total AsA (AsA + DHA) in light red fruits, only L2-4, L3-1, and L3-24 displayed significant differences with the L1 controls (Fig. 3C).

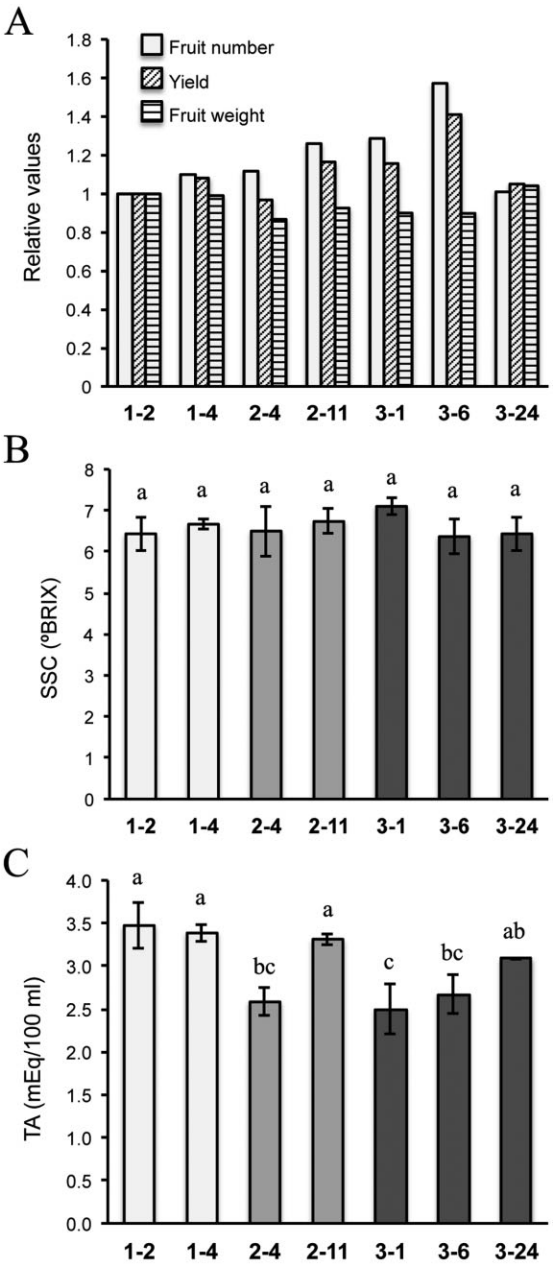

Figure 2. Characterization of *FaGalUR* transgenic lines. (**A**) Agronomic traits in transgenic and control lines. (**B**) Soluble solids content and (**C**) titratable acidity (TA) in red ripe fruits. The 10 plants of each line were evaluated as a bulk in (**A**). For (**B**) and (**C**), data points (mean ± SD) with different letters above bars indicate significant differences by least-squared-difference (LSD; $p < 0.05$; $n = 5$ for SSC and 3 for TA), using one-way analysis of variance (ANOVA).

In most plant tissues, AsA is the most abundant low molecular weight antioxidant [43], having an important contribution to the total antioxidant activity, which is commonly evaluated by measuring the TEAC [36]. Then, TEAC was evaluated in polar extracts of tomato light red fruits (Fig. 3D). Significant increases in antioxidant capacity were found for L2-4, L3-1, and L3-24, the three lines with the highest AsA content.

3.3 Metabolic changes in fruits and leaves of *FaGalUR* tomato lines

The content of certain primary metabolites, mostly glycolytic and tricarboxylic acids (TCA) intermediates, has been shown as a reliable diagnostic tool to assess the redox state of a plant organ [44]. Therefore, metabolite levels in light red fruits were determined by GC–MS [37]. Significant and consistent changes between *FaGalUR* and control lines were found for some groups of metabolites (Fig. 4; Supporting information, Table S1). An increase of erythritol and galactinol (up to five-fold) occurred in light red fruits of all transgenic lines compared to the controls. In addition, the disaccharides sucrose, maltose, and isomaltose were also increased in the transgenics. In contrast, glucose and fructose decreased in *FaGalUR* lines, although were only significant in L3 lines. A reduction higher than 50% of citrate content, an intermediate of the TCA cycle, was also observed in all transgenic lines. Other organic acids were slightly, but not significantly, reduced in transgenic fruits, which altogether explain the reduction in TA previously reported (Fig. 2C). The content of Asn and Glu, whose biosynthesis is directly connected to TCA intermediates, were reduced by 50 and 30%, respectively, in all transgenic lines. The only amino acid whose content increased in the transgenic fruits was Ala, although this increase was significant only in two lines.

