

RESEARCH PAPER

Regulation of L-ascorbic acid content in strawberry fruits

Eduardo Cruz-Rus¹, Iraida Amaya², José F. Sánchez-Sevilla², Miguel A. Botella¹ and Victoriano Valpuesta^{1,*}

¹ Instituto de Hortofruticultura Subtropical y Mediterránea, Universidad de Málaga-Consejo Superior de Investigaciones Científicas (IHSM-UMA-CSIC), Departamento de Biología Molecular y Bioquímica, Facultad de Ciencias, Universidad de Málaga, Campus de Teatinos s/n, E-29071 Málaga, Spain

² Área de Mejora y Biotecnología IFAPA-CIFA Málaga, Laboratorio de Bioquímica, Cortijo de la Cruz, E-29140 Málaga, Spain

* To whom correspondence should be addressed. E-mail: valpuesta@uma.es

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Abstract

Plants have several L-ascorbic acid (AsA) biosynthetic pathways, but the contribution of each one to the synthesis of AsA varies between different species, organs, and developmental stages. Strawberry (*Fragaria × ananassa*) fruits are rich in AsA. The pathway that uses D-galacturonate as the initial substrate is functional in ripe fruits, but the contribution of other pathways to AsA biosynthesis has not been studied. The transcription of genes encoding biosynthetic enzymes such as D-galacturonate reductase (*FaGalUR*) and myo-inositol oxygenase (*FaMIOX*), and the AsA recycling enzyme monodehydroascorbate reductase (*FaMDHAR*) were positively correlated with the increase in AsA during fruit ripening. Fruit storage for 72 h in a cold room reduced the AsA content by 30%. Under an ozone atmosphere, this reduction was 15%. Ozone treatment increased the expression of the *FaGalUR*, *FaMIOX*, and *L-galactose-1-phosphate phosphatase* (*FaGIPP*) genes, and transcription of the L-galactono-1,4-lactone dehydrogenase (*FaGLDH*) and *FaMDHAR* genes was higher in the ozone-stored than in the air-stored fruits. Analysis of AsA content in a segregating population from two strawberry cultivars showed high variability, which did not correlate with the transcription of any of the genes studied. Study of GalUR protein in diverse cultivars of strawberry and different *Fragaria* species showed that a correlation between GalUR and AsA content was apparent in most cases, but it was not general. Three alleles were identified in strawberry, but any sequence effect on the AsA variability was eliminated by analysis of the allele-specific expression. Taken together, these results indicate that *FaGalUR* shares the control of AsA levels with other enzymes and regulatory elements in strawberry fruit.

Key words: L-Ascorbic acid, D-galacturonic acid reductase, fruit ripening, gene expression, strawberry, oxidative stress.

Introduction

L-Ascorbic acid (AsA) is the common name for the six-carbon sugar derivative L-threo-hex-2-enono-1,4-lactone. Humans, non-human primates, and a few other mammals cannot synthesize AsA because the gene encoding the enzyme catalysing the last step in the biosynthesis, L-gulonono-1,4-lactone oxidase, is non-functional due to mutations (Chatterjee, 1973) (Fig. 1). This compound has important antioxidant and metabolic functions, making its incorporation into the human diet essential and is known as vitamin C. Plants, the main source of vitamin C for humans, also require this compound (Pastori *et al.*, 2003).

Strawberry fruits are particularly rich sources of vitamin C (Perkins-Veazie, 1995).

There are several known and proposed alternative pathways for AsA biosynthesis in plants (Fig. 1), but the prevalence of these pathways in different tissues and/or developmental stages is largely unknown (Davey *et al.*, 2000; Jain and Nessler, 2000; Valpuesta and Botella, 2004). The first AsA biosynthetic pathway to be described in plants proceeds through GDP-D-mannose and L-galactose (Wheeler *et al.*, 1998). In this pathway, D-glucose is converted to AsA through the following intermediate

Abbreviations: AsA, L-ascorbic acid; GME, GDP-mannose-3'-5'-epimerase; G1PP, L-galactose-1-phosphate phosphatase; GalUR, L-galacturonic acid reductase; GLDH, L-galactono-1,4-lactone dehydrogenase; MYOX, myo-inositol oxygenase; MDHAR, monodehydroascorbate reductase; DHAR, dehydroascorbate reductase.
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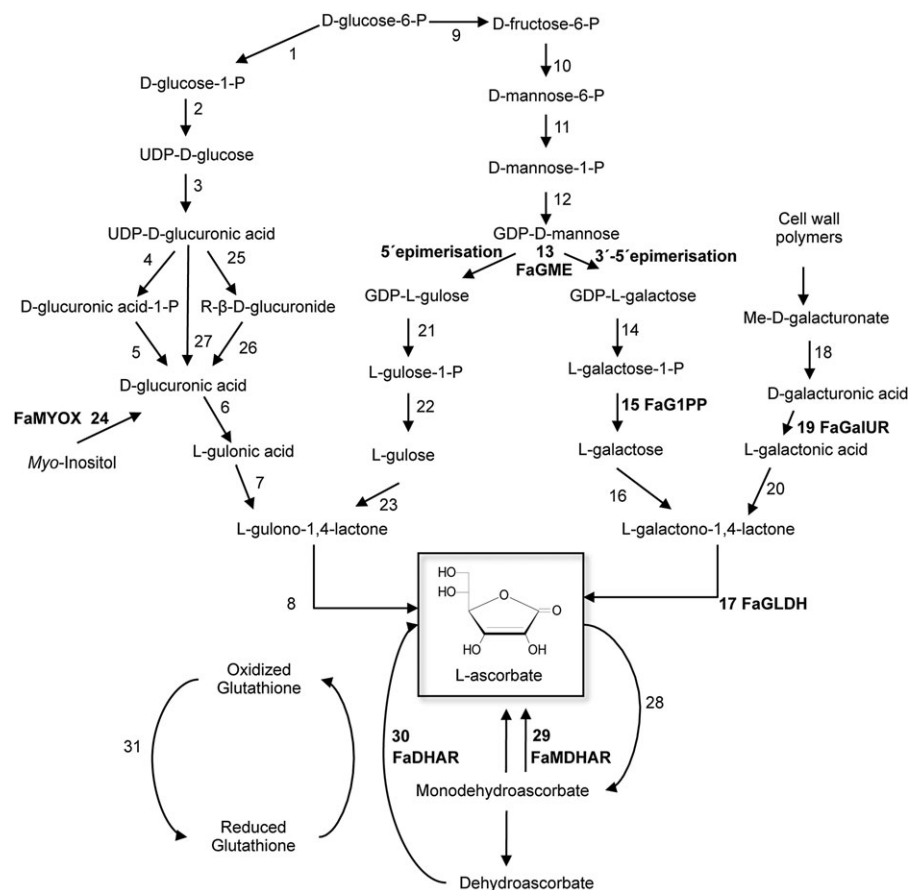