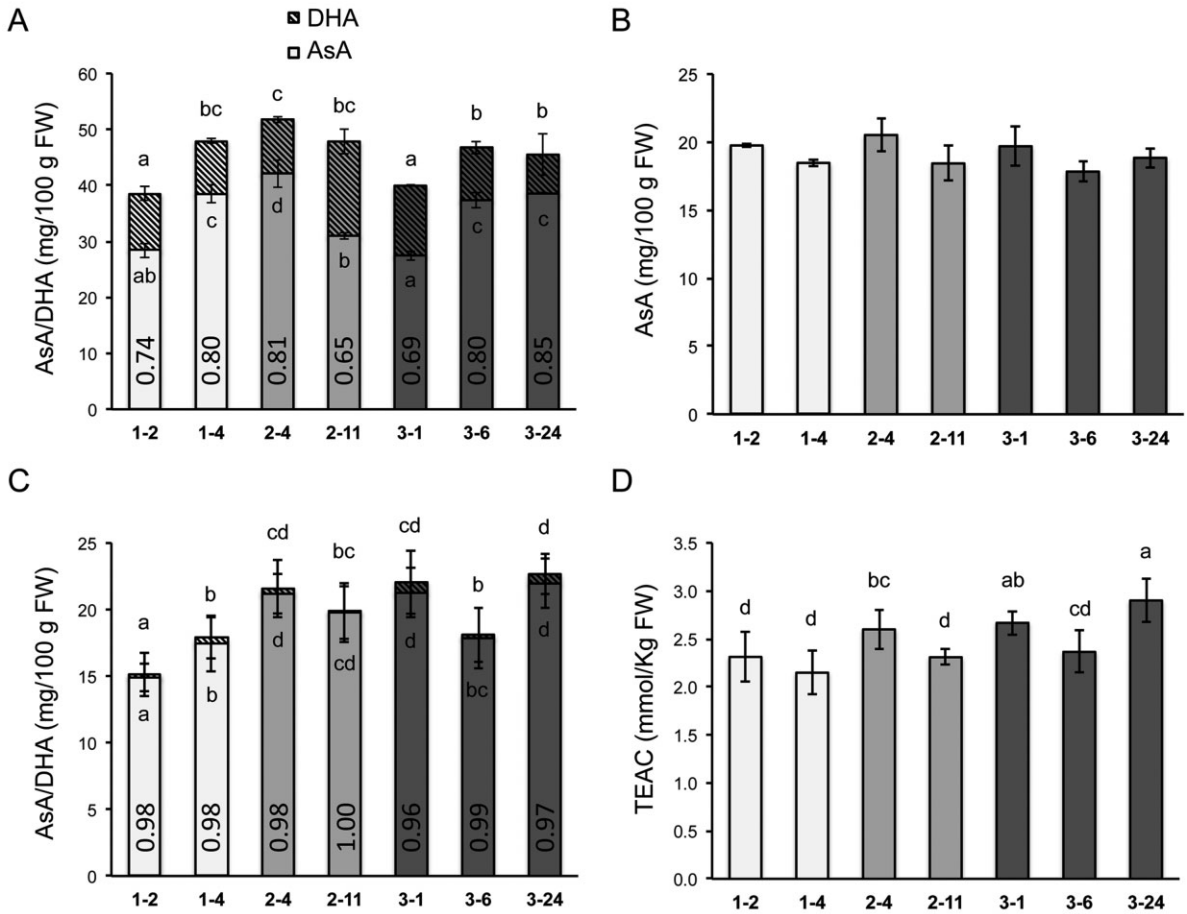

Figure 3. AsA content (mean of six replicates ± SD) in (**A**) leaf, (**B**) green, and (**C**) light red fruits of *FaGalUR* transgenic lines in comparison to control lines (1-2 and 1-4). Reduced AsA and total AsA (AsA and DHA) were measured for leaf and red fruits. (**D**) Total antioxidant capacity in the hydrophilic fraction of light red fruits from control and *FaGalUR* transgenic tomato lines. Different letters above bars indicate significant differences by least-squared-difference (LSD; $p < 0.05$; $n = 6$), using one-way analysis of variance (ANOVA); For (**A**) and (**C**), letters of total ascorbate (AsA + DHA) statistical analysis are shown on top, and below for AsA. The ratio AsA/AsA + DHA is shown inside the bars. FW, fresh weight.

Finally, we observed an increase higher than two-fold in 3-caffeoyl quinic acid (chlorogenic acid).

Although no consistent changes in AsA content were observed in leaves of transgenic lines compared to the controls, we analyzed whether overexpression of *FaGalUR* had an effect on other metabolites in this tissue. The analysis was performed in all transgenic lines, including the *PG-FaGalUR* lines that did not express the enzyme in vegetative tissues (Supporting information, Table S2). As expected, most changes were restricted to the *35S-FaGalUR* lines. The content of glucuronic/galacturonic acids increased two-fold, while there were no significant changes in the levels of any other organic acid. Regarding amino acids, the levels of oxaloacetate-derived Asn, -Ala, and Thr were significantly decreased in L2-4 and L2-11. Similar to fruits, the content of fructose and glucose was reduced in leaves of *35S-FaGalUR* lines, as well as the content of xylose. In the case of disaccharides, trehalose rather than sucrose, as occurred in fruits, was increased in *35S-FaGalUR*. As observed for fruits, the contents of erythritol and chlorogenic acid were also significantly increased in leaves of transgenic lines L2-4 and L2-11. Changes in a few leaf metabolites were observed in *PG-FaGalUR* lines, but in any case were significant in the three independent lines.

3.4 Leaves of *35S-FaGalUR* plants display higher non-photochemical quenching of chlorophyll fluorescence

We investigated whether the modified metabolism of transgenic plants was associated to changes in photosynthetic efficiency by determining in vivo chlorophyll fluorescence parameters in leaves of *35S-FaGalUR* (L2) and control plants grown at controlled irradiance. No significant differences were found neither on maximum photosynthetic PSII efficiency (F_v/F_m) nor intrinsic non-radiative decay measured at predawn (Φ_{NOpd}), suggesting that PSII activity or abundance was not altered in leaves of L2