Fig. 1. Proposed biosynthetic pathways of L-ascorbic acid in animals (reactions 1–8 and 25–27), plants (reactions 9–24) and the recycling pathway (reactions 28–31) are shown. The enzymes used in this work are in bold. The enzymes catalysing these reactions are the following: 1, phosphoglucomutase; 2, UDP-D-glucose pyrophosphorylase; 3, UDP-D-glucose dehydrogenase; 4, D-glucuronate-1-phosphate uridylyltransferase; 5, D-glucurono kinase; 6, D-glucuronate reductase; 7, aldono-lactonase; 8, L-gulono-1,4-lactone dehydrogenase; 9, D-glucose-6-phosphate isomerase; 10, D-mannose-6-phosphate isomerase; 11, phosphomannomutase; 12, GDP-mannose pyrophosphorylase (mannose-1-phosphate guanyltransferase); 13, GDP-mannose-3',5'-epimerase; 14, GDP-L-galactose phosphorylase; 15, L-galactose-1-phosphate phosphatase; 16, L-galactose dehydrogenase; 17, L-galactono-1,4-lactone dehydrogenase; 18, methyltransferase; 19, D-galacturonate reductase; 20, aldono-lactonase; 21, phosphodiesterase; 22, sugar phosphatase; 23, L-gulose dehydrogenase; 24, *myo*-inositol oxygenase; 25, UDP-glucuronosyltransferase; 26, β -glucuronidase; 27, UDP-glucuronidase; 28, ascorbate peroxidase; 29, monodehydroascorbate reductase; 30, dehydroascorbate reductase; 31, glutathione reductase.

compounds: GDP-D-mannose, GDP-L-galactose, L-galactose-1-phosphate, L-galactose, and L-galactono-1,4-lactone (Fig. 1). All the genes involved in this pathway (Man/Gal pathway) have been characterized in plants, including those encoding GDP-D-mannose pyrophosphorylase (Conklin *et al.*, 1999), GDP-D-mannose-3',5'-epimerase (Gibson *et al.*, 2008; Wolucka and Van Montagu, 2003), GDP-L-galactose phosphorylase (Dowdle *et al.*, 2007; Laing *et al.*, 2007), L-galactose-1-phosphate phosphatase (Laing *et al.*, 2004), L-galactose dehydrogenase (Gatzek *et al.*, 2002), and L-galactono-1,4-lactone dehydrogenase (Imai *et al.*, 1998; do Nascimento *et al.*, 2005). Biochemical studies indicated that GDP-L-gulose was an alternative product of the enzyme GDP-D-mannose-3',5'-epimerase (Wolucka and Van Montagu, 2003). This reaction product established both a new branch of the pathway in plants and a link to the animal pathway (Fig. 1). The subsequent steps in the branch, however, have not yet been described in plants. In

addition, a pathway initiating from D-galacturonate was proposed after the identification of a D-galacturonate reductase gene in strawberry (Agius *et al.*, 2003) (Fig. 1). Finally, a *myo*-inositol-based pathway was proposed after the cloning of *myo*-inositol oxygenase from *Arabidopsis* (Lorence *et al.*, 2004) (Fig. 1).

In plants, the AsA recycling pathway (Fig. 1) plays an important role in the response and adaptation to the stress (Stevens *et al.*, 2008) and also contributes to the regulation of AsA levels jointly with the processes of synthesis, degradation, and transportation (Yin *et al.*, 2010). Ascorbate peroxidase (APX) uses two AsA molecules to reduce H_2O_2 to water, with two molecules of monodehydroascorbate (MDHA) generated as by-products. MDHA is reduced back to AsA by monodehydroascorbate reductase (MDHAR) using NADH/NADPH as electron donors. In addition, because MDHA is an unstable radical, it can spontaneously disproportionate to AsA and dehydroascorbate (DHA). DHA is reduced to AsA

by dehydroascorbate reductase (DHAR) using reduced glutathione (GSH) as a reducing compound. Therefore, DHAR and MDHAR are key components in the maintenance of the reduced pool of AsA and are of paramount importance in oxidative stress tolerance (Eltayeb *et al.*, 2006)

In plants, AsA content has been shown to change with light (Tabata *et al.*, 2002; Badejo *et al.*, 2009; Li *et al.*, 2009), hour of the day (Chen and Gallie, 2004; Tamaoki *et al.*, 2003), age (Bartoli *et al.*, 2000), plant tissue (Lorence *et al.*, 2004), and cell compartment (Davey *et al.*, 2000; Zechmann *et al.*, 2011). A tightly regulated metabolic network of AsA biosynthesis, catabolism, and recycling might explain this fine modulation. Surprisingly, there have only been a few studies examining the various elements involved in this metabolic network (Linster and Clarke, 2008; Badejo *et al.*, 2009; Cruz-Rus *et al.*, 2010).

In strawberry, the nucleotide sequences for some of the genes encoding enzymes of the different AsA biosynthetic and recycling pathways are known. The AsA content of these fruits is known to vary and has also been reported to increase in ripe strawberry fruits after treatment with ozone (Pérez *et al.*, 1999). For example, there is a continuous increase in the content of AsA during fruit development and ripening (Agius *et al.*, 2003). Because of these observations, strawberry fruits were selected to study the correlation between the expression levels of the genes involved in the biosynthesis of AsA and the levels of AsA. In addition, the variability of AsA content in the ripe fruits of different cultivars of *Fragaria* × *ananassa* and species of *Fragaria* was evaluated to explore whether a relationship exists between AsA content and the expression and/or structure of D-galacturonate reductase.

Material and methods

Plant materials

Fragaria species and *F. × ananassa* varieties were sampled from the *Fragaria* Germplasm Collection (IFAPA, Churriana, Málaga, Spain). All plants were grown in a shade house under the typical prevailing field conditions of the area. In all cases, fruits were carefully selected at the same stage of ripeness.

The strawberry (*F. × ananassa*) mapping population (Y Zorrilla-Fontanesi *et al.*, unpublished results) consisted of a full-sib family of 95 F₁ progeny derived from an intraspecific cross between two IFAPA selection lines, 232 ('Vilanova' × Sel. 4-43) and 1392 ('Gaviota' × 'Camarosa'). These lines were chosen because they differ in important agronomic and fruit quality traits including production, time to flowering, fruit colour, acidity, and the content of sugars, anthocyanins, volatile compounds, and AsA.