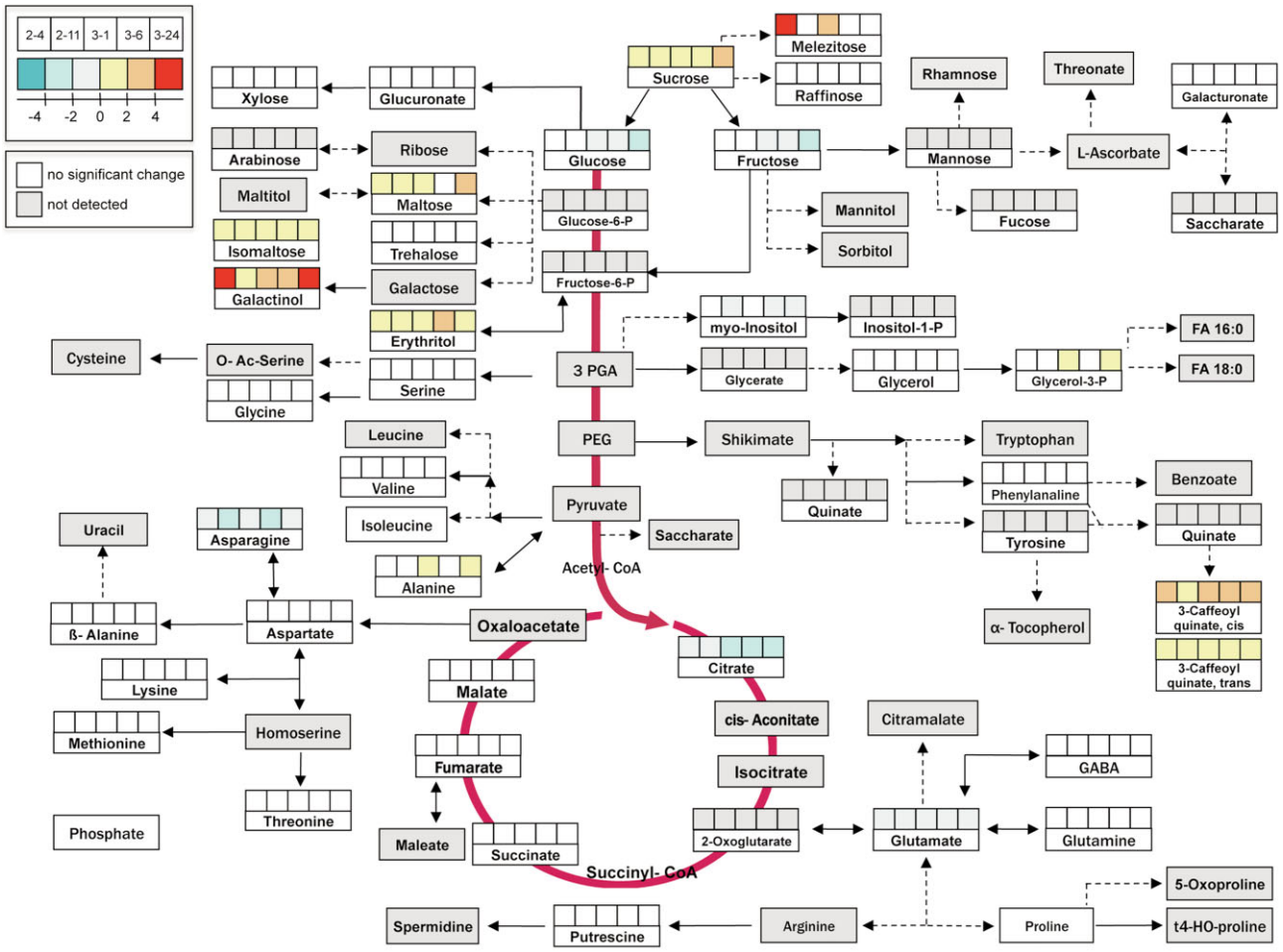

Figure 4. Schematic representation of metabolite changes occurring in light red fruit as a result of *FaGalUR* expression under the control of the *35S* promoter (lines 2-4 and 2-11) and the *PG* promoter (lines 3-1, 3-6, and 3-24). Each box represents fold change of a transgenic line related to control lines. Only changes that were significant in the statistical analysis (Supporting information, Table S1) are shown. Boxes in white and gray indicate no significant change and not determined, respectively.

plants (Fig. 5A). Values of F_v/F_m were within the approximate optimal value for most plant species, which ranges from 0.79 to 0.85 [45]. However, despite similar F_v/F_m ratios, analysis of non-photochemical quenching parameters revealed that, albeit not significantly, leaves of L2-4 and L2-11 dissipated more absorbed energy by non-photochemical pathways than the other lines (Φ_n and Φ_{NPQ} in Fig. 5B and NPQ in Fig. 5C). When plotting average NPQ values from each line against total AsA content, a significant and positive correlation was found in most of the lines with the exception of L1-4 and L3-6 (R^2 = 0.98; $p < 0.01$); those lines expressing *FaGaLUR* in leaves (L2) had higher NPQ values associated to higher total AsA content (Fig. 5C). Furthermore, when *35S-FaGalUR* lines were analyzed jointly as L2 and compared to L1 and L3 lines, L2 lines differed in the fraction of photons dissipated by photoprotective strategies at PSII (Supporting information, Fig. S4). This was shown by the significantly higher values of Φ_{NPQ} and NPQ compared to the rest of the lines (i.e. 1.2- and 1.4-fold increase compared with the L1 controls, respectively; Supporting information, Fig. S4A and S4B). No significant differences were found in the fraction of NPQ related with intrinsic non-radiative decay measured at midmorning (Φ_{NO}).

4 Discussion

4.1 Does metabolic plasticity buffer changes in AsA content in tomato?

Overexpression of *FaGalUR* in heterologous species such Arabidopsis and potato increased AsA between two and three-fold [20, 24]. In this work, we aimed to increase the content of AsA in tomato fruit by expression of *FaGalUR* under the control of two well-characterized promoters. Our data indicate that both *35S* and *PG* cause a similar accumulation of FaGalUR protein in light red fruits. While

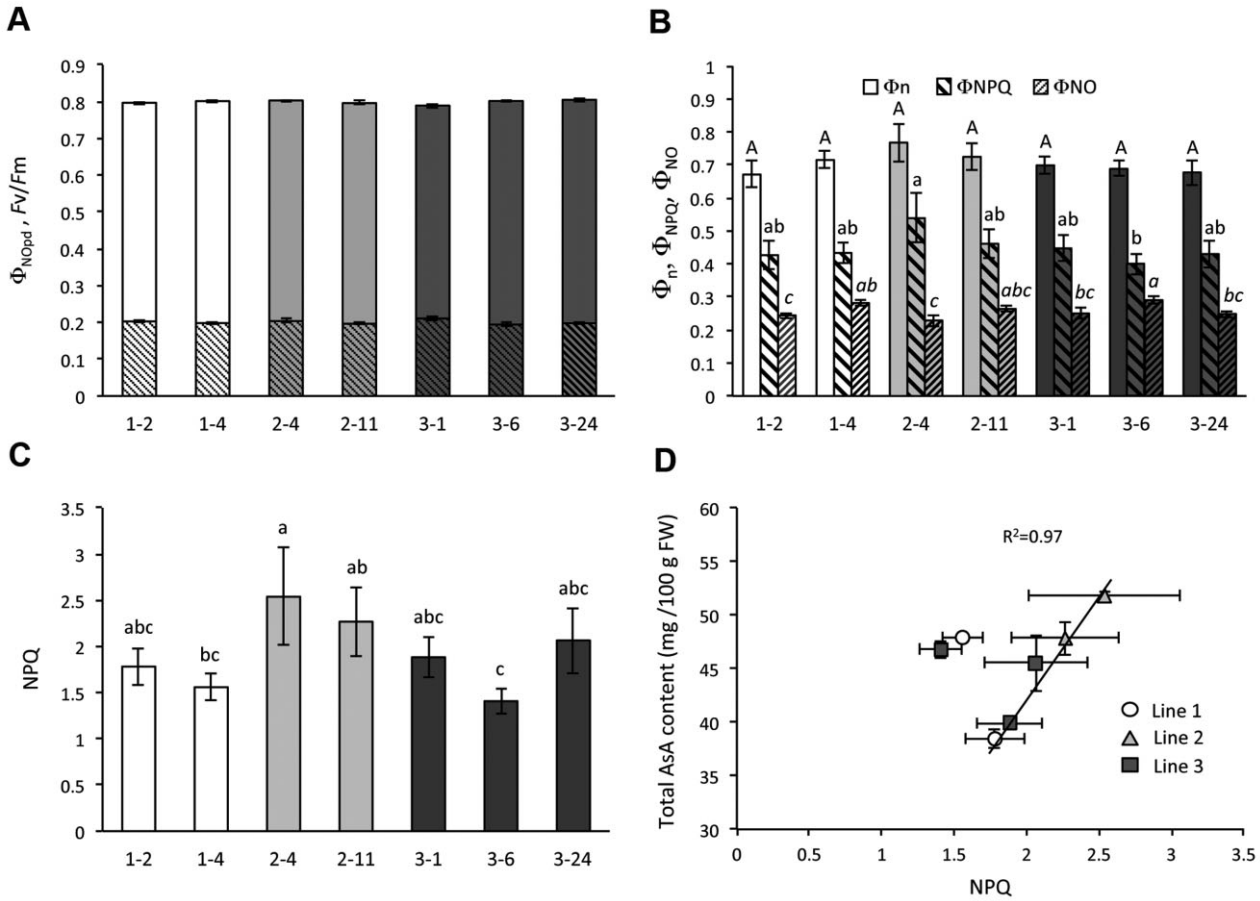

Figure 5. Effect of *FaGalUR* overexpression on chlorophyll fluorescence parameters in leaves of *35S-FaGalUR* lines (L2-4 and L2-11) compared to control (L1-2 and L1-4) and *PG-FaGalUR* plants (L3-1, L3-6, and L3-24), which do not express the transgene in leaves. (**A**) Maximal photochemical efficiency of PSII (F_v/F_m) and yield of non-regulated non-photochemical losses in PSII in the dark (Φ_{NOpd}). (**B**) Non-photochemical yield (Φ_n), regulated (Φ_{NPQ}) and non-regulated (Φ_{NO}) yields of non-photochemical losses in the steady-state light conditions. (**C**) Non-photochemical quenching (NPQ). (**D**) Relationship between NPQ and total AsA content in leaves of each of the individual lines. Lines 1-4 and 3-6 were not included in the regression line. Data are the means SE of $n = 7$–9 plants. Different letters indicate significant differences ($p < 0.05$; one-way ANOVA followed by LSD).