The strawberry population was planted at the end of October in the strawberry-producing area of Moguer (Huelva, Spain). A total of six plants of each parental line and four plants of each genotype of the progeny were established on mulched raised beds that were 35 cm high and 50 cm wide in a double row in a zigzag formation. The

population was grown under macro tunnels of polyethylene following conventional practices, with an inter-row distance of 30 cm and a distance of 25 cm between plants. Fruits for AsA determination were harvested at the same ripening stage for the whole population and immediately frozen in liquid nitrogen.

Ascorbic acid determination

The ascorbic acid content of strawberry fruits was determined by HPLC. Briefly, the fruits were harvested and pulverized in liquid nitrogen. Samples were suspended in 2.0% (w/v) metaphosphoric acid and 2 mM EDTA, chilled on ice for 20 min, and centrifuged at 13 000 g for 20 min at 4 °C, which corresponds to the protocol previously published (Davey *et al.*, 1996) with minor modifications. The supernatants were filtered through a 0.45 µm syringe filter, and 15 µl of the sample was injected into the HPLC system. The flow rate was 0.6 ml min⁻¹ with a mobile phase consisting of 0.1 M sodium phosphate monobasic, 0.2 mM EDTA (adjusted to pH 3.1 with orthophosphoric acid), and a Kromasil 100 C18 5 µm 25 × 0.46 column (Scharlab www.scharlab.com). The eluents were monitored with a UV detector at 254 nm (Harapanhalli *et al.*, 1993). Three biological repetitions, with three technical replicas for each, were performed for every sample. The AsA content was calculated by comparison with the values obtained from a standard curve.

Ozone treatment

Strawberry (*F. × ananassa* Duch., cv. Camarosa) plants were grown in the south of Spain (Moguer, Huelva) under the same conditions previously described for the mapping population. Fruits were harvested, selected for uniformity of size and colour, and packed in polypropylene berry baskets (500 g each). Ozone was produced using an ozone generator (Laboratory Ozonizer 300.7) to a level of 35 ppm and applied to one set of fruit. Both sets, the treated and untreated fruit, were placed in the same cold room at 2–4 °C and 90% relative humidity. After 3 d in the cold room, the fruits were frozen at –80 °C until used for analysis.

RNA isolation and gene expression analysis

Total RNA was isolated from the *Fragaria* fruits according to the method described by Manning (1991). RT-PCR of the different genes was performed starting from 1 µg of total RNA using the iScript Select cDNA Synthesis Kit (Bio-Rad, <http://www.bio-rad.com/>). Expression was analysed by quantitative RT-PCR (qRT-PCR) using a SYBR® Premix Ex Taq™ sample (Takara, <http://www.takara-bio.com/>), a Rotor Gene detection system (Qiagen, <http://www.qiagen.com>) according to the manufacturer's instructions, and gene-specific primers (see Supplementary Table S1 at *JXB* online). At least two biological and three technical replicates were performed for every sample. The absence of primer dimers was confirmed by an examination of the dissociation curves.

The results obtained were normalized against *FaRib413* expression, which had been shown by Northern blot analysis to be constitutive in different strawberry plant tissues, including fruits at different stages of development (Casado-Díaz *et al.*, 2006). The relative quantification of expression level was performed using the comparative C_t method (Pfaffl, 2001). Statistical analysis was performed using the StatGraphic Centurion program. Significant differences were determined by ANOVA. Differential allelic expression was determined using a standard PCR with specific primers for the open reading frame of *FaGalUR*. To differentiate between the distinct alleles, the PCR product was subsequently digested using the restriction enzymes *MbiI* and *Eco52I* (Fermentas, <http://www.fermentas.com>) according to the manufacturer's instructions, followed by the quantification of the bands using the Quantity One software (Bio-Rad, <http://www.bio-rad.com>).

Protein extraction and immunoblot analysis

The fruits were first pulverized in liquid nitrogen. Proteins were then extracted in 6× Laemmli buffer (Laemmli, 1970) and centrifuged for 5 min at 10 000 g. Protein separation and blotting were performed on 12% polyacrylamide gels and nitrocellulose membranes, respectively, as previously described by Cruz-Rus *et al.* (2010). The gels were stained with Coomassie blue to confirm that there was equal loading in all lanes. After being blocked in 5% milk, the membranes were incubated with polyclonal anti-*FaGalUR* antibodies (Agius *et al.*, 2003) followed by anti-rabbit antibodies conjugated to horseradish peroxidase. The immunoblots were developed using the ECL detection kit from Amersham (Amersham Biosciences, <http://www.gelifesciences.com/>).

Results

The sequences of the strawberry genes encoding *L-galactone-1,4-lactone dehydrogenase (FaGLDH)* (do Nascimento *et al.*, 2005) and *L-galacturonic acid reductase (FaGalUR)* (Agius *et al.*, 2003) have previously been published. A tBLASTn search of the strawberry EST database (Bombarely *et al.*, 2010) (<http://fresa.uco.uma.es/srs71/>), using the amino acid sequences previously described for enzymes of the AsA biosynthesis metabolic pathways in plants as queries, identified ESTs for *GDP-mannose-3',5'-epimerase (FaGME)*, *L-galactose-1-phosphate phosphatase (FaGIPP)*, and *myo-inositol oxygenase (FaMYOX)*.

The sequence identity between the strawberry genes and the homologues found in other species was high (Table 1). Strawberry *FaGME* showed an 88% identity with its corresponding *Arabidopsis* enzyme, with similar levels of identity with proteins from other members of the Rosaceae family, such as *Prunus persica* (89%) and *Malus domestica* (85%). Strawberry *FaGIPP* had identities of 63% and 90% with its corresponding enzymes from *Arabidopsis* and *Prunus persica*, respectively. *FaMYOX* had a 56% identity

with *Arabidopsis* MYOX4. As expected, the identity of each of the enzymes was highest when compared with the corresponding enzymes of other Rosaceae species.

In addition, the sequences of *monodehydroascorbate reductase (FaMDHAR)* and a chloroplastic *dehydroascorbate reductase (FaDHAR)*, two genes involved in AsA recycling, were identified in the strawberry EST database (Bombarely *et al.*, 2010). *FaMDHAR* showed identities of 76% and 90% with the corresponding enzymes of *Arabidopsis* and *Malus domestica*, respectively. *FaDHAR* had identities of 64% and 81% with the corresponding enzymes from *Arabidopsis* and *Malus domestica*.