an increase of AsA occurred in light red fruits for the majority of *FaGalUR* transgenic lines, an increase in leaf AsA was only obtained for L2-4. There are several possible explanations for this limited increase in AsA despite the high amount of FaGalUR protein achieved: (i) a shortage of substrate of FaGalUR, (ii) the presence of high endogenous GalUR activity, or (iii) an additional enzymatic activity limiting the synthesis of AsA.

Previous attempts to increase AsA content in tomato fruit by genetic engineering has rendered variable success [5]. Thus, the tomato *SlGME* gene only increased up to 1.6-fold the AsA content in fruits and up to 1.4 in leaves [21], while the kiwi *AdGGP* gene increased fruit AsA content up to six-fold but failed to significantly increase it in leaf [15]. The results point to GGP having higher flux control coefficient in the AsA biosynthetic pathways in fruit compared to GME and FaGalUR. Another study in tomato further pinpoint to GGP as a rate-limiting step for fruit AsA biosynthesis although alternative pathways and AsA recycling enzymes may have additional roles at specific developmental stages [46]. Additional examples suggest the existence of a metabolic readjustment aimed to maintain AsA content under tight control. A conspicuous example of this metabolic readjustment has been also reported in tomato fruits, by the manipulation of the *GalDH*, the last enzyme common to the two main pathways in plants. Silencing of *GalDH* reduced weakly leaf AsA content but did not change its level in fruit [47]. Furthermore, only modest increases of up to two-fold have been reported for over expression of this enzyme in tobacco, suggesting that this enzyme may also have a low flux control coefficient in the pathway [48, 49].

4.2 Metabolic analysis identifies a redox readjustment in *FaGalUR* transgenic fruits

The TCA cycle plays a central role in energy metabolism and it is highly sensitive to changes in the cell redox state

[44]. Citric acid has been reported as the most abundant intermediate of the pathway in tomato fruits [50]. The reduction in citric acid content in fruits expressing *FaGalUR* suggests either a reduced flux or a change in the flux mode, causing a concomitant reduction of oxaloacetate-derived Asn and 2-oxoglutarate-derived Glu. A reduction in the flux of carbon skeletons from TCA to nitrogen assimilation into amino acids has been previously established by reducing the expression of different TCA cycle enzymes [51–53].

Glucose, fructose, and sucrose are the most abundant sugars in tomato fruits [50]. During fruit ripening, there is a continuous increase of glucose and fructose from green to the light red stage, accompanied by a decrease in sucrose [22]. Expression of *FaGalUR* caused a significant reduction of glucose and fructose and an increase of sucrose in fruits. Transgenic tomato plants with diminished activity of an apoplastic ascorbate oxidase showed an increased hexose:sucrose ratio in fruits associated to a change in the sucrose phosphate synthase (SPS) activity that is sensitive to redox changes [54]. Whether this activity is also altered in *FaGalUR* fruits remains to be investigated.

The metabolite that showed the highest increase in red fruits of *FaGalUR* plants was galactinol, with a three- to five-fold increase in L2-4, L3-1, and L3-24. Galactinol is the substrate for raffinose family oligosaccharides (RFOs), which accumulate under stressful environmental conditions and have been implicated in mitigating the effects of stresses such as cold, heat, or dehydration [55, 56]. RFOs are synthesized from sucrose by the addition of activated Gal moieties donated by galactinol, which has recently been shown to scavenge hydroxyl radicals in vitro [57]. Besides, galactinol is formed from UDP-Gal and *myo*-inositol by the enzyme galactinol synthase. In agreement with this, the content of *myo*-inositol was reduced in fruits of most transgenic lines. The accumulation of galactinol and sucrose in transgenic lines, both substrates for RFOs synthesis, suggest an alteration in this pathway that protects plants from oxidative damage.