The content of AsA in the fruits of the strawberry cv. Camarosa increased over the course of development from the green immature stage to that of red ripe fruits, reaching a final concentration of 50 mg/100 g fresh weight (Fig. 2A). This level lies within the range of values previously reported for ripe fruits of other varieties of this species (Agius *et al.*, 2003). Notably, the increase in AsA levels with ripening is much higher on a dry weight basis. Thus, the AsA content rose from 23.9 mg/100 g dry weight in green fruits to 292.5 mg/100 g dry weight in ripe fruits, a greater than 12.2-fold increase.

In the same samples, the relative expression levels of the genes encoding the enzymes of the AsA biosynthetic pathways were evaluated by qRT-PCR (Fig. 2B). The expression pattern of both *FaGalUR* and *FaMYOX* was positively correlated with alterations in the levels of AsA. The increase associated with fruit development was higher for *FaGalUR*, changing more than 150-fold from the green to the red stage. By contrast, the expression of *FaGIPP* and *FaGME* was negatively associated with AsA levels, with the highest values in green fruits and the lowest in red fruit. The net changes were below 3-fold. With respect to the expression of *FaGLDH*, no significant change was observed among the three developmental stages.

The regulation of AsA levels in cells is tightly controlled, not only by its synthesis, but also by the recycling, degradation, and transport processes (Loewus, 1999; Pallanca and Smirnov, 1999; Horemans *et al.*, 2000). An examination of the expression patterns of the genes encoding monodehydroascorbate reductase (MDHAR) and dehydroascorbate reductase (DHAR) revealed contrasting associations. The *FaMDHAR* levels increased over 17-fold in the transition from green to ripe fruit (Fig. 2C), paralleling the increase in AsA levels. The levels of *FaDHAR*, however, were highest in green fruits, with decreasing levels in white and red fruits (Fig. 2C).

Strawberry is one of the most delicate and perishable fruits, being highly susceptible to mechanical injury, physiological deterioration, water loss, and fungal decay. Therefore, effective post-harvesting procedures are required to prevent deterioration during strawberry packing and transport. Installation of ozone generators in strawberry cooling rooms has been effectively used to increase the AsA content of the fruits (Pérez *et al.*, 1999). The ozone treatment was successfully reproduced here, and the effectiveness of the ozone treatment was confirmed by the appearance of the

Table 1. Strawberry sequences corresponding to genes encoding the enzymes of the L-ascorbic acid biosynthetic and recycling pathways

The identity at the amino acid level between each of the enzymes and those of the reference species are shown.

Accession number	Enzyme (EC)	Reference species	Identity (%)
GW402740	GDP-mannose-3',5'epimerase (EC 5.1.3.18)	<i>Arabidopsis thaliana</i> (At5g28840)	88
GT150979	L-Galactose-1-phosphate-phosphatase (EC 3.1.3.25)	<i>Arabidopsis thaliana</i> (At3g02870)	63
AY102631	L-Galactono-1,4-lactone dehydrogenase (EC 1.3.2.3)	<i>Fragaria × ananassa</i> (cv. Campineiro)	100
AF039182	D-Galacturonate reductase (EC 1.1.1.203)	<i>Fragaria × ananassa</i> (cv. Chandler)	100
GW402590	<i>myo</i> -Inositol oxygenase (EC 1.13.99.1)	<i>Arabidopsis thaliana</i> (At4g26260)	56
Cl248Cg1	Monodehydroascorbate reductase (EC 1.6.5.4)	<i>Arabidopsis thaliana</i> (At3g52880)	76
CO817488	Dehydroascorbate reductase (EC 1.8.5.1)	<i>Arabidopsis thaliana</i> (At1g19570)	64

fruits, which, when stored in air, were eventually naturally infected with fungi (*Rhizopus* sp.) while the ozone-treated fruits were not (results not shown). The AsA content was lower after 72 h in air than just after fruit detachment (0 h), but this reduction was significantly smaller in the ozone-treated fruits (Fig. 3A). Differences in expression between the air- and ozone-stored fruits were significant for each of the biosynthetic genes examined (Fig. 3B), ranging from 1.2-fold for *FaMIOX* to 5-fold for *FaGIPP*. With respect to the AsA recycling genes, the expression of *FaMDHAR* was 3-fold higher in fruits stored for 72 h in ozone compared with those stored in air, whereas this difference was not significant for the *FaDHAR* gene (Fig. 3C). In order to determine whether fungal infection influences plant responses, the same samples were analysed for the expression of *PR5* and *polygalacturonase-inhibiting protein (PGIP)* using semi-quantitative RT-PCR, genes that have been reported to be induced by infection in strawberry fruits (Mehli *et al.*, 2004; Osorio *et al.*, 2008). There were no significant differences in the expression of these genes between fruits stored either in ozone or air (see Supplementary Fig. S1 at *JXB* online) suggesting that the changes in expression of AsA biosynthetic and recycling genes previously reported was mainly an effect caused by the ozone rather than a consequence of the infection.

The search for a possible relationship between gene expression and AsA was extended to a *F. × ananassa* segregating population, which provided highly variable levels of AsA in a limited genetic background. This population consisted of 95 lines derived from a cross between selections 1392 (fruits with high AsA content) and 232 (fruits with a low-medium AsA content). The AsA content was measured in the red fruits of all lines, and the mean values of fruit AsA were normally distributed and varied from 20–58 mg/100 g FW (see Supplementary Fig. S2 at *JXB* online). In addition to the parental lines, two progeny individuals with high AsA levels (93-30 and 93-34), one with a medium

AsA level (93-29) and two with low AsA levels (93-67 and 93-72) (Fig. 4A), were selected for the analysis of the expression of genes encoding enzymes of the AsA biosynthetic pathways (Fig. 4B). No correlation was found between the expression of selected genes, including *FaGalUR*, and the levels of AsA. With respect to the genes involved in AsA recycling, the expression of *FaMDHAR* was generally higher in the high AsA content lines, although the differences were not significant (Fig. 4C).

Transcription studies can be of limited value because discrepancies between transcripts and encoded proteins can occur. Therefore, the amounts of FaGalUR protein and AsA were analysed to determine if there was a correlation between the two. There were no significant differences in the amount of FaGalUR protein among the analysed individuals of the 232×1392 population (Fig. 4D) despite the different levels of AsA. This study was then extended to other genotypes belonging to different species of the *Fragaria* genus, which should provide a wider range of variability in the levels of AsA than in the full-sib population. Moreover, it had previously been found that a *Fragaria moschata* genotype with lower levels of AsA than *F. × ananassa* also had lower levels of the GalUR protein (Agius *et al.*, 2003). The selected samples included three accessions of the wild diploid species *Fragaria vesca*, one accession of the diploid *Fragaria viridis*, three accessions of the hexaploid *Fragaria moschata*, three accessions of the octoploid *Fragaria chiloensis*, one accession of octoploid *Fragaria virginiana*, and four cultivars of octoploid *F. × ananassa*. As expected, the AsA content in the ripe fruits of these species was highly variable, not only between species, but also within them (Fig. 5A). The lowest values were found for *F. moschata* (nos 194, 591) and the highest for *F. × ananassa* cv. Vicontesse. Gene expression was evaluated at the protein level by Western blot analysis in the same fruit samples. Although there were some cases for which the levels of AsA and the GalUR protein were

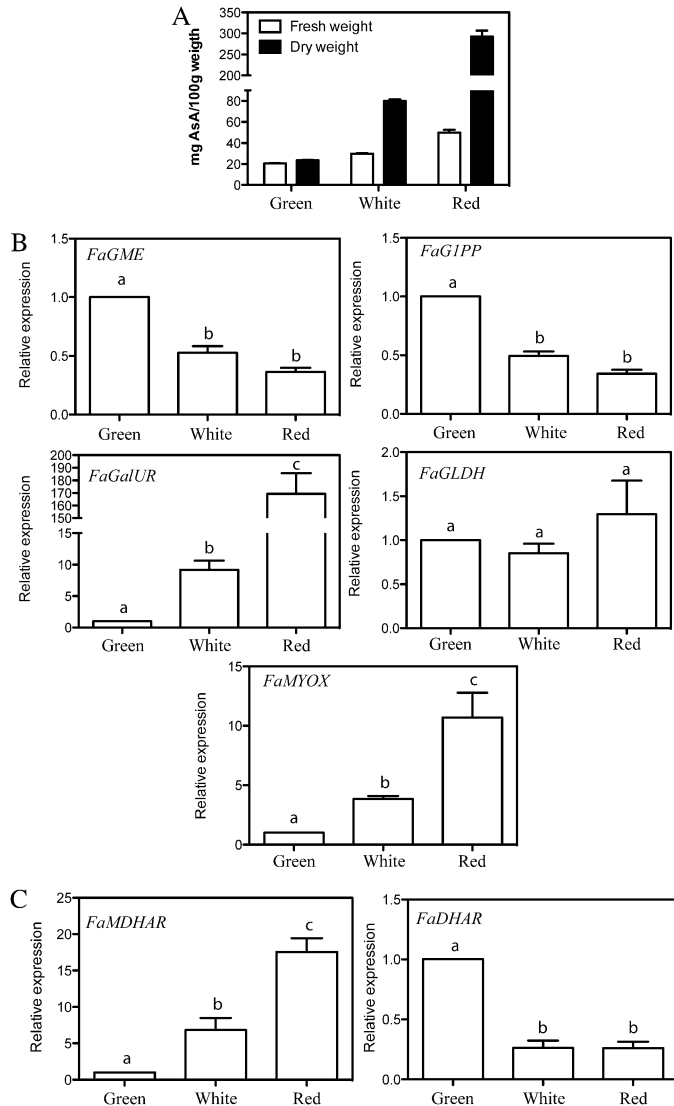


Fig. 2. (A) Changes in AsA levels and (B, C) mRNA levels of genes involved in AsA biosynthesis (*FaGME*, *FaG1PP*, *FaGalUR*, *FaGLDH*, and *FaMYOX*) and recycling (*FaMDHAR* and *FaDHAR*) in fruits during three ripening stages. The values were normalized against the abundance of *FaRib413*, using green fruit value as the reference (value 1). The bars represent the mean of at least two independent biological samples \pm SD. Different letters indicate a significant difference between samples according to the corresponding ANOVA ($P < 0.05$).

parallel, a tight correlation between these two parameters was not always found (Fig. 5B).

A striking discrepancy between the levels of AsA and the GalUR protein was found between the two *F. × ananassa* cultivars, Camarosa and Chandler. Therefore, the possibility of differences in the primary structure of the enzyme was explored by the sequence analysis of 12 genomic clones of *FaGalUR* from each of these two cultivars. The genomic structure of this gene included three introns and four exons (Fig. 6A). A SSR in the first intron that was polymorphic in the two cultivars was identified (Fig. 6B), and it had been previously reported to also be polymorphic in other