Interestingly, a significant and consistent accumulation of chlorogenic acid (3-caffeoyl quinic acid) was observed in L2 and L3 fruits and leaves of L2. Chlorogenic acid is the most abundant phenolic compound in tomato [58, 59]. Its synthesis occurs as a branch of the general phenylpropanoid pathway, being hydroxycinnamoyl CoA quinate transferase (HQT) the key enzyme. Fluxes directing the biosynthesis to different branches of the phenylpropanoid pathway are controlled by biochemical, genetic, environmental, and developmental cues [60]. We can speculate that the accumulation of chlorogenic acid could result from a general change in the cellular redox state in *FaGalUR* transgenic lines. Supporting this, it has been reported that constitutive overexpression of *GGP* in tomato and strawberry enhanced AsA content and also increased the content of total polyphenolic and hydroxycinnamic compounds in fruits of both species [15].

4.3 *35S-FaGalUR* lines exhibited higher photoprotective capacity against photo-oxidative stress

We have observed limited changes in AsA and DHA content in *35S-FaGalUR* leaves, and only L2-4 showed a significant increase in AsA while L2-11 displayed the highest increase in DHA. We could argue that this is due to a limitation of substrate, D-galacturonic acid, despite the high content of FaGalUR protein in this tissue and the increased content of glucuronic/galacturonic acid found in *35S-FaGalUR* transgenic leaves. Unfortunately, metabolite analysis does not allow distinguishing between these two metabolites. FaGalUR belongs to the family of alko-keto reductases that catalyze the NADPH-dependent reduction of a wide variety of carbonyl compounds, including the pentose xylose [61]. We cannot exclude that in the absence of substrate, FaGalUR activity in leaf might have a side-activity of carbonyl reduction, that in the case of xylose reductase could explain the diminished content of xylose in *35S-FaGalUR* lines. Other metabolic changes found in *35S-FaGalUR* transgenic leaves, such as diminution of Asn, Frc, and Glc, and the increase of erithritol and quinic acid-derivatives must also be a consequence of the overexpressed reductase activity. Thus, the analysis of non-photochemical related parameters revealed a higher photoprotection at the PSII antennae in leaves of *35S-FaGaLUR* lines, mainly achieved by thermal energy dissipation processes related with NPQ (Φ_{NPQ}) but to a lower extent with the contribution of basal dissipation (i.e. Φ_{NO}; Fig. 5B, 5C, and Supporting information, Fig. S4; [41, 62]). NPQ has been associated with the photoprotective role of the xanthophyll cycle and the binding of zeaxanthin to light harvesting complex of PSII (LHCII) proteins [63]. Therefore, the higher NPQ values observed on L2 lines point to a higher contribution of xanthophylls to the non-photochemical quenching. In this cycle, AsA plays an important role as cofactor of the violaxanthin de-epoxidase enzyme that catalyzes the cyclic conversion of violaxanthin into pigments such as zeaxanthin [64]. In agreement with this, the increased NPQ values observed in *35S-FaGaLUR* leaves can be associated with the increased total AsA content, which is consistent with a higher energy dissipation dependent on xanthophyll-cycle. Besides, increases in galactinol and chlorogenic acid could contribute to enhanced protection of the transgenic lines against oxidative damage. All together, these results would discard a diminished carbon assimilation capacity of transgenic lines and would favor an explanation based on increased sucrose synthesis and transport or starch production.

5 Conclusion

The metabolomic analyses here reported show that ectopic expression of *FaGalUR* is associated with changes in a number of antioxidant compounds such AsA, chlorogenic acid, and galactinol resulting in an increase in total antioxidant capacity in fruits. The data indicate that expression of *FaGalUR* in tomato has far-reaching effects on metabolic pathways such as TCA cycle, amino acid metabolism, sugars, and chlorogenic acid accumulation. Many of these alterations are associated with oxidative stress responses, as was reported previously for tomato lines differing in AsA content [33]. The results presented here pinpoint to a complex interconnection of AsA, either directly or through altering the redox status, with a wide range of metabolites and indicate that even though expression *FaGalUR* caused a limited increase in AsA, the overall plant fitness and redox state have been improved.

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