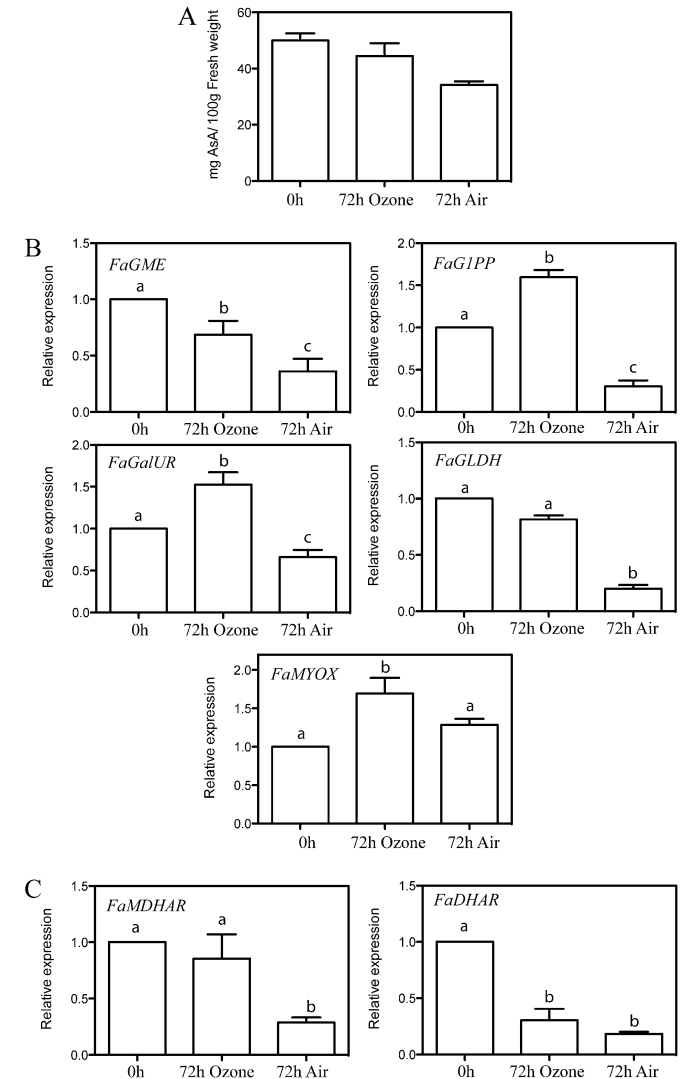


Fig. 3. (A) Changes in AsA levels and (B, C) mRNA levels of the genes involved in AsA biosynthesis (*FaGME*, *FaG1PP*, *FaGalUR*, *FaGLDH*, and *FaMYOX*) and recycling (*FaMDHAR* and *FaDHAR*) in fruits before (0 h) and after storage for 72 h in ozone or air atmosphere. The values were normalized against the abundance of *FaRib413*, using the 0 h value as the reference (value 1). The bars represent the mean of at least two independent biological samples \pm SD. Different letters indicate a significant difference between samples according to the corresponding ANOVA ($P < 0.05$).

cultivars of this species (Zorrilla-Fontanesi *et al.*, 2011). In addition, variations in a single nucleotide were found at four positions within the coding region (Fig. 6B). In summary, this analysis identified the same three alleles in both cultivars (see Supplementary Fig. S3 at *JXB* online). Only two of the substitutions in the coding region altered the amino acid sequence (Fig. 6B), with only the change in position 258, Gln/His, involving a non-conservative substitution. The location of this amino acid within the predicted tertiary structure indicated that it is not close to the active site but is located within to the C-terminal region, which is not highly conserved among the members of this

protein family (Sanli *et al.*, 2003). The possibility of a difference in the activity of the enzymes encoded by the different alleles, however, cannot be discarded. Therefore,

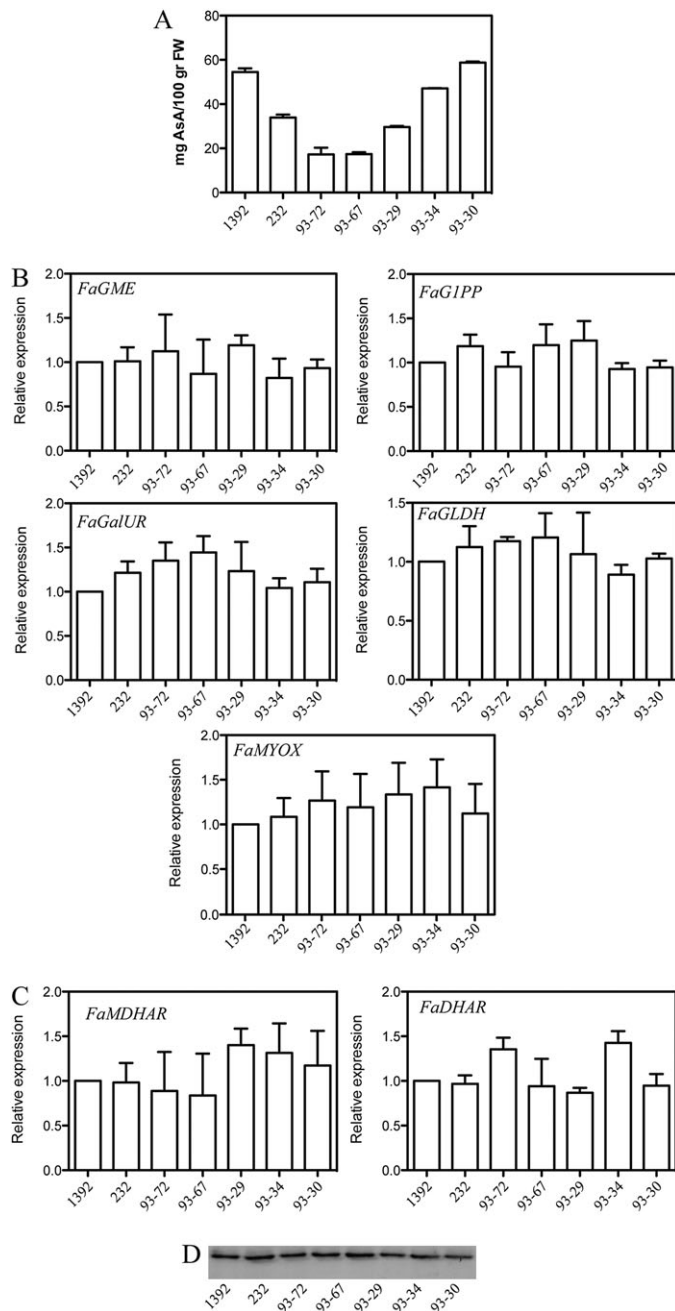


Fig. 4. (A) Changes in AsA levels and (B, C) mRNA levels of the genes involved in AsA biosynthesis (*FaGME*, *FaG1PP*, *FaGalUR*, *FaGLDH*, and *FaMYOX*) and recycling (*FaMDHAR* and *FaDHAR*) by QRT-PCR. (D) *FaGalUR* protein levels in the ripe fruits of the parentals (1392 and 232) as well as lines with high (93-30 and 93-34), medium (93-29), and low (93-67 and 93-72) AsA levels in the segregating population. The QRT-PCR values were normalized against the abundance of *FaRib413*, using the parental 1392 value as the reference (value 1). The bars represent the mean of at least two independent biological samples \pm SD. Different letters indicate a significant difference between samples according to the corresponding ANOVA ($P < 0.05$).

differential expression of the alleles between cv. Camarosa and cv. Chandler could explain the discrepancy between the total *FaGalUR* protein and AsA levels (Fig. 5A). The changes in two of the four positions produced new restriction sites that could be used to identify which allele was expressed in each cultivar. At the first nucleotide change (Fig. 6B), *Eco52I*-digestion of alleles 1 and 2 in the cDNA generated two fragments of 554 bp and 406 bp. At the second nucleotide change (Fig. 6B), *MbiI*-digestion of alleles 1 and 2 in the cDNA generated two fragments of 651 bp and 309 bp (see Supplementary Table S2 at *JXB* online). Accordingly, the restriction study of the cDNAs would distinguish the joint expression of alleles 1 and 2 from that of allele 3. Using RNA extracted from the ripe fruits of cv. Camarosa and cv. Chandler and *FaGalUR* specific primers, RT-PCR followed by digestion with the appropriate restriction enzyme showed that in both cultivars, the fragment pattern was consistent with the expression of the three alleles (Fig. 6C). Using densitometry on the bands, the relative expression of each allele within each lane was estimated (Fig. 6B). Basically, there were no significant differences between the cultivars with respect to the joint expression of alleles 1 and 2 and that of allele 3.

Discussion

Among those genes of the AsA biosynthetic pathways that were analysed, only two genes of the AsA biosynthetic pathways, *FaGalUR* and *FaMIOX*, showed an expression pattern that paralleled the increase of AsA in the strawberry fruit, and only one, *FaGalUR*, showed a striking increase (over 150-fold) during the development of the fruit from the green to the ripe stage. The AsA content in strawberry fruit

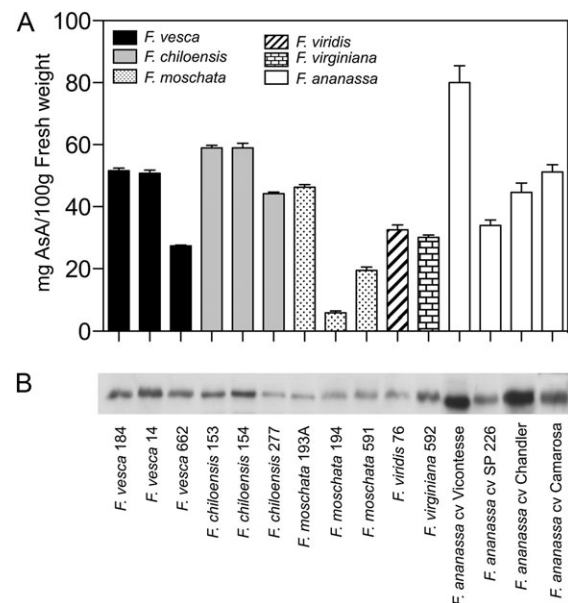


Fig. 5. (A) Changes in AsA levels and (B) GalUR levels by Western blot in the red fruits of different species from the genus *Fragaria* and different varieties of *F. ×ananassa*.

from cv. Chandler increased from the green immature to the ripe stage, with a ≥ 12.2 -fold increase on a dry weight basis. During this transition, the number of cells per fruit remains constant and the cell volume increases (Perkins-Veazie, 1995). The change in cell size is mainly due to an increased volume of vacuoles. Recent reports on AsA distribution in different cell compartments showed that the lowest levels were localized just in this compartment (Zechmann *et al.*, 2011). If this distribution pattern is also seen in strawberry fruit cells, the increase in the AsA levels in compartments other than the vacuole during ripening would be dramatic.

Two other genes encoding enzymes of the mannose/galactose pathway, *FaGME* and *FaGIPP* (Wheeler *et al.*, 1998), were down-regulated as fruit ripening proceeded from the green to the red stage. This observation suggests that this pathway is responsible for AsA biosynthesis in green fruits. The prevalence of one pathway over the others depending upon the developmental stage has previously been reported in the fruits of other plants such as grape (Cruz-Rus *et al.*, 2010) and tomato (Ioannidi *et al.*, 2009). This metabolic versatility toward AsA synthesis could be related to the different starting metabolites of each pathway, the availability of each of which is dependent on many environmental and/or developmental factors. In the mannose/galactose pathway, hexose phosphates are produced in the assimilatory biosynthetic pathways (Wheeler *et al.*, 1998), whereas in the GalUR pathway, methyl-D-galacturonate results from the breakdown of pectin polymers (Agius *et al.*, 2003). The first pathway is expected to be abundant in green fruits, whereas the second is expected to be abundant in the ripening fruits.

Interestingly, *FaMDHAR* expression was over 17-fold increased as the fruits ripened from green to red, whereas the expression of *FaDHAR* was higher in green fruits than in white and red fruits. These patterns indicate that *FaMDHAR* expression is associated with high AsA content in ripe fruit. The involvement of this enzyme in the reduction to AsA has been linked to the response of the plant to oxidative stress (Eltayeb *et al.*, 2006; Stevens *et al.*, 2008). Early transcriptional analysis during strawberry fruit ripening indicated that the strawberry ripening transcriptional programme is an oxidative, stress-induced process (Aharoni *et al.*, 2002). Therefore, *FaMDHAR* might contribute, along with other enzymes of these biosynthetic pathways, to the maintenance of high AsA content in the ripe fruit of strawberries. In green fruit, however, AsA recycling appears to be performed by DHAR. These observations are consistent with previous studies on tomato (Ioannidi *et al.*, 2009), kiwifruit (Bulley *et al.*, 2009) and acerola (Eltelib *et al.*, 2010). In these fruits, the two AsA recycling genes are differently regulated during ripening, depending on the plant species and the stage of ripening.

The ripe fruits stored under an ozone atmosphere suggest another scenario in which changes in the synthesis of AsA are expected. The most significant changes at the gene expression level were found in the *FaGIPP*, *FaGalUR*, and *FaGLDH* genes. This observation suggested the activation of both the mannose/galactose and the D-galacturonate

pathways under this treatment. In fact, decreases in sucrose, glucose, and fructose have been reported in ozone-treated fruits (Pérez *et al.*, 1999). The mannose/galactose pathway may consume these sugars. The analysis of the *FaGalUR* promoter revealed the presence of a G-box motif (Agius *et al.*, 2005), which is present in the ozone-responsive promoter region of the grape stilbene synthase gene (Schubert *et al.*, 1997). In addition, the 3-fold higher expression of *FaMDHAR* in the ozone-treated fruits compared with the air-stored fruits also supports a role of the corresponding enzyme in maintaining the AsA content in those fruits treated with ozone. In tomato fruits under stress conditions, a main role for MDHAR in maintaining the AsA levels has been reported (Stevens *et al.*, 2008).

Information on the regulation of the AsA biosynthetic pathways is still limited. In the mannose/galactose pathway, substrate availability has been suggested to be a limiting factor (Wheeler *et al.*, 1998). Other studies indicate that GME and/or GDP-L-galactose guanylyltransferase (GGT) (Bulley *et al.*, 2009; Gilbert *et al.*, 2009) play key roles in the regulation of ascorbate biosynthesis in plants. During tomato fruit ripening, GIPP has been proposed to play a main role in the accumulation of AsA (Ioannidi *et al.*, 2009). In the D-galacturonate pathway, a correlation between GalUR expression and AsA content was found (Agius *et al.*, 2003). This study has extended the analysis of the AsA pathways to ripe fruits of a segregating population from a cross between two *F. ananassa* parents with a very close genetic background that corresponded to cultivars with a Californian pedigree (Rousseau-Gueutin *et al.*, 2009). The lack of correlation between the transcriptional activity of the genes and the highly variable AsA content in the ripe fruits of this segregating population excluded the possibility of a single regulatory step and points to a coordinated action involving several of these genes.

Effectively, our results from the protein studies, both in different species of the *Fragaria* genus and in two closely related cultivars of *F. ananassa*, nullified the hypothesis that the enzyme GalUR had a unique regulatory role on AsA synthesis in ripe strawberry fruit. In plants, the AsA content in different tissues varies with environmental (Smirnoff and Wheeler, 2000; Tamaoki *et al.*, 2003) and developmental (Pallanca and Smirnoff, 1999; Bartoli *et al.*, 2000; Agius *et al.*, 2003) conditions, which requires tight control of its biosynthesis, catabolism, and reductive recovery from the oxidized form. Therefore, a number of regulatory steps should be expected. In agreement with this theory, plants transformed with the genes from different pathways have shown only a limited increase in their AsA content (Ishikawa *et al.*, 2006). This observation holds true for *GalUR* (Agius *et al.*, 2003; Hemavathi *et al.*, 2009), *L-galactose dehydrogenase* (Gatzek *et al.*, 2002), *GDP-L-galactose guanylyltransferase* (Bulley *et al.*, 2009), *GLDH* (Tokunaga *et al.*, 2005), and *DHAR* (Kwon *et al.*, 2003). In addition, genetic studies also support this complexity because several quantitative trait loci contributing to AsA content have been identified in apple and tomato (Davey *et al.*, 2006; Stevens *et al.*, 2007).

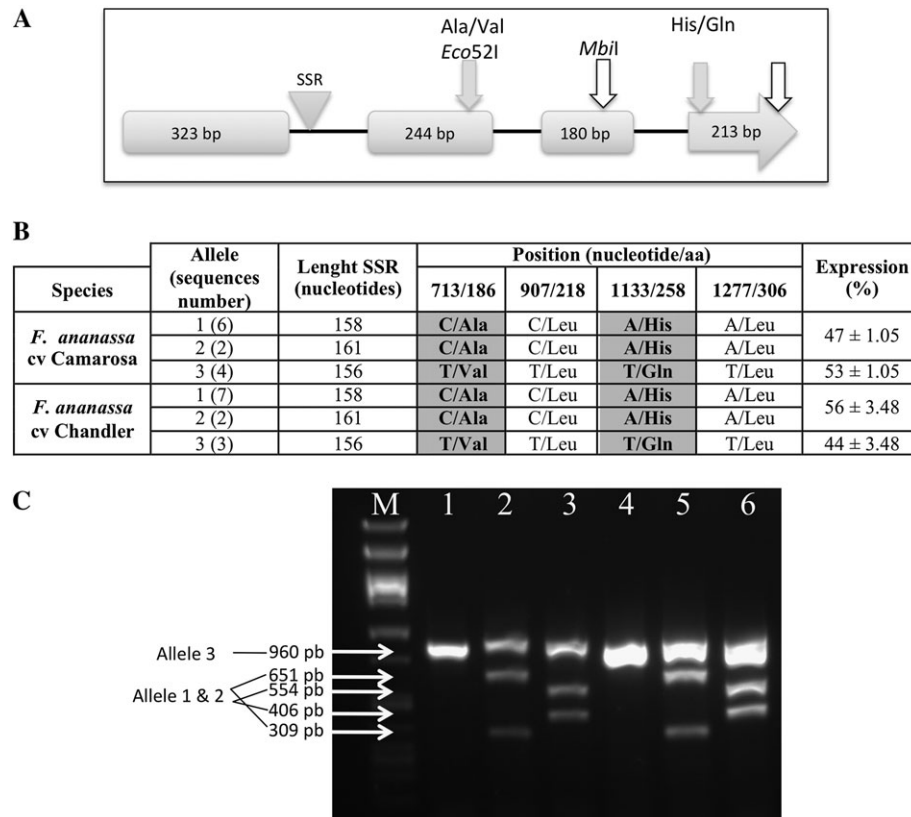


Fig. 6. (A) Schematic representation of the *GalUR* genomic DNA. The grey boxes represent exons, and the black lines represent introns. White arrows indicate synonymous mutations of a nucleotide, and grey arrows indicate non-synonymous mutations. The triangle marks the position of the SSR. (B) Changes in nucleotides and amino acids, highlighted in grey, as well as their positions in the different genomic clones of *GalUR* from strawberry cv. Chandler and cv. Camarosa are shown. The 'Allele' column indicates the abundance of the different alleles that were identified. The 'Expression' column indicates the expression of each allele in cv. Camarosa and cv. Chandler. The positions of nucleotide/amino acid correspond to Allele 3. (C) Digestion pattern of the open reading frame of *FaGalUR* from cv. Camarosa (1–3) and cv. Chandler (4–6) using specific primers and digested with *Eco52I* and *MbiI*. M, Marker; 1, *FaGalUR* from cv. Camarosa; 2, *FaGalUR* from cv. Camarosa digested with *MbiI*; 3, *FaGalUR* from cv. Camarosa digested with *Eco52I*; 4, *FaGalUR* from cv. Chandler; 5, *FaGalUR* from cv. Chandler digested with *MbiI*; 6, *FaGalUR* from cv. Chandler digested with *Eco52I*.

Overall, our results indicate that the *GalUR* enzyme plays a prominent role in the regulation of the AsA biosynthetic pathway in ripe strawberry fruits. This regulatory role, however, is probably limited and shared with the enzymes of other pathways and even with other components of this AsA pathway, which might include the substrate availability. As in other plant tissues, the metabolic control of AsA levels is not executed by a single element, enzyme or substrate. Rather, this control is likely to be shared by several components in a complex and still unknown regulatory network that must be studied using species/tissue/stage-based approaches.

Supplementary data

Supplementary data can be found at JXB online

Supplementary Table S1. Sequence of the specific primers used for RT-PCR expression studies.

Supplementary Table S2. Table with the different restriction sites and the fragment length of the different cuts in the different alleles.

Supplementary Fig. S1. Changes in mRNA levels of *PR5* and *PGIP*, two pathogen-induced genes (Osorio *et al.*, 2008) in fruits before (0 h) and after storage for 72 h in ozone or air atmosphere by semi-quantitative RT-PCR.

Supplementary Fig. S2. Frequency distributions of AsA for the 95 individuals from the population segregation derived from the cross between '232' and '1392'; arrows indicate the parental values.

Supplementary Fig. S3. Alignment of the three different alleles of *FaGalUR*

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